FORUM REVIEW ARTICLE

Metabolic Regulation of Redox Status in Stem Cells

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Abstract

Significance: Metabolism-dependent generation of reactive oxygen species (ROS) and associated oxidative damage have been traditionally linked to impaired homeostasis and cellular death. Beyond the adverse effects of ROS accumulation, increasing evidence implicates redox status as a regulator of vital cellular processes. **Recent Advances:** Emerging studies on the molecular mechanisms guiding stem cell fate decisions indicate a role for energy metabolism in regulating the fundamental ability of maintaining stemness versus undergoing lineage-specific differentiation. Stem cells have evolved protective metabolic phenotypes to minimize reactive oxygen generation through oxidative metabolism and support antioxidant scavenging through glycolysis and the pentose phosphate pathway. **Critical Issues:** While the dynamics in ROS generation has been correlated with stem cell function, the intimate mechanisms by which energy metabolism regulates ROS to impact cellular fate remain to be deciphered. **Future Directions:** Decoding the linkage between nutrient sensing, energy metabolism, and ROS in regulating cell fate decisions would offer a redox-dependent strategy to regulate stemness and lineage specification. *Antioxid. Redox Signal.* 21, 1648–1659.

Introduction

REACTIVE OXYGEN SPECIES (ROS) have historically been viewed as detrimental to cell function; however, new knowledge increasingly points to a critical contribution in regulating cellular physiology (84). The last decades have seen an immense evolution pertaining to the biology of ROS and their general impact on organ systems and disease states. Specifically, a growing body of work is evolving to delineate ROS and downstream signaling in control of cellular fate within the paradigm of stemness maintenance and lineage specification (51, 99). Accordingly, the main objective of this overview is to highlight metabolic regulation of redox status in the context of stem cell differentiation and dedifferentiation.

ROS Regulation

Cellular redox status is set by the balance of ROS production (Box 1) versus antioxidant defenses (Box 2). These processes are highly regulated, enabling a dynamic setting of the intracellular ROS milieu that is critical for both physiological and pathophysiological cellular functions.

Generation of ROS

ROS production occurs throughout the cellular environment *via* conserved biochemical reactions (Box 1), and can largely be divided into extra- and intramitochondrial processes. Extramitochondrial locations include nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase, uncoupled endothelial NO synthase, myeloperoxidase, lipoxigenase, cytochrome p450, heme oxygenase, and peroxisomes (67). Cellular respiration and metabolic processes, however, represent a major source of ROS, as O₂ is used as the ultimate electron acceptor during respiration, and carries the risk of generating intermediates with unpaired electrons due to the successive transfer of single electrons during reduction of oxygen. Although complex IV catalyzes the reduction of O₂ to H₂O, this complex does not contribute to mitochondrially derived ROS. Rather, mitochondria contain seven sites that have the ability to generate ROS; however, their relative contribution to physiological ROS generation remains uncertain (6). Specific sites within complexes I and III of the electron transport chain can contribute to mitochondrial ROS generation. Complex I couples oxidation of nicotinamide adenine dinucleotide (NADH) to proton pumping by passing

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liberated electrons through a series of redox centers, including flavin mononucleotide, eight Fe-S clusters, and ubiquinone. It has been established that complex I contributes to mitochondrial ROS production, although controversy still remains as to whether there is one or two separate sites of ROS production, partially due to lack of inhibitor specificity (6). Isolated complex I-based studies indicate that the predominant source of superoxide is the reduced flavin center; however, during forward respiration of NADH-linked substrates in isolated mitochondria, flavin remains relatively oxidized and superoxide generation remains low (44, 54). However, blocking the complex I ubiquinone binding site using rotenone causes electrons to back up and reduce the upstream redox centers, and significantly increases superoxide generation (6). The quinone-binding site in complex I represents an additional site for superoxide generation, which is particularly important during reverse electron transport through complex I from ubiquinol to NAD^+ when succinate and glycerol 3-phosphate are used as substrates. Indeed, superoxide production is reduced when reverse electron transfer is inhibited with rotenone (6, 44).

Superoxide is also readily generated by complex III as electrons are passed from ubiquinol to cytochrome C, which can only accept a single electron. Meanwhile, the additional electron from ubiquinol is recycled in the modified Q cycle to regenerate an additional ubiquinol molecule after two turns of the cycle, acting as a safeguard against radical generation (61). Despite this, in the presence of antimycin, complex III can produce high amounts of superoxide blocked using specific inhibitors of centre o of complex III such as stigmatellin, indicating that a build-up of semiquinone at this site is responsible for reducing oxygen to superoxide (8, 97). The impact of this site under physiological conditions, however, remains controversial (6).

Alternative sites for mitochondrial ROS generation exist, albeit their contribution are less well characterized. Similar to complex I, these enzymes are dependent on flavin and can generate superoxide as they cycle among reduced, oxidized, and partially reduced states (61). Although complex II mutations can result in ROS generation, the enzyme suppresses flavin radical production so that limited superoxide is produced compared with complex I or III (78). Electron transfer flavoprotein:ubiquinol oxidoreductase (89), dihydroorotate dehydrogenase (36), α -ketoglutarate (96), and pyruvate dehydrogenases (39) also can generate ROS, but their contributions are largely uncharacterized (6). Glycerol-3-phosphate dehydrogenase also is a source of mitochondrial-derived ROS and is capable of generating ROS on both the matrix and intermembrane space side of the inner mitochondrial membrane (24). Of the sites that produce mitochondrial ROS, only glycerol-3-phosphate dehydrogenase and complex III can supply superoxide to the intermembrane space, uniquely enabling them to cross the outer mitochondrial membrane and modulate extramitochondrial signaling (84).

ROS scavenging and cellular repair

Abundant ROS production is associated with detrimental cellular consequences. Cells have evolved complex mechanisms to neutralize ROS and maintain redox balance. Cells and body fluids contain molecules that neutralize or scavenge ROS, collectively termed antioxidants. Anti-

Box 1: ROS Primer

ROS is a generic term encompassing reactive oxygen intermediates, including neutral molecules (*e.g.*, hydrogen peroxide), ions (*e.g.*, superoxide anion), or radicals (*e.g.*, hydroxyl radicals). O_2 accepts one electron at a time during reduction to H_2O , and accordingly, reactive intermediates are observed as a cascade of transitions from superoxide ($\text{O}_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) to hydroxyl radical ($\text{OH}^\bullet$). Typically, the initial reaction is a one-electron transfer to oxygen to form $\text{O}_2^{\bullet-}$; the addition of another electron gives rise to the peroxide ion, which is a weaker acid and is protonated (spontaneously or catalyzed by SOD) to hydrogen peroxide (H_2O_2). The hydroxyl radical can be produced if a single electron is added to H_2O_2 by a reduced metal ion such as Fe^{2+} (Fenton reaction). The hydroxyl reaction is extremely reactive with a very short half life; thus, it reacts within the vicinity of its formation site. Superoxide does not readily cross membranes and is short lived and local in its effect, but SOD converts superoxide to longer-lasting and membrane-diffusible H_2O_2 (42).

Due to high reactivity, ROS readily react with virtually any type of biological molecules. Sustained high concentrations of ROS can, thus, cause damage to cellular and extracellular constituents, including DNA, proteins, and lipids (25). Of particular concern is DNA oxidation, which induces mutations and/or aberrant gene expression (107). mtDNA appears to be more sensitive to oxidative stress-induced mutations, because it lacks DNA repair enzymes. Further, oxidation of proteins may lead to the formation of insoluble protein aggregates. Such protein aggregates are the molecular basis of a number of diseases, particularly neurodegenerative pathologies. ROS are also involved in the formation of advanced glycosylation end products, which is an irreversible molecular change typically seen in glycosylated cell surface proteins.

oxidants include antioxidant enzymes that neutralize ROS by forming less toxic species and antioxidant small molecules—the latter comprising endogenous metabolites, biosynthesized cofactors, and exogenous diet-procured molecules (Box 2). Other defense mechanisms operate to reduce ROS production. In mitochondria, uncoupling proteins have been suggested to fulfill this function, producing mild uncoupling and limiting ROS generation by the respiratory chain (60). In addition, downregulation of substrate supply and a decrease of pO_2 would limit ROS production.

Oxidative damage occurs when ROS generation outpaces antioxidant defenses and buffering capacity. Cells maintain mechanisms that are capable of repairing some of this

Box 2: Antioxidant Defenses

A major component of the antioxidant system includes enzymes that metabolize ROS into more stable and less reactive molecules. SOD, which is represented by three isoforms in humans, including cytosolic Cu,Zn-SOD, mitochondrial matrix Mn-SOD, and extracellular SOD, catalyzes dismutation of superoxide to H_2O_2 and oxygen. However, accumulation of H_2O_2 in the presence of reduced metal ions would activate the Fenton reaction to generate highly reactive hydroxyl radicals; therefore, SOD should work in tandem with H_2O_2 -scavenging enzymes, such as peroxidedoxins, thioredoxins, and glutathione peroxidases in the mitochondria and catalase in the peroxisome (55). Mitochondrial-specific glutathione (mGSH) represents a major line of defense maintaining mitochondrial redox homeostasis. The importance of mGSH is based not only on its abundance, but also on its versatility to counteract H_2O_2 , lipid hydroperoxides, or xenobiotics, by serving as a cofactor of ROS degrading (62, 84). One such enzyme is *glutathione peroxidase* (GPx), which exists in selenium-dependent and independent forms and catalyzes degradation of both organic peroxides and H_2O_2 coupled to the oxidation of GSH to glutathione disulfide. To complete the cycle, GSH should be regenerated by *glutathione reductase* (GR) using NADPH as the reducing agent; the major source of NADPH for cytosolic GR is the pentose phosphate pathway, and NADH/NADP⁺ transhydrogenation in mitochondria.

A variety of small molecules can nonenzymatically react with ROS, offering a cellular buffering capacity. *Vitamin E*, which includes eight different derivatives with α -tocopherol being the preferentially absorbed form in humans, is a critical lipid-soluble antioxidant. It is distributed in all cellular membranes, including mitochondria as a function of lipid content (62), and mainly prevents lipid peroxidation. *Vitamin C* (ascorbic acid) is water soluble and cooperates with Vitamin E to regenerate α -tocopherol from tocopheroxyl radical produced during the Vitamin E radical scavenging activity; the product of the reaction is a very stable ascorbate radical. Although excessive doses of ascorbate may be pro-oxidant, physiological amounts have been demonstrated to be antioxidant even in the presence of metal ions (79). A number of metabolites within central metabolism also display ROS-buffering capacity, such as the α -keto acids of glycolysis and the tricarboxylic acid cycle.

damage once it occurs. A well-established example is DNA repair, including nucleotide excision repair and base excision repair, by which oxidative damage and other lesions are removed from chromosomal and mitochondrial DNA (mtDNA) to restore the original strand integrity (46). However, the mtDNA-repairing systems are considered less efficient than those operating in the nucleus, contributing to the higher mutagenic rate of mtDNA (21). However, oxidative damage in other molecules, particularly in amino-acid side chains of proteins, cannot be repaired. Accumulation of damaged molecules can have a profound impact on cellular function and homeostasis; thus, mechanisms exist that either

sequester these molecules or tag them for degradation. Oxidized proteins tend to be extensively ubiquitinated, and are directed to the proteasome for digestion; alternatively, these molecules can be degraded in the autophagosome (40). Since the mitochondria is a major source of ROS, mitochondrial proteins are especially exposed to oxidative modification and the elimination of oxidized proteins is crucial for maintaining the integrity of this organelle (98). Damaged cellular organelles can be recognized by the autophagy system and preferentially degraded, protecting the cell from accumulation of dysfunctional organelles.

Metabolic Regulation of ROS and Control of Cell Fate

Stem cells represent unspecialized cell types with the unique ability for asymmetric cell division, which, on one hand, ensures self-renewal and maintenance of the nascent stem cell pool, and, on the other hand, enables lineage-specific differentiation and tissue rejuvenation. Embryonic stem cells are the quintessential prototype derived from the inner cell mass of blastocysts, where a limited number of cells give rise to all tissues of the developing embryo. Hence, embryonic stem cells are referred to as pluripotent due to their ability to differentiate into three germ layers, namely the ectoderm, mesoderm, and endoderm. Recent advances in the technology of nuclear reprogramming has enabled the bioengineering of somatic cells back to a pluripotent state through the expression of primordial transcription factors yielding induced pluripotent stem cells (iPSC) (94). In contrast to natural or bioengineered pluripotent stem cells, adult stem cells, also known as resident stem cells as they reside in specific niches within differentiated tissues such as the blood, skin, intestine, and muscle, display a more limited differentiation capacity known as multipotency and typically are restricted to differentiation into specialized cells of the resident tissue. Growing evidence indicates that ROS are not simply a detrimental byproduct of energy metabolism but may play critical roles in regulating physiological cellular processes (48, 51, 99). In this context, the mechanism by which ROS regulates critical functions in stem cells, including their ability to self-renew and differentiate into specific lineages, is starting to be understood (10, 68, 73, 104).

Metabolic regulation of ROS and stemness maintenance

Pluripotent stem cells. Since the inner cells mass from which embryonic stem cells are derived is the source of all tissues of the developing organism, mechanisms are in place to avoid accumulation of DNA damage and secure repair, ultimately preventing propagation of mutations with tissue differentiation. Indeed, embryonic stem cells are endowed with superior maintenance and repair systems to ensure genomic stability through a combination of low levels of stress generation, high activity of stress defense, and fidelity of repair mechanisms coupled to efficient elimination of cells that accumulate mutations or DNA damage (9, 81, 82).

To mitigate risk of ROS-dependent DNA damage, pluripotent stem cells display a reduced dependence on mitochondrial oxidative metabolism (33, 77). Embryonic stem cells are characterized by low levels of mtDNA and sparse mitochondrial density with predominantly perinuclear

localization (4, 29, 34, 57, 76, 77, 88, 93, 110). Consistent with an immature mitochondrial ultrastructure and overall morphology, pluripotent stem cells display lower rates of oxygen consumption and a limited reserve capacity compared with differentiated cells, distinguishing features associated with reduced ATP levels and energy turnover (34, 76, 93, 101). In fact, to meet their energetic demands, pluripotent stem cells largely rely on glycolysis instead of oxidative energy metabolism, in contrast to their differentiated counterparts (34, 70). An increased metabolic flux through glycolysis and the pentose phosphate pathway promotes generation of NADPH, which is a critical cofactor for maintaining thioredoxin and glutathione in their reduced forms, thereby supporting the scavenging of ROS. Indeed, in cancer cells metabolizing glucose, ROS can inhibit pyruvate kinase by oxidizing cysteine 358, thereby directing metabolic flux into the pentose phosphate pathway to augment NADPH production and sustain glutathione and thioredoxin levels (3). That glycolysis-dependent metabolism affords protection from ROS-induced damage is illustrated in mouse embryonic fibroblasts which are immortalized through overexpression of the glycolytic enzymes phosphoglycerate mutase and glucophosphate isomerase and, as a consequence, display elevated rates of glycolysis and less oxidative damage as demonstrated by cytosolic ROS staining and 8-hydroxydeoxyguanosine quantification (52). Consistent with this observation, ablation of glucose-6-phosphate dehydrogenase, the rate-limiting step of the pentose phosphate pathway, results in elevated sensitivity of these cells to oxidative stress, with predilection for apoptosis occurring at ROS concentrations that are otherwise well tolerated by their wild-type counterparts (31). The preferential utilization of glycolysis over mitochondrial oxidative metabolism, thus, may represent a protective metabolic mechanism to preserve the genomic integrity of pluripotent stem cells (Fig. 1).

In utero the nascent environment of the inner cell mass of the blastocyst within oviductal and uterine lumen may potentiate this protective metabolic phenotype, as the natural environment is considerably hypoxic, with oxygen tension ranging from 1.5% to 9% (32). Since oxygen functions as the final electron acceptor of oxidative phosphorylation, limiting oxygen availability would impair oxidative metabolism and require a metabolic shift to anaerobic glycolysis to provide sufficient ATP generation for cellular homeostasis. Indeed, culturing embryos in 20% oxygen leads to greater arrest rates and fewer embryos developing to the blastocyst stage, associated with greater pyruvate uptake in the precompaction embryo and reduced glucose uptake in the postcompaction embryo (103). Although pluripotent stem cells can grow well under low O₂ (3%–5%), as well as high O₂ (20%) environments, consistent with their origin in the inner cell mass, the hypoxic environment promotes glycolysis (37) and maintenance of stemness and the self-renewal capacity (27, 64, 75, 106) and moreover, can increase the efficiency of nuclear reprogramming to the pluripotent state (109). This strongly supports the concept that metabolic modulation is critical for regulating key stem cell properties (Fig. 1).

Complementing reduced ROS generation supported by a nonoxidative metabolic phenotype, pluripotent stem cells also appear to have elevated antioxidant defenses. Indeed, cell lysates from embryonic stem cells display greater antioxidant capacity than their differentiated counterparts as

demonstrated by the ability to delay metmyoglobin/H₂O₂-mediated free radical generation (81). This is associated with a low level of peroxides in embryonic stem cells and appears dependent on a high expression of thioredoxin-glutathione reductase, glutathione peroxidases 2, 3, and 4, glutathione-S-transferase, and superoxide dismutase 2 (SOD2). Antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, are also elevated in circulating progenitor cells, suggesting a consistent role in reducing intracellular ROS levels across diverse stem cell species (22). To complement physiological mechanisms that reduce ROS accumulation, pluripotent stem cells also possess efficient stress response systems to prevent or repair DNA damage. These are exemplified by the high expression of the verapamil-sensitive multidrug efflux pump, heat shock protein expression, and DNA strand-break repair processes (63, 81, 82). As a final mechanism to prevent proliferation of mutations and ensure mutations are not passed onto subsequent generations, pluripotent stem cells display enhanced susceptibility to undergo apoptosis in response to genotoxic agents and DNA damage (2, 17, 87, 100).

Based on the indicated differences in energy metabolism and ROS production in pluripotent versus differentiated cells, it could be anticipated that regulation of these processes may also play an important role during nuclear reprogramming. Indeed, nuclear reprogramming induces a remodeling of the metabolic infrastructure that precedes the induction of pluripotent markers (34, 45), including a reduction in mtDNA (4, 76), as well as transcriptional and epigenetic regulation of nuclear genes related to oxidative and glucose metabolism. Broadly, this supports targeted upregulation of glycolytic enzymes and selective downregulation of electron transport chain subunits, and is associated with regression in mitochondrial density, distribution, and ultrastructure (34, 76, 101). Specifically, proteomic profiling has identified a stoichiometric remodeling of the mitochondrial electron transport chain, including a reduction in subunit expression of complex I and IV and an increase in II, III, and V (45). In contrast, the initial and final steps of glycolysis and the nonoxidative branch of the pentose phosphate pathway are upregulated, while the intermediate reactions in glycolysis are downregulated, suggesting preferentially flux through the pentose phosphate pathway in support of anabolic metabolism and antioxidant defenses (101). These changes support a functional metabolic switch away from oxidative metabolism in favor of glycolysis, with iPSCs displaying elevated glycolytic rates, low basal cellular respiration, and an inability to increase their oxygen utilization in response to electron transport chain uncoupling (34, 101). The switch is critical for reprogramming, as inhibition of glycolysis or stimulation of oxidative metabolism impairs reprogramming, while stimulation of glycolysis augments reprogramming efficiency (34, 113), consistent with the observation that somatic cells with low oxidative metabolism and high glycolytic rates have greater reprogramming efficiency (70).

These metabolic changes critical for nuclear reprogramming support a low ROS state due to reduced reliance on oxidative processes and support of antioxidant defenses through the pentose phosphate pathway. Similar to their embryonic counterparts, iPSCs display reduced generation of superoxide anions, which results in less oxidatively modified macromolecules, including DNA, proteins, lipids, and DNA,

FIG. 1. Metabolic regulation of reactive oxygen species (ROS) and impact on stem cell fate. Stem cells ensure genomic stability through minimizing stress generating molecules and maintaining high activity of stress defense mechanisms. This is partially accomplished by maintaining a glycolytic state, which supports stem cell maintenance through a lower reliance on oxidative metabolism, enabling a lower mitochondria mass, oxygen consumption, and oxidative stress generation, while supporting flux through the pentose phosphate pathway to facilitate NADPH formation and maintenance of antioxidant defenses. On differentiation cues, intracellular ROS levels increase and are required for differentiation into specific lineages. ROS may accumulate due to an increase in ROS generation, as well as a reduction in antioxidant defense, which may be partially due to a significant remodeling of the metabolic infrastructure, including an increase in mitochondrial mass, maturity, and intracellular networks to support a greater reliance on oxidative metabolism and an increase in ROS generation, while reducing the antioxidant supportive role of glycolysis and the pentose phosphate pathway.

even after exposure to hydrogen peroxide, supporting a low level of oxidative stress and greater antioxidant defense mechanisms (4, 76). Indeed, induction of ROS generation and DNA damage as a consequence of viral induction of reprogramming factors may act as a roadblock to efficient pluripotent induction (59). Indeed, SOD1 and 2 are upregulated in reprogramming factor-induced fibroblasts and inclusion of c-Myc in the reprogramming cocktail-reduced ROS levels and facilitate reprogramming efficiency. However, only inclusion of vitamin C, not other antioxidants such as n-acetylcysteine or resveratrol, in the reprogramming milieu improved reprogramming efficiency (26). This suggests that some antioxidant-independent mechanism may account for the beneficial effect of vitamin C, such as the partial alleviation of senescence which may be a roadblock for reprogramming (26), or *via* modulation of p53 levels and Ink4/Arf locus and attenuation of hypermethylation of Dlk1-Dio3 to support iPSC formation (90, 105). Recent reports have demonstrated that addition of antioxidants during reprogramming and initial clonal expansion has also been demonstrated to reduce genomic aberrations (50); however, the long-term impact of antioxidant treatment remains to be determined (58).

Adult stem cells. Adult stem cells reside in niches within differentiated tissues and support life-long tissue regeneration. Since these cells, in principle, reside for extended periods of time in the body, it is presumed that they are uniquely resistant to diverse stressors, ranging from chemical poisons, ultraviolet and ionizing radiation to oxidative stress. Adult stem cells are maintained in a quiescent state with limited cell-cycle progression that may serve to partially protect them (51). Indeed, the underpinnings of adult stem cell-associated stress defense mechanisms remain a fundamental issue in stem cell biology.

Similar to their embryonic counterparts, a low oxygen tension (1%–8% O₂) is maintained across a number of stem cell niches, resulting in the suppression of ROS generation and the prevention of stress damage (64, 92). The best-characterized stem cell niche is that associated with hematopoietic stem cells, that share the densely populated bone marrow microenvironment with stromal and progenitor cells, which may compete for oxygen and potentiate the hypoxic gradient within the common space (92). Quiescent hematopoietic stem cells with long-term reconstitution activity (LT) and slow cycle kinetics maintain a more robust ability to repopulate and engraft under hypoxic conditions, and avoid damage accumulation from oxidative stress. Hypoxia stabilizes hypoxia-inducible factor-1 α (HIF-1 α) through suppression of prolyl hydroxylation and subsequent ubiquitination and proteasomal degradation, enabling binding to hypoxia response elements in target genes (92). LT-hematopoietic stem cells have a high expression of HIF-1 α , which should be maintained within a narrow range as knockout of this pathway induces defective hematopoiesis and stress resistance, resulting in embryonic lethality, while excessive expression promotes destabilization and premature exhaustion. HIF-1 α promotes a switch from oxidative to glycolytic metabolism through transcriptional activation of genes that regulate glycolysis, including glucose uptake (GLUT1), pyruvate disposal (LDHA), and upstream regulatory proteins (PDK1) (92). Transcriptional activation of HIF-

1 α through the transcription factor Meis1 enables a similar LT-hematopoietic stem cells metabolic phenotype to other stem cell populations consisting of a dependence on glycolysis instead of mitochondrial oxidative metabolism, and potentially suppressing ROS production (86). Oxygen tensions as low as 3% are also observed below the dura (23), indicating that hypoxia may be an important component of the neurogenic niche required to maintain neural stem cells in the undifferentiated state (64). To this end, hypoxia has been demonstrated to promote the *in vitro* maintenance of the undifferentiated state and proliferation of a number of neural stem cell populations, including neural crest cells (65), mesencephalic precursors (91), and central nervous system precursors (80). Maintenance of neural stem cells in normoxia (20%) leads to precursor depletion and preferential differentiation into astrocytes (12, 74). Lack of oxygen and hypoxic signaling may, therefore, be more broadly applicable to other stem cell niches, as hypoxia prolongs lifespan, increases proliferative capacity, and reduces differentiation of additional stem cell population, including mesenchymal and pluripotent stem cells (27, 30, 64, 75, 106, 109).

Selective deletion of the metabolic sensor LKB1 in hematopoietic stem cells results in death due to impaired homeostasis and loss of quiescence, depletion of hematopoietic cell pools, and loss of reconstitution capacity (38, 41, 66). Disruption of LKB1 also significantly impairs energy homeostasis including reduced mtDNA copy number, mitochondrial membrane potential, and oxidative capacity, all of which are associated with reduced ATP levels (38, 41, 66). Metabolomic analysis indicated that LKB1-deficient cells display a prominent elevation of long-chain fatty acids and nucleotide metabolites with modifications in glycolytic and tricarboxylic acid cycle components, indicating an essential role of LKB1 in the metabolic homeostasis of hematopoietic stem cells (41). Although LKB1 deficiency reduces downstream AMPK phosphorylation (T172) and knockout AMPK α 1/ α 2 partially recapitulates the phenotype, pharmacological activation of downstream signaling pathways mediated by AMPK, mTOR, and FoxO does not rescue hematopoietic stem cell function, indicating an alternative LKB1 signaling pathway (38, 41, 66). A potential LKB1 signaling pathway may be through PGC1 α / β , which regulates mitochondria biogenesis and energy metabolism, as transcriptional analysis identified an LKB1 enrichment of PPAR γ genes and loss of LKB1 leads to a reduction in PGC1 α / β expression (38). LKB1 plays an essential role in hematopoietic stem cell homeostasis; however, the mechanism by which it impacts ROS generation and stem cell fate remains unknown.

Metabolic regulation of ROS and stem cell differentiation

Regulation of ROS generation during differentiation. Redox status is increasingly recognized to be a critical component of the stem cell differentiation program (68, 84). Intracellular ROS levels appear to increase early during stem cell differentiation and are required for differentiation into specific lineages, including cardiomyogenesis (4, 11, 18, 76). Physiologically, ROS may accumulate due to an increase in ROS generation, as well as a reduction in antioxidant defenses. To this end, extramitochondrial sources of ROS, including Nox, are upregulated during stem cell differentiation, with

inactivation of Nox-4 blocking stem cell cardiac specification (7, 56, 99). The impact of differentiation on expression of antioxidants is more variable and may be stage and lineage specific. Differentiation of pluripotent stem cells is associated with a downregulation of a number of antioxidant defenses such as *GSR* (glutathione reductase), *SOD2* (superoxide dismutase 2), *MGST1* (microsomal glutathione S-transferase 1), and *GPX2* (glutathione peroxidase 2) (4). In contrast, an increase in catalase and SOD without changes in other antioxidants were observed during osteogenic differentiation of mesenchymal stem cells (11). Despite this, supplementation of embryoid bodies during spontaneous cardiac differentiation with H_2O_2 results in an increased number of beating areas; meanwhile, addition of antioxidants, including n-acetylcysteine, catalase, and the Nox inhibitor diphenyleneiodonium, impairs beating activity (7, 83).

Stem cell differentiation is also associated with a remodeling of the mitochondrial and bioenergetic infrastructure to support the emerging energy requirement of increasingly specialized functions during lineage specification (33). Beyond energetic support to the nascent cell progeny, a greater reliance on mitochondrial-dependent oxidative processes will impact the cellular ROS load, which may, in turn, contribute to defining cellular fate (Fig. 1). On downregulation of stemness genes in response to differentiation stimuli, mtDNA levels are gradually increased due to acceleration of mtDNA replication in support of mitochondrial maturation and biogenesis (13, 15, 28, 29, 76, 88, 93). Indeed, differentiation induces a transformation of the spherical and cristae-poor mitochondria characteristic of stem cells into the tubular and cristae-rich structures of their differentiated progeny. Lineage specified cells also obtain extensive mitochondrial networks throughout the cytosol to ensure sufficient ATP supply to cellular sites of energy utilization, in contrast to a perinuclear localization of mitochondria in stem cells (13–16, 71, 72, 76, 88, 93). Concomitant with maturation of the mitochondrial infrastructure, key enzymes within mitochondrial-dependent metabolic pathways, including the tricarboxylic acid cycle and oxidative phosphorylation, are a hallmark of stem cell differentiation (13–15, 28). Collectively, these changes support oxygen-dependent catabolism of energy substrates, functionally manifested as an elevation in oxygen consumption, and reserve capacity to promote accelerated ATP production (13, 15, 52, 53, 112). This transition to mitochondria-dependent metabolism occurs at the expense of glycolysis, by which glycolytic gene expression and metabolic flux is concomitantly suppressed during differentiation (13–15, 28, 112). Acquisition of oxidative phosphorylation-dependent metabolic phenotype supports an elevated ROS state, due to a greater mitochondrial-dependent ROS generation in tandem with potential suppression of scavenging systems through reduced metabolic flux through the glycolysis/pentose phosphate pathway and downregulation of NADPH generation. A comparison of embryonic stem cells and their differentiated progeny, using high throughput metabolomics, has defined a higher degree of metabolite saturation (fewer carbon–carbon multiple bonds) on differentiation into neuronal or cardiac lineages (108). Generating energy from metabolites requires their oxidation, which implies that more reduced metabolites would enable generation of greater amounts of energy to support specialized functions of differentiated progeny. Supplementation of differentiation medium with saturated metabolites promotes dif-

ferentiation, while enhancing levels of unsaturated fatty acid through inhibition of the eicosanoid pathway impairs lineage specification (108). These metabolic changes were associated with changes in redox status as demonstrated by changes in key antioxidant levels, including ascorbic acid and the ratio of reduced to oxidized glutathione (108).

ROS exposure leading to accumulated damage affects stem cell maintenance proficiency and functional differentiation capacity. In this context, hematopoietic stem cells can be stratified in high versus low subpopulations based on a fluorescent ROS probe, 2,7-dichlorofluorescein diacetate (DCF-DA) (49). The fraction with high ROS content demonstrates an impaired reconstitution capacity in contrast to the low ROS fraction. Beyond reconstitution capacity, ROS abundance also defines which lineages these progenitor subpopulation can differentiate to, with the high fraction preferentially differentiating into myeloid lineage, a phenotype associated with aging of the stem cell population. This phenotype can be rescued by treatment with antioxidants, rapamycin, or inhibitors of p38 mitogen-activated protein kinase, which suppress ROS levels.

Metabolic conversion observed during *in vitro* stem cell differentiation may reflect an evolutionarily optimized process. Indeed, *in utero* embryonic development is guided by distinct changes in cellular microenvironment. The pre-implantation embryo transitions from a relatively hypoxic environment of the oviduct and uterus, and during initial implantation, to a gradually more oxygen and substrate-rich environment supported by vasculogenesis/angiogenesis and exchange with the maternal circulation (85, 102), which coincides with development of specialized tissues. However, these observations are not universal, as low oxygen concentrations have been suggested to promote differentiation into specific lineages that usually reside in hypoxic niches within the body, such as embryonic, mesenchymal, and adult stem cell differentiation into chondrocytes (1) and neural stem cells into dopaminergic and serotonergic neurons (19, 102). In addition, some specialized cells types may preferentially utilize glycolysis despite significant oxygen availability, such as endothelial cells, which enables them to transfer more oxygen to perivascular cells, protects them from low oxygen availability during vessel sprouting, and minimizes ROS production (20).

Initial studies in *Drosophila* demonstrated that increasing ROS production beyond basal levels through deletion of *SOD2* or suppression of the ND75 subunit of complex I in multipotent hematopoietic progenitors induced premature differentiation into mature blood cells; meanwhile, reducing ROS through overexpression of ROS scavenger proteins GTPx-1 or catalase impairs differentiation (69). Indeed, expression of GTPx-1 can counteract the impact of ND75 disruption, providing a causal link between ROS production and the differentiation phenotype. Consistent results have been observed during differentiation of human mesenchymal stem cells into adipocytes, by which reduction in ROS through use of mitochondrial-targeted antioxidants MitoCP/MitoCTPO, or knockdown of the Rieske Fe-S protein of complex III impaired adipocyte lineage specification (95). The differentiation propensity of mesenchymal stem cells can be rescued through either enhancing downstream H_2O_2 production or activating PPAR γ 2, indicating that adipocyte differentiation may be dependent on a signaling axis initiated through

generation of mitochondrial-derived superoxide, and propagated by conversion to hydrogen peroxide and activation of the PPAR γ 2 transcriptional network (95). However, a recent study did not observe an increase in ROS production at day 7 of adipocyte differentiation, but observed an increase in the antioxidants catalase and SOD, potentially indicating that ROS generation is critical during early differentiation before antioxidant enzyme expression can be upregulated (112).

ROS signaling also appears to modulate stem cell function and development *in vivo*. The mitochondrial permeability transition pore (mPTP), a nonselective conduit below 1.5 kDa in the inner mitochondrial membrane, regulates mitochondrial metabolism and ROS generation, which significantly impacts cardiomyocyte maturation and differentiation in the early embryo (35, 47). Day 9.5 embryo (e9.5) cardiomyocytes display a greater mPTP open probability associated with few immature mitochondria displaying lower mitochondrial membrane potential and higher ROS generation compared with e13.5 cardiomyocytes, where mPTP is closed. Promotion of early mPTP closure at e9.5 promoted structural/functional maturation of mitochondria and led to cardiomyocyte differentiation, while artificially sustained open probability of mPTP impairs subsequent development (47). Consistent with a downstream consequence of mPTP opening/closing on ROS production, a reduction in ROS using antioxidants at e9.5 promoted cardiomyocyte differentiation, while supplementation with stable antioxidants impairs differentiation (47). Selective deletion of the mitochondrial transcription factor A (TFAM) within keratinocytes reduces expression of electron transport chain subunits, leading to a reduction in oxygen consumption, superoxide generation, and DCF-DA fluorescence (43). This phenotype significantly impairs differentiation of isolated keratinocytes *in vitro*, which can be rescued through increasing hydrogen peroxide production, and, ultimately, leads to progressive loss of hair follicles, an epidermal barrier defect, and premature death with a median survival of only 13 days (43). Loss of TFAM also impairs mitochondrial ATP production and although TFAM-deficient keratinocytes were able to meet the metabolic demands of proliferation through glutamine-dependent reductive carboxylation, it remains unclear whether they can meet the very different metabolic demands associated with differentiation (33, 111), which could potentially account for the observed phenotype. In addition, these results are inconsistent with a previous report indicating that epidermal specific deletion of TFAM does not affect keratinocyte proliferation, overall epidermal differentiation or lead to an epidermal barrier defect, despite observing mtDNA depletion, absence of respiratory chain complexes, and growth retardation associated with short lifespan (5). The reasons for the differences between these studies remain unclear.

Concluding Remarks

Beyond traditional adverse effects of ROS accumulation, increasing evidence implicates redox status as a regulator of stem cell function. ROS appear to function in both a temporal and a concentration-dependent manner; with a low ROS state supporting stemness maintenance versus a moderate ROS state enabling stem cell differentiation. Indeed, stem cells minimize reactive oxygen generation through adoption of nonoxidative metabolic phenotypes and deploying glycolysis/

pentose phosphate pathway-dependent antioxidant scavenging. In contrast, on differentiation, oxidative metabolism and ROS are quickly upregulated and induce ROS-dependent signaling, which is critical for lineage specification. Although ROS and stem cell function are well correlated, the molecular mechanisms by which ROS regulate cell fate remain to be elucidated. Therefore, future studies are required not only to map the time and concentration-dependent changes in energy metabolism and ROS but also to define the molecular mechanisms by which ROS interact with stem cell fate regulators to understand the underlying basis of redox-dependent regulation of stem cell function, and, ultimately, enable the development of novel strategies that are aimed at modulating stem cell function and lineage-specific commitment.

Box 3: Stem Cell Glossary

- *Cell fate determination*: the process in which a previously undifferentiated cell is programmed to become a specific cell type by following a specified path during differentiation.
- *Differentiation*: the developmental-based process by which cells evolve from relatively generalized to specialized structures and functions.
- *Lineage commitment*: site of developmental divergence between specific cell lineages beyond which cells are committed to a specific cell fate.
- *Multipotent*: the ability to differentiate into a limited number of cell fates within a closely related family of cell types.
- *Pluripotent*: the ability to differentiate into any cell type within an organism except extraembryonic cells.

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Abbreviations Used

GPx = glutathione peroxidase
 GR = glutathione reductase
 H₂O₂ = hydrogen peroxide
 HIF-1 α = hypoxia inducible factor-1 α
 iPSC = induced pluripotent stem cells
 mGSH = mitochondrial-specific glutathione
 mPTP = mitochondrial permeability transition pore
 mtDNA = mitochondrial DNA
 NADH = nicotinamide adenine dinucleotide
 NADPH = nicotinamide adenine dinucleotide phosphate
 O₂[•] = superoxide
 OH[•] = hydroxyl radical
 ROS = reactive oxygen species
 SOD = superoxide dismutase
 TFAM = mitochondrial transcription factor A