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Reactive Oxygen Species in Normal and Tumor Stem Cells

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Abstract

Reactive oxygen species (ROS) play an important role in determining the fate of normal stem cells. Low levels of ROS are required for stem cells to maintain quiescence and self-renewal. Increases in ROS production cause stem cell proliferation/differentiation, senescence, and apoptosis in a dose-dependent manner, leading to their exhaustion. Therefore, the production of ROS in stem cells is tightly regulated to ensure that they have the ability to maintain tissue homeostasis and repair damaged tissues for the life span of an organism. In this chapter, we discuss how the production of ROS in normal stem cells is regulated by various intrinsic and extrinsic factors and how the fate of these cells is altered by the dysregulation of ROS production under various pathological conditions. In addition, the implications of the aberrant production of ROS by tumor stem cells for tumor progression and treatment are also discussed.

1. INTRODUCTION

About 2.5 million years ago, cyanobacteria evolved to gain the ability to produce oxygen (O₂) as a by-product of photosynthesis. O₂ is a paramagnetic gas that readily reacts with other elements like hydrogen, carbon, copper, and iron. As O₂ accumulated, it is thought to have converted the early reducing atmosphere into an atmosphere more conducive to oxidation reactions. Also, as atmospheric O₂ levels rose, many new organisms evolved and flourished after developing antioxidant defense systems to protect against the toxicity of by-products related to O₂ metabolism. Moreover, early aerobic organisms continued evolving to become multicellular organisms by taking selective advantage of efficient O₂ utilization in various vital metabolic processes, such as employing O₂ as the terminal electron acceptor for mitochondrial electron transport chain (ETC) activity during oxidative phosphorylation (OXPHOS), allowing for the efficient production of energy (Halliwell & Gutteridge, 2007).

However, utilizing O₂ in many essential metabolic processes by living systems came at an evolutionary price, because O₂ metabolism can lead to the production of reactive oxygen species (ROS) (Boveris, 1977; Buettner, 1993; Chance, Sies, & Boveris, 1979; Forman &

Kennedy, 1974, 1975; Fridovich, 1978). Fortunately, living systems are normally maintained in a nonequilibrium steady-state that is highly reducing and is exemplified by the reduced glutathione (GSH)/glutathione disulfide (GSSG) redox couple that oscillates between about -200 and -240 mV (Schafer & Buettner, 2001). This highly reducing intracellular environment keeps steady-state ROS at relatively low levels that oscillate with changes in metabolic activity, which can communicate normal shifts in oxidative metabolism to signaling and gene expression pathways that control many diverse cellular functions including cell proliferation, circadian rhythms, differentiation, immunological functions, tissue remodeling, and vascular reactivity (Beckman & Koppenol, 1996; Kessenbrock, Plaks, & Werb, 2010; Menon & Goswami, 2007; Oberley, Oberley, & Buettner, 1980, 1981; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Rutter, Reick, Wu, & McKnight, 2001). If the metabolic production of ROS exceeds the capacity of the endogenous antioxidant defense systems, oxidative stress can occur (Sies, 1991; Spitz, Azzam, Li, & Gius, 2004). Depending on the severity of oxidative stress, an organism may adapt by increasing its antioxidant capacity, increasing the capacity to repair oxidative damage, or shifting metabolic processes away from oxidative metabolism towards glycolytic metabolism. If the cellular adaptive processes that are induced in response to chronic metabolic oxidative stress cannot mitigate the accumulation of oxidative damage to critical biomolecules, potentially pathological conditions can develop due to increasing oxidative damage to DNA, proteins, and lipids. It is this gradual accumulation of oxidative damage to critical biomolecules that is believed to contribute to most if not all degenerative diseases associated with aging and cancer (Droge, 2002; Finkel, 2005).

Although all cells in an organism can be affected by the accumulation of oxidative damage, the effects of ROS on stem cells (or pluripotent cells) in most self-renewing tissues are of particular interest to the processes of aging and cancer development because of their undifferentiated state and longevity of replicative potential (Kobayashi & Suda, 2012; Oberley et al., 1980, 1981; Shyh-Chang, Daley, & Cantley, 2013). Stem cells can exist in a completely undifferentiated state, such as pluripotent embryonic stem cells (ESCs), or can be more committed to a particular lineage in a tissue as tissue stem cells or adult stem cells (ASCs). All normal stem cells appear to be highly sensitive to oxidative stress because of their relatively undifferentiated state with a long division potential for accumulating genetic damage. Accumulation of oxidative damage in normal stem cells can lead to cell transformation and tumorigenesis or cause tissue injury, loss of function, enhanced senescence, and loss of division potential associated with degenerative diseases associated with aging (Shyh-Chang, Daley, et al., 2013). Therefore, in this chapter, we will focus our discussions on the role of metabolic ROS in stem cell physiology and pathology and discuss strategies to exploit the differences in normal and tumor stem cell (TSC) sensitivities to oxidative stress for selectively protecting normal ASCs while sensitizing TSCs including leukemia stem cells (LSCs) and cancer stem cells (CSCs) to oxidative damage induced during leukemia and cancer therapy.

2. ROS

2.1. Common biological ROS

ROS is a collective term for oxygen-containing species that are more reactive than molecular O_2 . The most likely ROS to be produced initially during the metabolism of O_2 by living systems were proposed to derive from the superoxide anion ($O_2^{\bullet-}$) because it represents the one-electron reduction product of O_2 (Boveris, 1977; Buettner, 1993; Chance et al., 1979; Fridovich, 1978). $O_2^{\bullet-}$ is a relatively weak oxidant but is an excellent reductant for transition metal ions such as Fe^{+3} , Mn^{+3} , and Cu^{+2} leading to the formation of Fe^{+2} , Mn^{+2} , and Cu^{+1} . Interestingly, approximately 0.3% of $O_2^{\bullet-}$ in cellular aqueous environments is believed to exist in the protonated form as the hydroperoxyl radical ($HO_2^{\bullet}$). In contrast to $O_2^{\bullet-}$, $HO_2^{\bullet}$ can diffuse more readily through hydrophobic environments because it is uncharged. In addition, $HO_2^{\bullet}$ is a much more aggressive oxidant than $O_2^{\bullet-}$, capable of damaging a wide variety of potentially critical biomolecules such as polyunsaturated fatty acids to initiate lipid peroxidation chain reactions forming ROOH and aldehydic by-products of lipid oxidation (Buettner, 1993).

The other common ROS derived from reactions of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ in biological matrices include hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), organic hydroperoxyl radicals ($RO_2^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), and the hydroxyl radical ($HO^{\bullet}$) (Buettner, 1993). H_2O_2 forms from the spontaneous dismutation of $O_2^{\bullet-}$ with a rate constant of $10^5 \text{ mol}^{-1} \text{ s}^{-1}$ or through enzymatic dismutation of $O_2^{\bullet-}$ at a rate of $10^9 \text{ mol}^{-1} \text{ s}^{-1}$ in the presence of the superoxide dismutase (SOD) enzymes (Fridovich, 1978). ROOH are formed from the oxidation of organic molecules such as during lipid peroxidation reactions (Buettner, 1993; Buettner, Ng, Wang, Rodgers, & Schafer, 2006; Fridovich, 1978). More highly reactive O_2 radicals are also formed from Fenton-type reactions involving transition metals such as Fe^{+2} and Cu^{+1} with H_2O_2 and ROOH, respectively. These reactions result in the oxidation of the transition metals (to Fe^{+3} and Cu^{+2}) and the formation of $HO^{\bullet}$ and organic hydroperoxyl and alkoxyl radicals ($RO_2^{\bullet}$). $HO^{\bullet}$, $RO_2^{\bullet}$, and $RO^{\bullet}$ are potentially very damaging species because they are formed in close proximity to sites of metal ion binding to biomolecules and they readily react with many critical biological macromolecules including lipids, proteins, and nucleic acids. For this reason, $HO^{\bullet}$, $RO^{\bullet}$, and $RO_2^{\bullet}$ are thought to significantly contribute to the accumulation of oxidative damage to critical biomolecules leading to cytotoxic and mutagenic responses involved in carcinogenesis and aging (Droge, 2002; Fridovich, 1978; Gius & Spitz, 2006; Jorgenson, Zhong, & Oberley, 2013; Oberley et al., 1980, 1981; Spitz et al., 2004).

2.2. Sources of ROS

2.2.1 Mitochondria—The mitochondria have been considered as a major source of cellular-derived ROS based on the fact that most cellular O_2 consumption occurs as a result of mitochondrial ETC activity in mammalian cells (Balaban, Nemoto, & Finkel, 2005; Boveris, 1977; Chance et al., 1979; Finkel & Holbrook, 2000; Forman & Kennedy, 1974, 1975; Fridovich, 1978). Mitochondrial ETCs consist of four multiprotein complexes (complexes I–IV) assembled on the inner mitochondrial membrane that contains a series of redox catalysts (i.e., pyridine nucleotides, flavoproteins, iron sulfur proteins, ubiquinone,

and cytochromes) that utilize electrons obtained from glycolysis and the tricarboxylic acid cycle to generate energy via the process of OXPHOS (Lehninger, 1976; Voet, Voet, & Pratt, 1999) (Fig. 1.1). The ETC proteins are arranged according to their redox potentials, and the flow of electrons down the ETC chains is accompanied by the pumping of protons out of the inner mitochondrial membrane creating a proton gradient (-140 to -180 mV) across the inner membrane that is coupled to the production of ATP from ADP+Pi as the protons reequilibrate through the ATP synthase (also known as ETC complex V) (Fig. 1.1). O_2 is the terminal electron acceptor at complex IV (cytochrome oxidase) where it undergoes a 4-electron reduction to form two water molecules in a tightly regulated process that is not believed to result in the production of $O_2^{\bullet-}$ or H_2O_2 . Despite the efficiency of the 4-electron reduction of O_2 to form H_2O at complex IV ($\approx 99\%$ of all mitochondrial O_2 consumption), there is believed to be a finite probability that 0.1–1.0 % of all O_2 consumed by mitochondria can undergo 1-electron reductions at sites in complexes I, II, and III of the ETCs to form $O_2^{\bullet-}$ (Ahmad et al., 2005; Boveris, 1977; Boveris & Cadenas, 1982) (Fig. 1.1). Once formed, $O_2^{\bullet-}$ is converted to H_2O_2 via the spontaneous or enzymatically driven dismutation of $O_2^{\bullet-}$. Results using isolated mitochondria from normal tissues suggest that the majority of $O_2^{\bullet-}$ and H_2O_2 originate from complexes I and III (Boveris, 1977). Again, based on the data using isolated mitochondria from normal tissues, complex II is typically found to produce small amounts of $O_2^{\bullet-}$ (1% or less of the total) (Boveris, 1977) and was thought to be a minor contributor to overall $O_2^{\bullet-}$ and H_2O_2 production in normal cells (Fig. 1.1).

Recently, evidence is accumulating to suggest that cancer cell mitochondria may produce greater steady-state levels of $O_2^{\bullet-}$ and H_2O_2 , relative to normal cells (Aykin-Burns, Ahmad, Zhu, Oberley, & Spitz, 2009). Several recent studies have also shown that mutations in complex II subunits C and D increase $O_2^{\bullet-}$ and H_2O_2 production as well as causing genomic instability and cancer induction (Aykin-Burns et al., 2011; Ishii et al., 1998; Owens et al., 2012; Slane et al., 2006). Furthermore, familial forms of two human cancers (paraganglioma and pheochromocytoma) have also been found to be associated with mutations in genes coding for subunits B, C, and D in complex II (Fliedner et al., 2012; Gimm, Armanios, Dziema, Neumann, & Eng, 2000). In addition, the mitochondria of malignant human tumor cells and rodent tumors have been shown to exhibit histological abnormalities characterized by unusual arrangements of mitochondrial cristae, mitochondrial hypertrophy, and mitochondrial fragmentation when compared to normal human cells (Bize, Oberley, & Morris, 1980; Springer, 1980). Furthermore, many tumors, including human epithelial cancers (i.e., colon and breast), have been shown to have high rates of mitochondrial DNA (mtDNA) mutations (relative to normal human tissues), and this has been suggested to contribute to increased $O_2^{\bullet-}$ and H_2O_2 production (Penta, Johnson, Wachsmann, & Copeland, 2001; Yankovskaya et al., 2003). Furthermore, there are now several reports measuring $O_2^{\bullet-}$ and H_2O_2 in normal versus cancerous human cells, suggesting that cancer cell mitochondria produce higher steady-state levels of $O_2^{\bullet-}$ and H_2O_2 , relative to normal cell mitochondria (Ahmad et al., 2005; Aykin-Burns et al., 2009).

Given this mounting evidence of increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 in cancer versus normal cells (Aykin-Burns et al., 2009; Dhar & St Clair, 2012; Droge, 2002; Yankovskaya et al., 2003), it is reasonable to hypothesize that mitochondria from cancer

cells demonstrate alterations in the proper assembly of ETC complexes such that there is an increase in the residence time and/or accessibility of electrons on sites capable of mediating increased 1-electron reductions of O_2 (relative to normal cells) resulting in increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 . This increased flux of ROS from cancer cell mitochondria could contribute to uncontrolled growth, the inability of cancer cells to differentiate, genomic instability, and disease progression. This hypothesis was originally proposed by Oberley et al. and forms the basis for the continuously evolving free radical theory of cancer (Oberley & Buettner, 1979; Oberley et al., 1980, 1981; Spitz, Sim, Ridnour, Galoforo, & Lee, 2000).

2.2.2 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs)

—An increasing body of evidence indicates that another major cellular source of ROS can be generated from a family of tightly regulated NOXs that are homologues of the phagocyte NOX (e.g., Phox or NOX2) originally discovered in neutrophils (Bedard & Krause, 2007; Lambeth, 2007). The NOX family of enzymes (NOXs 1–5, DUOX1, and DUOX2) represent a diverse and widely distributed group of redox signaling proteins that play physiological and pathophysiological roles in biology and medicine by transferring electrons from NAD(P)H to molecular O_2 to form $O_2^{\bullet-}$ and H_2O_2 . In this regard, NOX enzymes can potentially transduce redox signals regarding the availability of NAD(P)H, which is necessary for many biosynthetic and detoxification processes necessary for maintaining redox homeostasis, to redox-sensitive signaling pathways that govern growth and survival. In addition to their originally proposed role in phagocytic cells and inflammatory cells, NOXs have also been shown to mediate redox signaling for a wide variety of normal cellular functions such as cell division, differentiation, wound healing, fibrosis, induction of progrowth/prosurvival pathways, and angiogenesis (Bonner & Arbiser, 2012; Coso et al., 2012; Kamata, 2009). Recently, it has also been suggested that the activation of NOXs in cancer cells could play an important role in oncogene-mediated cancer cell survival that could be targeted to inhibit prosurvival/progrowth pathways in order to inhibit cancer cell growth and progression (Weyemi, Redon, Parekh, Dupuy, & Bonner, 2013). In this regard, NOX enzymes appear to represent a major source of ROS-producing enzymes that can govern biological responses relevant to both cancer biology and therapy including cancer cell survival and normal tissue responses.

2.2.3 Other sources—In addition to mitochondria and NOX enzymes, there are a number of different pathways by which ROS can be formed during oxidative metabolism including peroxisomal metabolism, cytochrome P450 metabolism, inflammatory reactions, and the metabolism of lysyl oxidases (LOXs) that may be relevant to cancer biology (Barker, Cox, & Erler, 2012; Gonda, Tu, & Wang, 2009; Nordgren & Fransen, 2014; Pani, Galeotti, & Chiarugi, 2010; Rusyn et al., 2004; Seitz & Stickel, 2006). Peroxisomes are the site of many fatty acid and other enzymatic oxidation reactions that generate ROS containing antioxidant enzymes that metabolize ROS including catalase that is very efficient at dealing with high levels of H_2O_2 (Antonenkova, Grunau, Ohlmeier, & Hiltunen, 2010). In fact, it appears that peroxisomes are highly compartmentalized for the specific purpose of keeping the enzymes that generate large amounts of H_2O_2 sequestered from the rest of the cellular compartments. Peroxisomal enzymes that produce ROS include many FAD-dependent (or FMN-dependent)

oxidases that generate $O_2^{\bullet-}$ and/or H_2O_2 as reaction by-products including xanthine oxidase, acyl-CoA oxidases, urate oxidase, 2-hydroxyacid oxidases, polyamine oxidases, pipecolic acid and sarcosine oxidases, D-amino acid and D-aspartate oxidases, palmitoyl-CoA oxidase, and pristanoyl-CoA oxidase. These enzymes oxidize fatty acids, amino acids, purines, and nitrites and are important sources of breakdown products that represent building blocks for salvage pathways in metabolism. These enzymes necessarily generate high levels of H_2O_2 as a by-product of their enzymatic activity, which is believed to be the reason why catalase is peroxisomally located. Compounds that cause an increase in peroxisomal activity (termed peroxisomal proliferators) are thought to contribute to hepatocellular carcinoma development by increasing H_2O_2 -mediated oxidative stress and DNA damage, leading to disturbances in redox signaling causing inflammation and aberrant cell proliferation and neoplastic transformation (Bosgra, Mennes, & Seinen, 2005).

Cytochrome P450 enzymes and their partner P450 reductases are xenobiotic-metabolizing enzymes that have also been associated with increased production of $O_2^{\bullet-}$ and/or H_2O_2 and carcinogenesis (Imaoka et al., 2004). Phenobarbital, 3-methylcholanthrene, and ethanol are strong inducers of cytochrome P450 enzymes that have been associated with carcinogenesis and tumor promotion. The induction of cytochrome P450 and P450 reductase by phenobarbital has also been shown to induce ROS production from liver microsomes that is capable of causing oxidative damage to DNA and lipids. This cytochrome P450 reductase-mediated ROS and subsequent oxidative stress have been suggested to account for the tumor-promoting activity of phenobarbital. It has also been proposed that genetic polymorphisms of cytochrome P450 enzymes associated with leukemia may also result in elevated ROS, which could contribute to genomic instability and uncontrolled growth signaling, but the causal involvement of ROS in these phenomena needs further confirmation (Irwin, Rivera-Del Valle, & Chandra, 2013).

The hypothesis that oxidative stress induced by chronic inflammation could mediate neoplastic transformation of stem cells arose from the realization that chronic inflammation was accompanied by the copious production of ROS, nitric oxide (*NO), and hypohalous acids (such as $HOCl$) by immune-competent cells that had become activated by a variety of stressors including autoimmune disease, xenobiotic exposure, viral infection, radiation exposure, obesity, asbestos exposure, and smoking (Archer, 1979; De Marco, 2013; Nathan & Cunningham-Bussel, 2013; Ohshima, Tatemichi, & Sawa, 2003; Saeidnia & Abdollahi, 2013). In this regard, inflammatory reactions may contribute to the induction of cancer caused by many if not all environmental stresses. Furthermore, ROS associated with inflammation are also causally associated with all the steps of carcinogenesis including initiation, promotion, and progression (De Flora & Ferguson, 2005; De Marco, 2013; Gukovsky, Li, Todoric, Gukovskaya, & Karin, 2013; Nathan & Cunningham-Bussel, 2013; Ohshima et al., 2003). In this regard, chronic inflammation appears to be a complete carcinogen that is capable of contributing to stress-induced and aging-associated carcinogenesis (Cannizzo, Clement, Sahu, Follo, & Santambrogio, 2011).

The extracellular LOX enzymes are regulated by hypoxia-inducible factor-1 (HIF-1)/HIF-2 transcription factors and utilize the lysyl tyrosylquinone cofactor to convert lysine residues to the aldehydic product, allysine, that is required for cross-link formation in the

stabilization of collagen and elastin. The LOX catalytic cycle also produces H_2O_2 as a by-product. LOX activity is believed to contribute to metastasis by facilitating the cross-linking of collagen fibers, which is required for the recruitment of bone marrow (BM)-derived cells believed to be required for the formation of metastatic niches for CSCs to colonize distant organs (Semenza, 2013). In addition, the H_2O_2 produced as a by-product of LOX activity has been hypothesized to lead to enhanced migration and cell-matrix adhesion formation of invasive breast cancer cells via activation of the FAK/Src signaling pathway. These data suggest that inhibition of pathways leading to the activation of LOXs may represent viable targets for limiting the progression of aggressive breast cancers (Payne et al., 2005).

2.3. Antioxidants and antioxidant enzymes

2.3.1 Cellular antioxidants—An intricate system of nutritional antioxidants (i.e., vitamin C, vitamin E, lycopenes, and selenium), low-molecular-weight reducing cofactors and peptides (i.e., GSH, thioredoxin (Trx), and NADPH), and antioxidant enzymes [i.e., SOD enzymes, catalase, glutathione peroxidases (GPx), and peroxiredoxins (Prx)] regulates the oscillations in intra- and extracellular redox state that occur normally during oxidative metabolism and provides protection from ROS-induced damage during conditions of oxidative stress (Fig. 1.2) (Fridovich, 1978; Halliwell & Gutteridge, 2007; Rhee, Chae, & Kim, 2005; Sies, 1991; Spitz et al., 2004). During normal steady-state oxidative metabolism, periodic fluctuations in the intra- and extracellular redox environment are believed to coordinately regulate signal transduction and gene expression through redox-sensitive kinases, phosphatases, and transcription factors to maintain the cell in a nonequilibrium steady-state supporting most normal cellular functions including proliferation, differentiation, epigenetic regulation, and quiescence (Fig. 1.2) (Cyr & Domann, 2011; Cyr, Hitchler, & Domann, 2013; Droge, 2002; Gius & Spitz, 2006; Hitchler & Domann, 2012; Jorgenson et al., 2013; Li, Wicha, Schwartz, & Sun, 2011; Monfort & Wutz, 2013; Oberley et al., 1980, 1981; Ogasawara & Zhang, 2009; Sarsour, Kumar, Chaudhuri, Kalen, & Goswami, 2009; Spitz et al., 2000, 2004). Since cancer cells, relative to normal cells, are believed to demonstrate increased steady-state fluxes of ROS due to disruptions in oxidative metabolism, it is logical to propose that they would adapt by upregulating fluxes through reductive antioxidant pathways to avoid the potentially growth-inhibitory and cytotoxic accumulation of oxidative damage (Blackburn et al., 1999; Spitz et al., 2000). This proposition has led to the hypothesis that glucose metabolism is increased in cancer versus normal cells to (1) increase levels of pyruvate from glycolysis because pyruvate reacts with H_2O_2 through a deacetylation reaction to form H_2O and (2) regenerate NADPH from NADP⁺ in the pentose cycle to provide reducing equivalents for GPx and Prx enzymes to detoxify hydroperoxides (Fig. 1.2) (Blackburn et al., 1999; Spitz et al., 2000). This logic has also led to the hypothesis that increased fluxes of electrons through both prooxidant and antioxidant pathways in cancer cells, relative to normal cells, might represent a significant target for selectively manipulating cancer cell defects in oxidative metabolism to limit the growth and progression to the malignant phenotype while sparing sensitive normal tissues.

2.3.2 SOD and catalase—The first indication of an altered antioxidant profile in cancer versus normal cells was observed when the activity of the mitochondrial matrix form of the SOD enzyme, MnSOD, and sometimes catalase was found to be decreased in many

transformed versus normal cells (Dionisi, Galeotti, Terranova, & Azzi, 1975; Oberley, Bize, Sahu, Leuthauser, & Gruber, 1978; Oberley & Buettner, 1979; Peskin, Koen, Zbarsky, & Konstantinov, 1977; Sahu, Oberley, Stevens, & Riley, 1977). The significance of fluctuations in the expression of MnSOD in cancer cells is still controversial (Dhar & St Clair, 2012), but a preponderance of recent evidence has supported the conclusion that during the initial stages of tumor formation, MnSOD is generally downregulated, but as cancer cells progress, the enzyme is upregulated contributing to the enhanced ability to achieve the metastatic potential seen in later-stage disease (Dhar & St Clair, 2012). This transition between lowered and increased expression of MnSOD during cancer progression has been shown to correlate with an apparently metabolic reprogramming of cancer cells that may relate to differences in steady-state H_2O_2 levels caused by the activity of MnSOD (Buettner, 2011; Dhar & St Clair, 2012; Li, Yan, Yang, Oberley, & Oberley, 2000).

Increased fluxes of H_2O_2 in cancer cells could be driven by the MnSOD-catalyzed conversion of $2\text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$, since the rate constant for this reaction is nearly diffusion-limited at $\approx 10^9 \text{ mol}^{-1}\text{s}^{-1}$. If the forward rate constant (k_f) for a given protein (such as ubiquinone, $\text{Q}^\bullet$, in complex III; Fig. 1.1) to reduce O_2 to form $\text{O}_2^{\bullet-}$ ($\text{Q}^\bullet + \text{O}_2 \rightarrow \text{Q} + \text{O}_2^{\bullet-}$) is less than the reverse rate constant (k_r) of back reaction ($\text{O}_2^{\bullet-} + \text{Q} \rightarrow \text{Q}^\bullet + \text{O}_2$), then having high levels of MnSOD could actually lead to increased steady-state H_2O_2 formation by pulling the reaction of $\text{Q}^\bullet + \text{O}_2$ in the forward direction towards $\text{O}_2^{\bullet-}$ being rapidly converted to H_2O_2 (Buettner, 2011; Buettner et al., 2006; Kaewpila, Venkataraman, Buettner, & Oberley, 2008).

It has been postulated that the growth-inhibitory effects of MnSOD over-expression in cancer cells can be explained in part by increased fluxes of H_2O_2 (Dhar & St Clair, 2012; Oberley, 2005; Wang et al., 2005). Also, from this line of thinking, it is logical to hypothesize that once cancer cells have adapted to higher fluxes of H_2O_2 by increasing their capacity to detoxify hydroperoxides through increasing glycolytic metabolism and the activities of catalase, GPx, and Prx enzymes (Fig. 1.2), then the H_2O_2 -mediated growth-inhibitory effects of MnSOD may no longer be a selective pressure against progression to metastasis (Dhar & St Clair, 2012; Oberley, 2005). Furthermore, increasing MnSOD activity in cancer cells when they have adequate peroxide removal systems in place may actually help to accelerate cancer cell progression to metastasis phenotype by limiting the reactions of $\text{O}_2^{\bullet-}$ with redox-active metals such as Fe^{+3} and Cu^{+2} , which would in turn limit the ability of these metal ions and $\text{O}_2^{\bullet-}$ to participate in free radical production from hydroperoxides through Haber–Weiss and Fenton chemistry (Koppenol, 2001).

Throughout the 30- to 40-year history of the paradoxical reports of alterations in SOD and catalase activities in cancer cells, the clear pattern that has emerged is that the regulation of SODs, catalase, GPx, and Prx is nearly universally altered in cancer versus normal cells (Buettner, 2011; Dhar & St Clair, 2012; Oberley, 2005). These alterations in $\text{O}_2^{\bullet-}$ – and H_2O_2 -metabolizing enzymes in cancer versus normal cells are thought to be indicative of disruptions in cancer cell oxidative metabolism leading to the inability of cancer cells to coordinately regulate one-electron signaling (directed by reactions of $\text{O}_2^{\bullet-}$ with metal ions in ETC chains and metal ions required for governing HIFs and Jumonji protein activity) with two-electron signaling pathways (directed by reactions of H_2O_2 with sulfhydryl and

methionine residues on kinases, phosphatases, and acetylases) (Blackburn et al., 1999; Buettner, 2011; Cyr & Domann, 2011; Cyr et al., 2013; Hitchler & Domann, 2009, 2012; Spitz et al., 2000). In this regard, if CSCs are experiencing higher fluxes of ROS, relative to normal stem cells, in a background of an immortalized phenotype, the inability to regulate one-electron versus two-electron signaling could represent a fundamental metabolic abnormality rendering CSCs unable to properly control cell division and normal differentiation leading to the inevitable progression to malignancy.

2.3.3 GSH—GSH is the most abundant (1–10 mM) intracellular low-molecular-weight soluble thiol and is thought to act as a major intracellular redox buffer. GSH is a tripeptide containing a redox-active cysteine residue capable of providing reducing equivalents to the GPx enzymes to detoxify a wide variety of hydroperoxides including H_2O_2 and organic hydroperoxides (Fig. 1.2). Once oxidized to GSSG, GSH can be regenerated using electrons from NADPH by the glutathione reductase enzyme (Fig. 1.2). NADPH is then regenerated by metabolizing glucose in the pentose phosphate cycle (PPC) (Fig. 1.2). GSH can also be used by the glutathione transferase enzymes in conjugation reactions to detoxify a wide variety of electrophiles formed during oxidative stress (Spitz, Malcolm, & Roberts, 1990; Spitz, Sullivan, Malcolm, & Roberts, 1991). GSH is synthesized by transporting cystine into the cell via the xCT transporter; the cystine is then reduced to cysteine, which is conjugated to glutamate by the action of glutamate–cysteine ligase to form γ -glutamyl cysteine. γ -Glutamyl cysteine is then conjugated to glycine to form GSH by the action of glutathione synthase.

Given that cancer cells demonstrate increased steady-state fluxes of hydroperoxides relative to normal cells, it is not surprising that cancer cell GSH metabolism is usually dramatically upregulated (Spitz et al., 2000). Interestingly, CSCs have also been suggested to upregulate glutathione-dependent metabolism. (Herault et al., 2012; Ishimoto et al., 2011; Nagano, Okazaki, & Saya, 2013; Pei et al., 2013; Sato et al., 2013; Tamada et al., 2012). Since CSCs appear to rely heavily on the reducing equivalents in GSH to maintain redox homeostasis in the face of metabolic oxidative stress, manipulation of GSH by limiting its synthesis and/or inhibiting its redox recycling has been shown to be an effective means of sensitizing CSC clonogens to cell killing using a number of different pharmacological and genetic manipulations (Fath, Ahmad, Smith, Spence, & Spitz, 2011; Hadzic et al., 2010; Herault et al., 2012; Ishimoto et al., 2011; Nagano et al., 2013; Pei et al., 2013; Sato et al., 2013; Scarbrough et al., 2012; Simons, Ahmad, Mattson, Dornfeld, & Spitz, 2007; Simons et al., 2009; Sobhakumari et al., 2012; Tamada et al., 2012). Many of these pharmacological approaches are well tolerated in animals and show great promise for the development of combined-modality cancer therapies designed to selectively enhance metabolic oxidative stress in cancer versus normal stem cells.

2.3.4 Trx—Trx1 and Trx2 are 12 kDa proteins with a vicinal thiol moiety at their active site that acts as an electron donor in many redox signaling/gene expression pathways (Wei et al., 2000), ROS detoxification pathways (Rhee et al., 2005), and protein activation pathways where maintaining protein thiols in the reduced state is necessary for proper function (Arner & Holmgren, 2006; Du, Zhang, Zhang, Lu, & Holmgren, 2013; Lee, Kim, & Lee, 2013; Lu

& Holmgren, 2014; Nakamura, Nakamura, & Yodoi, 1997; Ueda et al., 2002). Trx1 is primarily cytosolic and can be transported to the nucleus to activate redox-sensitive transcription, and Trx2 is located in the mitochondria and is believed to keep functional proteins in the reduced state. The vicinal thiol moiety of Trx contains two redox-active cysteine residues capable of providing reducing equivalents to the Prx enzymes (aka Trx peroxidases) to detoxify a wide variety of hydroperoxides including H_2O_2 and organic hydroperoxides (Fig. 1.2). In this regard, the Trx system in many cellular compartments is redundant with the GSH/GPx system, highlighting the importance of controlling hydroperoxide levels in cancer and normal cells. Once oxidized from $\text{Trx}(\text{SH})_2$ to $\text{Trx}(\text{S})_2$, $\text{Trx}(\text{S})_2$ can be regenerated using electrons from NADPH via the action of Trx reductases (TrxRs) (Fig. 1.2). NADPH is again regenerated by metabolizing glucose in PPC (Fig. 1.2) (Arner & Holmgren, 2006; Lee et al., 2013; Nakamura et al., 1997). Given that cancer cells demonstrate increased steady-state fluxes of hydroperoxides, relative to normal cells, it is logical to hypothesize that cancer cell Trx metabolism is generally significantly upregulated (Arner & Holmgren, 2006; Du et al., 2013; Lee et al., 2013; Lu & Holmgren, 2014; Powis, Mustacich, & Coon, 2000). Since CSCs are believed to require the reducing equivalents in Trx and GSH to maintain redox homeostasis in the face of metabolic oxidative stress, manipulation of Trx activity by inhibiting its redox recycling has been shown to be an effective means of sensitizing CSC clonogens to cell killing using a number of different pharmacological manipulations including TrxR inhibitors such as auranofin combined with inhibitors of glutathione metabolism such as buthionine sulfoximine (BSO) (Du, Zhang, Lu, & Holmgren, 2012; Fath et al., 2011; Scarbrough et al., 2012; Simons et al., 2009; Sobhakumari et al., 2012). Again as with manipulation of GSH, many of the pharmacological approaches used to manipulate the Trx system, such as auranofin, are well-tolerated in humans and show promise for the development of combined-modality cancer therapies designed to selectively enhance metabolic oxidative stress-induced cell killing in cancer versus normal stem cells (Nguyen, Awwad, Smart, Spitz, & Gius, 2006; Pennington et al., 2007; Scarbrough et al., 2012).

2.3.5 Coordinate regulation of intracellular redox environment—A functionally interdependent relationship exists between metabolic and genetic processes necessary for maintaining living systems in the non-equilibrium steady state within which they propagate and reproduce their species (Fig. 1.3). Mammals are O_2 -breathing organisms that represent complex higher-order biological structures that derive their life force from the ability to extract, store, and move high-energy electrons. The genetic material coded for in the DNA of mammalian cells maintains the blueprint for transcription and translation of the functional proteins necessary for replicating and maintaining the living biological structure of each organism (Fig. 1.3). Oxidative metabolism by the biological structures removes high-energy electrons from the food sources and moves them onto electron carriers such as NADH, NADPH, and FADH_2 . These high-energy electron carriers are used to generate energy via OXPHOS where O_2 acts as the terminal acceptor of the electrons once the energy from the electrons, in the form of proton motive force across the inner mitochondrial membrane, has been harnessed to synthesize ATP (Figs. 1.1 and 1.3). The high-energy electron carriers are also utilized as reducing equivalents for biosynthetic enzymes to make and break chemical bonds in organic molecules to maintain the differentiated structure of the living system,

accomplish normal cellular functions, and drive the replication and maintenance of the living system (Fig. 1.3).

The biochemical basis of living systems that are based on O_2 metabolism is very efficient, and greater than 99% of the electrons fluxing through the biological structure leave the system as H_2O via the four-electron reduction of O_2 . However, small amounts of one-electron reduction products of O_2 in the form of $O_2^{\bullet-}$ can be produced during metabolism that together with redox-active metal ions and $\bullet NO$ can form a variety of highly reactive ROS and RNS (Fig. 1.3). The levels of these potentially damaging reactive species are regulated by cellular and nutritional antioxidants and low-molecular-weight redox cofactors necessary for antioxidant enzymes to function. The normal fluctuations in the oxidized to reduced ratios of electron carriers and thiol redox couples and the fluctuations in the reactive species act as redox signaling molecules that allow signal transduction proteins and gene expression pathways to “taste” the fluxes of electrons moving through all the metabolic pathways in order to coordinately regulate metabolism and gene expression for the execution of normal cell division, differentiation, and adaptive responses necessary to maintain homeostasis of the living system (Figs. 1.1–1.3). There is believed to be a small but finite probability that the reactive species produced as by-products of oxidative metabolism can evade the antioxidant pathways to cause covalent alterations in the genetic material in the form of base damage, adducts, and deletions to the DNA. This damage is usually repaired with high fidelity, but slowly, as a function of time, some of this damage escapes the high-fidelity repair pathways and can lead to heritable changes in the genetic material of the cell. When this damage accumulates to a level where the assembly and efficient function of the metabolic machinery begin to be affected, the metabolism of the living system is believed to become compromised leading to an acceleration of the production of reactive species and the accumulation of damage to the genetic material as metabolism continues to proceed. At this point in the life span of the organism, the slow accumulation of damage accelerates in an exponential fashion due to the increasing inefficiency that is introduced into the metabolic processes as damage to the metabolic machinery accumulates. The living system then begins to deteriorate in its ability to function normally as an exponential function of the age of the organism. This process of deterioration of the biological structure and function then manifests itself as degenerative diseases associated with aging and increased cancer induction. This biochemical scenario forms the basis for the free radical theories of aging and cancer that have been proposed for the last 60 years (Harman, 2003, 2006; Jorgenson et al., 2013; Oberley & Buettner, 1979; Spitz et al., 2000, 2004).

As the deterioration of oxidative metabolism begins to affect the stem cell compartments of living tissues, the stem cells are believed to either undergo senescence or lose their ability to regenerate normally functioning tissues or undergo neoplastic transformation where they become immortalized and begin the progression towards malignant metastatic phenotypes. Since during the transformation process CSCs presumably carry the same faulty oxidative metabolic process forward during their progression to malignancy, oxidative metabolism may represent a significant target for selectively inactivating cancer versus normal stem cells. From this theoretical construct, it is logical to propose that if the pathways (such as glucose, Trx, and GSH metabolism) that the CSCs must upregulate to survive their defect in oxidative metabolism could be simultaneously inhibited, then they would be selectively

sensitized to cell killing by agents capable of inducing oxidative stress. Metabolic oxidative stress in CSCs could therefore represent an “Achilles heel” for developing novel combined-modality cancer therapies based on fundamental defects in cancer cell oxidative metabolism.

3. ROS AND NORMAL STEM CELLS

3.1. Types of major normal stem cells

As concluded by Leydig “*Omne vivum ex vivo* (all life [is] from life),” life neither ends nor begins but continues (Sell, 2004). A new life is formed by the union of an oocyte and a sperm after fertilization, which generates the first stem cell, for example, the zygote, in an organism. The zygote is a totipotent stem cell. It undergoes cleavage, proliferation, and differentiation to produce pluripotent ESCs and multipotent trophoblast stem cells. ESCs continue proliferating and differentiating to generate various multipotent tissue stem cells. These tissue stem cells have the ability to self-renew and produce various specialized cell types in a tissue or an organ during embryogenesis in an embryo and organogenesis in a fetus after they differentiate into transit-amplifying cells (TACs) or progenitor cells. In addition, they persist in various tissues in an adult, termed ASCs. ASCs are responsible for maintaining tissue homeostasis and repairing tissue injury by replenishing senescent and damaged cells for the entire life of an organism. Somatic cells can become pluripotent stem cells after their nuclei are transferred into enucleated oocytes (Gurdon, 1962; Gurdon, Elsdale, & Fischberg, 1958) or reprogrammed by ectopic expression of a panel of defined transcription factors (such as Oct4, Sox2, Klf4, and Myc) (Takahashi et al., 2007; Takahashi & Yamanaka, 2006), incubation with selective small molecules (Hou et al., 2013), or a combination of both (Zhu et al., 2010). These induced pluripotent stem cells (iPSCs) exhibit some of the same properties as ESCs, including expressing certain ESC genes and proteins, forming embryoid body *in vitro* and teratoma *in vivo*, and differentiating into various somatic cells in culture and in a tetraploid blastocyst complementation assay. Therefore, “*Omne vivum ex vivo* (all life [is] from life)” through stem cells (Fig. 1.4).

3.1.1 ESCs—ESCs can be derived from the inner cell mass (ICM) or an epiblast of an early embryo, for example, a blastocyst. The first ESC lines were derived from mouse embryos in 1981 independently by Evans and Kaufman (1981) and Martin (1981). It took another 17 years for Thomson et al. to derive the first human ESC lines from human blastocysts (Thomson et al., 1998). ESCs exhibit an almost unlimited capacity of cell proliferation *in vitro* but retain a normal karyotype. They have the potential to differentiate into all lineages of cells in an embryo body *in vitro* and in teratomas after ectopic transplantation into mice. After being injected into blastocysts, ESCs can contribute to all cell types including the germ cells in chimeric mice (Bradley, Evans, Kaufman, & Robertson, 1984) but poorly to the trophectoderm and extraembryonic endoderm (Beddington & Robertson, 1989). Therefore, ESCs are pluripotent cells. They are different from the totipotent zygote and blastomeres because the zygote and blastomeres can turn into both embryos and the placenta and other extraembryonic tissues via ESCs and trophoblast stem cells, respectively.

3.1.2 ASCs—ASCs originated from ESCs during embryogenesis with the ability to differentiate into multiple types of cells (multipotency) down to a single cell type (unipotency) in a tissue (Sell, 2004). ASCs can be found not only in fetal tissues but also in various tissues of children and adults because they can self-renew and persist through adulthood. However, unlike ESCs, ASCs cannot grow indefinitely in culture and divide infrequently *in vivo* to generate one stem cell and a TAC that then undergoes a limited number of cell divisions before terminally differentiating into mature cells in a tissue. ASCs are supposed to play an important role in the maintenance of tissue homeostasis and tissue repair after injury by replenishing aged and damaged cells in a body. Therefore, they can be found in many tissues with a high rate of cell turnover, such as those in the BM (Becker, Mc, & Till, 1963; McCulloch & Till, 1960; Till & Mc, 1961), intestine (Barker et al., 2007; Jung et al., 2011), and skin (Nowak, Polak, Pasolli, & Fuchs, 2008; Sun & Green, 1976). In addition, they can also be found in tissues undergoing a slow rate of cell turnover, such as brain (Reynolds & Weiss, 1992; Uchida et al., 2000), heart (Beltrami et al., 2003; Oh et al., 2003), and skeletal muscle (Lipton & Schultz, 1979; Mauro, 1961). Since hematopoietic stem cells (HSCs) are the first ASCs that have been isolated in high purity and the best characterized ASCs in our body, we will use HSCs as an example to discuss some of the fundamental biology of ASCs.

In the early 1960s, Till and McCulloch showed that mouse BM HSCs can self-renew and give rise to multiple lineages of progeny after transplantation into lethally irradiated animals (Becker et al., 1963; McCulloch & Till, 1960). This landmark discovery laid the foundation for modern stem cell biology and hematology research. However, as demonstrated by Till and McCulloch in their pioneering works, the cells that were originally believed to be HSCs identified in their colony-forming unit-spleen (CFU-S) assay were heterogeneous, because they had variable capacity for self-renewal (Becker et al., 1963; McCulloch & Till, 1960; Till & Mc, 1961). This finding provoked a series of investigations, aiming to prospectively identify and isolate HSCs for characterization. Through decades of research, HSCs and their progeny, including multipotent progenitors (MPPs) and various hematopoietic progenitor cells (HPCs), can now be prospectively isolated in high purity using multiparameter flow cytometry and a large array of monoclonal antibodies against various cell surface molecules not only in mice but also in humans (Fig. 1.5).

Murine HSCs, MPPs, and HPCs are lineage-negative (Lin^-) hematopoietic cells, because they are immature hematopoietic cells and express no detectable levels of lineage cell surface markers, such as B220, CD4, CD8, Gr-1, Mac-1, and Ter-119. Both HSCs and MPPs express c-Kit and Sca1, and thus, they are collectively called LSK ($\text{Lin}^- \text{sca1}^+ \text{c-kit}^+$) cells. In contrast, HPCs express c-Kit but not Sca1 and thus are termed $\text{LS}^- \text{K}^+$ ($\text{Lin}^- \text{sca1}^- \text{c-kit}^+$) cells (Kondo et al., 2003). Later, Kiel et al. demonstrated that the expression of CD150 and CD48 can be used to separate HSCs and MPPs, because HSCs are $\text{CD150}^+ \text{CD48}^- \text{LSK}$ cells and MPPs are $\text{CD150}^{+/-} \text{CD48}^+ \text{LSK}$ cells (Kiel, Yilmaz, Iwashita, Terhorst, & Morrison, 2005). Alternative strategies using other cell surface markers and dye effluxing have also been used to identify and isolate mouse HSCs. These include identification of HSCs as CD34-LSK cells (Osawa, Hanada, Hamada, & Nakauchi, 1996), $\text{Thy1}^{\text{lo}} \text{Flk-2-LSK}$ cells (Christensen & Weissman, 2001), and the Hoechst-effluxing side population (SP) cells

(Goodell, Brose, Paradis, Conner, & Mulligan, 1996). More recently, according to the expression of CD34, CD150, and CD48, HSCs can be further differentiated into long-term or dormant HSCs (CD34⁺CD135⁺CD150⁺CD48⁺LSK cells) and short-term HSCs (CD34⁺CD135⁺CD150⁺CD48⁺LSK cells) (Wilson et al., 2008) (Fig. 1.5).

The first marker to enrich human HSCs, for example, CD34, was discovered by Civin et al. (1984). It has been widely used in clinic to harvest human HSCs from BM, mobilized peripheral blood, or cord blood for transplantation since its discovery. However, the majority of Lin⁺CD34⁺ cells are committed progenitors. Therefore, extensive studies have been done over the years to identify additional markers that can be used to prospectively isolate and characterize human HSCs. These studies were facilitated by the use of long-term culture-initiating cell (LTC-IC) assay and various severe combined immunodeficiency (SCID) mice as transplantation recipients. For example, Lansdorp, Sutherland, and Eaves (1990) found that CD34⁺CD45RA⁺ cells were enriched for LTC-ICs. In addition, using a humanized SCID mouse model, Baum, Weissman, Tsukamoto, Buckle, and Peault (1992) and Murray et al. (1995) showed that Lin⁺CD34⁺Thy-1 (CD90)⁺ cell population from human fetal BM and mobilized peripheral blood contains more multipotent hematopoietic progenitors than Lin⁺CD34⁺CD90⁺ cells. Subsequently, Bhatia, Wang, Kapp, Bonnet, and Dick (1997) demonstrated that CD38 can be used as an additional marker to differentiate primitive human hematopoietic cells from more committed progenitors and Lin⁺CD34⁺CD38⁺ cells represent a population of highly purified SCID-repopulating cells. More recently, Notta, Doulatov, et al. (2011) found that the expression of integrin $\alpha 6$ (CD49f) can be used to differentiate human HSCs and MPPs. Thus, through almost two decades of research, human HSCs can be identified as Lin⁺CD34⁺CD38⁺CD45RA⁺CD90⁺CD49f⁺ cells, whereas Lin⁺CD34⁺CD38⁺CD45RA⁺CD90⁺CD49f⁺ cells are MPPs and Lin⁺CD34⁺CD38⁺ cells contain various committed progenitors or HPCs (Fig. 1.5).

In both rodents and humans, the hematopoietic system is organized in a hierarchical manner (Fig. 1.5). The rare HSCs reside at the top of the hierarchy and have the ability to self-renew, proliferate, and differentiate into different lineages of peripheral blood cells through MPPs and HPCs (Reya, 2003; Weissman, Anderson, & Gage, 2001). HSCs are quiescent under steady-state conditions and serve as a reserve that protects the hematopoietic system from exhaustion under various stress conditions (Wilson, Laurenti, & Trumpp, 2009). In contrast, MPPs and HPCs are proliferating cells with limited and no self-renewal ability, respectively. The proliferation and differentiation of MPPs and HPCs meet the needs of normal hematopoiesis and also allow the hematopoietic system to react swiftly and effectively to meet demands for increased production of mature cells during hematopoietic crises, such as loss of blood, hemolysis, and infection. Quiescent HSCs can be activated in response to severe hematopoietic damage when MPPs and HPCs are depleted by an exogenous stressor. Under such circumstances, activated HSCs can undergo self-renewing proliferation and differentiation to repopulate MPPs and HPCs and restore homeostasis. However, if HSCs are injured or their self-renewing ability is impaired, HSCs could be exhausted, leading to BM failure and death of the organism (Wang, Schulte, & Zhou, 2006).

The majority of HSCs are assumed to reside in the osteoblastic niche adjacent to the endosteal bone surface (Calvi et al., 2003; Zhang et al., 2003). The osteoblastic niche

provides HSCs with a special environment that supports their self-renewal. This is likely achieved in part by extensive interactions between HSCs and the niche via a variety of soluble factors, such as Wnt (Sugimura et al., 2012), bone morphogenetic proteins (BMPs) (Goldman et al., 2009), angiopoietin-1 (Arai et al., 2004), osteopontin (Nilsson et al., 2005; Stier et al., 2005), thrombopoietin (TPO) (Yoshihara et al., 2007), interleukin-3 (IL-3), and IL-6 (Barria, Mikels, & Haas, 2004); various adhesion molecules, including CXCL12–CXCR4 (Sugiyama, Kohara, Noda, & Nagasawa, 2006) and N-cadherin (Haug et al., 2008); and different signaling pathways, for example, stem cell factor (SCF)/c-Kit (Broudy, 1997) and Jagged/Notch (Mercher et al., 2008). These intricate interactions promote HSC self-renewal not only by increasing HSC survival but also by keeping them quiescent in a hypoxic environment to prevent HSCs from exhaustion. In addition, sinusoidal endothelial cells (SECs) in BM have been revealed to function as an alternative HSC niche called the vascular niche (Kiel et al., 2005). The vascular niche plays an important role in hematopoietic development during embryonic and fetal development. It is also involved in regulation of HSC/HPC mobilization, proliferation, and differentiation in response to hematopoietic stress (Hattori et al., 2002). Therefore, HSCs may use either osteoblasts or endothelial cells as their niche under different circumstances to maintain a fine balance between quiescence and proliferation or self-renewal and differentiation and to respond to stress (Trumpp, Essers, & Wilson, 2010). Furthermore, recent studies have identified additional types of cells that contribute to the compositions of the HSC niche, which include arteriolar pericytes (Kunisaki et al., 2013), perivascular cells, CXCL12-abundant reticular (CAR) cells, mesenchymal stem cells, macrophages, regulatory T cells, and Schwann cells along with sympathetic nerve fibers (Chow et al., 2011; Fujisaki et al., 2011; Mendez-Ferrer et al., 2010; Omatsu et al., 2010; Yamazaki et al., 2011). These cells can also regulate various HSC activities directly and/or indirectly via affecting the function of the osteoblastic and vascular niche.

3.2. Role of ROS in stem cell physiology

3.2.1 ROS and stem cells—In the beginning of the twentieth century, Warburg (1908) observed a respiratory burst immediately after the sea urchin eggs were fertilized by the sperms to generate the zygotes. However, the biological significance of the increased O₂ consumption by the fertilized eggs was unknown until nearly 100 years after the initial observation by Warburg (Wong, Creton, & Wessel, 2004; Wong & Wessel, 2005). Wong and her colleagues revealed that the oxygen burst of the fertilized eggs is attributed to the activation of Udx1, a dual NOX, which converts O₂ to H₂O₂. H₂O₂ produced by Udx1 not only functions as a substrate of ovoperoxidase to participate in fertilization envelop cross-linking to prevent polyspermy but also acts as signaling molecule to regulate the zygote cleavage. Since then, accumulating evidence demonstrates that ROS plays a very important role in determination of the fate of various stem cells in a concentration-dependent manner. For example, Ezashi, Das, and Roberts (2005) showed that human ESCs (hESCs) underwent spontaneous differentiation when they were cultured under a normoxic condition (e.g., 21% O₂). When the cells were cultured under a physiological O₂ tension (3–5% O₂), the amount of spontaneous cell differentiation was significantly reduced without significant change in the rate of cell proliferation. However, further reduction in O₂ concentration (to 1%) inhibited the cell proliferation. The effects of O₂ on hESC differentiation and proliferation

are likely in part attributable to the production of ROS, because the treatment of hESCs with BSO that increases ROS production by reducing intracellular levels of GSH promoted hESC differentiation via mitogen-activated protein kinase (MAPK) signaling (Ji et al., 2010). Similar findings were also observed in mouse ESCs (mESCs) in which ROS also acts as a signaling molecule to promote mESC cardiovascular differentiation (Sauer & Wartenberg, 2005).

The effects of ROS on ASCs, particularly on HSCs, have also been extensively studied. Low levels of ROS are permissive for HSCs to maintain their normal functions, including proliferation, differentiation, and mobilization (Juntilla et al., 2010; Kinder et al., 2010; Lewandowski, Sheehan, Bennett, & Boswell, 2010; Owusu-Ansah & Banerjee, 2009). For example, it was reported recently that HSCs from *Akt1/2* double-knockout mice exhibit a defect in long-term hematopoietic reconstitution after transplantation (Juntilla et al., 2010). The defect is attributable to the reduced production of ROS, as moderate elevation of ROS in HSCs by incubation of the cells from the knockout mice with low doses of the glutathione depleting agent, BSO, increased their clonogenicity. This is in agreement with another recent observation that ROS-dependent proliferation of HSCs also plays an important role in the early steps of hematopoietic reconstitution after HSC transplantation (Lewandowski et al., 2010). However, increased production of ROS can be detrimental to HSCs. Jang and Sharkis (2007) showed that mouse BM HSCs could be separated into ROS^{high} and ROS^{low} populations based on the intensity of dichlorodihydrofluorescein (DCF) staining. ROS^{high} HSCs exhibited a reduced ability to form the colony-forming unit granulocyte–erythrocyte–monocyte–megakaryocyte (CFU-GEMM) and LTC-ICs *in vitro* and to produce long-term engraftment after transplantation in comparison with ROS^{low} HSCs. ROS^{high} HSCs, but not ROS^{low} HSCs, became exhausted after the third transplantation. These defects in ROS^{high} HSCs were associated with an increased activation of p38 MAPK (p38) and mammalian target of rapamycin (mTOR), which could be attenuated by the treatment with an antioxidant, a p38 inhibitor, or rapamycin. Furthermore, incubation of mouse BM HSCs with high concentrations of BSO resulted in a dramatic reduction in HSC clonogenicity (Juntilla et al., 2010; Rodriguez et al., 2011). In addition, increased production of ROS has been found to have a cause and effect relationship with HSC defects in various pathological conditions. For example, deletion of the ataxia-telangiectasia mutated (*Atm*) gene in mice increases ROS production, which comprises the ability of HSCs to self-renewal and leads to HSC premature exhaustion by disrupting HSC quiescence and stimulating HSC cycling (Ito et al., 2004, 2007). Treatment of *Atm*^{-/-} mice with N-acetylcysteine (NAC) can restore the function of HSCs and prevent the development of BM failure (Ito et al., 2004). Subsequently, it was shown that the number of HSCs and their long-term repopulating activity were markedly reduced in association with an increased production of ROS in HSCs after the deletion of the genes encoding the O subclass of the forkhead family of transcription factors, for example, *Foxos* (*Foxo1*, *Foxo3*, and *Foxo4*) in mice (Tothova et al., 2007). These defects were associated with an increased production of ROS in HSCs and ameliorated by the treatment with NAC. In addition, increased production of ROS is also associated with HSC defect in several other pathological conditions, including deletion of *Bmi1* (Park et al., 2003; Schuringa & Vellenga, 2010), the mouse double minute 2 homologue gene (*Mdm2*) (Abbas et al., 2010), and the tuberous sclerosis 1 (*Tsc1*) gene

(Chen et al., 2008); Fanconi anemia mutation (Du, Adam, Rani, Zhang, & Pang, 2008); aging (Ito et al., 2006); and post-ionizing radiation (IR) exposure (Shao et al., 2013; Wang et al., 2010).

3.2.2 Hypoxia and metabolism of stem cells—In general, stem cells are more sensitive to the adverse effects of ROS than their progeny. Increased production of ROS can inhibit stem cell self-renewal not only by promoting stem cell differentiation but also via induction of stem cell senescence and/or apoptosis as discussed in the next section. To maintain stem cell stemness, both ESCs and ASCs have developed various abilities to minimize the impact of ROS on the cells. Since O₂ is an important ingredient for the production of ROS, ESCs and various ASCs preferentially reside in a hypoxic environment to reduce ROS production (Eliasson & Jonsson, 2010; Ezashi et al., 2005; Mohyeldin, Garzon-Muvdi, & Quinones-Hinojosa, 2010; Sauer & Wartenberg, 2005; Suda, Takubo, & Semenza, 2011). ESCs are derived from ICM or epiblast of a blastocyst before implantation. They are adapted to the low levels of O₂ in the uterus where these cells reside *in vivo* (Fischer & Bavister, 1993) and undergo less differentiation when they are maintained in a hypoxic condition *in vitro* (Ezashi et al., 2005). In addition, ESCs preferentially use glycolysis as a main source of energy, which also reduces ROS production through mitochondrial OXPHOS. This is because undifferentiated ESCs possess small and immature mitochondria with lower levels of mitochondrial mass, membrane potential, and OXPHOS activity compared with the mitochondria in mature or differentiated cells (Rafalski, Mancini, & Brunet, 2012; Shyh-Chang, Daley, et al., 2013). As ESCs differentiate and commit to a specific cell fate, the cells gain more mitochondria with a more mature phenotype (such as an elongated morphology with swollen cristae and dense matrices) and exhibit increased copy numbers of mtDNA per cell and elevated levels of O₂ consumption and ATP production (Facucho-Oliveira & St John, 2009). In contrast, a switch from OXPHOS back to glycolysis occurs when differentiated cells are converted to iPSCs during reprogramming (Folmes et al., 2011; Shyh-Chang, Locasale, et al., 2013). In addition, elevation of glycolysis promotes the induction of iPSCs, whereas stimulation of OXPHOS or inhibition of glycolysis reduces the induction (Shyh-Chang, Daley, et al., 2013). These findings suggest that increased glycolysis and reduced OXPHOS are the common metabolic characteristics of pluripotent stem cells, which can help stem cells to maintain their stemness by reducing ROS production.

The endosteal osteoblastic niches of HSCs are also considered hypoxic, as they are relatively remote from blood flow (Winkler et al., 2010). It is estimated that the concentration of O₂ in these niches is below 1%. In the mouse BM, HSCs show lower blood perfusion as determined by low Hoechst 33342 (Hoe) staining after the dye injection (Parmar, Mauch, Vergilio, Sackstein, & Down, 2007). In addition, HSCs can be enriched in the BM cell populations with the most pimonidazole staining after administration of pimonidazole, a chemical marker for hypoxia (Parmar et al., 2007). Furthermore, human cord blood CD34⁺ cells became hypoxic within a few weeks after transplantation into NOD/SCID interleukin-2 receptor γ chain knockout mice (Shima et al., 2010). These findings suggest that hypoxia is an important component of the HSC niche, which can protect HSCs from oxidative damage by reducing the production of ROS in HSCs. Similarly, other ASCs

are also believed to reside in a hypoxic environment to be preserved in an undifferentiated state (Mohyeldin et al., 2010). In addition, hypoxia increases the expression and activity of HIF-1 α in HSCs (Simsek et al., 2010; Takubo et al., 2010). Increased expression of HIF-1 α and activity of HIF-1 alters the metabolism of HSCs by upregulating glycolysis while downregulating OXPHOS, leading to reduced production of ROS (Simsek et al., 2010; Takubo et al., 2010). In addition, HIF-1 also plays a critical role in the maintenance of HSC quiescence not only via inhibiting ROS production but probably also via antagonizing the effect of c-Myc to upregulate the expression of p21 (Koshiji et al., 2004). Similar to ESCs, quiescent HSCs possess low mitochondria mass and immature mitochondrial morphology and thus primarily utilize glycolysis rather than OXPHOS for ATP production (Piccoli et al., 2005; Simsek et al., 2010). Therefore, HSCs are presumably better protected from oxidative stress to maintain their ability to self-renew by residing in a hypoxic environment and being in a quiescent state in the endosteal osteoblastic niches.

3.2.3 Regulation of ROS production in stem cells—Since ROS plays an important role in the determination of the fate of stem cells, its production in stem cells has to be tightly regulated. This is achieved in part via a network of transcription factors and signaling transduction pathways as discussed in the succeeding text (Fig. 1.6).

HIF-1: HIF-1 is a heterodimeric transcription factor. It consists of two basic helix-loop-helix proteins: an oxygen-sensitive HIF-1 α subunit and a constitutively expressed HIF-1 β subunit (Semenza, 1999, 2004; Wang & Semenza, 1995). HIF-1 activity is regulated by posttranslational modification of the oxygen-dependent degradation (ODD) domain in the α subunit by a prolyl hydroxylase (PHD) at the proline residues 402 and/or 564 (Kaelin & Ratcliffe, 2008). Prolyl-hydroxylated HIF-1 α is ubiquitinated by the E3-ubiquitin-ligase von Hippel–Lindau (VHL) tumor suppressor protein, leading to its subsequent degradation by the 26S proteasome. Under hypoxic conditions, HIF-1 α proteins are stabilized due to suppression of PHD and reduced HIF-1 α hydroxylation (Kaelin & Ratcliffe, 2008). The stabilized HIF-1 α dimerizes with HIF-1 β to form heterodimers that can bind to hypoxia response elements (HREs) to activate the transcription of numerous target genes (Semenza, 2004). The downstream target genes of HIF-1 include glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase 1 (PDK1) (Semenza, 1999, 2004). LDHA converts pyruvate into lactate, whereas PDK1 can phosphorylate and inactivate pyruvate dehydrogenase (PDH) that catalyzes the conversion of pyruvate into acetyl-CoA. Increase in LDHA and PDK1 expression shunts pyruvate away from OXPHOS. Therefore, HIF-1 activation induces the switch of cellular metabolism from OXPHOS to glycolysis, resulting in reduced O₂ consumption and ROS production.

Knockout of HIF-1 α or HIF-1 β in mice is embryonically lethal (Keith & Simon, 2007). Coculture of hESCs with human fetal liver stromal cells over-expressing HIF-1 α inhibits hESC differentiation in association with an increased expression of Oct4 and Nanog (Ji et al., 2009). In addition, HIF-1 can potentially affect ESC self-renewal and differentiation via regulation of Notch and Wnt signaling (Keith & Simon, 2007; Mazumdar, Dondeti, & Simon, 2009). However, the roles of HIF-1 in the regulation of ESC hypoxic response, metabolism, and ROS production have yet to be investigated. In contrast, the effect of HIF-1

on HSCs has been extensively studied. It was shown that HSCs express higher levels of HIF-1 α mRNA and protein than their progeny (Simsek et al., 2010; Takubo et al., 2010). The expression of HIF-1 α in HSCs is regulated by the transcriptional factor Meis 1 (Kocabas et al., 2012; Simsek et al., 2010; Unnisa et al., 2012). Conditional knockout of Meis 1 or HIF-1 α in HSCs induced a shift of HSC metabolism from glycolysis to OXPHOS. This resulted in increases in HSC ROS production, cell cycle entry and proliferation, and apoptosis and decreases in the ability of HSCs to reconstitute the hematopoietic system during serial BM transplantation and to tolerate stress such as 5-fluorouracil administration. These HSC defects could be attenuated by treatment of the knockout mice with NAC, knockdown of VHL, or conditional monoallelic knockout of VHL (Kocabas et al., 2012; Simsek et al., 2010; Takubo et al., 2010; Unnisa et al., 2012). However, overactivation of HIF-1 in HSCs induced by conditional biallelic knockout of VHL or inhibition of PHD is detrimental to HSCs and can lead to HSC premature exhaustion (Eliasson et al., 2010; Takubo et al., 2010). These findings demonstrate that HIF-1 α plays an essential role in the maintenance of HSCs. Moderate levels of HIF-1 activity are beneficial for HSCs by restricting OXPHOS and ROS production and keeping HSCs in quiescence, whereas excessive levels of HIF-1 activity are harmful to HSCs. The mechanism by which excessive levels of HIF-1 activity adversely affect HSCs remains to be investigated.

FOXOs: The forkhead box O (FOXO) family of transcription factors consists of four members: FOXO1, FOXO3, FOXO4, and FOXO6 (Eijkelenboom & Burgering, 2013). They can regulate the expression of a large array of genes that affect many cellular processes, including metabolism, cell cycle regulation, differentiation, survival, and response to stress. The activities of FOXOs are normally suppressed in proliferating cells by insulin and growth factor signaling that activates phosphatidylinositol 3-kinases (PI3Ks) to increase the production of phosphatidylinositol-3-phosphate (PIP3). PIP3 functions as a second messenger to recruit and activate phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB or Akt). Active Akt translocates to the nucleus and phosphorylates FOXO proteins at three consensus phosphorylation sites. Phosphorylated FOXOs are then exported from the nucleus to the cytoplasm by 14-3-3 proteins, resulting in reduced expression of target genes. In addition, the activities of FOXOs can be regulated by diverse signaling pathways and a variety of stresses. Accumulating evidence suggests that FOXOs play an important role in stem cell maintenance. For example, FOXO1 is essential for the maintenance of ESC pluripotency by regulating the expression of OCT4 and SOX2 (Zhang et al., 2011). More importantly, it was found that FOXOs maintain HSC self-renewal because the numbers of BM HSCs in *Foxo1*^{-/-}/*Foxo3a*^{-/-}/*Foxo4*^{-/-} triple-knockout mice were dramatically reduced and their ability to produce long-term engraftment after transplantation was significantly lower than the cells from wild-type mice (Tothova et al., 2007). These HSC defects were associated with reduced expression of SOD2, catalase, and ATM, increased expression of p16^{Ink4a} (p16) and production of ROS, accelerated cell cycling, and decreased cell survival. Similar findings were also observed in *Foxo3a* knockout mice, as BM HSCs from this knockout mice exhibited high levels of ROS, activation of p38, increased expression of p16 and Arf, and loss of self-renewal (Miyamoto et al., 2007). Administration of the antioxidant NAC to the knockout mice could rescue the defects of HSCs. These findings demonstrate that FOXOs maintain HSC homeostasis

primarily via transcriptional regulation of anti-oxidants in addition to regulation of cell quiescence and DNA damage repair (Tothova & Gilliland, 2007).

Bmi1: Bmi1 is a member of the Polycomb family of transcriptional repressors that assemble in multimeric complexes and transcriptionally repress target genes through histone modifications (Sauvageau & Sauvageau, 2010). It is essential for the maintenance and self-renewal of not only normal ASCs such as HSCs but also LSCs (Lessard & Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). *Bmi1*^{-/-} mice develop progressive BM hypoplasia and die from BM failure shortly after birth (about 2 months), even though they have normal levels of HSCs in their fetal liver. However, *Bmi1*^{-/-} fetal liver HSCs can only transiently reconstitute the hematopoietic system after transplantation into lethally irradiated recipients (Lessard & Sauvageau, 2003; Park et al., 2003). This is because *Bmi1*^{-/-} HSCs undergo premature senescence and apoptosis. The induction of HSC premature senescence and apoptosis by *Bmi1* knockout was initially attributed to the derepression of the *Ink4a/Arf* locus that encodes two important tumor suppressors, p16 and Arf, because ectopic expression of p16 and Arf in HSCs induces cell cycle arrest and apoptosis, respectively, whereas deletion of both *Ink4a* and *Arf* partially rescues the ability of *Bmi1*^{-/-} HSCs to self-renew (Oguro et al., 2006). However, a more recent study suggests that Bmi1 also regulates mitochondrial function and redox homeostasis, as the cells from *Bmi1*^{-/-} mice produce increased levels of ROS and DNA damage and exhibit activation of the DNA damage response (DDR) pathway (Liu et al., 2009). The treatment of the knockout mice with the antioxidant NAC or the interruption of the DDR by *Chk2* knockout improves the function of HSCs and prolongs the life span of *Bmi1*^{-/-} mice. These findings demonstrate that Bmi1 promotes HSC self-renewal at least in part by regulating mitochondrial function to inhibit ROS production and p16 expression to prevent HSCs from undergoing premature senescence. In addition, Nakamura et al. showed that over-expression of Bmi1 in HSCs confers resistance to oxidative stress (Nakamura et al., 2012). However, the mechanism by which Bmi1 regulates mitochondrial function and ROS production and confers resistance to oxidative stress has yet to be elucidated.

ATM: ATM is a serine/threonine kinase that plays a central role in coordinating the repair of DNA double-strand breaks (DSBs) and the cellular response to DNA damage (Shiloh & Ziv, 2013). Although ATM is primarily located in the nucleus, a fraction of ATM is localized in the cytoplasm, particularly in the mitochondria (Ditch & Paull, 2012; Valentin-Vega et al., 2012). Recent studies suggest that the cytoplasmic ATM also functions as a redox sensor and regulator that controls the levels of ROS in a cell. ROS can directly oxidize ATM; and oxidized ATM then can form an active dimer via intermolecular disulfide bonds (Guo, Kozlov, Lavin, Person, & Paull, 2010). Activated ATM can phosphorylate BID to inhibit mitochondrial production of ROS (Maryanovich et al., 2012; Valentin-Vega et al., 2012). Alternatively, ATM may regulate ROS production via phosphorylation of HIF-1 α and/or liver kinase B1 (LKB1) (Ditch & Paull, 2012). Therefore, ATM-deficient cells produce increased levels of ROS and have reduced levels of GSH biosynthesis, and *Atm*^{-/-} mice exhibit signs of oxidative stress in different cells and multiple tissues (Ito et al., 2004; Kamsler et al., 2001; Meredith & Dodson, 1987). Particularly, it was found that *Atm*^{-/-} mice exhibit a progressive decline in hematopoiesis with age because their HSCs show a

defect in HSC self-renewal (Ito et al., 2004). This defect is associated with increased production of ROS, disruption of HSC quiescence, activation of p38, and elevated expression of p16. Treatment of *Atm*^{-/-} mice with NAC or a p38 inhibitor can restore the function of HSCs and prevent the development of BM failure (Ito et al., 2004, 2006).

mTOR: mTOR is a serine/threonine kinase belonging to the PI3K-related kinase family that has an important role in regulating cell growth and metabolism (Laplane & Sabatini, 2012). It interacts with several proteins to form two distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), proline-rich Akt substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor), whereas mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase-interacting protein (mSin1), protein observed with Rictor-1 (Protor-1), mLST8, and Deptor. Rapamycin inhibits mTORC1 but not mTORC2. mTORC1 plays an important role in sensing environmental cues, such as growth signals, nutrient and energy status, and O₂ supply, to regulate cell metabolism, growth, proliferation, autophagy, and other functions. mTORC2, which is less investigated than mTORC1, regulates cell survival, cell metabolism, and cytoskeletal organization. Multiple upstream signaling pathways including the PI3K–Akt pathway can regulate the activity of mTORC1 but converge at the tuberous sclerosis 1 (TSC1) and TSC2 complex that functions as a GTPase-activating protein (GAP) for the Ras homologue enriched in brain (Rheb) GTPase. As a Rheb GAP, TSC1/2 negatively regulates mTORC1 activity by converting GTP-bound Rheb into its inactive GDP-bound state. Initially, it was reported that deletion of phosphatase and tensin homologue (*Pten*), a negative regulator of the PI3K–Akt pathway, from HSCs in adult mice promotes HSC proliferation, resulting in HSC depletion and leukemic transformation (Yilmaz et al., 2006; Zhang et al., 2006). The effect of *Pten* deletion on HSCs could be ameliorated by the treatment with rapamycin, indicating that mTORC1 is involved in regulation of HSCs. Subsequently, it was shown that overactivation of mTORC1 by conditional deletion of *Tsc1* stimulates HSC proliferation, impairs HSC self-renewal, and eventually causes HSC exhaustion in association with an increased production of ROS (Chen et al., 2008; Gan et al., 2008). Treatment with rapamycin or NAC can rescue HSC defects in *Tsc1* knockout mice, demonstrating that ROS are primarily responsible for mediating mTOR overactivation-induced HSC dysfunction.

Although each of these transcription factors and signaling pathways described in the preceding text can regulate HSC maintenance individually via regulating the production of ROS, there are extensive cross talks among them (Fig. 1.6). For example, ATM can regulate mTORC1 indirectly via phosphorylating Akt and LKB1/AMP-activated protein kinase (AMPK) (Ditch & Paull, 2012). In addition, ATM-mediated phosphorylation of HIF-1α can not only directly modulate the activity of HIF-1 but also indirectly downregulate mTORC1 (Cam, Easton, High, & Houghton, 2010). FOXO1 is a potential target of ATM (Matsuoka et al., 2007), whereas FOXO3a can increase ATM expression in HSCs (Yalcin et al., 2008) and directly interact with ATM to promote ATM activation in response to DNA damage (Tsai, Chung, Takahashi, Xu, & Hu, 2008). In contrast, FOXO1 can inhibit mTORC1 via

TSC2-dependent and TSC2-independent mechanisms (Chen et al., 2010). Furthermore, there are numerous other molecules and pathways that also participate in the maintenance of HSCs via regulating ROS production (Liu, Cao, & Finkel, 2011; Suda et al., 2011). Due to space limitations, we are regrettably unable to discuss all of them here in this chapter.

3.2.4 Cellular sources of ROS in stem cells—As discussed in Section 2, cellular sources of ROS include the mitochondrial respiratory chain, NOXs, other oxidases, and various oxygenases. Different stem cells can use ROS derived from diverse cellular origins to perform unique functions. For example, the zygote of the sea urchin utilizes Udx1 (a dual NOX) to convert O₂ to H₂O₂ to prevent polyspermy and regulate cleavage (Wong et al., 2004; Wong & Wessel, 2005). hESC differentiation is associated with mitochondrial biogenesis, which leads to increases in mitochondrial mass and production of ATP and ROS (Cho et al., 2006; Saretzki et al., 2008). However, cardiovascular differentiation of ESCs depends on various NOXs for increased production of ROS (Ateghang, Wartenberg, Gassmann, & Sauer, 2006; Li et al., 2006; Sauer & Wartenberg, 2005; Schmelter, Ateghang, Helmig, Wartenberg, & Sauer, 2006). The mitochondria have frequently been considered to be the main source of cellular-derived ROS in HSCs. It has been shown that cells, including HSCs, from *Bmi1*^{-/-} mice exhibit abnormal mitochondrial function, resulting in increased production of ROS (Liu et al., 2009). In addition, increased production of ROS in HSCs from *Tsc1*^{-/-} mice has been attributed to the elevation of mitochondrial biogenesis and oxidative activities (Chen et al., 2008). However, compared to their progeny, HSCs are dormant and have fewer mitochondria and primarily utilize glycolysis rather than OXPHOS for ATP production (Piccoli et al., 2005; Simsek et al., 2010). Thus, it has yet to be determined whether the mitochondria are the major cellular source of ROS in HSCs. In contrast, the expression of NOX1, 2 and 4 and various regulatory subunits of NOXs has been detected in human HSCs (Piccoli et al., 2005, 2007). It was estimated that NOX-mediated extramitochondrial O₂ consumption accounts for about half of the endogenous cell respiration in human HSCs (Piccoli et al., 2005). Interestingly, our recent studies showed that NOX1, 2, and 4 are also expressed in mouse BM HSC-enriched LSK cells, whereas HPCs, Lin⁻ cells, and mononuclear cells from mouse BM express NOX1 and 2, but not NOX4, suggesting that the expression of NOX4 is downregulated upon HSC differentiation and that NOX4 may play an important role in regulation of HSC function (Wang, Liu, et al., 2010). Moreover, exposure to IR induces persistent increases in ROS production in both human and murine HSCs (Wang, Liu, et al., 2010; Yamaguchi & Kashiwakura, 2013). However, the increases in ROS production in HSCs is not associated with mitochondrial dysfunction nor elevated mitochondrial O₂^{•-} production (Yamaguchi & Kashiwakura, 2013), but is related to upregulation of NOX4 expression (Wang, Liu, et al., 2010). Inhibition of NOX4 activity with diphenyliodonium inhibits IR-induced elevation of ROS production in HSCs, suggesting that NOX4 is likely one of the main cellular sources of ROS in HSCs after radiation injury (Wang, Liu, et al., 2010).

3.3. Role of ROS in stem cell pathology

Although low levels of ROS production are required for stem cells to undergo self-renewing proliferation and proper differentiation (Ezashi et al., 2005; Juntilla et al., 2010; Kinder et al., 2010; Lewandowski et al., 2010; Owusu-Ansah & Banerjee, 2009; Sauer & Wartenberg,

2005), increased production of ROS is detrimental to stem cells and has been implicated in the pathogenesis of many pathological conditions and diseases by causing damage to stem cells. The pathological effects of ROS on stem cells are dose-dependent. A moderate increase in ROS production can impair stem cell self-renewal by promoting stem cell proliferation and differentiation, resulting in premature exhaustion of stem cells (Chen et al., 2008; Gan et al., 2008; Miyamoto et al., 2007; Tothova et al., 2007). Further increase in ROS production can induce stem cell senescence through the redox-dependent activation of the p38–p16 pathway (Shao, Wu, & Zhou, 2012). Finally, an acute and excessive increase in ROS production can induce stem cell apoptosis by activating the DNA damage response and p53 pathway (Shao et al., 2010; Yu et al., 2010) (Figs. 1.6 and 1.7).

3.3.1 ROS and stem cell differentiation—One of the fundamental characteristics of stem cells is their ability to self-renew and differentiate into different lineages of cells. However, these two functions have to be tightly regulated in order to maintain a proper balance between stem cell self-renewal and differentiation to prevent stem cells from premature exhaustion. Furthermore, stem cell differentiation to different lineages of cells has to be fine-tuned to prevent lineage skewing. It has been well documented that increased production of ROS can promote stem cell differentiation. However, the mechanisms by which ROS promotes stem cell differentiation have not been well established. It was shown that ROS enhances hESC differentiation to mesendodermal lineage by the activation of the members of the MAPK family and downregulation of the expression of pluripotent transcription factors, such as Oct4, Nanog, and Sox2 (Ji et al., 2010). The activation of selective members of the MAPK family was implicated in mediating ROS-induced cardiovascular differentiation of ESCs (Sauer & Wartenberg, 2005; Schmelter et al., 2006). In addition, Xiao et al. reported that NOX4-derived H₂O₂ promoted mESC differentiation to smooth muscle cells by the upregulation and activation of serum response factor (SRF) via phosphorylation (Xiao et al., 2009). However, the mechanisms by which H₂O₂ upregulates and activates SRF have yet to be investigated. Increased production of ROS not only promotes HSC differentiation but also causes lineage skewing (Pervaiz, Taneja, & Ghaffari, 2009; Shao, Lou, & Zhou, 2014; Suda et al., 2011). Jang and Sharkis showed that ROS^{high} HSCs exhibited accelerated exhaustion and myeloid skewed differentiation after serial transplantation (Jang & Sharkis, 2007). Similar findings were also observed in HSCs from sublethally irradiated mice (Li, Wang, Pazhanisamy, et al., 2011; Wang et al., 2012). The myeloid skewing of HSCs induced by IR was associated with increases in ROS production and DNA damage, which could be corrected after treatment with a SOD mimetic antioxidant (Li, Wang, Pazhanisamy, et al., 2011). In addition, Wang et al. showed recently that DNA damage induced by IR and telomere dysfunction promoted lymphoid differentiation of HSCs, resulting in depletion of HSCs with the capacity of lymphoid differentiation and accumulation of myeloid-biased HSCs (Wang et al., 2012). The enhanced lymphoid differentiation was attributed to the activation of the granulocyte colony-stimulating factor (G-CSF)-/Stat3-/BATF-dependent differentiation checkpoint in HSCs. *Gcsf* knockout, *Stat3* knockdown, or *Batf* deletion improved HSC self-renewal and function in response to IR or telomere shortening. However, it has yet to be determined whether ROS mediates IR-induced activation of this differentiation checkpoint in HSCs.

3.3.2 ROS and stem cell senescence—Most somatic cells have a finite growth potential, which was discovered by Hayflick and Moorhead in the early 1960s (Hayflick & Moorhead, 1961). The intrinsic replicative life span of a cell is determined by telomere length (Campisi, Kim, Lim, & Rubio, 2001). Without the expression of telomerase, telomeric sequences shorten after each time DNA replicates. When the telomeres reach a critically short length (~4 kb) after a certain number of cell doublings, known as the Hayflick limit, cells stop dividing and are irreversibly arrested at the G₁ phase, entering replicative senescence (Campisi et al., 2001; Marcotte & Wang, 2002). ESCs and the majority of tumor cells do not undergo replicative senescence because they express high levels of telomerase to prevent telomere erosion (Campisi et al., 2001; Marcotte & Wang, 2002; Zeng, 2007). Expression of telomerase is also important for ASCs such as HSCs to maintain their function (Allsopp, Morin, DePinho, Harley, & Weissman, 2003; Goytisolo et al., 2000; Greenwood & Lansdorp, 2003; Samper et al., 2002), because a deficiency in telomerase activity can lead to telomere shortening and reduction in HSC transplantation ability as seen in the late generations of telomerase RNA component (*Terc*)-null mice (Samper et al., 2002). In addition, the development of aplastic anemia or BM failure has been observed in patients with telomerase deficiency, due to mutations in telomerase reverse transcriptase (*Tert*) or *Terc* (Yamaguchi et al., 2005). In addition, many human and animal cells undergo premature senescence after exposure to oxidative and genotoxic stress or subjected to oncogenic mutations and/or aberrant activation of the p38 pathway (Serrano & Blasco, 2001). Premature senescent cells have a shortened intrinsic replicative life span without significant erosion in telomeres but are morphologically indistinguishable from replicatively senescent cells and exhibit many of the characteristics ascribed to replicatively senescent cells (Campisi et al., 2001; Marcotte & Wang, 2002; Serrano & Blasco, 2001). These changes include an enlarged and flattened appearance, increased senescence-associated β -galactosidase (SA- β -gal) activity, and elevated expression of p16 (Campisi et al., 2001; Marcotte & Wang, 2002; Serrano & Blasco, 2001). Moreover, premature and replicative senescence share common induction pathways (Campisi et al., 2001; Marcotte & Wang, 2002; Serrano & Blasco, 2001). Although ESCs are resistant to oxidative stress-induced senescence (Guo, Chakraborty, Rajan, Wang, & Huang, 2010), HSCs are highly sensitive to the induction of premature senescence by ROS. For example, it was shown that *Atm*^{-/-} mice exhibit progressive failure of hematopoietic function with aging (Ito et al., 2004). The failure is attributed primarily to HSC premature senescence resulting from an increased production of ROS, as treatment of *Atm*^{-/-} mice with NAC can restore the function of HSCs and prevent the development of BM failure. Increased production of ROS also contributes to the induction of HSC senescence in mice with genetic deletion of *Bmi1* (Lessard & Sauvageau, 2003; Park et al., 2003), *Foxos* (Miyamoto et al., 2007; Tothova et al., 2007; Yalcin et al., 2008), *Mdm2* (Abbas et al., 2010), and *Tsc1* (Chen et al., 2008); Fanconi anemia mutation (Du et al., 2008); aging (Ito et al., 2006); and post-IR exposure (Li, Wang, Pazhanisamy, et al., 2011; Wang, Liu, et al., 2010; Zhang, Zhai, et al., 2013). Human HSCs exhibit increase in ROS production, accumulation of oxidative DNA damage, and impairment in self-renewal and long-term repopulation after serial transplantation into immunodeficient mice (Yahata et al., 2011). These defects can be attenuated by the treatment with NAC. Interestingly, the effects of ROS on HSCs appear not to be a nonspecific oxidative effect as previously hypothesized, but at least in part mediated by the

redox-dependent activation of the p38–p16 pathway as described in the succeeding text (Wang, Liu, & Zhou, 2011).

p38: p38 is a member of the MAPK family of signal transduction kinases (Kyriakis & Avruch, 2001). It can be activated by ROS via apoptosis signal-regulating kinase 1 (ASK1) (Matsuzawa & Ichijo, 2008) and/or inactivation of protein tyrosine phosphatases (PTPs) such as MAPK phosphatases (Keyse, 2008; Patterson, Brummer, O'Brien, & Daly, 2009). Normally, ASK-1 forms an inactive complex with the repressor protein Trx in a cell. The formation of this complex is dependent on the presence of a reduced form of an intramolecular disulfide bridge between two cysteine residues of Trx. Oxidation of Trx by ROS causes dissociation of ASK-1 from Trx, resulting in the activation of ASK1 by oligomerization, interaction with TNF receptor-associated factor-2/TNF receptor-associated factor-6, and threonine autophosphorylation (Matsuzawa & Ichijo, 2008). It has been shown that ROS production from NOX4 can activate p38 via activation of ASK-1 (Chiang et al., 2006). In addition, oxidation of the catalytic cysteine of PTPs by ROS can reversibly inactivate PTPs (Shouse, Warren, & Whipple, 1931), which in turn can increase p38 activity. It remains to be determined whether ROS can activate p38 in HSCs through any of these mechanisms.

Activation of p38 regulates a variety of cellular processes such as inflammation, cell cycle arrest, and apoptosis in a cell type-specific manner. There is an increasing body of evidence demonstrating that p38 plays a critical role in the induction of senescence in response to a variety of stimuli via upregulating p16 (Antonchuk, Sauvageau, & Humphries, 2002; Geest & Coffey, 2009). For example, it was shown that a high level of Ras or Raf activation in human normal fibroblasts induced senescence by stimulating a sustained activation of p38, which in turn upregulated the expression of p16 (Antonchuk et al., 2002). Activation of the p38 pathway also contributes to the induction of p16 and cellular senescence after DNA damage resulting from exposure to genotoxic and oxidative stress and telomere shortening due to extensive replication (Kirito, Fox, & Kaushansky, 2003; Madlambayan et al., 2005; Waegell, Higley, Kincade, & Dasch, 1994). Furthermore, activation of p38 by ectopic transfection of mitogen-activated protein kinase kinase 3 (MKK3) and/or MKK6 increases p16 expression and induces senescence. In contrast, inhibition of p38 activity or downregulation of p38 expression attenuates the induction of p16 and cellular senescence by oncogenic stress, DNA damage, and telomere shortening (Kirito et al., 2003; Madlambayan et al., 2005; Waegell et al., 1994).

In addition, activation of p38 has been implicated in BM suppression in various pathological conditions associated with HSC dysfunction, including aplastic anemia and myelodysplastic syndromes (Navas et al., 2006; Zhou, Opalinska, & Verma, 2007). Furthermore, recently, it was shown that mutation of the *Atm* gene and knockout of the *Foxo3* gene induced premature senescence/exhaustion of HSCs (Ito et al., 2007; Miyamoto et al., 2007; Tothova et al., 2007). The induction of HSC senescence/exhaustion was associated with an elevated production of ROS, a selective activation of p38, and an upregulation of p16 in HSCs. Pharmacological inhibition of p38 activity rescued the defects of HSCs from *Atm* mutants and *Foxo3* knockout mice (Ito et al., 2007; Miyamoto et al., 2007; Tothova et al., 2007). These findings indicate that p38 plays an important role in regulation of HSC self-renewal

and its activation by oxidative stress can mediate the induction of HSC senescence via upregulation of p16 (Ito et al., 2006). Similar findings were also observed in mice after exposure to a sublethal dose of TBI (Li, Wang, Wu, et al., 2011; Wang et al., 2011). In these irradiated mice, we found that p38 was selectively activated in irradiated hematopoietic cells and this activation sustained up to 5 weeks after IR in a long-term BM cell culture assay. Inhibition of p38 activity with a specific inhibitor attenuated IR-induced suppression of BM hematopoietic cell function in association with a significant reduction in p16 expression and SA- β -gal activity. Moreover, our *in vivo* data show that inhibition of p38 attenuated IR-induced residual BM suppression. These results suggest that p38 activation plays a role in mediating IR-induced hematopoietic cell senescence and BM suppression and that pharmacological inhibition of the p38 pathway with a specific inhibitor can be further exploited for amelioration of IR-induced residual BM injury (Li, Wang, Wu, et al., 2011; Wang et al., 2011).

p16 and Arf: The *Ink4a/Arf* locus encodes two tumor suppressors, p16 and Arf (Lowe & Sherr, 2003; Sharpless & DePinho, 1999). The transcripts for these proteins have different first exons (α for p16 and β for Arf) but share exons 2 and 3. However, there is no amino acid sequence similarity between these two proteins due to the use of alternative reading frames for their translation. p16 is a potent cyclin-dependent kinase (CDK) 4/6 inhibitor. By inhibiting CDK4/6 activity, p16 causes retinoblastoma protein (Rb) hypophosphorylation and suppresses the expression of E2F-dependent genes, resulting in restriction of G1/S cell cycle progression and formation of senescence-associated heterochromatic foci (SAHF) (Lowe & Sherr, 2003; Narita et al., 2003; Sharpless & DePinho, 1999). Once SAHF are formed after the engagement of the p16–Rb pathway, the cells become permanently growth-arrested and senescent. It has therefore been suggested that diverse stimuli can induce cellular senescence via various upstream signal transduction cascades, including the p38 and p53–p21 pathways, but converge on the p16–Rb pathway, whose activation provides an inescapable barrier preventing senescent cells from reentering the cell cycle. This suggestion is supported by the finding that activation of p53 and induction of p21 in cells undergoing senescence are transient events that occur during the onset of senescence and then subside when the expression of p16 starts rising (Campisi, 2005; Robles & Adami, 1998; te Poele, Okorokov, Jardine, Cummings, & Joel, 2002). Inactivation of p53 prior to upregulation of p16 can prevent senescence induction. However, once p16 is highly expressed, cell cycle arrest becomes irreversible by downregulation of p53, indicating that activation of the p53–p21 pathway plays an important role in the initiation of senescence, but induction of p16 is required for the maintenance of senescence (Beausejour et al., 2003; Campisi, 2005). In agreement with this suggestion, we found that IR induced p53 activation and p21 expression in HSCs prior to the induction of p16 (Li, Wang, Pazhanisamy, et al., 2011; Wang, Schulte, LaRue, Ogawa, & Zhou, 2006). While p53 activation and p21 upregulation gradually declined within a few weeks after IR, p16 expression in irradiated HSCs remained elevated and the cells subsequently became senescent, exhibiting positive SA- β -gal staining. In contrast, the biological action of Arf relies on the p53 pathway. This is because Arf can directly bind to MDM2 and cause the accumulation of p53 by segregating MDM2 from p53 and by inhibiting MDM2's E3 ubiquitin protein ligase activity for p53 (Lowe & Sherr, 2003; Sharpless & DePinho, 1999; Sherr & Weber, 2000). Therefore, activation of p53 by Arf can

induce not only cellular senescence but also apoptosis, depending on which gene downstream of p53 is induced following its activation.

Upregulation of p16 and Arf has been implicated in mediating the induction of cellular senescence in HSCs. For example, increased expression of p16 and Arf was found in HSCs from *Bmi1*^{-/-} mice (Park et al., 2003). However, it appears that p16 but not Arf plays an important role in mediating the induction of *Bmi1*^{-/-} HSC senescence (Park et al., 2003). In addition, it has been found that knockout of both the *p16* and *Arf* genes in mice significantly increases the clonal expansion of HSCs *in vitro* but modestly promotes HSC self-renewal *in vivo* (Lessard & Sauvageau, 2003; Molofsky et al., 2003). However, knockout of the *Arf* gene alone does not provide any advantage for HSC/HPC expansion and self-renewal (Molofsky et al., 2003). In contrast, knockout p16 increases the life span of HSCs by promoting HSC self-renewal (Janzen et al., 2006; Stepanova & Sorrentino, 2005). Furthermore, mutation of the *Atm* gene also results in the upregulation of p16 and Arf in HSCs (Ito et al., 2007; Molofsky et al., 2003). Inactivation of the p16–Rb pathway by retroviral transfection of HPV E7 proteins restores the reproductive function of *Atm*^{-/-} HSCs, while inhibition of the Arf–p53 pathway by E6 transfection has no such effect (Ito et al., 2004). These findings suggest that p16 plays a more significant role than Arf in the regulation of HSC self-renewal and induction of HSC senescence, even though both proteins are overexpressed in senescent HSCs. Increased expression of p16 and Arf has been found in IR-induced senescent LSK cells (Li, Wang, Pazhanisamy, et al., 2011; Wang, Schulte, LaRue, et al., 2006). However, their roles in mediating IR-induced HSC senescence and long-term BM suppression remain to be investigated.

3.3.3 ROS and stem cell apoptosis—Apoptosis is an orderly and regulated form of cell death via a genetically controlled process (Kerr, Wyllie, & Currie, 1972; Majno & Joris, 1995). The characteristics of an apoptotic cell include externalization of phosphatidylserine on the outer leaflet of the plasma membrane, cell shrinkage, condensation of the nuclear chromatin, fragmentation of the nucleus and DNA, and cellular membrane blebbing (Kerr et al., 1972; Majno & Joris, 1995). It is well established that exposure of cells to oxidative stress induces DNA damage, particularly DSBs, which activates ATM (Shiloh & Ziv, 2013). Alternatively, ROS can directly activate ATM (Ditch & Paull, 2012). Activation of ATM causes accumulation and activation of p53. Activated p53 then translocates into nucleus to transcriptionally activate the expression of various proapoptotic factors such as Fas, DR5, Puma, and Bax (Riley, Sontag, Chen, & Levine, 2008; Villunger et al., 2003) or into mitochondrial to directly interact with Bax (Chipuk et al., 2004; Chipuk, Maurer, Green, & Schuler, 2003). The interaction of Fas or DR5 with their respective ligands can directly activate the initiator caspase-8 to trigger apoptosis through mitochondria-independent and mitochondria-dependent mechanisms, whereas Puma and Bax can cause the mitochondria to release caspase-activating factors, such as cytochrome c and Apaf-1 that, in turn, activate another initiator caspase, for example, caspase-9. All these diverse upstream apoptotic pathways converge at the effector caspases (e.g., caspase-3, caspase-6, and caspase-7) whose activation leads to the final stage of cell self-destruction (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999).

Although ESCs are highly resistant to senescence, they are extremely sensitive to the induction of apoptosis by ROS and other genotoxic insults (Filion et al., 2009; Guo, Chakraborty, et al., 2010; Qin et al., 2007). This is because ESCs either express low levels of p53 or are incapable of expressing cell cycle inhibitors and proapoptotic proteins in response to p53 activation (Qin et al., 2007; Zeng, 2007). However, activated p53 can induce rapid ESC apoptosis by translocating into mitochondria to directly interact with Bax (Han et al., 2008). The high sensitivity of ESCs to p53-mediated mitochondrial apoptosis may be attributable to ESC mitochondrial priming or the expression of constitutive active form Bax at the Golgi (Dumitru et al., 2012; Liu et al., 2013). These differential sensitivities to the induction of senescence and apoptosis by ROS and other insults may be an important mechanism for ESCs to maintain their genomic integrity to prevent the propagation of undesirable mutations to the resulting somatic and germ cell lineages.

In coordination with cell proliferation and differentiation, apoptosis contributes to the maintenance of hematopoietic homeostasis by regulating the size of hematopoietic lineages (Wickremasinghe & Hoffbrand, 1999). Dysregulation of apoptosis in HSCs and their progeny can result in many pathological conditions (Wickremasinghe & Hoffbrand, 1999). For example, it has been well established that induction of apoptosis in HPCs and HSCs is primarily responsible for the induction of acute radiation syndrome after exposure to a high or moderate dose of total body irradiation that induces acute and intense oxidative stress (Domen, Gandy, & Weissman, 1998; Mauch et al., 1995). Overexpression of an antiapoptotic protein, bcl-2, throughout the hematopoietic compartment protects mice against IR-induced hematopoietic failure and death, because HSCs isolated from *bcl-2* transgenic mice are more resistant to IR-induced apoptosis (Domen et al., 1998). In contrast, bcl-2 deficiency sensitizes murine HSCs to IR (Hoyes, Cai, Potten, & Hendry, 2000). The induction of HSC apoptosis by IR is mediated by the activation of the p53 pathway, because HSCs from p53-deficient mice are less sensitive to IR than are those from wild-type mice (Cui et al., 1995; Hirabayashi et al., 1997; Lee & Bernstein, 1993) and treatment with a p53 inhibitor protected mice from IR-induced lethal damage by suppression of p53-dependent apoptosis (Komarov et al., 1999). More recently, several groups of investigators reported that Puma, a downstream target of p53 and a proapoptotic BH3-only protein, plays a critical role in mediating IR-induced HSC apoptosis (Shao et al., 2010; Yu et al., 2010). They showed that Puma was selectively induced by IR in LSK cells and LSK cells from *Puma* knockout mice were insensitive to IR-induced apoptosis. As such, Puma deficiency in mice confers resistance to high-dose IR in a hematopoietic cell-autonomous manner. In contrast, other p53 targets, such as Bim and Noxa, play a minor or moderate role in IR-induced apoptosis in hematopoietic cells (Erlacher et al., 2005; Labi et al., 2010). These findings indicate that targeting the p53–Puma pathway may represent a novel strategy to protecting HSCs from IR injury, particularly considering that transient inhibition of p53 activity with an inhibitor did not increase IR-induced carcinogenesis, while Puma knockout actually reduced IR-induced tumorigenesis in mice (Christophorou, Ringshausen, Finch, Swigart, & Evan, 2006; Labi et al., 2010; Michalak et al., 2010).

4. ROS AND TSCS

4.1. TSCs

Like their normal tissue counterparts, tumors contain phenotypically and functionally heterogeneous populations of tumor cells (Beck & Blanpain, 2013; Nguyen, Vanner, Dirks, & Eaves, 2012). These cells are structured in a hierarchical manner in some, but not all, tumors (Magee et al., 2012). Undifferentiated tumor cells that are at the apex of the hierarchy have the ability to propagate the tumor by generating all of the cells in a tumor, including undifferentiated and differentiated tumor cells and other cells in the tumor, including endothelial cells (Ricci-Vitiani et al., 2010; Wang, Chandalavada, et al., 2010) and pericytes (Cheng et al., 2013). Therefore, these cells have been named TSCs or tumor-initiating cells. Furthermore, TSCs in leukemia are called LSCs and those in solid tumors CSCs. The first definitive evidence to demonstrate the existence of TSCs was observed by Furth and Kahn (1937). They showed that leukemia could be transmitted to a recipient mouse after receiving injection of a single leukemia cell. Later, Pierce and his colleagues showed that teratocarcinomas contain undifferentiated cells that are highly tumorigenic and can differentiate into multiple types of differentiated and nontumorigenic cells (Pierce & Speers, 1988). However, the true identity of TSCs was not known until the landmark studies by Dick and his colleagues in the 1990s. They showed that the peripheral blood of acute myeloid leukemia (AML) patient contained the leukemia-initiating cells or LSCs in the CD34⁺CD38⁻ population (Bonnet & Dick, 1997; Lapidot et al., 1994). These cells were rare but capable of differentiating into leukemic blasts to initiate human AML in NOD/SCID mice. It took almost another 10 years for Clarke and his colleagues to identify the first CSCs (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). They prospectively identified and isolated CD44⁺CD22^{-/low}/lineage⁻ CSCs from nine breast cancer patients. They showed that as few as 100 CD44⁺CD22^{-/low}/lineage⁻ tumorigenic cells could form tumors in a xenograft transplantation mouse model and the tumors contained both newly generated CD44⁺CD22^{-/low}/lineage⁻ CSCs and the phenotypically diverse mixed populations of nontumorigenic cells present in the initial tumor after serial passages. Since then, CSCs have been identified and prospectively isolated from many other cancers, including brain tumors and colorectal, lung, pancreatic, prostate, and ovarian cancers (Beck & Blanpain, 2013; Nguyen et al., 2012).

ASCs may represent an ideal cellular origin of TSCs because they have the ability to self-renew and are long-lived cells to allow the accumulation of mutations for transformation. Therefore, it has been suggested that some TSCs may be derived from ASCs because TSCs share some of the same characteristics of normal ASCs, such as self-renewal. This suggestion is supported by the studies on chronic myelogenous (or myeloid) leukemia (CML). CML is caused by the oncogenic Bcr-Abl fusion gene generated by a reciprocal translocation between chromosomes 9 and 22 in HSCs. The Bcr-Abl fusion gene only initiates CML in HSCs but not in committed murine hematopoietic progenitors (Huntly et al., 2004). However, TSCs can also originate from more differentiated progenitors that acquire the ability to self-renew by accumulation of genetic mutations and epigenetic abnormalities. For example, the oncogenic fusion genes MLL-ENL, MLL-AF9, and MOZ-TIF2 found in human AML patients can also transform mouse committed myeloid

progenitors for the induction of AML (Cozzio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006). More importantly, even when a tumor-initiating mutation occurs first in ASCs, the functional TSCs become detectable in their progeny (Jamieson et al., 2004; Miyamoto, Weissman, & Akashi, 2000).

TSCs may play an important role in tumor progression, metastasis, resistance to therapy, and relapse after treatment (Bacelli & Trumpp, 2012; Clevers, 2011). Therefore, strategies that can specifically target TSCs may offer a cure or a better treatment for tumors. However, TSCs appear a moving target that is difficult to kill. This is because unlike normal ASCs, TSCs are genetically unstable. They can constantly generate new subclones that are different from the initial “parental” clones of TSCs by obtaining additional mutations as revealed by the studies on LSCs from human acute lymphoblastic leukemia (ALL) (Anderson et al., 2011; Notta, Mullighan, et al., 2011). These different clones of LSCs may compete, resulting in clonal evolution during tumor progression, metastasis, and treatment. Furthermore, TSCs and some other tumor cells have high plasticity. TSCs may change their phenotype, and nontumorigenic cells can be reprogrammed or “dedifferentiate” into TSCs in response to various environmental cues (Tang, 2012). Therefore, we need to have a better understanding of the fundamental differences between TSCs and normal ASCs to develop more effective treatments for tumors. Since ROS plays an important role in the determination of the fate of normal ASCs, it would be of a great interest to determine whether regulation of ROS production in TSCs can be exploited for therapeutic gains against leukemia and cancer as discussed in the succeeding text.

4.2. ROS and LSCs

A large body of evidence demonstrates that leukemia and solid tumor cells produce increased levels of ROS compared with normal cells (Ogasawara & Zhang, 2009; Shi, Zhang, Zheng, & Pan, 2012; Trachootham, Alexandre, & Huang, 2009; Zhou, Shen, & Claret, 2013). The increased production of ROS is attributable to the activation of oncogenes (such as Ras, Bcr–Abl, and c-Myc) and/or inactivation of tumor suppressor genes (such as p53), resulting in mitochondrial dysfunction, aberrant metabolism, and alteration in antioxidant production. In addition, inflammation and exposure to IR and genotoxic stress can also increase ROS production. Increased production of ROS has been implicated in tumorigenesis because ROS is a potent carcinogen that causes DNA damage and gene mutations, which may eventually lead to induction of genomic instability and cell transformation. Therefore, antioxidant therapy has been extensively exploited as a preventive strategy to reduce carcinogenesis and tumorigenesis. So far, this strategy has yielded mixed results, indicating that more studies are needed to gain a better understanding of redox biology and cancer biology. In addition, because tumor cells produce higher levels of ROS, thus they are assumed to be more sensitive to oxidative stress than their normal counterparts. Therefore, the promotion of ROS production has the potential to selectively kill tumor cells without causing significantly collateral damage to normal tissues. Since tumors consist of undifferentiated tumorigenic TSCs and various differentiated nontumorigenic tumor cells and TSCs are primarily responsible for the initiation and propagation of a tumor, recent studies have started focusing on ROS in TSCs (Ogasawara & Zhang, 2009; Shi et al., 2012; Zhou et al., 2013).

It has been well established that Bcr–Abl oncoprotein stimulates CML cell production of ROS in a kinase-dependent manner (Koptyra et al., 2006; Nowicki et al., 2004). The increase production of ROS may result from the activation of Rac2 GTPase that alters mitochondrial membrane potential and electron flow through respiratory chain complex III (Nieborowska-Skorska et al., 2012). The increased production of ROS induces genomic instability by causing chronic DNA damage and inhibiting DNA damage repair in chronic-phase CML LSCs, which may contribute to the progression, drug resistance, and relapse of the disease (Slupianek et al., 2013). To our surprise, a study reported very recently by Lagadinou et al. (2013) showed that the majority of LSCs from AML patients are defined as ROS^{low} cells. However, this finding is in agreement with an earlier observation that the expression of GPx3, an enzymatic antioxidant scavenger of ROS, correlates with the abundance of LSCs in Hoxa9+Meis1-induced AML (Herault et al., 2012). These findings suggest that LSCs from AML and CML in chronic phase have different capacity of producing ROS. It remains to be determined whether the difference is related to the different phases of leukemia studied and/or underlying causes by the different oncogene mutations. In addition, it is also not known whether the production of ROS by LSCs is comparable to that of normal HSCs, ideally from the same individuals. However, AML LSCs appear more sensitive than normal HSCs to drugs that elevate the production of ROS. For example, Guzman et al. (2005) and Guzman et al. (2007) showed that parthenolide and its analogs can selectively eradicate AML and CML stem and progenitor cells. Similar findings were also observed when AML LSCs were treated with niclosamide and fenretinide (Jin et al., 2010; Zhang, Mi, et al., 2013). Interestingly, all these compounds also inhibit NF-κB, suggesting that increased production of ROS may induce synthetic lethality selectively in LSCs with NF-κB inhibition (Guzman et al., 2005, 2007; Jin et al., 2010; Zhang, Mi, et al., 2013). This is because NF-κB can protect cells against oxidative stress by regulating the expression of SOD2, several cell cycle inhibitors, and various anti-apoptotic proteins (Ahmed & Li, 2008).

4.3. ROS and CSCs

Diehn et al. (2009) were the first to report that CD44⁺/CD22^{–/low} CSCs from some human and murine breast tumors produce lower levels of ROS than corresponding nontumorigenic cancer cells. The decreased production of ROS by breast CSCs may be attributed to their increased expression of antioxidant genes, including GCLM and FOXO1, because GCLM encodes the regulatory subunit of glutamate–cysteine ligase that catalyzes the rate-limiting step of GSH synthesis and FOXOs have been shown to regulate the expression of SOD2 and catalase. In addition, high levels of expression of CD44 in CSCs may also contribute to the lower production of ROS, because CD44 can promote the uptake of cysteine for the synthesis of GSH via interaction with xCT, a glutamate–cystine transporter (Ishimoto et al., 2011). More recently, Dong et al. (2013) showed that Snail can mediate metabolic reprogramming of basal-like breast cancer cells to CSC-like cells. These CSC-like cells express decreased levels of fructose-1,6-biphosphatase (FPB1) due to the Snail–G9a–Dnmt1 complex-mediated epigenetic silencing of FPB1 via promoter methylation. Decreased expression of FPB1 increases glycolysis and NADPH production by PPC while reducing OXPHOS and ROS production. The lower levels of ROS production in CSCs not only may be important for the maintenance of CSCs but also can confer resistance to therapy, because lower ROS levels in CD44⁺/CD22^{–/low} breast CSCs are associated with less DNA damage

and a higher rate of cell survival after exposure to IR and pharmacological depletion of GSH with BSO sensitizes CSCs to IR (Diehn et al., 2009). Similar findings were also observed in CD13⁺ human liver CSCs (Haraguchi et al., 2010; Kim et al., 2012). Therefore, pharmacologically increasing ROS production has the potential to be used as an adjuvant therapy to more effectively eradicate CSCs. However, this strategy requires further investigation, because of concerns that increased production of ROS may also cause damage to normal ASCs.

5. CONCLUSION

Oxidative stress resulting from an increase in ROS production and/or a reduction in antioxidant capacity has been implicated in the pathogenesis of many diseases and aging. Although all cells in an organism can be affected by oxidative stress, the effects of ROS on stem cells have the greatest impact on the body, because they have the ability to self-renew and generate/replenish all other cells for the life span of the organism. Unfortunately, stem cells are more sensitive to oxidative stress than their differentiated progeny. Increased production of ROS can lead to stem cells exhaustion by induction of stem cell proliferation/differentiation, senescence, and/or apoptosis, which can be a major contributor to aging, degenerative diseases, and normal tissue injury induced by conventional cancer treatment and also limits the use of stem cells for regenerative medicine. Recent studies have provided a better understanding of the mechanisms whereby the production of ROS is regulated in stem cells and those by which the fate of the cells is affected by oxidative stress as discussed in this chapter, which may lead to the development of new interventions for aging-associated diseases and cancer therapy-induced normal tissue injury and novel strategies to harness the healing power of stem cells to treat diseases. In addition, an accumulating body of evidence demonstrates that many tumors contain TSCs that play an important role in tumor progression, metastasis, resistance to therapy, and relapse after treatment. The production of ROS in some TSCs is dysregulated, resulting in aberrant production of ROS, which can be exploited for the design of a better therapy for cancer and leukemia in the future.

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Figure 1.1.

Mitochondrial electron transport chains can form superoxide and hydrogen peroxide that can act as signaling molecules transducing redox signals from metabolic processes to the nucleus. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; VDAC, voltage-dependent anionic channel; Ref-1, redox factor 1; FMN, flavin mononucleotide; mPTP, mitochondrial permeability transition pore; MnSOD, manganese superoxide dismutase; CuZnSOD, copper/zinc superoxide dismutase; Cyt-C, cytochrome C.

Figure 1.2.

Theoretical model outlining redox couples and antioxidant pathways that coordinately regulate the flow of electrons from metabolism to redox-sensitive signaling and gene expression pathways contributing to the maintenance of the non-equilibrium steady state necessary for normal cellular functions during growth and development. TCA, tricarboxylic acid cycle; PPC, pentose phosphate cycle; GR, glutathione reductase; TR, thioredoxin reductase; GSH/GSSG, glutathione/glutathione disulfide; TrxS₂H₂/TrxSS, thioredoxin reduced and oxidized; GPX, glutathione peroxidase; Prx, peroxiredoxin; sites I–IV, electron transport chain complexes I–IV; Ref-1, redox factor 1.

Figure 1.3.

Theoretical model describing the interrelationship between metabolic and genetic processes necessary for life and death of mammalian organisms. ROS, reactive oxygen species; RNS, reactive nitrogen species.

Figure 1.4. Life cycle through stem cells. ICM, inner cell mass; ESCs, embryonic stem cells; iPSCs, inducible pluripotent stem cells.

Figure 1.5.

A hierarchical model of the hematopoietic system. LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term HSC; HSC, hematopoietic stem cell; MPP, multipotent progenitor; LMPP/MLP, lymphoid-primed multipotent progenitor/multilymphoid progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/monocyte progenitor.

Figure 1.6.

Theoretical model illustrating the regulation of ROS production in HSCs and the effects of ROS on HSCs. HSCs, hematopoietic stem cells; IR, ionizing radiation; NOXs, NADPH oxidases; mTORC1, mammalian target of rapamycin complex 1; HIF-1, hypoxia-inducible factor-1; OXPHOS, oxidative phosphorylation; SOD, superoxide dismutase; ROS, reactive oxygen species; FOXOs, forkhead box O transcription factors; DSBs, DNA double-strand breaks; ATM, ataxia-telangiectasia mutated.

Figure 1.7.

A diagram illustrating the relationship of cell metabolism, ROS production, and HSC self-renewal under increasing concentrations of oxygen and after exposure to IR and stress. HSC, hematopoietic stem cell; IR, ionizing radiation; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species.