**PRIMER**

**Stem cells and the impact of ROS signaling**

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**ABSTRACT**

An appropriate balance between self-renewal and differentiation is crucial for stem cell function during both early development and tissue homeostasis throughout life. Recent evidence from both pluripotent embryonic and adult stem cell studies suggests that this balance is partly regulated by reactive oxygen species (ROS), which, in synchrony with metabolism, mediate the cellular redox state. In this Primer, we summarize what ROS are and how they are generated in the cell, as well as their downstream molecular targets. We then review recent findings that provide molecular insights into how ROS signaling can influence stem cell homeostasis and lineage commitment, and discuss the implications of this for reprogramming and stem cell ageing. We conclude that ROS signaling is an emerging key regulator of multiple stem cell populations.

**KEY WORDS:** Hematopoietic stem cells, ROS, Embryonic stem cells, Metabolism, Mitochondria

**Introduction**

Reactive oxygen species (ROS) have been increasingly implicated in the physiological regulation of crucial developmental processes, e.g. the emergence of embryonic blood stem cells or differentiation of embryonic cardiomyocytes (Harris et al., 2013; Hernández-García et al., 2010; Hom et al., 2011). There is also increasing evidence that ROS are implicated at many distinct levels of biological processes, from gene expression and protein translation to protein-protein interactions, etc. (Holmström and Finkel, 2014). They function in cellular signaling, propagating signals from one tissue to the next, and in translating environmental cues into cellular responses in order to balance cellular input, e.g. nutrients and cytokines, with the appropriate cellular response. ROS may function as a rheostat to coordinate various cellular processes and adjust cellular activity to the available bioenergetic sources (Liang and Ghaffari, 2014). With the advances in genomics and proteomics, there is also increasing information about various ways in which ROS are balanced and control cellular processes. Particularly in stem cells, changes in oxidation state, otherwise known as redox regulation, might be responsible for the communication between mitochondria and the nucleus (Gomes et al., 2013; Mouchiroud et al., 2013; Rimmé et al., 2014). Redox-mediated mitochondrial-nucleus crosstalk could explain the coordination of cellular metabolism with chromatin remodeling, gene expression, cell cycling, DNA repair and cell differentiation. ROS have also been implicated in the ageing process but less is known about whether and how ROS might be involved in the ageing of stem cells (Beckman and Ames, 1998; Harman, 1972). As slight variations in ROS content may have profound effects on stem cell fate (Ito et al., 2004, 2006; Jang and Sharkis, 2007), elucidating mechanisms whereby ROS metabolism influences stem cell fate could reveal how stem cell ageing relates to age-associated diseases. In this Primer, we review what ROS are, how they function and what is known about their role in different stem cell populations, both embryonic and adult. We describe the various sources of ROS in stem cells, as well as what is known about oxygen metabolism in stem cells and how this might influence stem cell fate. The role of ROS in regulating stem cell dynamics has implications for various diseases, including cancer and age-related illnesses. We conclude by considering the potential role of oxygen metabolism in stem cell ageing and discuss how the properties of ROS signaling can be exploited to manipulate stem cell fate.

**What are ROS and how are they generated?**

ROS arise from the one-electron reduction of molecular oxygen (Fig. 1). Intracellular ROS exist primarily in three forms: superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH^-$). The superoxide anion contains an unpaired electron that impacts high reactivity and necessitates a rapid reduction to $H_2O_2$ by the antioxidant enzyme superoxide dismutase (SOD) (Dröge, 2002). $H_2O_2$ can be further reduced to $H_2O$ and $O_2$ by various cellular antioxidants (Fig. 1A). ROS can be detected intracellularly using a range of techniques; however, most assays for ROS do not discriminate between different ROS species (Box 1). Although ROS were originally thought to be merely a harmful byproduct of metabolism, accumulating evidence demonstrates a role for ROS in cell fate signaling, as discussed below (Finkel, 2003; Janssen-Heininger et al., 2008). $H_2O_2$ is thought to be the main ROS species involved with intracellular signaling, and in specific contexts can act directly as a second messenger, integrating environmental cues and passing them to downstream signal transduction cascades. This is due mostly to the longer half-life of $H_2O_2$ and its ability to diffuse easily through membranes relative to other types of ROS (Holmström and Finkel, 2014).

Under normal physiological conditions, the generation of ROS is tightly regulated by the ROS scavenging system. ROS scavengers are antioxidant enzymes that can neutralize ROS by directly reacting with and accepting electrons from ROS. When ROS production outpaces ROS scavenging, an excessive accumulation of ROS occurs, leading to oxidative stress and producing adverse effects on multiple cellular components, including proteins, lipids and nucleotides. To counteract this, the cell contains multiple types of antioxidants that are specific to different species of ROS, which helps to prevent pathological levels of ROS and to repair oxidative damage to cellular components. These include superoxide dismutase (SOD), catalase, peroxiredoxins (PRX), thioredoxin (TRX), glutathione peroxidase (GPX) and glutathione reductase (GR). Glutathione, a tripeptide, is one of the most abundant antioxidants synthesized by the cell. Oxidized proteins and $H_2O_2$ are reduced by glutathione through the glutaredoxin and
ROS are generated from mitochondrial respiration through the premature electron flow to O₂, mainly through electron transport chain complexes I and III (red arrows), leading to the generation of O₂⁻ (red). −

Mitochondria

NADH

NADP⁺

O₂

O₂⁻

H₂O₂

OH⁻

SOD

Catalase

Glutathione system

Box 1. Tools for ROS detection and their limitations

There are a great variety of reactive oxygen species (ROS) probes that allow analysis by flow cytometry or microscopy (Murphy et al., 2011; Winterbourn, 2014). However, most of these are not specific to any specific ROS species, are unstable and can be affected by other factors distinct from the oxidants. Therefore, data generated from the use of these probes should be carefully interpreted. Below is a brief description of the types of probes that are currently used.

Oxidized fluorescent probes

These are the most widely used probes. They diffuse through the cell membrane as non-fluorescent esterified compounds and fluoresce upon oxidation in the cytoplasm. The most common are dihydrodichlorofluorescein (DCFH₂) and dihydrorhodamine, widely used to measure hydrogen peroxide levels. These probes, however, are not directly oxidized by H₂O₂, but require a peroxidase or a metal catalyst for the reaction to occur. As such, any change in fluorescence might simply indicate a change in catalyst levels.

Non-oxidized fluorescent probes

These are composed of fluorophores protected by a blocking group that is released upon oxidation, allowing them to fluoresce. The most commonly used form are the boronate-conjugated probes, which display a higher sensitivity than oxidized probes, although they are still not specific to any particular ROS species. Some other conjugates, such as benzene sulfonylester or benzyl groups, have shown some specificity for H₂O₂. Future manipulation and protocol establishment for the use of these non-oxidized probes seem very promising.

Redox-sensitive green fluorescent proteins (GFP)

These are GFP protein variants in which redox-sensitive cysteines are incorporated in the beta-barrel of GFP (e.g. roGFP and HyPER). These probes constitute the most promising probes for in vivo analysis as they can be used, when combined with tissue-specific promoters, to generate transgenic animals. The disadvantage of these probes is that in freshly isolated primary cells, including stem cells, their use might be limited because of the need to introduce the reporter plasmids into the cells (Guzman et al., 2010).

ROS signaling: molecular targets and downstream pathways

ROS were originally shown to have signaling properties when they were found to act as secondary messengers in growth factor and oncogenic signaling (Chandel et al., 1998; Irani et al., 1997; Lee, 1998; Salmeen et al., 2003; Sundaresan et al., 1995; Toledano and Leonard, 1991). However, not all ROS can be employed in signaling events. Only ROS with a substrate specificity that generates reversible oxidation, such as H₂O₂, are likely to trigger signaling cascade in in vivo physiological settings (Janssen-Heininger et al., 2008).

ROS can signal directly to proteins via amino acid oxidation (Box 2), the most common reaction being oxidation of cysteine residues. ROS signaling to amino acids can cause functional changes in range of different proteins (Table 1) and thus these types of modifications have established ROS as crucial regulators of cellular signaling. Such proteins are known as redox sensors, meaning that they are directly modified by ROS, undergoing a conformational change as a result of the oxidative modification (Box 2); this change influences their function, stability, subcellular localization, interactions with other proteins and other crucial processes (summarized in Table 1).
### Table 1. Crucial regulators of ROS and redox sensor molecules

<table>
<thead>
<tr>
<th>Molecule</th>
<th>How do ROS affect its activity?</th>
<th>What effect does it have on ROS?</th>
<th>Outcome in stem cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
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<tr>
<td>AKT kinases</td>
<td>Cys oxidation abrogates AKT phosphorylation and, therefore, function (Murata et al., 2003)</td>
<td>Modulates ROS levels through the negative control exerted over FOXO TFs (Juntilla et al., 2010)</td>
<td>AKT1 and AKT2 knockouts display reduced ROS, causing HSC arrest in quiescence and a defect in differentiation (Juntilla et al., 2010)</td>
</tr>
<tr>
<td>Apurinic/apirimidinic (AP) endonuclease 1/redox factor 1 (APE/REF1)</td>
<td>Oxidized APE/REF1 binds TFs (HIF1α, AP1, NRF2 and p53) to keep them in their reduced form (Liu et al., 2005; Walker et al., 1993)</td>
<td>Decreases ROS levels and binds to oxidized TFs (Angkeow et al., 2002)</td>
<td>Compromised self-renewal of HSCs and NSCs in Atm⁻/⁻ mice (Ito et al., 2004; Maryanovich et al., 2012)</td>
</tr>
<tr>
<td>Ataxia telangiectasia mutated (ATM)</td>
<td>Activated by Cys oxidation (Guo et al., 2010b)</td>
<td>Redox homeostasis; mediates BID phosphorylation, causing ROS reduction (Maryanovich et al., 2012)</td>
<td>Tsc1⁻/⁻ (negative regulator of mTOR) HSCs display high ROS levels and compromised function (Chen et al., 2008)</td>
</tr>
<tr>
<td>Mammalian target of rapamycin (mTOR)</td>
<td>Activated by Cys oxidation (Sarbassov and Sabatini, 2005; Yoshida et al., 2011)</td>
<td>mTOR overactivity increases mitochondrial biogenesis and ROS production (Chen et al., 2008)</td>
<td></td>
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<tr>
<td>p38 mitogen-activated protein kinase (MAPK)</td>
<td>Possesses a reactive Cys; oxidation activates p38-MAPK</td>
<td>Mediator of ROS-regulated stem cell self-renewal (Ito et al., 2006)</td>
<td>p38-MAPK activation results in loss of quiescence in HSCs (Ito et al., 2006), and regulation of self-renewal in ESCs and NSCs (Ding et al., 2008; Kim and Wong, 2008). The balance between MSC proliferation and differentiation is controlled by p38-MAPK (Bhandari et al., 2010)</td>
</tr>
<tr>
<td>Phosphate and tensin homolog (PTEN)</td>
<td>Oxidation of Cys in PTEN catalytic sites causes inactivation (Lee et al., 2002)</td>
<td>Modulation of the PI3K/AKT pathway</td>
<td>PTEN inactivation by NOX-generated ROS increases PI3K-AKT activation in NSCs (Le Belle et al., 2011)</td>
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<tr>
<td>Sirtuins (SIRTs)</td>
<td>Cys oxidation inhibits SIRT1 activity (Zee et al., 2010)</td>
<td>SIRTs are NAD⁺-dependent deacetylases that modulates the activity of FOXO TFs (Brunet et al., 2004; Kobayashi et al., 2005; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004)</td>
<td>SIRT3 controls ROS levels during HSC ageing (Brown et al., 2013); SIRT1 induces HSCs accumulate ROS, show DNA damage and display an aged-like phenotype (Rimmelé et al., 2014). Regulation of SIRT1 p53 and NANOQ expression and/or activity via ROS in mouse ESC (Han et al., 2003)</td>
</tr>
<tr>
<td>Thioredoxin (TRX) system (complex: TRX-TRX reductase-NADPH oxidase)</td>
<td>Increased ROS induce TRX dissociation from ASK1, allowing ASK1 to activate JNK and p38, and inducing apoptosis (Saitoh et al., 1998)</td>
<td>Offer reduction power to several molecules by being kept reduced when in complex with thioredoxin reductase and NADPH oxidase</td>
<td>TRX1 and TRX2 modulate proliferation and survival of human MSCs (Song et al., 2011); TRX reduces and modulates OCT4 transcription activity in ESCs (Guo et al., 2004)</td>
</tr>
<tr>
<td>Forkhead homeobox type O proteins (FOXOs)</td>
<td>FOXOs possess 5-10 reactive Cys; the FOXO4 signaling switch from cell cycle arrest to apoptosis is redox mediated (direct); redox modulation of PTEN and AKT impact FOXOs negatively or positively, respectively (indirect) (Dansen et al., 2009)</td>
<td>Can regulate the transcription of antioxidant enzymes, such as SOD2, catalase and GPX1</td>
<td>Maintenance of redox balance is crucial for stemness and self-renewal of HSCs and NSCs (Myamoto et al., 2007; Renault et al., 2009; Tothova et al., 2007; Yalcin et al., 2008; Yeo et al., 2013)</td>
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<td>Hypoxia inducible factors (HIFs)</td>
<td>ROS-mediated modulation of HIFs can either stabilize (normoxia) or inhibit (hypoxia) HIF activity (Epstein et al., 2001)</td>
<td>HIFs mitigate ROS levels by modulating cell metabolism (glycolytic over oxidative phosphorylation);</td>
<td>Maintenance of HSC cell cycle quiescence and metabolic phenotype (Simsek et al., 2010; Takubo et al., 2010)</td>
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<td>Nuclear factor erythroid 2-related factor 2 (NRF2)</td>
<td>Indirectly modulated by APE/REF1 and KEAP1, the oxidation of which results in NRF2 activation and inactivation, respectively (Itoh et al., 1999; Motohashi and Yamamoto, 2004)</td>
<td>Can regulate the transcription of antioxidant enzymes (Venugopal and Jaiswal, 1996)</td>
<td>Control of stem cell fate by protecting NSCs, ISCs and MSCs from oxidative damage (Hochmuth et al., 2011; Tsai et al., 2013)</td>
</tr>
<tr>
<td>p53</td>
<td>Redox sensor whose DNA-binding capacity is impaired by oxidation (direct) or maintained by interaction with oxidized APE/REF1 (indirect) (Parks et al., 1997)</td>
<td>Regulates transcription of antioxidants and pro-oxidant enzymes (Polyak et al., 1997)</td>
<td>p53 controls ROS levels in postnatal BM (Abbás et al., 2010); p53 activity regulates stem cell fate and self-renewal in HSCs and ESCs (Liu et al., 2009a; Tekippe et al., 2003)</td>
</tr>
</tbody>
</table>

Different types of enzymes and transcription factors are regulated by ROS and can, in turn, regulate ROS with varying outcomes in different stem cell populations. AKT, protein kinase B; ASK1, mitogen-activated protein kinase kinase kinase 5 (MAP3K5); BID, BH3 interacting domain death agonist; BM, XXXXXX; Cys, cysteine; ESC, embryonic stem cell; GPX1, glutathione peroxidase 1; HSC, hematopoietic stem cell; ISC, XXXX; JNK, Jun kinase; KEAP1, kelch-like ECH-associated protein 1; NOX, NADPH oxidase; NRF2, nuclear factor erythroid 2; MSC, mesenchymal stem cells; NSC, neural stem cell; OCT4, POU domain, class 5, transcription factor 1 (POUSF1); p53, transformation related protein 53; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; TFs, transcription factors.
Box 2. Types of oxidative modification

Oxidation of the cysteine thiol group is the most extensively characterized type of protein modification that transduces reactive oxygen species (ROS) signaling. This results in sulfur-containing products, including disulfide bridges. In addition, a growing list of amino acids modifications by reactive oxygen and nitrogen species are described as knowledge on free radicals extends (Finkel, 2011).

Cysteine oxidation

Cysteines possess a reactive sulfur atom, the oxidation of which accounts for 1.9% of all protein modifications by ROS. Reactive cysteines can be easily converted to a sulphenic form (SOH) and reconverted to their reduced form, modulating protein activity. Oxidation of the cysteine thiol group is the most extensively characterized type of protein modification that transduces ROS signaling.

Cysteine nitrosylation

This is the reversible modification by nitric oxide (NO) that is substrate specific.

Cysteine glutathionylation

This process involves converting the reactive cysteines in proteins to the intermediate molecule SOH. These can be conjugated to glutathione to form a G-glutathionylated intermediate that subsequently modulates protein activity.

Protein carboxylation

This occurs through direct oxidation of side chains of lysines, arginines, prolines and threonines, or by covalent attachment of products from lipid peroxidation (e.g. unsaturated aldehydes). Carboxylation is an irreversible protein modification that leads to protein inactivation that is unlikely to mediate physiological cellular signaling.

Methionine oxidation

Methionines, like cysteines, possess reactive sulfur atoms and are therefore modified in a similar way to cysteines. The rate constant for methionine oxidation is much slower than for Cys oxidation.

Protein hydroxylation

Hydroxylation occurs on valines, leucines and lysines residues. The elevated oxidant property of the hydroxyl radical can even lead to the modification of these amino acids such that they have fewer reactive side chains. Hydroxyvalines and hydroxylysines are common markers in advanced stages of human diseases, such as atherosclerosis.

Although ROS can modify protein function, the opposite is also true: a growing network of proteins have been shown to modulate ROS levels (Fig. 2). Interestingly, many of these redox sensor proteins (Table 1) that are directly modulated by ROS in response to oxidative stress are also found to be crucial regulators of stem cell fate (Fig. 2). Among these proteins are transcription factors that have been connected to the regulation of cellular antioxidant machinery. These include members of the forkhead box O (FOXO) family, nuclear factor erythroid 2 (NRF2), PR domain containing 16 (PRDM16) and the p53 (TRP53) tumor suppressor (Chuikov et al., 2006; Tothova et al., 2007; Yalcin et al., 2008). Modulations of ROS levels and is crucial for mitochondrial function (Lessard and Sauvageau, 2003; Liu et al., 2009b; Molofsky et al., 2003). However, whether BMI1 is also directly modulated by ROS or whether BMI1 regulates mitochondria in HSCs remains to be determined. It is noteworthy that the redox-sensing property of many, if not all, of the proteins discussed above was established in somatic cells and often in cultured lines; whether these reactions also occur in primary stem cells or have a similar outcome remains to be established.

As well as a role in redox regulation, ROS might also function to alter the epigenetic landscape, which plays a particularly pertinent role in regulating stem cell fate (Challan et al., 2012; Mishra et al., 2014; Rimmele et al., 2014; Singh et al., 2013; Trowbridge et al., 2009; Will et al., 2013). Many metabolic intermediates are necessary substrates for the post-translational modifications of histones that together establish the epigenetic landscape of stem cells. As the activity of glycolysis and oxidative phosphorylation can directly influence ROS, leading to changes in the concentrations of various metabolic intermediates, this might represent a potential mechanism of ROS-mediated epigenetic regulation, albeit indirect (Gut and Verdin, 2013; Sutendra et al., 2014; Xiao et al., 2012). For example, acetylation of the lysine tails of histones cannot occur without acetyl CoA, the TCA cycle metabolite, while deacetylation by sirtuin proteins (SIRTs) requires activation by nicotinamide adenine dinucleotide (NAD). Similarly, methylation of CpG islands in DNA requires the substrate SAM (s-adenosylmethione), which is generated through threonine metabolism, a highly upregulated pathway in embryonic stem cells (ESCs) (Wang et al., 2009). Demethylation occurs through a series of hydroxylations of the methyl group catalyzed by TET (ten eleven translocase) enzymes and requires αKG (α-ketoglutarate) and O2 as substrates (Kohli and Zhang, 2013; Tahilian et al., 2009). Both
SIRT1 and TET enzymes are crucial factors in regulating hematopoietic stem cells (Moran-Crusio et al., 2011; Rimmelé et al., 2014; Singh et al., 2013). However, how flux through various metabolic pathways and nutrient availability control the concentrations of substrates required by histone-modifying enzymes has not yet been fully explored in stem cells.

**ROS in stem cell metabolism**

Cellular metabolism is the sum of catabolic and anabolic processes that involve the chemical conversion of carbon substrates to generate energy in the form of ATP and reducing co-factors (catabolic), or to produce macromolecular precursors in the form of nucleotides, lipids and amino acids (anabolic). The balance of catabolic and anabolic processes can shift depending on the cellular process being executed. Processes such as cellular growth and proliferation require mostly anabolic processes to generate building blocks for DNA, protein and membranes.

Manipulating metabolic pathways used by stem cells with either genetic approaches or drugs can directly affect whether stem cells remain quiescent, self-renew or differentiate (Takubo et al., 2013; Yu et al., 2013; Zhang et al., 2011b). One of the major ways in which metabolism can affect signaling pathways is through alterations of ROS levels. In turn, ROS can directly react with various proteins, such as kinases, phosphatases or transcription factors, to alter processes that regulate cell cycle progression, apoptosis, quiescence or differentiation (Dansen et al., 2009; Guo et al., 2010b; Vela et al., 2007). Furthermore, ROS can also directly modify metabolic enzymes or proteins that participate in nutrient-sensing pathways to direct the metabolic flux (Anastasiou et al., 2011; Brunelle et al., 2005; Sarbassov and Sabatini, 2005). In these contexts, ROS can be considered as signaling molecules that take part in the crosstalk between metabolism and stem cell fate decisions. Importantly though, metabolism can affect cell fate through multiple ROS-independent mechanisms or via mechanisms where the influence of ROS on metabolism is less obvious. Such mechanisms include changes in the epigenetic landscape brought about by metabolite abundances, as well as the ‘moonlighting’ functions of metabolic enzymes beyond their role in catalyzing metabolic reactions (De Bock et al., 2013; Gut and Verdin, 2013; Ritterson Lew and Tolan, 2013; Sutendra et al., 2014; Yang et al., 2011). However, compared with the effects of ROS, these other methods of crosstalk between metabolism and cell fate have not been as well characterized in stem cells.

**Embryonic stem cells**

Embryonic stem cells (ESCs) originate from the inner cell mass of the mammalian blastocyst and possess the ability to differentiate into all three germ layers of the embryo under defined conditions. ESCs self-renew rapidly due to a shortened G1 cell cycle phase. The maintenance of HSCs is also highly dependent on glycolysis, similar to ESCs (Unwin et al., 2006). Examination of metabolic parameters of HSCs showed that mitochondrial respiration and abundance are decreased relative to downstream progenitors (Norddahl et al., 2011; Simsek et al., 2010). In addition, HSCs show low levels of ROS and are enriched for glycolytic metabolites (Norddahl et al., 2011; Simsek et al., 2010). Similar analyses in neural stem cells (NSCs) and mesenchymal stem cells (MSCs) also revealed a preference for aerobic glycolysis and repression of the precursors for nucleotide biosynthesis. Both ATP and nucleotides are required to power the rapid proliferation and DNA replication of ESCs (Ward and Thompson, 2012). The glycolytic requirement became apparent after studies that compared multiple metabolic parameters between ESCs and differentiated cells revealed increased lactate production and an uncoupling of electron transport chain flux from ATP production in ESCs, as well as immature mitochondrial morphology and a more-reduced redox environment (Yanes et al., 2010; Zhang et al., 2011b) (Fig. 3). Furthermore, forced activation of oxidative phosphorylation led to loss of stem cell properties and increased differentiation or apoptosis (Zhang et al., 2011b). This was shown by knock down of the uncoupling protein 2 (UCP2), a gatekeeper of pyruvate entry into the mitochondrial oxidative phosphorylation pathway (Fig. 3), as well as by delivery of metabolites that stimulate this pathway. Conversely, enhancing glycolysis through hypoxia-mediated HIF activation and inhibition of oxidative phosphorylation improved proliferation and maintenance of ESCs, while repressing differentiation (Mandal et al., 2011; Zhou et al., 2012). Both results also lead to concomitant decrease in ROS levels with improved stem cell maintenance. In mouse ESCs, endogenous ROS are elevated by a SIRT1-mediated inhibition of p35 antioxidant function (Han et al., 2008). In addition, SIRT1-mediated regulation of ROS in ESCs is central in coordinating p53 activity with the expression of pluripotency factor NANOG (Han et al., 2008). Evidence also suggests that SIRT1 is an important player in the regulation of ESC mitochondria (Ou et al., 2014). Together, these findings support the idea that stem cell fate may be directly modified by metabolism through ROS. They also support the notion that, in ESCs, the need for glycolysis meets the biosynthetic demands of highly proliferative cells, similar to the Warburg effect in cancer cells (Ward and Thompson, 2012). Interestingly, glucose metabolism increases the generation of hematopoietic stem cells via ROS-mediated HIF stabilization, a mechanism that might be implicated in leukemia in children exposed to high glucose in general (Harris et al., 2013; Hjalgrim et al., 2004).

**Adult stem cells**

During foetal life and later after birth, adult stem cells continue to replenish damaged and lost tissue. The potency of adult stem cells is limited to a subset of lineages, which necessitates a specialized stem cell that is specific to different tissue types, as well as a specialized niche in which the stem cell resides. Unlike ESCs, adult stem cells are mainly highly quiescent, a property that is crucial for their self-renewal capacity (Foudi et al., 2009; Saito et al., 2010; Wilson et al., 2008; reviewed by Liang and Ghaffari, 2014). Despite their quiescence, adult stem cells are empowered by an intrinsic potential to proliferate quickly in order to regenerate tissue within a limited time in response to damage or loss. This requires metabolic plasticity in order to adapt to either quiescence or to the highly proliferative state. Thus, adult stem cells such as HSCs require a delicate balance between the maintenance that prevents exhaustion of the stem cell pool and the differentiation that continually replenishes downstream lineages.

The maintenance of HSCs is also highly dependent on glycolysis, similar to ESCs (Unwin et al., 2006). Examination of metabolic parameters of HSCs showed that mitochondrial respiration and abundance are decreased relative to downstream progenitors (Norddahl et al., 2011; Simsek et al., 2010). In addition, HSCs show low levels of ROS and are enriched for glycolytic metabolites (Norddahl et al., 2011; Simsek et al., 2010). Similar analyses in neural stem cells (NSCs) and mesenchymal stem cells (MSCs) also revealed a preference for aerobic glycolysis and repression of...
oxidative phosphorylation (Funes et al., 2007; Paik et al., 2009; Yeo et al., 2013). The dependence on glycolysis and the pentose phosphate pathway of adult stem cells, and more specifically of HSCs, may be due to multiple factors, such as their location within a hypoxic niche, the low energy requirements of quiescence and the need to minimize oxidative stress from mitochondrial ROS (Jang and Sharkis, 2007; Kunisaki et al., 2013). Evidence of this comes from genetic ablation of HIFs, which causes activation of oxidative phosphorylation and an increase in ROS, resulting in the subsequent loss of quiescence and the self-renewal properties of HSCs (Rouault-Pierre et al., 2013; Takubo et al., 2010). In HSCs, MEIS1 regulates both HIF1α and HIF2α (Simsek et al., 2010; Kocabas et al., 2012). Loss of MEIS1 results in a phenotype almost identical to Hif−/− HSCs that is entirely reversible by ROS scavenging (Kocabas et al., 2012), suggesting that MEIS1 is an important regulator of HSC metabolism upstream of HIF. More recently, conditional deletion of the M2 isoform of pyruvate kinase 2 (PKM2) or lactate dehydrogenase A (LDHA), both crucial enzymes of glycolysis, further emphasizes the key function of glycolytic metabolism for normal HSCs and leukemic stem cells.
Mitochondrial ROS in stem cells

The role of mitochondria in regulating stem cell fate appears more complex than the role of aerobic glycolysis. Mitochondria are highly dynamic organelles at the center of major signaling pathways. They control cellular processes such as apoptosis, Ca$^{2+}$ signaling, oxidative phosphorylation and ROS production, to name a few. As such, mitochondria can manifest in many different morphologies and subcellular localizations, depending on their activity. Normally, actively respiring mitochondria exist as a filamentous network, with elongated shapes, and are densely packed with cristae (Fig. 4). Cristae are the folds made up by the inner mitochondrial membrane and allow for greater amounts of surface area to house the electron-transport chain complexes (Youle and van der Bliek, 2012). In ESCs, the mitochondrial network is punctate, with individual mitochondria that are small and round with low numbers of swollen cristae (Prigione et al., 2010; Zhang et al., 2011b). These features of mitochondria are indicative of an immature and inactive mitochondrial network. When compared with fibroblasts, ESC mitochondria have a lower respiratory capacity but a higher mitochondrial membrane potential, an important component of the proton motive force (Chung et al., 2007; Holmes et al., 2011; Zhang et al., 2011b). High mitochondrial membrane potential can be an indicator of increased electron transport chain activity, whereas low mitochondrial membrane potential is associated with lower amounts of respiration; complete loss of mitochondrial membrane potential can trigger apoptosis (Vander Heiden et al., 1997). Similar to ESCs, HSCs also contain relatively immature mitochondria, suggesting that HSCs contain mitochondria with low levels of activity. This is supported by a lower respiratory rate and a low mitochondrial membrane potential when compared with downstream progenitors (Du et al., 2014; Simsek et al., 2010). The difference in mitochondrial membrane potential between ESCs and HSCs may represent the proliferative and ‘primed to differentiate’ nature of ESCs, in contrast to HSCs (which are mostly quiescent). It is therefore conceivable that it is the mitochondrial membrane potential and not the type of metabolism that is indicative of the degree to which stem cells are primed to differentiate; however, this requires further investigations (Schieke et al., 2008).

Compared with more differentiated cells, the mitochondria of stem cells are relatively metabolically inactive in terms of ATP production. Nonetheless, functional mitochondria are still required for proper maintenance of adult stem cells. In mice, deficiencies or mutations in important genes for mitochondrial function, such as Lkb1 (Stik11 – Mouse Genome Informatics), BID, mortalin (Hspa9 – Mouse Genome Informatics), Dj-1 (Park7 – Mouse Genome Informatics) and Tsc1 (tuberous sclerosis 1), have been associated with loss of HSC quiescence and transplantation capacity (Chen et al., 2008; Gan et al., 2010; Gurumurthy et al., 2010; Maryanovich et al., 2012; Nakada et al., 2010; Tai-Nagara et al., 2014) (Fig. 4). Although all the models in these studies showed increased ROS levels, albeit to varying degrees, only the Lkb1−/− HSC phenotype was not rescued with N-acetyl-cysteine (NAC), a glutathione precursor able to reduce levels of oxidative stress. Together, these results point towards ROS as a major marker through which stem cells can sense mitochondrial health and activity, although this is not the only mechanism. The need to survey and maintain constantly the health and numbers of mitochondria within stem cells is emerging as a key aspect of stem cell biology (Joshi and Kundra, 2013). Based on this hypothesis, mitophagy machinery that regulate clearance of damaged mitochondria, and transcription factors such as PGC1α, a regulator of the mitochondrial biogenesis, may have important functions in regulating stem cells.

Given the complexity of the biochemical pathways and reactions that occur within mitochondria, it is likely that there are multiple metabolic checkpoints that regulate cell fate. Recently, mitochondrial fatty acid oxidation mediated by the promyelocytic leukaemia protein (PML)-peroxisome proliferator activator receptor δ (PPARδ) axis was shown to be necessary for the self-renewal of HSCs by promoting asymmetrical cell division (Ito et al., 2012). Given that mitochondrial ATP production is reduced in HSCs compared with committed progenitors, it has been proposed that the fatty acid oxidation in HSCs supports acetyl CoA generation (Ito et al., 2012). Acetyl CoA is fed into the TCA cycle to generate downstream substrates that are subsequently shuttled into the cytosol as citrate to reduce NADP to NADPH, a co-factor in replenishing reduced
ROS as a mediator of stem cell fate and reprogramming

One of the eventual applications of stem cell biology is the generation of healthy differentiated cells to repair damaged or deteriorated tissues and organs. Given that ROS may influence a vast array of biological processes, and that we are limited in our knowledge of which species of ROS are implicated in any given physiological setting, it seems an immense challenge to explore how ROS metabolism can be manipulated to generate stem cells and influence stem cell fate. However, the study of metabolism and ROS mediated mechanisms of stem cell fate regulation has led to improved differentiation and reprogramming protocols. Differentiation of ESCs towards the cardiac lineage has been shown to rely on H$_2$O$_2$ signaling induced by NOX4 upregulation (Wang et al., 2007; Xiao et al., 2009). In the case of the cardiac lineage, not only are ROS important for differentiation, but the exclusive use of oxidative phosphorylation in cardiomyocytes compared with pluripotent stem cells (PSCs) can be taken advantage of to improve purification and differentiation efficiency (Chung et al., 2007; Tohyama et al., 2012). Interestingly, the degree of activation of mitochondrial metabolism is related to mouse ESC fate determination (Schieke et al., 2008). Finally, a recent study in human HSCs demonstrated that glutamine metabolism and pentose phosphate pathway-mediated generation of nucleotides is required for erythroid lineage commitment (Oburoglu et al., 2014). Chemical inhibition of these metabolic pathways led to commitment towards myeloid and granulocytic fates. Notably, as in ESCs, differentiation of MSCs towards adipocytes or neuron-like cells has also been shown to employ NOX4-mediated H$_2$O$_2$ signaling, as well as mitochondrial ROS (Kanda et al., 2011; Tormos et al., 2011). Further studies are required to reveal whether manipulation of ROS through metabolic pathways or directly can direct differentiation of other types of stem cells to various lineages (Box 3).

In contrast to differentiation, induced pluripotency occurs when a cell is reprogrammed to revert to a pluripotent state and becomes what is called an induced pluripotent stem cell (iPSC) (Takahashi and Yamanaka, 2006). The generation of iPSCs from differentiated cells has also benefited from careful regulation of ROS levels and metabolic flux. Metabolic rewiring from oxidative phosphorylation to glycolysis may even precede the activation of other key steps in the process of reprogramming (Holmes et al., 2011). Consistent with this, the key reprogramming factor OCT4 transcriptionally targets multiple metabolic genes (Kang et al., 2009). Moreover, new methods of small molecule-mediated iPSC generation have been shown to modulate the transition to aerobic glycolysis (Zhu et al., 2010). Although the precise effect of ROS on signaling pathways during the reprogramming process has not been evaluated, levels of ROS appear to increase during reprogramming and to cause damage to DNA, which can be minimized by the addition of NAC (Ji et al., 2014). The efficiency of reprogramming and continued maintenance of iPSCs can also be improved under low O$_2$ conditions (Ezashi et al., 2005). Based on the fact that mitochondrial consumption of O$_2$ is suppressed under hypoxia, leading to diminished levels of ROS, and that in iPSCs many ROS scavenging pathways are enhanced, it is logical to assume that increased levels of ROS might be detrimental to reprogramming efficiency (Armstrong et al., 2010). In light of these findings, it will also be interesting to evaluate the effects of FOXO factors, which are essential for the maintenance of pluripotency in ESCs, have been implicated in iPSC reprogramming, and are crucial for the regulation of ROS and cellular metabolism (Yeo et al., 2013; Zhang et al., 2011a, 2014).

###Box 3. Manipulation of ROS levels for eliminating the cancer stem cell

Cancer stem cells (CSCs) are defined as cells within a tumor that have acquired stem cell properties enabling them to reconstitute the whole tumor months or years after therapy (Baccelli and Trumpp, 2012). These cells are found in solid tumors such as prostate and breast cancers, as well as in leukemias. CSCs, in opposition to the bulk of cancer cells and similar to normal stem cells, display very low levels of reactive oxygen species (ROS), mainly due to increased activity of the antioxidant machinery and to their metabolic properties, which rely mainly on aerobic glycolysis. Leukemic stem cells (LSCs) are highly vulnerable to increases in ROS levels (Diehn et al., 2009; Kim et al., 2013). Subtle differences between normal cells and CSCs in their sensitivity to ROS can be exploited to target CSCs in therapy. Below, we summarize studies in which targeting ROS resulted in the efficient elimination of CSCs.

**Targeting glutathione metabolism**

Glutathione metabolism is central for ROS scavenging and glutathione peroxidase 3 (GPX3) expression correlates positively with the severity of acute myeloid leukemia. Knockdown of GPX3 or the use of the pharmacological inhibitor panthenolide, which depletes GPX1, efficiently induces apoptosis in LSCs and breast CSCs (Herault et al., 2012; Pei et al., 2013).

**Increasing ROS by targeting mitochondrial energy production**

Recently, BCL2 inhibition was shown to disrupt mitochondrial energy production, which increased ROS and induced apoptosis in LSCs, with little or no impact on normal stem cells (Lagadinou et al., 2013).

**ROS dynamics in stem cell homeostasis**

In *Drosophila*, multipotent hematopoietic progenitors that are similar to mammalian myeloid progenitors display high ROS levels that decline upon differentiation (Owusu-Ansah and Banerjee, 2009). Modulation of ROS levels has been shown to direct the differentiation of *Drosophila* multipotent hematopoietic progenitor cells, supporting a signaling role for ROS in regulating hematopoietic cell fate (Owusu-Ansah and Banerjee, 2009). An increase in ROS is associated with mammalian blood stem cell differentiation and with increased production of their immediate progenitors, in which ROS mediate cell cycle progression (Jang and Sharkis, 2007). Consistent with this, increased ROS mediate myeloproliferation in *Foxo3* mutant mice and in a mouse model of human myeloproliferative disorder (Marty et al., 2013; Yalcin et al., 2010).

In contrast to myeloid progenitors, HSCs with a long-term competitive repopulation capacity (LT-HSC) found within bone marrow compartments have been shown to have low levels of ROS. Indeed, a decrease in blood stem cell activity occurs within regions of bone marrow that have increased levels of ROS (Jang and Sharkis, 2007). Consistent with this, genetic ablation in mice of ataxia telan giectasia mutated kinase (*Atm*), *Foxo1/3/4* (forkhead box O 1/3/4) transcription factors or just *Foxo3*, resulted in an accumulation of ROS in HSCs, which compromised their activity (Ito et al., 2004; Miyamoto et al., 2007; Tothova et al., 2007; Yalcin et al., 2008). The defects in *Foxo*~−/−~ or *Foxo3*~−/−~ HSC activity were suggested to be due to elevated ROS levels that resulted from the decreased expression of antioxidant enzymes, including catalase and superoxide dismutase 2 (SOD2); however, the source of increased ROS in *Atm* mutant HSC remains unclear (Ito et al., 2004; Miyamoto et al., 2007; Tothova et al., 2007; Yalcin et al., 2008; Zhang et al., 2011c). Nonetheless, mice with a *Sod2* mutation do not exhibit any blood stem cell defects, which might indicate some potential functional redundancy between antioxidant enzymes of the ROS scavenging system (Friedman et al., 2014).
The defective Atm$^{-/-}$ HSC activity is attributed to the ROS-mediated activation of p16$^{ink4a}$ and of the retinoblastoma pathway (Ito et al., 2004). By contrast, the Foxo3 mutant HSC defects are likely to be mediated by ROS-induced activation of p53 tumor suppressor (Yalcin et al., 2008) (Fig. 5) or the activation of p38-MAPK (Miyamoto et al., 2007). In addition, the activation of p38-MAPK by elevated ROS compromises HSC self-renewal potential and impairs their engraftment (Ito et al., 2004, 2006; Yahata et al., 2011). ATM enzymatic activity and expression are regulated by FOXO3, but the extent to which ATM might contribute to the Foxo3 mutant HSC phenotype is unknown (Yalcin et al., 2008; Tsai et al., 2008). FOXO3 redox balance and transcriptional control of metabolic genes is also implicated in the maintenance of neural stem cells (NSCs) (Renault et al., 2009; Yeo et al., 2013). Nonetheless, highly proliferative NSCs require high ROS to maintain their self-renewal and neurogenesis properties (Le Belle et al., 2011). ROS generated by NADPH oxidases are also important for the self-renewal of spermatogonial stem cells (SSCs). However, elevated ROS in MSCs reduce their engraftment potential and induce apoptosis after transplantation (Morimoto et al., 2013; Rodrigues et al., 2012).

**Redox modulation and ageing of stem cells**

Stem cell function is compromised with increasing age (Liu et al., 2011; Signer and Morrison, 2013). The free radical theory of ageing proposes that it is caused by ROS-mediated damage to macromolecules (Harman, 1972). Although this has been challenged recently (Lapointe and Hekimi, 2010), increasing evidence implicates mitochondria in the ageing process of the whole organism (Gomes et al., 2013; Mouchiroud et al., 2013). However, the possible role of mitochondria in ageing is not necessarily due to generation of free radicals. Indeed, there is little evidence to suggest free radicals are involved in the ageing of adult stem cells and, furthermore, mitochondrial DNA mutations are not involved in the declining homeostasis of blood stem cells with age (Norddahl et al., 2011).

Although mitochondria have been implicated in whole organism ageing, it remains unclear whether mitochondrial metabolism is directly implicated in stem cell ageing. The NAD that serves as a redox regulator of oxidoreduction reactions in the cell has been recently implicated in the organismal ageing process, and thus could potentially be involved in stem cell ageing (Gomes et al., 2013; Mouchiroud et al., 2013). The NAD/NADH ratio is a measure of cellular redox status and is important for the maintenance of the glycolytic flux. Importantly, NAD serves also as an activator of several enzymes, including SIRT family deacetylases that regulate histones and non-histone proteins (Haigis and Sinclair, 2010). This activation is crucial for mitochondrial homeostasis, as SIRT1 regulates the expression of oxidative phosphorylation enzymes and PGCl1, which are crucial for mitochondrial gene expression (Gomes et al., 2013). The NAD/SIRT pathway is also involved in the regulation of worm lifespan, although the underlying mechanism may be distinct and mediated by FOXO (Mouchiroud et al., 2013). These results show that communication between the nucleus and mitochondria mediated by NAD is crucial for decelerating the ageing process. In this context, recent findings implicating SIRT proteins in the regulation of blood stem cells and their ageing are notable (Brown et al., 2013; Rimmelé et al., 2014; Singh et al., 2013). Although SIRT3 is critical for the maintenance of the blood stem cell pool in old mice or under stress, SIRT1 is key to the maintenance of blood stem cells in young adult mice during both steady-state and stress conditions (Brown et al., 2013; Rimmelé et al., 2014; Singh et al., 2013). Loss of SIRT1 results in an ageing-like phenotype associated with defective lineage specification, as well as other hallmarks of stem cell ageing, including the accumulation of ROS and DNA damage in young adult mice, some of which is mediated by relative loss of FOXO3 activity in the Sirt1 mutant HSC (Rimmelé et al., 2014). Together, these findings raise the possibility that modulations of NAD might be important for the stem cell ageing process. In addition, they point to a potential function for SIRT/FOXO in the regulation of adult stem cell ageing. As well as regulating FOXO3 in blood stem cells, SIRT1 has many targets, including p53, PGC1 and HIF1, which suggests that SIRT1 may regulate stem cells through a panel of critical stem cell proteins (Lim et al., 2010; Rodgers et al., 2005; Vaziri et al., 2001). It will be necessary to devise reliable methods for the measurement of NAD levels in cell populations that contain few adult stem cells and, in this context, to clarify whether and how SIRT1 and/or SIRT3 regulation of mitochondria contribute to the correct lineage specification and/or ageing of stem cells.

**Concluding remarks**

Work in the past decade has uncovered the importance of redox signaling to the biology of stem cells. ROS signal the metabolic state of stem cells and, in doing this, can impact stem cell fate. Whether ROS globally impact the stem cell epigenome is not known; however, given the ability of metabolic intermediates to modify epigenetic machinery, it certainly appears possible. As is the case in cancer cells, the exact underlying mechanisms of metabolic regulation of stem cell epigenetics remains unknown, representing an exciting avenue for future exploration.

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