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Forum Review

Regulation of Reactive Oxygen Species and Genomic Stability in Hematopoietic Stem Cells

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Abstract

Hematopoietic stem cells (HSCs) are defined by their ability both to self-renew and to give rise to fresh blood cells throughout the lifetime of an animal. The failure of HSCs to self-renew during aging is believed to depend on several intrinsic (cell-autonomous) and extrinsic (non–cell-autonomous) factors. In this review, we focus on how dysregulation of reactive oxygen species (ROS) and disruptions of genomic stability can impair HSC functions. Recently, it was shown that long-term self-renewing HSCs normally possess low levels of intracellular ROS. However, when intracellular ROS levels become excessive, they cause senescence or apoptosis, resulting in a failure of HSC self-renewal. Repression of intracellular ROS levels in HSCs by treatment with an antioxidant that scavenges ROS can rescue HSC functions, indicating that excess ROS levels are at the root of HSC failure. Products of numerous genes that are involved in either DNA-damage responses or longevity-related signaling contribute to the maintenance of the HSC self-renewal capacity. Further investigations on the molecular mechanisms of ROS regulation and on the manipulation of excess ROS levels could lead to the development of novel therapeutics for hematopoietic diseases, regenerative medicine, and the prevention of leukemia. *Antioxid. Redox Signal.* 10, 1883–1894.

Introduction

APPROPRIATE intracellular levels of reactive oxygen species (ROS) play a vital role in regulating several biologic phenomena, including those that involve the activation of signaling pathways in response to cytokines and the gene expression elicited by this signaling. However, excessive production of ROS or inadequacy in a normal cell's antioxidant defense system (or both) can cause the cell to experience oxidative stress. Major endogenous sources of ROS are primary oxidative metabolism in the mitochondria, metabolic processes, and inflammation [reviewed in (6)]. Chemically, ROS include the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical. All these molecules contain unpaired electrons and thus can act as free radicals. The superoxide dismutases Mn-SOD and Cu/Zn-SOD contribute to the conversion of superoxide anion into hydrogen peroxide (Fig. 1). In addition, transition metals such as Fe^{2+} and Cu^{+} catalyze the conversion of hydrogen peroxide to the highly reactive hydroxyl radical *via* the Fenton or Harber–Weiss reactions (6). The hydroxyl radical can cause DNA damage. A cell attempts to defend itself from ROS-mediated oxidative stress by expressing enzymes such as catalase or selenium-dependent glutathione peroxidase (GPx), which detoxify hydrogen peroxide by converting it to water.

Stem cells are defined by their ability both to produce stem cells, a property known as self-renewal, and to give rise to differentiated progeny. Tissue stem cells are responsible for the generation of differentiated somatic cells in many tissues of higher eukaryotes (Fig. 2). Although hematopoietic stem cells (HSCs) give rise to blood cells of all hematopoietic lineages throughout the lifetime of an animal, a primary hallmark of these cells is their ability to remain in the nondividing G_0 phase of the cell cycle and to maintain a state termed quiescence. Cheshier and colleagues (13) estimated that ~75% of HSCs present in an animal are in the G_0 phase. Several studies have reported the intriguing result that knockout mice deficient for genes implicated in ROS regu-

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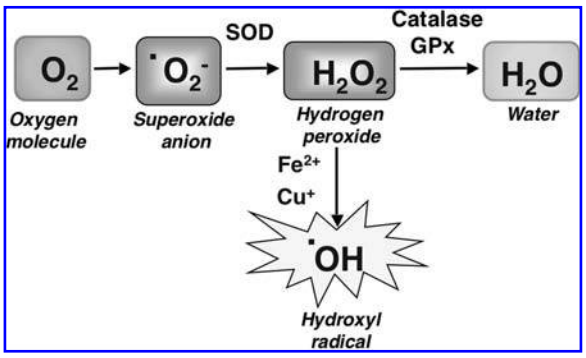

FIG. 1. Production of reactive oxygen species (ROS). Molecular oxygen can be partially reduced to superoxide anion *via* several biologic phenomena (6). Superoxide anion is converted by superoxide dismutases (Mn-SOD and Cu/Zn-SOD) to hydrogen peroxide. Catalase or selenium-dependent glutathione peroxidase (GPx) can detoxify hydrogen peroxide to water. Transition metals, such as Fe^{2+} and Cu^+, catalyze the conversion of hydrogen peroxide to the highly reactive hydroxyl radical by the Fenton or Harber-Weiss reactions.

lation, such as ataxia–telangiectasia mutated (*Atm*) and *Foxo*, have HSCs that cannot maintain quiescence and are defective in self-renewal (58). In this review, we focus on how the regulation of ROS levels and the maintenance of genomic stability affect HSC functions.

Regulation of ROS Levels Within HSCs

A stem-cell niche is a spatial structure and "comfortable" microenvironment that protects tissue stem cells and allows them to self-renew in the absence of differentiation (Fig. 2). An appropriate association between HSCs and their niche is essential for the maintenance of the HSC compartment. Mutant mice in which osteoblast numbers are increased also show an expansion of the HSC pool, indicating that osteoblasts are important components of the HSC niche (10, 88). Supporting this notion, it was reported that angiopoietin-1 regulates the quiescence of HSCs in the osteoblastic niche (4). Conversely, it also was shown that HSCs are maintained in locations adjacent to vascular cells, the so-called vascular niche (46). The migration of HSCs to the vascular niche is regulated by the chemokine CXCL12 (75). Although it remains unclear how HSCs are supported by their various niches *in vivo*, a finely tuned relation between HSCs and niche factors is crucial for the maintenance of HSC numbers and functions.

A relevant niche factor may be the effect of niche conditions on HSC intracellular ROS levels. Jang and Sharkis (40) reported a novel mouse HSC phenotype based on the intracellular ROS content of these cells. The mouse HSCs in this study could be separated into two fractions, ROS^{low} HSCs and ROS^{high} HSCs, although both populations had identical profiles of HSC cell-surface markers (*i.e.*, $CD34^-$, Lineage $marker^-$, Sca-1^+, c-Kit^+). Interestingly, ROS^{low} HSCs retained their long-term self-renewal ability throughout a serial transplantation assay, whereas this capacity gradually decreased in serially transplanted ROS^{high} HSCs. Treatment with the antioxidant *N*-acetyl-L-cysteine (NAC) was able to restore the functional activity of ROS^{high} HSCs. Jang and Sharkis concluded that intracellular ROS levels control the long-term self-renewal ability of HSCs. Taken together with these findings, these data suggest that the maintenance of low ROS levels in HSCs may depend on the interaction of HSCs with factors in their niche. Although it is still murky exactly how HSCs regulate their intracellular ROS levels, it is clear that ROS are a novel means of controlling the homeostasis and functions of this important cell population.

DNA Damage Responses in HSCs

Functional DNA-damage responses and regulation of ROS are crucial for maintaining chromosomal stability

The genomic DNA of normal cells is under continuous assault from intrinsic insults, such as oxidative stress, and extrinsic insults, such as ultraviolet (UV) light and ionizing radiation (IR). In dividing cells, the DNA may also suffer from the introduction of errors during the replication required for mitosis. Cells have therefore had to evolve mechanisms to maintain genomic stability. In response to DNA damage, a cell may trigger a checkpoint response that induces cell-cycle arrest and allows the cell time to repair the DNA damage before cell-cycle progression is resumed. Alternatively, if the DNA damage is too severe, the cell becomes senescent or undergoes apoptosis (Fig. 3). Thus, a defect in a DNA damage checkpoint response can result in unchecked mutation and genomic instability.

Two of the most important genes involved in maintaining genomic stability are *ATM* and "*ATM* and Rad3-related" (*ATR*), which are both members of the phosphatidylinositol 3-kinase–related kinase (PIKK) family. In humans, mutational inactivation of the *ATM* gene causes the autosomal recessive disorder ataxia-telangiectasia (A-T), which is characterized by immunodeficiency, progressive cerebellar ataxia, oculocutaneous telangiectasia, defective spermatogenesis, premature aging, and a high incidence of lymphoma (57). The frequency of tumors in A-T patients and *Atm*-deficient mice is a graphic demonstration that the maintenance of genomic stability is crucial for prevention of tumorigenesis. With respect to *ATR*, a splicing mutation of this gene has been identified in humans with Seckel syndrome, an autosomal recessive disorder characterized by intrauterine growth retardation, dwarfism, microcephaly, and mental retardation (62). Patients with Seckel syndrome are often at risk of developing myelodysplasia and acute myelogenous

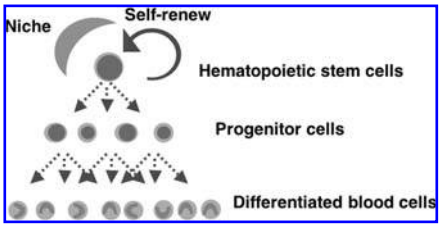

FIG. 2. Maintenance of hematopoietic homeostasis by the stem cell system. Hematopoietic stem cells (HSCs) have the ability to self-renew and to give rise to differentiated blood cells *via* progenitor cells. The self-renewal ability of HSCs is sustained by their "comfortable" microenvironment, termed the stem cell niche.

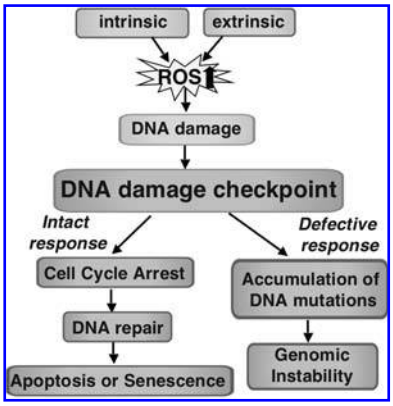

FIG. 3. Roles of DNA-damage checkpoint responses. ROS generated by intrinsic (*e.g.*, oxidative metabolism) or extrinsic insults (*e.g.*, environmental defects, inflammation, UV, IR) may cause DNA damage. DNA-damage checkpoint responses allow a cell time to repair the DNA damage before cell-cycle progression is resumed. If the DNA damage in a cell is too extensive, the DNA-damage checkpoints trigger the cell to become senescent or undergo apoptosis. Defective DNA-damage checkpoint responses result in accumulation of DNA mutations, leading to genomic instability.

leukemia (AML). At the molecular level, the kinases encoded by the *ATM* and *ATR* genes contribute to the maintenance of genomic stability by activating a cell-cycle checkpoint in response to DNA damage or stalled replication (57, 74). Thus, ATM and ATR functions are essential for the maintenance of genomic stability.

Support for the theory that excessive ROS can compromise genomic stability comes from studies of *Ku-86*$^{-/-}$ mice, which have a defect in the nonhomologous DNA end-joining (NHEJ) pathway of DNA-damage repair. Primary fibroblasts from *Ku-86*$^{-/-}$ mice show a marked increase in spontaneous chromosome breaks compared with wild-type (WT) mice. To investigate the source of these breaks, *Ku-86*$^{-/-}$ fibroblasts were cultured under normoxia (20% oxygen) or hypoxia (3% oxygen) (44). The frequency of chromosome breaks in the absence of Ku-86 was significantly reduced under hypoxia, suggesting that the oxygen levels present under normoxic conditions synergize with the defective DNA-repair system in these mutant cells to enhance chromosomal breakage. Studies of Cu/Zn superoxide dismutase (SOD1), an enzyme that plays a major role in the metabolism of oxygen radicals, also bolstered the ROS–genomic instability connection (44). SOD1 converts superoxide radicals to H_2O_2, and *SOD1* transgenic mice show elevated levels of H_2O_2 and hydroxyl radicals in neurons and hematopoietic cells (5, 65). Primary fibroblasts from *SOD1* transgenic mice show increased levels of ROS that enhance the frequency of chromosome breaks. Thus, endogenous DNA damage observed in *Ku-86*$^{-/-}$ cells may arise predominantly from the oxidative stress caused by excess ROS.

Atm and Atr are essential for the self-renewal of HSCs

The products of the genes involved in the DNA-damage checkpoint machinery and the maintenance of genomic stability also regulate the ability of HSCs to self-renew. Studies of *Atm*-deficient mice have demonstrated that Atm kinase controls the self-renewal capacity of HSCs (36). *Atm*-deficient mice older than 24 weeks showed progressive bone marrow failure due to a functional defect that is present in HSCs but absent from differentiated cells. Significantly, the defects in the faulty HSCs were closely associated with elevated levels of ROS. Ito *et al.* (37) showed that *Atm* deficiency induces an elevation of ROS that activates p38 mitogen–activated protein kinase (MAPK) (37), and that p38 MAPK in turn triggers upregulation of the cyclin-dependent kinase (cdk) inhibitors and tumor suppressors $p16^{Ink4a}$ and $p19^{Arf}$ (Fig. 4). This increase in $p16^{Ink4a}$ and $p19^{Arf}$ levels presumably inhibits the division of HSCs required for self-renewal.

Brown and colleagues (71) developed a mouse system in which the *Atr* gene is conditionally ablated in adult mice (null mutation of *Atr* is embryonic lethal). The elimination of the *Atr* gene in adult mice leads to defects in tissue homeostasis and the rapid appearance of several aging-related phenotypes, including hair graying, alopecia, kyphosis, and osteoporosis. Notably, a significant decline in HSCs is observed in aged *Atr*$^{-/-}$ mice compared with control mice. In addition to HSC exhaustion, these mutants have a decrease in HSC regenerative capacity that may be related to the deterioration of their osteoblastic niche. The volume of trabecular bone, a site that has been proposed as an HSC niche, is reduced in conditional *Atr*-deficient mice compared with the WT. Thus, like *Atm*, *Atr* contributes to the maintenance of the HSC self-renewal capacity. These observations indicate that intrinsic HSC exhaustion, plus the extrinsic influence of defective cell-cycle checkpoint responses within a niche, may act synergistically to produce a decline in HSCs. In any case, the maintenance of genomic stability by Atm and Atr kinases is crucial for the self-renewal ability of HSCs (Table 1).

Potential molecular mechanisms by which Atm may regulate ROS levels

Although the suppression of intracellular ROS levels by Atm is crucial for maintaining the self-renewal ability of HSCs, many questions remain regarding the actual mechanism by which Atm controls ROS. Does Atm directly induce the transcriptional activation of genes that are implicated in ROS detoxification? Or are such ROS-detoxification genes activated by p53 instead? What other factors contribute to ROS production and regulation? Further studies are needed to un-

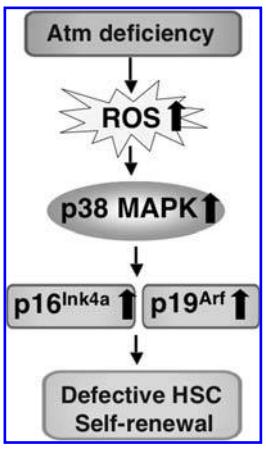

FIG. 4. Role of ATM in controlling ROS accumulation and preserving HSC self-renewal capacity. Atm kinase is activated by an elevation of ROS, so that loss of Atm function leads to an accumulation of ROS that occurs *via* unknown mechanisms. This excessive level of ROS induces p38 MAPK activation, which in turn triggers upregulation of the cdk inhibitors $p16^{Ink4a}$ and $p19^{Arf}$. These cdk inhibitors block the cell cycle and thereby impair HSC self-renewal.

Table 1. Summary of Consequence of Gene Mutations in HSCs

Mouse gene	*Normal role*	*HSC phenotypes in mutant mouse*
Atm	Cell cycle checkpoint Regulation of ROS levels	Defect in self-renewal capacity Elevation of ROS levels Activation of p38MAPK Up-regulation of $p16^{Ink4a}$ and $p19^{Arf}$
Atr	Cell cycle checkpoint	Defect in self-renewal capacity Deterioration of osteoblastic niche
XPD	Nucleotide excision repair	Defect in self-renewal capacity in aged mice
Ku-86	NHEJ	Defect in self-renewal capacity in aged mice Accumulation of DNA damage during aging
Lig4	NHEJ	Defect in self-renewal capacity in aged mice Accumulation of DNA damage during aging
Terc	Telomere elongation	Defect in self-renewal capacity in aged mice
Foxo	Transcription factor	Defect in self-renewal capacity Elevation of ROS levels Reduced expression of Mn-SOD and catalase
Pten	Lipid phosphatase Suppression of PI3K-AKT/PKB	Defect in self-renewal capacity Development of leukemia-initiating cells

derstand the relation between ROS levels, the maintenance of genomic stability, and HSC self-renewal.

Some progress has been made in identifying downstream effectors of Atm that may affect ROS composition. In mice, Li *et al.* (52) demonstrated that *Atm* deficiency alters the responses of osteoblasts to oxidative stress by inducing the antioxidant protein peroxiredoxin I (Prx I). This Prx I induction depends on an accumulation of Nrf2, a basic leucine-zipper transcription factor that occurs in a protein kinase C (PKC)-δ–dependent manner. In humans, studies of cells derived from A-T patients have revealed that glutathione (GSH) synthesis and catalase expression are impaired in the absence of ATM (43, 53). It would be interesting to examine the possible roles of all these molecules in ATM-mediated antioxidative functions in HSCs.

DNA damage accumulates in HSCs during aging

As an animal ages, its HSCs gradually lose the ability to self-renew and show an accumulation of endogenous DNA damage [reviewed in (69)]. It has been proposed that this loss of self-renewal capacity is linked to an age-related decrease in the ability to repair damaged DNA. Two studies have contributed important information on the role of DNA repair in the maintenance of HSC self-renewal capacity in aging animals (61, 68). Rossi *et al*. (68) showed that endogenous DNA damage accumulates in HSCs with age. These workers also reported that a deficiency of XPD, telomerase RNA component (Terc), or Ku-86 resulted in HSC dysfunction under stress conditions. XPD, Terc, and Ku-86 are involved in the maintenance of genomic stability *via* the nucleotide excision-repair pathway, telomere maintenance, and the NHEJ-repair pathway, respectively. In a similar vein, Nijnik *et al*. (61) reported that a hypomorphic mutation of Lig4, which is essential for DNA repair by NHEJ, caused a progressive loss of HSCs and bone marrow cellularity during aging. Taken together, these data suggest that unrepaired DNA damage is largely to blame for the loss of HSC function that occurs during aging.

Telomere Maintenance in HSCs

In addition to DNA repair, the maintenance of chromosomal telomeres is essential for the HSC self-renewal capacity. Studies *in vitro* have indicated that cultured human diploid fibroblasts undergo a finite number of cell divisions, termed the Hayflick limit (32). The Hayflick limit is imposed when the telomeres in a dividing cell reach a critically reduced length. It is now well established that molecules regulating telomere stability are involved in cellular senescence [reviewed in (7, 11, 18)]. Intriguingly, fibroblasts cultured under hypoxic conditions (2–10% oxygen) can resist cellular senescence (63). In contrast, fibroblasts cultured in hyperoxic conditions (40% oxygen) show accelerated telomeric shortening and premature replicative senescence (82). These results suggest that excess levels of ROS cause not only genome-wide DNA damage but also telomere dysfunction.

Telomere biology

Mammalian telomeres consist of repetitive TTAGGG duplex sequences and terminate in single-strand 3′ G-rich overhangs that form lariat-like structures called T-loops (9, 20, 26). The T-loop structure is protected from being recognized as a DNA double-strand break by the shelterin complex that contains TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 (9, 19). In most somatic cells, telomere length shortens with each cell division. In cells with a high capacity for self-renewal, such as stem cells and tumor cells, routine telomere shortening is prevented by expression of telomerase complex with reverse transcriptase activity. The telomerase complex can elongate telomeric repeats by catalyzing the addition of TTAGGG sequences to the 3′ ends of chromosomes. The telomerase complex consists of telomerase reverse transcriptase (TERT); dyskerin, encoded by the dyskeratosis congenita *(DKC1)* gene; and the RNA template (TERC) (9).

Telomere function in HSCs

It has been postulated that HSCs actively maintain telomere lengths throughout the many cell divisions that occur

during an animal's lifespan. Indeed, telomerase activity is readily detectable in mouse HSCs (56). HSCs obtained from adult human bone marrow, however, showed shorter telomeres than HSCs isolated from fetal human liver or cord blood, suggesting that a progressive decline in telomere length occurs in HSCs with aging (80). Consistent with this hypothesis, telomere length in mouse bone marrow cells shortens during serial transplantation (1). Therefore, the expression levels of telomerase are lower and are unable to compensate for the loss of telomeric DNA that occurred during successive cell divisions.

Dyskeratosis congenita (DC) is an inherited human syndrome that is characterized by bone marrow failure, mucocutaneous abnormalities, and tumorigenesis before age 50 years. Cells from DC patients frequently show a mutation in the *DKC1, hTERC,* or *hTERT* genes (9, 33, 47), indicating that DC is a disease of defective telomere maintenance. The bone marrow failure of DC patients has been attributed to the accelerated telomere shortening that occurs in their hematopoietic cells because of the defective telomerase components (83). Hao *et al.* (31) generated a mouse model of DC consisting of $mTerc^{+/-}$ mice on the CAST/EiJ background. The heterozygosity of the *mTerc* gene in these mutants results in progressive telomere shortening and insufficient bone marrow–reconstitution capacity. These observations suggest that defective telomere function due to accelerated telomere shortening results in hematopoietic failure. Thus, telomere function plays an essential role in the maintenance of HSC self-renewal ability (Fig. 5).

Interestingly, it has been reported that deletion of $p21^{Waf1/Cip1}$ prolongs the lifespan of $mTerc^{-/-}$ mice, which have dysfunctional telomeres (14). $p21^{Waf1/Cip1}$ is encoded by *Cdkn1a*, a gene proven essential for maintenance of HSC quiescence (12). Nevertheless, deletion of *Cdkn1a* improves the self-renewal capacity of HSCs in the fourth generation of $mTerc^{-/-}$ mice. Thus, $p21^{Waf1/Cip1}$ is a key effector mediating the failure of self-renewal in HSCs with shortened telomeres. These results implicate $p21^{Waf1/Cip1}$ as a potential therapeutic target for improving HSC regeneration and treating aging-related HSC disorders characterized by telomere dysfunction.

WT murine HSCs can be serially transplanted at least 4 times into recipients, although telomere lengths gradually decrease during successive transplantations. However, HSCs from $mTerc^{-/-}$ or $mTert^{-/-}$ mice can be serially transplanted for only two rounds (2, 73). Thus, telomerase activity is required to slow the telomere shortening of mouse HSCs that occurs during serial transplantation. To examine whether telomere shortening is also the cause of the defect in WT HSC bone marrow–reconstitution capacity that is observed after five transplantations, transgenic mice overexpressing *mTert* in HSCs were established (3). Serial transplantation of HSCs from *mTert*-transgenic mice showed that, even though telomere lengths were stable, the HSCs could be serially transplanted no more than 4 times. These results suggest that the lifespan of HSCs after a fifth transplantation is not dependent on telomere length *per se* but rather on the cumulative stress encountered during the repetitive removal of HSCs from their niche.

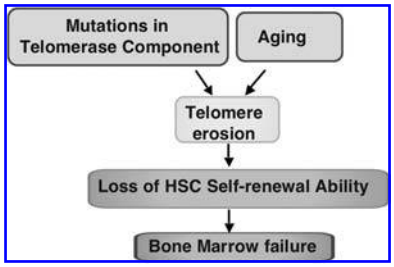

FIG. 5. The role of telomere function in HSCs. Telomere erosion due to aging or mutations in telomerase components causes loss of HSC self-renewal ability and results in bone marrow failure.

Activation of DNA-Damage Checkpoint Responses in HSCs by Telomere Dysfunction

Recognition of dysfunctional telomeres as DNA damage

Several lines of evidence have demonstrated that, when telomeres are rendered dysfunctional either through replicative attrition of the telomeric DNA or by inhibition of the shelterin complex, cells activate the same checkpoint responses as triggered by UV- or IR-induced DNA damage. First, DNA-damage checkpoint responses are activated in senescent human fibroblasts, in which telomere lengths have become critically short (17). Second, inhibition of the shelterin complex that protects telomeres results in the activation of ATM- or ATR-dependent DNA-damage responses, even in the absence of telomere shortening. Inhibition of TRF2, a telomeric protein that binds to double-stranded DNA, leads to the presence of dysfunctional uncapped telomeres and the activation of the ATM-dependent checkpoint (21, 45). Inhibition of the shelterin protein TPP1 has the same effects (27). In contrast, inhibition of POT1, which binds to the 3′ single-stranded G-rich telomeric overhang, activates ATR-dependent DNA damage responses (21). As well as inducing the ATM-dependent checkpoint response, inhibition of TRF2 promotes the accumulation of ATM as well as the DNA-damage response factors 53BP1, Rad17, and Mre11 in structures termed "telomere dysfunction–induced foci" (TIFs) (76). These observations indicate that telomeres that are dysfunctional due to the loss of the shelterin complex induce DNA-damage checkpoint activation, even in the absence of obvious telomere shortening. Thus, dysfunctional telomeres, regardless of length, have the same effects on cells as conventional DNA damage.

Cooperation of DNA-damage checkpoint responses and telomere function in HSCs

Laboratory mice have unusually long telomeres, so that no obvious phenotypes are observed in early generations of $mTerc^{-/-}$ mice. The telomeres of $mTerc^{-/-}$ mice, however, shorten drastically with each successive generation, leading to premature aging (8, 50, 70). In addition, chromosomal end-to-end fusions, which are a hallmark of dysfunctional telomeres, develop rapidly in $mTerc^{-/-}$ mice. Notably, deletion of the *Atm* gene aggravates the aging phenotype of $mTerc^{-/-}$ mice (86). Metaphase spreads of primary bone marrow cells from the fourth generation of $mTerc^{-/-}$ $Atm^{-/-}$ mice showed a significant increase in numbers of chromosomal end-to-end fusions and fragmented chromatids/chromo-

somes compared with samples from littermate *mTerc*$^{-/-}$ *Atm*$^{+/+}$ mice. Thus, a defect in DNA-damage checkpoint activity further attenuates genomic stability in HSCs with shortened telomeres. These results indicate that DNA-damage checkpoint responses and telomere function cooperate to maintain the self-renewal ability of HSCs.

Effects of Microenvironment on HSCs

Extrinsic factors in a telomere-dysfunctional environment can influence HSC self-renewal

Telomere dysfunction induces intrinsic checkpoint responses that limit the HSC self-renewal capacity. However, extrinsic factors present in microenvironments associated with telomere dysfunction may also influence HSC self-renewal. Ju *et al.* (42) reported that a telomere-dysfunctional environment can limit the engraftment of transplanted WT HSCs. These workers observed a significant defect in HSC engraftment when WT bone marrow cells were transplanted into aged *mTerc*$^{-/-}$ recipients, but not when these cells were transplanted into *mTerc*$^{+/-}$ recipients (42). To investigate possible extrinsic factors that could explain this difference, cytokines were analyzed in plasma samples from aged *mTerc*$^{+/-}$ and *mTerc*$^{-/-}$ mice. It has been postulated that cytokines are extrinsic factors that influence HSC function by mediating cross-talk between stem cells and their niche. Indeed, levels of granulocyte colony-stimulating factor (G-CSF) increase more with age in *mTerc*$^{-/-}$ mice than in *mTerc*$^{+/-}$ mice (42). In addition, administration of G-CSF to WT mice significantly impairs the engraftment of transplanted WT bone marrow cells, indicating that G-CSF treatment of WT mice can mimic the HSC transplantation defect seen in untreated, aged *mTerc*$^{-/-}$ mice. In line with this observation, inhibition of G-CSF by treatment with neutralizing anti–G-CSF antibody improved WT HSC engraftment in aged *mTerc*$^{-/-}$ mice (42). These results suggest that cytokines such as G-CSF are extrinsic components in the surrounding microenvironment that can influence HSC function. Notably, G-CSF stimulation has been shown to induce ROS production *via* the phosphatidylinositol-3-OH kinase (PI3K)-AKT/PKB signaling pathway (90), and inhibition of PI3K and AKT/PKB abrogates G-CSF–induced ROS production. Thus, G-CSF produced in a microenvironment associated with telomere dysfunction might induce ROS production that causes HSC failure.

Production of ROS in response to inflammatory signaling

ROS are widely implicated in the inflammatory process (49), and experiments with metabolic mutant mice have bolstered a link between inflammation and HSC self-renewal. Fanconi anemia (FA) is a genetic-instability syndrome that occurs in humans, and murine FA HSCs show reduced repopulating ability (29, 30). Zhang *et al.* (89) reported that the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) inhibits the self-renewal of HSCs from *FA complementation group C* (*Fancc*) gene-deficient mice (89). This TNF-mediated inhibition of HSC self-renewal was shown to be due to excessive ROS production. Furthermore, TNF-α treatment of bone marrow HSCs from *Fancc*$^{-/-}$ mice induced premature senescence that was associated with ROS accumulation. Neutralization of TNF-α or deletion of the *TNF receptor 1* gene in *Fancc*$^{-/-}$ mice (*Fancc*$^{-/-}$ *Tnfr1*$^{-/-}$ mice) prevented both excessive ROS production and hematopoietic senescence. Pretreatment of *Fancc*$^{-/-}$ mice with the ROS scavenger NAC significantly reduced TNF-mediated HSC senescence. However, overexpression of *mTert* failed to rescue the senescence of TNF-treated *Fancc*$^{-/-}$ HSCs, implying that, at least in *Fancc*$^{-/-}$ HSCs, ROS may promote senescence by the infliction of genome-wide DNA damage rather than by telomere shortening. These results indicate that production of ROS in response to inflammatory signaling can induce premature HSC senescence.

Regulation of ROS in HSCs by Longevity-Related Molecules

Longevity-related intracellular signaling pathways

In *Caenorhabditis elegans*, the insulin/insulin-like growth factor (IGF) signaling (IIS) pathway plays a vital role in the regulation of metabolism, reproduction, and lifespan. Mutations of *daf2*, the orthologue of mammalian insulin/IGF type 1 receptor (IGF-1R), induces the dauer stage with its characteristic diapause arrest, and results in a prolonged lifespan. This extension in lifespan induced by *daf-2* mutation can be prevented by null mutation of *daf-16*, the orthologue of mammalian FOXO [reviewed in (78)]. The IIS pathway is also involved in lifespan regulation in *Drosophila melanogaster*. *D. melanogaster* lifespan is extended either when the insulin-like receptor (InR) or its receptor substrate Chico is mutated (15, 77), or when dFoxo is overexpressed (23, 35). *D. melanogaster* lifespan is also increased by constitutive activation of stress-responsive Jun-N-terminal kinase (JNK), which induces nuclear localization of dFoxo (84).

IGF-1R is a tyrosine kinase receptor that regulates energy metabolism. Although *Igf-1r* null mice are not viable, female *Igf-1r*$^{+/-}$ mice live longer than their WT littermates and display greater resistance to oxidative stress. (34). Thus, heterozygous inactivation of *Igf-1r* significantly lengthens murine lifespan and increases resistance to ROS (34).

The Forkhead O (FOXO) subfamily of mammalian transcription factors includes FOXO1, FOXO3a, FOXO4, and FOXO6, all of which are important downstream targets of the PI3K-AKT/PKB signaling pathway (25). When a growth factor or insulin binds to the appropriate cell-surface receptor, the PI3K-AKT/PKB signaling pathway is activated. Activated AKT/PKB directly phosphorylates FOXOs at the conserved three phosphorylation sites, resulting in their nuclear export by 14-3-3 protein and subsequent degradation in the cytoplasm. In the absence of stimulation by growth factors or insulin, FOXOs are present in an active state in a cell's nucleus and freely induce their transcriptional targets. These targets have been implicated in diverse physiological processes, including cell-cycle arrest, stress resistance, induction of apoptosis, and detoxification of ROS (16) (Fig. 6).

In mice, Foxo is also involved in the mitochondrial longevity pathway (28). A mouse's lifespan can be significantly extended and the animal's resistance to ROS increased by homozygous inactivation of the p66 isoform of the *Shc* gene (54). p66^{Shc} is a redox enzyme that generates mitochondrial ROS (particularly hydrogen peroxide) as signaling molecules to induce apoptosis (24). Phosphorylation of p66^{Shc} is mediated by PKC-β, which is activated by oxidative conditions within a cell. Phosphorylated p66^{Shc} is rec-

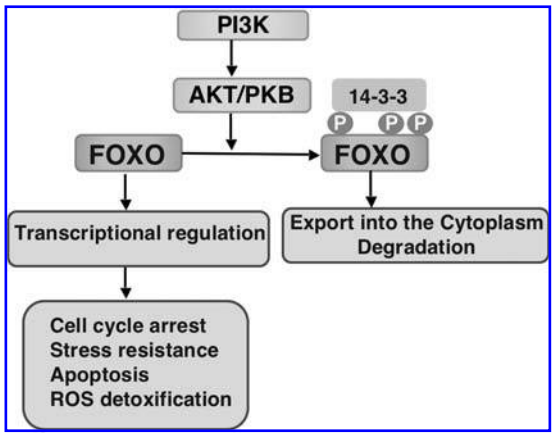

FIG. 6. Regulation of FOXO by the PI3K-AKT/PKB-FOXO signaling pathway. In the absence of PI3K-AKT/PKB activation, FOXO transcription factors are retained in the nucleus and drive transcription of their target genes that are involved in cell-cycle arrest, stress resistance, apoptosis, and detoxification of ROS. When a growth factor or insulin binds to the appropriate cell-surface receptor, the PI3K-AKT/PKB signaling pathway is activated, and the activated AKT/PKB directly phosphorylates FOXOs at the conserved three phosphorylation sites, resulting in their nuclear export by 14-3-3 protein and subsequent degradation in the cytoplasm.

ognized by the prolyl isomerase Pin1 and accumulates in the mitochondria (66). In response to an increase in intracellular ROS, phosphorylated $p66^{Shc}$ suppresses the activity of Foxo3a (60). In WT mouse embryonic fibroblasts (MEFs), stimulation with either insulin or hydrogen peroxide leads to a rapid and significant increase in the phosphorylation and thus inactivation of Foxo3a. In contrast, in $p66^{Shc-/-}$ MEFs, no phosphorylation of Foxo3a is observed in response to oxidative stress, whereas Foxo3a phosphorylation is observed in response to insulin stimulation. These results indicate that $p66^{Shc}$ is a key suppressor of Foxo under conditions in which ROS are produced. Activation of Foxo may contribute to extension of lifespan in $p66^{Shc}$-deficient mice through suppression of ROS levels. In contrast, it has been reported that activation of FOXO4 through phosphorylation at C-terminal lesion by JNK contributes to suppression of ROS in response to increased levels of ROS (22). Thus, FOXO transcription factors are intimately involved in many biologic responses that act to prevent oxidative stress.

Foxo regulates the self-renewal ability of HSCs

As well as being important for responses to oxidative stress, the Foxo transcription factors are key regulators of HSC self-renewal. In quiescent murine HSCs, Akt/PKB is not activated, and Foxo proteins are localized in the nucleus (87). On cytokine stimulation of HSCs *in vitro*, Akt/PKB becomes activated and induces the export of Foxo proteins from the nucleus to the cytoplasm. In conjunction with this export, the HSCs lose their repopulating capacity. Attenuation of cytokine signaling by lipid-raft inhibitors maintains both HSC quiescence and nuclear localization of Foxo proteins (87). These findings suggest that cytokine signaling can influence Foxo localization, which in turn governs HSC quiescence. Examination of the Foxo factors themselves has supported the relation between Foxo function and HSC self-renewal. In mice with triple conditional deletions of the *Foxo1*, *Foxo3a,* and *Foxo4* genes, a marked decrease in the immature hematopoietic cell population, including HSCs and progenitors, was observed (79). Furthermore, the HSCs of mice lacking only *Foxo3a* showed defective self-renewal and decreased expression of $p27^{Kip1}$ and $p57^{Kip2}$ (55). These results suggest that the loss of Foxo3a directly reduces the expression of multiple negative regulators of the cell cycle, leading to a defect in the maintenance of HSC quiescence. As well, HSC numbers were significantly decreased in aged *Foxo3a*-deficient mice compared with littermate controls, indicating that Foxo3a plays a pivotal role in maintaining the HSC pool (55). These data implicate the *Foxo* genes in the maintenance of HSC quiescence and functions (Fig. 7).

Regulation of ROS levels by the PI3K-AKT/PKB-FOXO signaling pathway

Several lines of evidence suggest that the activation of the PI3K-AKT/PKB-FOXO signaling pathway is involved in regulating intracellular ROS levels. First, as described earlier, G-CSF stimulation increases ROS production *via* the PI3K-AKT/PKB signaling pathway (90). Second, it has been demonstrated that Foxo proteins play essential roles in the expression of the *SOD* genes, which are involved in ROS detoxification. MnSOD is a downstream target of the PI3K-AKT/PKB signaling pathway, and Foxo3a can induce the expression of the *MnSOD* gene in a cultured cancer cell line (48). Third, the HSCs from *Foxo*-deficient mice not only exhibit a self-renewal defect but also show elevated ROS. To investigate whether the triple *Foxo*-deficient phenotype was associated with increased ROS, microarray assays were performed comparing gene expression by WT and triple *Foxo*-deficient HSCs (79). The expression of SOD genes was impaired in triple *Foxo*-deficient HSCs. In addition, $Foxo3a^{-/-}$ HSCs exhibit increased levels of ROS, indicating that deficiency of a single Foxo factor is sufficient to induce excess

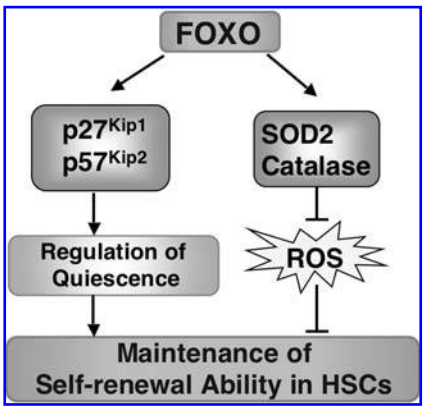

FIG. 7. Roles of FOXO in HSC self-renewal. FOXO transcription factors drive the transcription of SOD2 and catalase, as well as the expression of the cdk inhibitors $p27^{Kip1}$ and $p57^{Kip2}$. When ROS accumulate in response to a stimulus or because of loss of FOXO function, the elevation in ROS disrupts HSC quiescence and drives p38 MAPK activation, which impairs HSC self-renewal (as described in Fig. 4).

ROS production. *Foxo3a*$^{-/-}$ HSCs also showed reduced expression of the Foxo target genes *SOD2* and *catalase*. Thus, the FOXO transcription factors are responsible for both regulating ROS levels and maintaining HSC self-renewal capacity (Fig. 7).

Effects of the Inhibition of ROS and P38 MAPK on HSCs

ROS-mediated activation of p38 MAPK induces upregulation of p16^{Inka} and p19^{Arf}

In HSCs of *Atm*-deficient mice, elevation of ROS induces upregulation of the cdk inhibitors p16^{Ink4a} and p19^{Arf} through activation of p38 MAPK (37) (Fig. 3). A similar upregulation of p16^{Ink4a} and p19^{Arf} expression has been reported in HSCs of *Bmi-1*–deficient mice. Bmi-1 is a member of the Polycomb family, which is implicated in epigenetic gene silencing *via* chromatin remodeling. Disruption of the *Bmi-1* gene leads to increased expression of p16^{Ink4a} and p19^{Arf} and defects in HSC self-renewal (39, 51, 64), indicating that suppression of p16^{Ink4a} and p19^{Arf} by Bmi-1 is essential for HSC self-renewal. Consistent with this observation, age-dependent upregulation of p16^{Ink4a} has been shown to limit the lifespan of HSCs (41).

The Antioxidant NAC suppresses ROS elevation in HSCs and preserves HSC self-renewal

As described earlier, *Atm*-deficient HSCs exhibit reduced repopulating capacity associated with elevated ROS (Fig. 3). In an effort to manipulate intracellular ROS concentrations, *Atm*-deficient HSCs have been treated with NAC, which acts as an antioxidative agent that scavenges ROS (36). Treatment of *Atm*-deficient HSCs with NAC significantly decreases intracellular ROS levels and restores the repopulating capacity of *Atm*-deficient HSCs, preventing bone marrow failure. NAC treatment also abrogates p16^{Ink4a} and p19^{Arf} upregulation in *Atm*-deficient HSCs, preserving this stem cell population. These observations suggest that elevated levels of ROS are the cause of the defective HSC self-renewal in *Atm*$^{-/-}$ mice.

HSCs from triple *Foxo*-deficient mice also show a decrease in *in vivo* long-term repopulation capacity that correlates with increased ROS levels (79). To determine whether increased ROS were causally implicated in the defects of the HSCs from these mutants, Tothova *et al.* (79) treated triple *Foxo*-deficient mice with NAC in an attempt to reverse the HSC failure. Daily NAC treatment *in vivo* reduced ROS levels in the HSC compartment of these mutants and led to reversion of the HSC phenotypes. The treated animals showed increased numbers of HSCs, recovery of myeloid colony-forming ability *in vitro*, and restoration of day 12 of colony-forming units–spleen (CFU-S) activity *in vivo* (79). These results confirm that increased ROS levels are responsible for the HSC defects in triple *Foxo*-deficient mice.

As noted earlier, ROS^{low} HSCs have a greater long-term repopulating capacity in serial bone marrow transplantation assays compared with ROS^{high} HSCs (40). Treatment of mice with NAC for 20 weeks decreased the ROS^{high} population in total bone marrow cells compared with untreated mice (40). Moreover, NAC treatment rescued the *in vitro* repopulating capacity of ROS^{high} cells, as evaluated in a long-term culture–initiating cell (LTC-IC) assay that assesses the ability of primitive hematopoietic cells to expand *in vitro*. Significantly, NAC treatment also suppressed phosphorylated p38 MAPK levels, consistent with the fact that increased phosphorylated p38 MAPK correlates well with increased intracellular ROS.

Taken together, these examples of NAC-mediated recovery of HSC function imply that a reduction of ROS *via* antioxidant treatment could have a therapeutic effect based on p38 MAPK regulation and the protection of HSCs from oxidative stress. In particular, A-T patients might benefit from the pharmaceutical administration of antioxidant agents. Antioxidant treatment might also reduce the incidence of malignancies, as NAC administration has been shown to prevent cancer development in tumor-prone mouse models, including in *Atm*-deficient mice and *p53*-deficient mice (38, 67, 72).

p38 MAPK inhibition can rescue defective HSC self-renewal

Like NAC treatment, treatment with a p38 MAPK inhibitor can rescue defects in HSC self-renewal. p38 MAPK is a kinase involved in the regulation of numerous cellular processes, including cell-cycle arrest, apoptosis, and senescence. p38 MAPK is activated by cellular stresses such as ROS, UV, IR, and inflammatory cytokines. Inhibition of p38 MAPK rescues the ROS-induced defects in HSC self-renewal and quiescence seen in *Atm*-deficient HSCs. In serial transplantation experiments with WT HSCs, prolonged treatment with a p38 MAPK inhibitor extended HSC lifespan (37). Treatment of *Foxo3a*$^{-/-}$ HSCs, which exhibit increased p38 MAPK phosphorylation, with the p38 MAPK inhibitor SB203580 restored normal HSC functions *in vitro* (55). In humans, treatment with the p38 MAPK inhibitors, including SB203580 and SCIO-469, restored hematopoiesis in patients with aplastic anemia or one of the myelodysplastic syndromes (MDS) *in vitro* (59, 81).

Exposure of mice to a sublethal dose (6.5 Gy) of total body irradiation reduces the HSC compartment (85). The HSCs from such irradiated mice exhibit increased expression of the cellular senescence biomarkers p16^{INK4a} and senescence-associated β-galactosidase (SA-β-gal), demonstrating that IR exposure induces HSC senescence *in vivo*. An important outstanding issue is to determine whether it is the accumulation of ROS or the activation of p38 MAPK that is involved in the DNA-damage–induced HSC exhaustion exhibited in this case.

ROS^{high} HSCs, which show defective self-renewal ability, exhibit higher levels of phosphorylated p38 MAPK activity than do ROS^{low} HSCs (40). Like NAC, treatment with the p38 MAPK inhibitor SB203580 restores LTC-IC activity to ROS^{high} HSCs. However, treatment with the JNK inhibitor SP600125 or the MEK inhibitor U0126 did not rescue the LTC-IC activity of ROS^{high} HSCs (40). Thus, p38 MAPK is a downstream mediator that is specifically activated in HSCs in response to high ROS levels. These results indicate that control of intracellular ROS and normal function of p38 MAPK are crucial for the maintenance of HSC self-renewal capacity.

Conclusions

In this review, we have examined how regulation of ROS levels and genomic stability are critical for the maintenance of

HSC self-renewal capacity. Products of numerous genes involved in DNA-damage checkpoint responses and longevity-related signaling pathways regulate intracellular ROS levels and thus HSC self-renewal. However, precisely how elevated ROS impairs HSC self-renewal is still not clear. We must further expand our knowledge of the molecular mechanisms regulating ROS levels in HSCs, and in so doing, hope to identify targets for novel therapeutics aimed at treating hematopoietic diseases. Successful manipulation of ROS levels in HSCs may also contribute to improvements in bone marrow transplantation, anti-aging regimens, or regenerative medicine.

The links between the maintenance of HSC self-renewal ability and the development of leukemia stem cells from defective HSCs should also be explored in greater depth. It seems that mutational changes within the signaling pathways that regulate HSC self-renewal are involved in leukemia development. Compared with differentiated blood cells, HSCs are exposed to oxidative stress for much longer periods. This oxidative stress may generate ROS that can cause mutations in critical tumor-suppressor genes or in checkpoint genes responsible for maintaining genomic stability. Because of their long lifespans, HSCs may accumulate sufficient ROS-induced mutations to cause malignant transformation and the generation of leukemia stem cells. Thus, the investigation of the parallel and distinct roles of the genes controlling ROS and DNA-damage checkpoints in normal HSCs and leukemia cells will be important for the design of novel leukemia therapies. Further understanding of how ROS levels are regulated in HSCs should aid us to understand not only HSC self-renewal but also leukemogenesis.

Abbreviations

AML, acute myelogenous leukemia; A-T, ataxia–telangiectasia; ATM, ataxia–telangiectasia mutated; ATR, ataxia–telangiectasia mutated and Rad3-related; CDK, cyclin-dependent kinase; CFU-S ; colony-forming units–spleen, DC, dyskeratosis congenita; DKC1, dyskeratosis congenita 1; FA, Fanconi anemia; FANCC, Fanconi anemia complementation group C; G-CSF, granulocyte colony-stimulating factor; GPx, glutathione peroxidase; GSH, glutathione; HSCs, hematopoietic stem cells; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor type 1 receptor; IIS, insulin IGF signaling; JNK, Jun-N-terminal kinase; LTC-IC; long-term culture–initiating cell; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; MDS; myelodysplastic syndrome; NAC, *N*-acetyl-L-cysteine; NHEJ, nonhomologous DNA end joining; PI3K, phosphatidylinositol-3-OH kinase; PIKK, phosphatidylinositol 3-kinase–related kinase; PKC, protein kinase C; POT1, protection of telomeres 1; Prx I, peroxiredoxin I; ROS, reactive oxygen species; SA-β-gal, senescence associated–β-galactosidase; SOD, superoxide dismutase; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase; TIFs, telomere dysfunction-induced foci; TNF-α, tumor necrosis factor-α; TPP1, TINT1 PTOP1 PIP1; TRF1, telomeric repeat binding factor 1; TRF2, telomeric repeat binding factor 2; WT, wild-type.

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