HHS Public Access

Author manuscript

Free Radic Biol Med. Author manuscript; available in PMC 2023 February 09.

Published in final edited form as:

Free Radic Biol Med. 2021 June ; 169: 224–237. doi:10.1016/j.freeradbiomed.2021.04.022.

The FOXO signaling axis displays conjoined functions in redox homeostasis and stemness

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Abstract

Previous views of reactive oxygen species (ROS) depicted them as harmful byproducts of metabolism as uncontrolled levels of ROS can lead to DNA damage and cell death. However, recent studies have shed light into the key role of ROS in the self-renewal or differentiation of the stem cell. The interplay between ROS levels, metabolism, and the downstream redox signaling pathways influence stem cell fate. In this review we will define ROS, explain how they are generated, and how ROS signaling can influence transcription factors, first and foremost forkhead box-O transcription factors, that shape not only the cellular redox state, but also stem cell fate. Now that studies have illustrated the importance of redox homeostasis and the role of redox signaling, understanding the mechanisms behind this interplay will further shed light into stem cell biology.

Keywords

Pluripotent stem cell; Adult stem cell; Oxidative stress; Forkhead box O; Stemness; Reactive oxygen species

1. Introduction

Stem cells are essentially the raw materials of the body as they engender differentiated cells that are the building blocks of tissues and organs [1]. These unspecialized cells are present in embryonic, fetal, and adult phases of mammalian development. Interestingly, all stem cells do not behave in the same manner. Pluripotent stem cells (PSCs) can produce cell types from each of the three embryonic germ layers: the endoderm, mesoderm, and ectoderm [2–4]. The most prominently used PSCs in research are the embryonic stem cells (ESCs) that are derived from the inner cell mass of the mammalian blastocyst. The

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Declaration of interest

None.

newest addition to the PSC pool are the induced pluripotent stem cells (iPSCs). iPSCs are not derived from the embryo, but mimic ESCs in many properties [5–7]. Instead, these cells are *in vitro* engineered by 'reprogramming' adult mouse fibroblasts, which represent a fully differentiated cell, to a pluripotent state. This is achieved through co-expression of four genes: *Oct-3/4*, *Sox-2*, *c-Myc* and *Kfl4* under ESC culture conditions [5]. Research illustrated that iPSCs were synonymous to ESCs in their morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell markers, ability to differentiate into cells of the three germ layers, and telomerase activity [5,6].

PSCs differ from adult stem cells (ASCs) in that the latter can only differentiate into tissue-specific cells and are derived from adult tissues instead of the embryo but are not able to self-renew indefinitely. However, they are present throughout the life of the organism and produce committed progenitors that promote several functions of terminally differentiated cells thereby exhibiting the capacity to replace dying cells and restore damaged tissues.

No matter ASCs or PSCs, three defining features make stem cells inimitable. These are: (1) their ability to self-renew - a special kind of proliferation, which allows them to regenerate the stem cell pool, (2) their clonality – the fact that all progeny is ascending from a single cell, (3) and their potency – the ability to specialize into different cell types. The balance between self-renewal and differentiation remains a key area of study needed to fully understand the role of stem cells in development, disease, and medicine. This is invigorating because the regenerative nature of stem cells has been tightly linked to reactive oxygen species (ROS) (see Fig. 1) as shall be discussed here in depth.

2. Molecular control of stemness and differentiation induction

In the body, ASCs reside in so-called niches, in which cell-matrix and cell-cell adhesion factors (i.e. integrins), soluble signals and the orientation of the mitotic spindle determine their stemness state. Quiescent for most of their lifetime, ASCs re-enter the cell cycle in response to a variety of growth factors, chiefly among them WNT signaling [8–11].

However, unlike PSCs that have high cell cycle activity, ASCs are mainly in a quiescent state and spend most of their lifetime removed from the cell cycle in the G0 phase. Quiescence is achieved by high activation of prominent cyclin dependent kinase inhibitors (CKIs), such as $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which inhibit the binding of cyclin-dependent kinases to cyclins to block cell cycle progression. Deletion of any of these CKIs in hematopoietic stem cells (HSCs) initially leads to the expected impairment of self-renewal, a loss of quiescence, and high proliferation levels [12–14]. Long-term effects of CKI ablation, however, led to premature stem cell exhaustion [15]. Also, retinoblastoma (Rb) proteins are mostly inactive and as a result, cell division genes are primarily "off". Cells generated during the first few divisions after exit from the niche act as transit amplifying cells, which have a significant proliferative ability that is utilized to generate the many daughter cells needed for tissue homeostasis or repair. In culture and in the body, ASCs exhibit a limited lifespan and senesce after a pre-determined number of divisions, a state that is molecularly characterized by even higher levels of $p21^{cip1}$ and p53, accumulation of actin stress fibers and loss of differentiation potential [16].

Pluripotent cells, in turn, are found around the peri-implantation period and represent a transitory state only as their natural tendency in the embryo is to differentiate. In the lab, however, these cells exhibit an unsurpassed self-renewal potential coupled with an ability to differentiate into more than 200 different cell fates. These properties rest on a specialized cell cycle characterized by a shortened G1/S transition, continuously phosphorylated Rb and coordination of gene expression by a triad of transcription factors. Together, this triad composed of NANOG, OCT-4, and SOX-2 co-occupies many chromatin loci and is repressive on differentiation-associated loci and activating on pluripotency associated loci [17–19]. Thus, it is not surprising that the levels of these transcription factors control the exit from pluripotency.

For cells to preserve their pluripotency, they must self-renew not only through extensive proliferation, but also through the inhibition of differentiation [20]. In the culture dish, this is achieved through the addition of Leukemia Inhibitory Factor (LIF) [21,22], a cytokine belonging to the interleukin-6 family that binds to a heterodimeric receptor that is comprised of a LIF-receptor subunit and a glycoprotein 130 (gp130) subunit. Binding of LIF initiates a signal cascade that starts with activation of janus kinase (JAK) and ends with the dimerization of phosphorylated STAT3, which translocates to the nucleus to direct the regulation of a variety of genes required for pluripotency [23,24], making STAT3 a crucial and necessary component of the pluripotency machinery.

Unfortunately, an ‘unwanted’ LIF effect results from the activation of secondary kinase cascades down-stream of the gp130 subunit, which lead to the ultimate activation of extracellular signal-regulated kinase 1/2 (ERK1/2). This secondary pathway is linked to exit from pluripotency [25]. Thus, a dual activation of two separate arms of the LIF pathway marries the desired pluripotency maintenance effect to the inapt initiation of differentiation [24], which seems to explain the sometimes not negligible portion of murine ESCs that prematurely differentiate when cultured with LIF. Additional signaling molecules have since been identified that shift the balance to pluripotency including BMPs and WNTs [26–31]. Identification of a proper batch of serum that contains the correct levels of such additional growth factors helps with keeping murine ESCs in an undifferentiated state.

Also downstream of the LIF receptor is the activation of protein kinase B (AKT), an important survival kinase that participates in active cellular proliferation, an integral aspect of self-renewal. ESCs expressing a myristoylated, active form of AKT maintain pluripotency even in the absence of LIF [30]. While published studies generally investigate AKT without distinguishing between individual isoforms, there is an indication that not all AKT isoforms are equally involved. During iPSC generation, enhancing AKT1 and AKT2 promoted complete reprogramming mainly through increased activation of STAT3 in concert with LIF, and to a lesser extent, through promotion of colony formation. However, blocking AKT1 but not AKT2 expression prohibited cell proliferation and reprogramming [32].

Interestingly, once human ESCs (hESCs) had been derived, it became evident that there were differences between hESCs and murine ESCs (mESCs). At first, these differences were thought to be due to species-specific variation. However, it soon became apparent that human ESCs did not maintain pluripotency in response to LIF [4,33]. Instead, they

self-renewed upon addition of basic fibroblast growth factor (bFGF) and TGFβ/Activin A [34–37], culture additives typically used to culture murine epiblast stem cells (EpiSCs) [38,39]. Hence, the hypothesis formed that hESCs rather resembled a post-implantation pluripotent or epiblast-like state, which is nowadays referred to as “primed” pluripotency. On the other hand, mESCs derived from the ICM are referred to as “naïve” [40,41].

Transitory in the embryo, this naïve state can be propagated by combining LIF supplementation with an ERK inhibitor in the absence of serum in culture. While blocking the differentiation into the neuroectodermal lineage [42], ERK inhibition is not free from infelicitous side effects. Since ERK typically activates the ribosomal S6 kinase RSK which in turn catalyzes the phosphorylation and inactivation of the Bcl-2 pro-apoptotic family member protein BAD [43,44], ERK suppression consequently diminishes cell division. The result of ERK inhibition thus is a pluripotent cell that divides slowly. To resolve this issue a second kinase inhibitor is included in the so-called 2i + LIF medium [41], which targets glycogen-synthase kinase 3 beta (GSK-3β) to stabilize beta-catenin [45]. The beta-catenin target cyclin D1 [46,47] then ultimately moves the cell cycle forward. Subsequent work revealed that both murine EpiSCs and hESCs can be reprogrammed to revert into a more naïve state.

Studies pertaining to stem cell pluripotency and differentiation have mainly focused on gene networks and transcription factors, even though it is becoming more apparent that ROS in synchrony with metabolic pathways are also key regulators of stem cell identity to the effect that they not only control the switch between pluripotency and differentiation, but also the transition between naïve and primed states.

3. What are reactive oxygen species?

ROS are a group of highly reactive unstable oxygen containing molecules that readily react with other molecules in the cell. ROS can be demarcated into two different classes including nonradical and free radical ROS, the first class consisting of hydrogen peroxide (H_2O_2), ozone, peroxynitrite (ONOO-), and hydroxide and the latter of the prevalent superoxide anion ($O_2^{\bullet -}$), nitric oxide (NO), and HO [48]. In normal physiological conditions, the cell produces healthy levels of ROS. However, unregulated high levels of intracellular ROS can be detrimental and increase oxidative stress [49], a state in which the balance between free radicals, ROS, and endogenous antioxidant defense mechanisms is interrupted [50]. In simplistic terms, it is the disturbance in the equilibrium between oxidant-antioxidant states in which the oxidant condition is favored. Excessive ROS may oxidize nucleic and amino acids, carbohydrates, proteins, and lipids leading to damage to DNA, RNA, and proteins and ultimately senescence or apoptosis [51]. Therefore, the prevailing thought has been that ROS were detrimental as they played a vast role in stress and diseases [52].

As of late, however, the balance amongst oxidant and antioxidant species has been suggested to be beneficial to several cellular processes such as gene expression, protein translation, cellular signaling, and coordinating cellular activity in response to nutrients [53–55]. In stem cells, it is becoming more apparent that ROS homeostasis and changes in the redox state influence self-renewal and cell fate. Many studies on this topic suggest that the

amount of ROS present will determine the cellular destiny. For instance, low levels of intracellular ROS seem beneficial to perpetuate stemness and self-renewal. As such, low NO concentrations seem to increase expression of pluripotent markers *Nanog*, *Oct4*, and *Sox 2* and maintain stemness [56]. Mechanistically, this impediment to differentiation seemed dependent on activation of beta-catenin and PI3K/AKT, which is additionally consistent with the reinforcement of proliferation and self-renewal executed by these molecules.

Conversely, when provided with high concentrations of ROS, differentiation of hESCs into bi-potent mesendodermal cell lineages is promoted [57]. Similarly, high levels of NO induce differentiation as evident through the detection of early differentiation markers and repression of Nanog [58,59]. Indeed, as ESCs exit from pluripotency naturally they sharply increase the levels of endogenously generated ROS [60,61], a process that favors the generation of cardiomyocytes and osteoblasts [61–64]. Since this increase in ROS is accompanied by the expression of the pan-mesodermal T-Brachyury and supplementation with an NO donor can enhance T-Bra mRNA as well as the number of T-BRA positive cells [61], it is quite possible that NO augmentation shortly after pluripotency exit can support the generation of other mesodermally-derived cell types. While a concomitant up-regulation of beta-catenin nuclear activity is noted in this scenario also, this time it is elicited by high NO concentrations not low concentrations as in undifferentiated pluripotent cells. This evokes the notion of a reversed mechanistic response to ROS in pluripotent versus differentiating cells.

In point of fact, ROS and NO are implicated also in later differentiation events. For instance, increased levels of ROS augmented the expression of neuronal differentiation markers in NT2 cells, a pluripotent human embryonal carcinoma cell line, and human adipose tissue-derived multipotent stem cells demonstrating how increased levels of ROS may push differentiation towards a neural phenotype [65,66]. Our own group was able to demonstrate how exogenous NO augmented calcification when supplemented at the apt time during osteogenic maturation [64].

While it is becoming increasingly clear that ROS affect self-renewal as well as differentiation capacity of stem cells, the molecular underpinnings of such phenotypic responses remain under investigation. Understanding how oxidative stress affects numerous stem cells is imperative not only to better control stem cells in a dish, but also since oxidative dysregulation is attributed to disease states resulting from stem cells dysfunction, such as neurodegenerative, cardiovascular, and inflammatory diseases and cancer [67–72]. To further shed light on the molecules and pathways potentially implicated, we will next review sources of ROS and how cells typically balance their oxidant with their antioxidant capacity.

4. Energy metabolism as a source of ROS

Three major sources of ROS production come from the endoplasmic reticulum, the membrane-bound NADPH oxidase, and the mitochondria electron-transport chain, whereby the latter represent the principal source of ROS production amounting to approximately 90% of all intracellular ROS [55,73]. Hypoxanthine/xanthine oxidase, lipoxygenase and

cyclooxygenase and gamma-glutamyl transpeptidase epitomize further sources of ROS [60]. For a breakdown of intracellular locations of antioxidant systems and details of chemical reactions the reader is referred to a review by Kurutas [74].

Given that the majority of ROS are generated during mitochondrial aerobic energy production, it comes to no surprise that the preferential utilization of glycolysis over mitochondrial oxidative metabolism by stem cells may represent a mechanism to preserve genomic integrity by limiting ROS-mediated damage of nuclear and mitochondrial DNA as well as reducing oxidation of proteins and lipids [75]. In addition, PSCs are known to have immature mitochondria with lower levels of mtDNA and mitochondrial mass [76] again supporting the notion that the metabolic preference of the pluripotent stem cell is to glycolysis. It needs to be pointed out that glycolysis occurs in the presence of oxygen and is followed by lactate production, which prevents glucose from entering the TCA cycle [77,78]. Shared with cancer cells, this is known as the ‘Warburg effect’ and allows for the support of ATP generation without accumulation of high ROS levels supporting rapid cell growth and preventing differentiation (reviewed in Ref. [79]).

As pluripotent cells begin to differentiate, both in the embryo and *in vitro*, the mitochondria mature, increase in numbers, and a metabolic switch from glycolysis to oxidative phosphorylation (OxPhos) occurs [80–83]. Consequently, all somatic cells rely more heavily on OxPhos. Should these fully differentiated cells accumulate oxidative damage, the repercussions are insignificant as they have a limited lifespan and are shed periodically to be replaced by new cells.

During the conversion of somatic cells to iPSCs the reverse switch is observed and with interesting timing: the up-regulation of glycolysis precedes the activation of pluripotency markers [84–86]. While Shyh-Chang and colleagues [86] speculate that the timing of glycolysis reactivation suggests that glycolysis may not be specific to a pluripotent but rather to a rapidly proliferating cell, it is also possible that glycolysis is specifically selected as it may control pluripotency through mandating low ROS levels. This possibility seems pertinent given that ROS governs multiple transcription factors that not only play a role in ROS removal but have also recently been implicated in the governance of pluripotency.

Intriguingly however, not all pluripotent cells exhibit the same metabolic pattern. As such, the naïve ground-state pluripotency state distinguishes itself from the primed state through an alternate metabolic phenotype: metabolic flux in naïve ESCs is bivalent with both glycolysis and OxPhos being utilized for ATP generation, and mitochondrial respiration is reduced in primed pluripotency [87–89], as has also been proposed to occur *in utero* [88]. This may potentially explain the genomic instability often associated with the naïve state and the inability to stably culture naïve cells for prolonged periods of time [90].

Although ASCs represent a more committed population than PSCs, they also rely primarily on glycolysis. This state contributes to the protection of the cells from oxidative stress damage to promote lifelong renewal. For example, the most characterized ASC, hematopoietic stem cells, have low numbers of mitochondria, lower levels of oxygen consumption and intracellular ATP levels [91]. Specifically, as a variety of ASCs reside

in hypoxic niches, the reliance on anaerobic glycolysis rather than aerobic glycolysis to generate ATP is commonly seen [91–93]. Similar to the benefits of the PSC reliance on aerobic glycolysis is the minimal production of ROS needed to maintain ASCs in their quiescent state [94]. A deviance from the norm are the more recently identified $Lgr5^+$ columnar base cells in the crypt of the small intestine, which in contrast to the long known stem cells at position +4, mainly use OxPhos [92,95]. Their OxPhos is fueled by pyruvate that is provided by the adjacent Paneth cells and generates mitochondrial ROS. The differential reliance on OxPhos may potentially be explained by the difference in function between position +4 intestinal stem cells and $Lgr5^+$ CBCs, whereby the latter is rapidly dividing and participating in tissue homeostasis, and the former is highly quiescent and represents a population of 'reserve' stem cells that may divide only when the actively dividing CBCs are challenged, for example due to stress or injury [96–99]. It remains to be seen if such additional rapidly cycling stem cells also exist in other tissues and whether their reliance is more on OxPhos than on glycolysis.

Due to the relationship between metabolic flux and ROS formation laid out above, it is indisputable that variations to the concentration of glucose in the extracellular milieu influence stemness and differentiation. *In vivo* such milieu changes occur for example in diabetic patients and impact vascular and multiorgan function as well as bone micro-architecture [100–104]. Interestingly, newer studies have put forth the idea that the long-term side-effects of high blood glucose are mediated by stressed ASCs. For example, the long-term changes in the diabetic cellular microenvironment incite intrinsic dysfunction of the bone marrow stem cell niche ultimately resulting in mesenchymal stem cell (MSC) failure to mobilize and differentiate into osteoblasts [105]. This fits well with the *in vitro* evidence suggesting that hyperglycemia promotes MSC aging characterized by up-regulation of p53, actin stress fibers and loss of osteogenic differentiation potential [16].

In mESCs, culture in hyperglycemia elicited greater $O2^{\bullet -}$levels along with H_2O_2 than culture in normoglycemia, however, over time these levels declined back to baseline [106]. This may point to the efficient ability of PSCs to counteract their levels of oxidative stress. Nonetheless, the initial activation of c-jun N-terminal kinase (JNK) downstream of glucose-induced oxidative stress was sufficient to increase the expression of $p21^{cip}$ and $p27^{kip}$ and with that dampen the proliferative rate of the cells [106], despite the greater availability of nutrients.

Mechanistically, glucose stress may coax the mitochondrial electron transport chain into overproduction of $O2^{\bullet -}$ due to increased diversion of pyruvate into the mitochondria (reviewed in Ref. [102] (compare Fig. 2). This scenario is often observed in differentiated cells [107], which consequently would demand the action of an antioxidant mechanism that can defeat mitochondrial ROS, for instance superoxide dismutase 2 (SOD2), also known as manganese-dependent superoxide dismutase (MnSOD). Alternatively, plasma membrane-associated NADPH oxidases have also been described to couple glucose stress and $O2^{\bullet -}$ formation [108,109]. It may be possible that the latter mechanism is primarily activated in the response of mESCs to hyperglycemia as these cells make little use of OxPhos (Fig. 2). This remains speculative since not much research has been done on the topic. In point of fact, a recent paper suggests that deficiency in p38 MAPK in mESCs increases NOX2

levels which is accompanied by a rise in *Fgf 5*, an mRNA that associates with a primed pluripotency state [110]. However, p38 MAPK inhibition is exploited to return primed hESCs to a naïve state [111], which is either out of line with our argumentation or suggests that p38 holds a role in naïve cells that is independent of NOX expression or activity.

This leaves the following question to be answered: Why are naïve cells pluripotent and differentiating cells committing to a destined cell fate if they both use OxPhos? First, as mentioned above, naïve cells generate ATP both through OxPhos and aerobic glycolysis, while differentiating cells use mostly OxPhos. Secondly, differentiating cells contain a greater number of mitochondria and thus their capacity for respiration and generation of mitochondrial ROS seems higher, again exposing a potential role for oxidative stress in differentiation.

5. Prevention of oxidative damage

5.1. Maintaining a proper niche environment

The first line of defense to prevent oxidative damage is to avert contact with harmful external stimuli that may enter the cell and wreak intracellular havoc. ASCs achieve this by living in niches characterized by low oxygen tension (1%–8% O_2). This necessitates the use of anaerobic glycolysis as a mechanism for energy production resulting in the blockage of OxPhos, suppression of ROS generation, and the prevention of stress damage and differentiation [53,112–115] (for an illustration see Fig. 2). As the pre-implantation blastocyst develops under low oxygen conditions at approximating at 2–9% O_2 [116], it becomes important to mimic this microenvironment also when culturing PSCs *in vitro*. A variety of studies illustrate the significant benefit in terms of cell proliferation using low oxygen tensions [117,118]. As such, primed hESCs appear to grow more efficiently under low oxygen concentrations when compared to room air, even long-term [119,120] (Fig. 2). Oxygen conditions equivalent to what is found in the early embryo not only help to maintain pluripotency and reduce spontaneous differentiation in ESCs [119,121,122], but also to maintain two active X chromosomes, to preserve methylation status and improve chromosome stability [123–125]. Thus, low oxygen conditions have become mainstream for naïve stem cell generation and maintenance because of their stabilizing conditions [87,111,126,127].

5.2. Cell-endogenous defense mechanisms

A point of principle then is how cells may be capable of returning to a beneficial redox status in cases where cell-internal oxidative stress *cannot* be prevented. To return to the appropriate homeostatic balance stem cells will recruit antioxidants, activate their redox sensors and kinases, and ultimately shuttle transcription factors involved with such changes. Antioxidants may be endogenous or consumed with the diet, but share the common feature of being readily absorbable, capable of eliminating ROS and the free radicals generated from them, as well as function well in both aqueous and lipid (i.e. membrane) domains. Generally, there are two different antioxidant systems that multicellular organisms have developed: enzymatic or non-enzymatic, which work together to protect the cells against radical damage.

Those classified as non-enzymatic mechanism include the antioxidants Vitamin E and C, thiol (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, and natural flavonoids [74] and are often the substrates for the enzymatic reactions as they have the capability to cycle between oxidized and reduced states. Pretreatment with vitamin E in MSCs leads to increased survival in oxidative stress conditions due to increased scavenging of ROS [128]. In addition, vitamin E partially rescued an adipogenic differentiation defect otherwise noted in high levels of ROS [129]. In mESCs, addition of vitamin E and glutathione ethyl ester can squelch the rise in O2•⁻ registered in response to hyperglycemia and prevent the decline in cell proliferation [106]. The addition of ascorbic acid (vitamin C) into the normoxic culture of MSCs, which would otherwise induce senescence, promoted MSC proliferation [130]. Due to its reducing power, it is sometimes selected as culture additive specifically to mimic hypoxia or also with hypoxia, for example in naïve pluripotency media [89,111]. Not only does ascorbic acid exert a favorable effect on stemness but may be included into differentiation media: it enhances cardiomyocyte production and osteoblast yield from ESCs and iPSCs [6,131–134].

The major enzymatic mitochondrial defender against $O_2^{\cdot -}$ is SOD2. Situated in the mitochondrial matrix it functions to protect mitochondrial targets against $O_2^{\cdot -}$ produced on the cytosolic side of the inner mitochondrial membrane [135,136]. It converts the highly chemically reactive $O_2^{\cdot -}$ with its unpaired electron to hydrogen peroxide (H_2O_2) [53, 137]. Due to the high concentrations of mitochondrial superoxide dismutase, the mitochondrial O_2^- levels are kept at low steady state levels and only H_2O_2, which can permeate the mitochondrial membrane [138], may escape to the cytoplasm.

H_2O_2 can assume many fates, but one can be the conversion into highly toxic hydroxyl radical through the attainment of an electron [53]. If this is not regulated in a proper homeostatic manner, this could be problematic in stem cells. Luckily, cells have adopted numerous alternative ways to ameliorate unwanted H_2O_2. One of them is the conversion of hydrogen peroxide to harmless water and oxygen through catalase [139]. In mESCs, attainment of redox balance after glucose-induced oxidative stress is initiated by SOD2 and catalase [106], yet is accompanied by a reduction of cells in the S-phase of the cell cycle. In this context, when nutrient availability is higher than physiological, dampened proliferation seems the preferred and healthier state over a superproliferative outcome that potentially may become uncontrolled and turn into cancer (Fig. 3). In contrast, when nutrient availability is at the appropriate steady-state level, inhibition of cell proliferation through overexpression of SOD2 and catalase [140,141] might seem contraindicated, spurring the notion that ROS are needed as “life signals” that preserve cellular division [60].

H_2O_2 is also reduced by the glutathione peroxidase family of proteins (GPx) to water and lipid alcohols, respectively, with participation of reduced glutathione (GSH) which has been identified as an election donor. GSH, in turn, can be regenerated from its oxidized disulfide form (GSSG) by glutathione reductase (GR) using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. In PSCs glutathione reductase is increased throughout ESC differentiation [142]. However, it is not clear whether the increase

in GR expression precedes differentiation initiation, or whether GR activation is a byproduct of the switch to OxPhos.

Given its mechanism of action, GPx classifies as a “redox sensor”, a molecule which reads ROS levels. Such sensors possess highly conserved free cysteine (Cys) residues in which the functional thiol groups (RSH) are the targets of ROS that reversibly oxidize them to disulfides. In contrast to typical Cys residues, Cys targets of reversible modification routinely possess a low-pKa sulphahydryl group which supports susceptibility to oxidation [143]. They are recycled back to their original thiol states either through enzymatic or non-enzymatic dethiolation [144,145]. Classically, the level of Cys oxidation controls protein folding, as well as multimerization. For a detailed review of disulfide bond creation and biology the reader is referred to Ref. [146].

In addition to the GPx cycle, thioredoxin (Trx) is another classic redox sensor and part of the Trx system, which is comprised of enzymatic and non-enzymatic antioxidants. Trx also acts to reduce H_20_2 and oxidize proteins by reducing oxidized cysteines and cleaving disulfide bonds. In ESCs Trx was able to restore OCT4 DNA binding activity by reducing the cysteines in its DNA binding domain, indicating that these antioxidants play a role in maintaining stem cell pluripotency and in early embryonic development by maintaining proper OCT4 activity [147]. In addition, thioredoxin interacting protein (TXNIP) maintained HSCs and ROS levels by increasing p53 activity [148]. Further amplifying this point is that *Txnip* knockout mice showed a decrease in antioxidant enzyme expression elevating ROS levels and reducing HSC number.

Peroxiredoxin (Prx) can be identified as a thiol-specific antioxidant that is reduced to thioredoxin (Trx) after the reduction of peroxide forming oxidized Prx and water [149] and thus also acts as a modulator of the redox balance [150]. Prx has been linked to preservation of ESC stemness during neurogenesis by the suppression of ROS-sensitive signaling [151] again underlining the notion that increased ROS purport differentiation events. Like *Prx 1* regulation in ESCs, where levels drop with differentiation, the levels of another Prx isoform, *Prx6*, also decrease during osteogenic differentiation of human dental pulp stem cells [150]. Whether there is an ASC-specific Prx isoform that increases with differentiation as seen with Prx 2 in ESCs or Prx6 exerts a seemingly contrasting effect in ASCs still needs to be explored.

6. Transcription factors as redox sensors

The most prevalent of the redox sensors that contain modifiable cysteines are the antioxidant enzymes discussed above [152,153]. However, and importantly for the topic of this review, transcription factors, which have the ability to set new gene expression programs in motion that alter cell fate, may also contain redox-responsive cysteines and form disulfide double bonds. In the classical understanding of redox regulation, the oxidation of these cysteines should exchange the thiols to create disulfide bonds, moderating their dissociation from DNA. However, this is not always observed in stem cells. Instead, the actual ability of the transcription factors to bind DNA seems dependent on additional regulation through phosphorylation, availability of secondary redox sensors that modulate their oxidative state,

recruitment of histone modifiers and co-activators as well as overall cellular approach to generate energy. As such, H_2O_2 has been identified as the main ROS mediator of cellular signaling. It has the capacity to inhibit tyrosine phosphatases through oxidation of cysteine residues in their catalytic domain, which in turn activates tyrosine kinases and downstream signaling [154]. The de facto response thus is highly cell-context -specific and seems to vary between ASCs, PSCs and fully differentiated cells. As such, many transcription factors, including HIF-1α, Nrf2, STAT-3, p53, and NFκB are upregulated both by the hypoxic niche in ASCs and in response to ROS in PSCs [86,155–166]. The individual circumstances that might explain the transactivation state of these transcription factors seem to depend on their function as transcriptional repressors or activators, and the consequence of this regulation might be activation or repression of gene expression programs. To complicate matters further, these proteins cannot only be positively or negatively regulated by oxidative stress but act as redox sensors to inhibit ROS levels.

7. Governance of stemness is linked to forkhead box-O transcription factors

Transcription factors of the Forkhead box-O (FOXO) family have initially been reported to get activated in response to oxidative stress. They are the focus of this review since more recent reports also implicate them in development, differentiation, and maintenance of the stem cell state [167]. Intriguingly, their regulation through cysteine modification has not been fully elucidated for all family members, yet they participate in the resetting of the redox-state through transcriptional activation of antioxidant enzymes that lower ROS levels.

FOXO3a was the first of the FoxO family members to be implicated in the regulation of oxidative stress, followed by FOXO4 [168,169]. In humans, FOXO3a and FOXO4 have five cysteines, FOXO1 has seven, and FOXO6, identified as a neural-specific FOXO [170], has ten. Two cysteines in the four FOXOs are conserved: one is within a small, conserved region in the transactivation domain near the C-terminus. This cysteine is Cys 477 in FOXO4 which represents the main site for disulfide formation with acetyltransferase p300/cAMP-response element binding protein (CREB)-binding protein (CBP) [159,171]. The binding of these two cofactors with FOXO is necessary for downstream FOXO transcriptional regulation as p300 and CBP act to weaken histone DNA interactions for target gene expression [172]. In contrast, FOXO3a transcriptional activity is repressed by deacetylation with NAD-dependent deacetylase Sirt1 (Sirtuin 1) [173]. Of note, Sirt6 is a FOXO transcriptional target [174], representing an epigenetic feed-forward loop.

The FOXO targets important for ROS regulation are the ROS scavengers catalase, MnSOD, and Prx [106,175]. Upon irradiation or serum starvation, FOXOs can also promote expression of genes involved in DNA repair and cell cycle arrest such as DNA damage-inducible gene 45α (*GADD45α*), cyclin D2, and the cell cycle inhibitors $p21^{cip1}$ and $p27^{kip1}$, glucose metabolism (*Glc6p, Pepck*) and apoptosis (*Bim, FasL*) [106,176–181]. The decision which set of target genes to activate seems dependent on the type of insult, but also on the severity of the insult and what kind of co-factor binding is fortified. In conditions of weak oxidative stress, FOXO3a has been found to be captured by p53, suppressing FOXO3a

activity and leading to low levels of pro-apoptotic genes [182]. In turn, intermolecular interaction with p53 does not seem to affect cell cycle genes cyclin G2 and $p27^{kip1}$. Extensive oxidative stress disrupts the binding and restores the individual transactivation capacity of both transcription factors and - since expression of their pro-apoptotic gene targets is enhanced - seems to increase their affinity to such loci.

The other conserved cysteine, in turn, is likely implicated in regulation through AKT, since it is situated next to the threonine close to the N-terminus that is phosphorylated by AKT and part of the AKT consensus site [182]. Consequent to phosphorylation by AKT, controlled by the insulin signaling pathway and P13K, FOXOs are unbound from DNA and exported from the nucleus [175]. Additionally, phosphorylation of FOXO3a by the serum- and glucocorticoid-regulated kinase SGK [183] also causes cytoplasmic translocation [184]. Cytoplasmic FOXO proteins are then ubiquitinated and degraded in the proteasome [185]. In contrast, c-jun N-terminal kinase (JNK) phosphorylates FOXO4 at a distinct position to induce nuclear translocation [169,173]. FOXO3a also has such a potential JNK phosphorylation site in the N-terminus, which may promote nuclear translocation. In fact, our group was able to confirm that FOXO3a nuclear translocation in murine ESCs in response to glucose as shown in Fig. 4 is indeed mediated by JNK [106]. None of the other cysteines in the FOXOs are conserved between any of two family members, posing possibilities for differential regulation of FOXO family members through cysteine oxidation, which might help to explain some seemingly controversial observations in stem cells.

Among the FOXOs, FOXO1, FOXO3a, and FOXO4a have been shown to play a role in stem cell maintenance [181], with most studies focusing on the first two. FOXO1 and FOXO3 knockdown lead to differentiation of intestinal stem cells into goblet and paneth cells [186]. Similarly, HSCs deficient in all three FOXOs display uncontrolled ROS levels caused by decreased SOD1/3 and activation of $p53/p21^{cip1/waf1}$ leading to changes in HSC cell cycling, exit from quiescence, and eventually into arrest [187–189]. In neural stem cells (NSCs), FOXO1 knockdown increases neurogenic differentiation while constitutive activity inhibits differentiation [190]. Similarly, FoxO3 activity was also higher in NSCs undergoing self-renewal rather than differentiation. Additionally, ablation of FoxO3 in mutant mice led to a decrease in the NSC population. Interestingly, without FoxO3, the NSCs potentially lose capability to re-enter quiescence after they divide. This could result in an increase in progenitors and the exhaustion of the NSC pool *in vivo* [191]. Lastly, isolation of NSCs from $FoxO3^{-/-}$ mutant mice illustrated the downregulation of genes involved in oxidative stress resistance (*Selenbp1*), and in glucose metabolism and transport (*Pdk1*, *Slc2a3*) [191]. All these studies conclusively proof that the role of FOXOs is to maintain ASC stemness (Fig. 4).

However, the control of cell cycle genes or maintenance of the redox balance does not seem to exhaustively describe FOXO action. In an elegant study, Webb and colleagues conducted a ChiP-seq analysis in NSCs, which for the first time identified an additional cluster of FOXO3a controlled loci that demarcate a neural phenotype and confirmed the expression of the associated genes in activated NSCs and primary mouse neural progenitor cells [192]. Furthermore, binding of FOXO3 and Achaete-scute homolog 1 (ASCL1), a proneural

transcription factor activated in response to Notch inhibition, was seen at enhancers of genes involving Wnt and Notch signaling, which both have roles in NSC maintenance and homeostasis [193–195]. Their results thus suggest another alternative mechanism for modulation of FOXO3a action by a secondary transcription factor. While the fact that FOXO3a shares loci with ASCL1 and inhibits expression of a group of ASCL1 neurogenic targets could be interpreted to mean that FOXO3a is repressive on those loci, the authors instead conclude that FOXO3a affects ASCL dependent transcription rather than binding to such target genes [192]. The repressive nature of FOXOs, potentially dependent on co-factors, thus remains likely, but needs to be experimentally proven.

Interestingly, FOXO1 and FOXO3a seem to behave differently from each other in PSCs, which consequently portends that one of them is oppositely regulated in PSCs than in ASCs (Fig. 4). This seems to be FOXO1. First, FOXO1 is highly expressed in undifferentiated primed H1 hESCs, while FOXO3a expression was negligible [160]. Secondly, FOXO1 knockdown did not significantly regulate proliferation or apoptosis and did not provoke changes in expression of FOXO targets involved in antioxidant or stress response. However, FOXO1 did regulate pluripotent gene expression [160]. Specifically, ChIP revealed that endogenous FOXO1 bound to sequences within the regulatory regions of *OCT4* and *SOX2*. However, the cells were not stressed with any insult that would increase oxidation to activate FOXO, therefore the conclusion that in undifferentiated hESCs FOXOs do not function as redox regulators cannot be ultimately drawn.

Indeed, both FOXO3a and FOXO1, while expressed at the mRNA and protein level, are mostly cytoplasmic in mESCs [106] (Fig. 4). In response to glucose-induced oxidative stress, mESCs shuttle both FOXO3a and FOXO1 into the nucleus (Fig. 4). In the nucleus, FOXO3a and FOXO1 bind to the transcriptional co-activator beta-catenin (CTNNB1), which is also enriched in the nucleus upon glucose stimulation. The more abundant FOXO3a/CTNNB1 complex then stimulates $p21^{cip1}$, $p27^{kip1}$ and *Sod2* transcription [106] explaining the ability of the cells to recuperate from oxidative stress. Our so far unpublished results further suggest that glucose-induced oxidative stress comes with a down-regulation of *Oct4* and *Nanog* mRNA and acquisition of a more committed, potentially primed, state (unpublished observation). Thus, we speculate that in the antioxidant process nuclear FOXO3a may enhance its occupancy at pluripotency-associated promoters and contribute to their repression, but this hypothesis needs to be experimentally confirmed.

It is quite possible that the differential activity and localization of FOXOs observed in hESCs and mESCs hinges on the distinct pluripotent states these cells represent, which in turn are controlled by distinguishable redox states and divergent signals. Especially given that only FOXO3a, but not FOXO1 seems to bear cysteines within the DNA binding domain that are modulated in dependence of redox state [170], the differential contribution of either transcription factor to the glucose-dependent phenotype will also not surprise.

7.1. Upstream FOXO regulators with roles in stemness

7.1.1. AKT and JNK—AKT, as the negative regulator of FOXO, can also undergo redox modification. During oxidative stress, a disulfide bond between Cys 297 and Cys 311 can enhance dephosphorylation which will lead to loss of AKT activity [196]. Upstream of AKT

is phosphatase and tensin homolog (PTEN), which - depending on the redox state of the cell - can stimulate AKT activity to negatively regulate FOXOs. The catalytic cysteines within the active site of PTEN can undergo oxidation upon exposure to ROS and this modification can potentiate AKT activity [197,198].

As mentioned earlier, JNK is the main kinase that mediates FOXO nuclear shuttling in response to ROS. JNK achieves FOXO nuclear entry through phosphorylation of FOXO3 at Ser 574 and FOXO1 at Thr447 and Thr451 [199,200]. JNK also phosphorylates the same threonines on FOXO4, which disrupts the interaction with 14–3–3 proteins allowing for FOXO nuclear entry [169,201,202]. Activation of FOXO by JNK can lead to upregulation of antioxidant genes needed to control ROS levels as well as of those involved in apoptotic signaling. Aside from FOXOs JNK can phosphorylate BCL-2 to prevent premature senescence in both tumor and normal cells [203]. In response to DNA damage, JNK phosphorylates p53 and also stabilizes p73 leading to the expression of Bax and Puma pro-apoptotic molecules [204,205].

As further piece of evidence supporting the hypothesis that FOXO3a activation would induce differentiation, JNK activity has been shown to control exit from pluripotency. However, this might entail proliferation separately from differentiation inhibition. Current evidence suggests it might be the JNK2 isoenzyme that negatively controls proliferation: Wild-type and *Jnk1*$^{-/-}$ mESCs showed similar proliferation rates whereas *Jnk2*$^{-/-}$ and *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mESCs proliferated more rapidly than the wild-type [206]. Indeed, this is in line with our findings that causally relate ROS to JNK activation, subsequent FOXO1/3a nuclear localization, p21^{cip1}/p27^{kip1} transcriptional activation and decelerated proliferation [106]. Despite not having distinguished between different JNK isoforms, but considering the studies of our colleagues, it seems feasible to speculate then that this effect was mediated by JNK2.

The relationship between JNK activation and stem cell proliferation seems less clear in ASCs and one can find examples for positive and negative regulation. For example, JNK inhibition by JNK–IN–8, which inhibits all three isoenzymes, increased self-renewal of human HSCs [207], whereas gut specific JNK1 activation stimulated the cell cycle in intestinal stem cells and differentiation [208]. Such divergent response is not easily reconciled as both effects were moderated by c-JUN phosphorylation [207,208].

As these examples exemplify, JNK function can be through FOXO or other transcription factors, most relevant such of the AP-1 transcription factor family. Intriguingly, the selection of co-factors seems to also dictate the end outcome of JNK activation regarding stem cell differentiation: *Jnk1*$^{-/-}$ and *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$, but not *Jnk2*$^{-/-}$ mESCs display elevated *Sox 17* and *Hnf1* gene expression, both of which are crucial for differentiation of definitive and visceral endoderm, respectively [206]. Likewise, when treated with JNK inhibitor II (SP600125) and JNK inhibitor III (inhibits JNK/c-Jun), hESCs displayed decreased *Oct4* and *Nanog* expression [209], suggesting that JNK signaling has a role in maintaining the capacity to differentiate.

While this has not been experimentally investigated, JNK may further dictate stem cell state through its regulation of metabolism. Challenging HeLa cells with high concentrations of pyruvate increased the activity of JNK1, but not JNK2, via enhanced ROS production. The ROS-JNK1 axis then activated the ribosomal kinase p70S6K, which normally represses glycogen synthase kinase-3β. The result is increased activity of glycogen synthase, which converts glucose to glycogen, effectively sending the oversupply of nutrients into storage rather than into the mitochondria [210]. In cortical neurons, activation of JNK3 represses pyruvate metabolism in mitochondria and promotes pyruvate conversion to lactate in the cytosol [211]. Interestingly, JNK may also act to promote glycolysis in conditions of severe stress that require life and death decisions through the activation of phosphofructokinase-1, a key enzyme that catalyzes glycolysis, via phosphorylation of Bad (a BH3-only pro-apoptotic Bcl-2 family protein) [212]. In all noted examples glucose or glucose by-products are diverted from the mitochondria to promote glycolysis. Thus, it is conceivable that activation of JNK in PSCs could promote a shift from naïve pluripotency to primed pluripotency, since the latter cells increase their glycolytic rate over the former.

7.1.2. AMPK and mTOR complexes—Another link between energy production, stemness and FOXO activation is through AMP-dependent protein kinase (AMPK). AMPK is a key regulator of energy metabolism; its activity is regulated by a plethora of physiological conditions, many anti-diabetic drugs as well as lack of glucose availability. Under glucose starvation, with low levels of ATP, AMPK is activated to decrease anabolism and increase catabolism [213, 214]. While AMPK acts as the main sensor of AMP concentrations [2015], it can also be positively regulated by ROS levels. Exposure to H_2O_2 can result in the oxidation or S-glutathionylation of the cysteine residues in the α- and β-subunits of AMPK, which leads to an increase in kinase activity [216,217]. This then, based on the argumentation above, would suggest that activated AMPK would be detrimental to stemness and induce differentiation.

However, multiple examples shall be listed in support of the opposite. In HSCs, some level of AMPK is active, which can lead to FOXO activation to maintain the stem cell at low ROS levels and promote self-renewal [218,219]. Under glucose deprivation, AMPK can inhibit the activity of mammalian target of rapamycin (mTOR), which positively regulates protein synthesis [218,220], a cellular process that is in high demand as cells change their identity during differentiation [221]. The resulting decrease in protein and ribosome synthesis reduces ROS levels [220,222]. Indeed, the opposite activation of mTOR led to enhanced mitochondrial biogenesis, which together with ROS accumulation, caused a loss of HSC quiescence [218,219]. Specifically, increased ROS strengthen the interaction between mTOR and regulatory-associated protein of mTOR (Raptor), which complex together in mTOR complex 1 (mTORC1), in consequence of which ROS levels and oxidative stress increase further [218,219]. This feed-forward loop may be contributing to the reinforcement of the exit from self-renewal and preventing a reversion back to the stem cell state once differentiation has begun.

A similar phenotype is found upon deletion of TSC1 (tuberous sclerosis 1), which normally represses mTORC1 signaling. Due to increased mitochondrial biogenesis proliferation of long-term mouse HSCs increased, which eventually compromised hematopoiesis [218].

Excessive mTOR signaling has also been shown to cause adult epidermal stem cell exhaustion and progressive hair loss in mice [223]. Similarly, loss of LKB1 (serine/threonine protein kinase 11), which is upstream of AMPK in differentiated somatic cells [224], results in a domino effect in which AMPK activity, quiescence, and long-term repopulating HSC pools are lost, ultimately impairing hematopoiesis [187,225,226]. Together these examples manifest that some level of AMPK activation and inhibition of mTORC1 may benefit ASC stemness.

However, relatively little is known about whether AMPK hardwires nutrient supply, survival and differentiation in PSCs. Since mTORC1-mediated protein translation needs to be tightly controlled to prevent exit from pluripotency, one might speculate AMPK activation to be beneficial also for PSC self-renewal. Indeed, AMPK activators such as AICAR and metformin increase mRNA expression of pluripotency markers and decrease mRNA expression of differentiation markers in murine ESCs [227], an observation that we have confirmed in our laboratory (unpublished). Pursuant to the notion that AMPK activation might correlate to a less committed naïve pluripotent state, another study found that AMPK may enhance OxPhos and mitochondria biosynthesis [228,229]. However, crosstalk between metabolism, ROS, and cell fate is something that needs to be further clarified. For example, AMPK can directly and indirectly post-translationally modify histone tails to regulate the activity of histone deacetylases and histone acetyl transferases by controlling substrate availability [229], making clear that it is not only the network of redox-sensitive kinases and transcription factors that may control stem cell state in response to oxidative stress, but that additional layers of epigenetic regulation are at play that have not been considered here.

AMPK activation is often coupled with inhibition of AKT phosphorylation [230]. This inverse relationship between the two spawns from the differential activation of a secondary mTOR complex, mTOR complex 2 (mTORC2), in which mTOR associates with Rictor, and which is inversely regulated than mTORC1. In fact, down-regulation of mTORC1 induces a feedback regulation to enhance PI3K signaling, thus indirectly activating mTORC2 [231]. AMPK may therefore exert its effects on proliferation/differentiation through its inverse relationship with AKT/FOXO in an mTORC1 mediated fashion. However, this is not in line with our findings that report higher AKT activity levels in mESCs cultured in physiological glucose concentrations [106], which would suggest a positive correlation between AMPK and AKT. Indeed, such positive relationship does exist in somatic cells in conditions of energetic stress, where AMPK has been shown to associate directly with mTORC2, phosphorylating mTOR at Ser^{1261} and increasing activity of the complex toward AKT [232]. The hallmark of mTORC2 activation is increased phosphorylation of AKT at Ser 473. It is quite possible that PSCs lend themselves to this stress mechanism to control stemness and differentiation. The latter scenario would also explain how AMPK activation and FOXO nuclear exclusion could co-exist in PSCs, while AMPK is described to directly enhance FOXO3 transcriptional activity by recruiting CBP and p300 in other cells [167,215].

In conclusion, we would like to mention another interesting thought that may pertain to FOXO regulation and could potentially extend to all redox-sensitive transcription factors. Due to their nuclear localization and nuclear exit signals, they have the ability to shuttle in and out of the nucleus. While some are simply non-active when in the cytoplasm, others

may translocate to many diverse cellular compartments and fulfill separate roles depending on their location. For example, mitochondrial p53 participates in apoptosis induction [233], while in the cytoplasm it is susceptible to degradation [234] and binds to DNA in the nucleus. The concentrations of some redox regulators however, i.e. glutathione and Trx, are greater in the nucleus than in the cytoplasm [235–237], especially in times of stress [238,239]. Entry of the redox-sensitive FOXOs into the nucleus, therefore, may enhance their reduction and therewith DNA-binding ability, ultimately fine-tuning their response to ROS. Similarly, redox-sensitive cysteine residues may directly be involved in nuclear export, as has been shown for Cys 199 in human Nrf2 [240].

8. Conclusion

It is becoming increasingly apparent that the mechanism behind stem cell renewal is not solely reliant on the nucleus but also the crosstalk between the mitochondria and the nucleus, with redox sensors, redox-sensitive transcription factors and kinases relaying the message. Therefore, connecting metabolic pathways, ROS levels, and stem cell maintenance remains an important area to study. The exact mechanism of ROS mediated stem cell renewal clearly seems oppositely regulated in adult stem cells versus pluripotent stem cells, with even additional differences between naïve and primed PSCs. Full network analyses of the possibly involved molecules introduced above, coupled with a thorough investigation of the metabolic cell state as well as the redox state of the involved proteins would help shed light on the fascinating interplay between redox homeostasis and stem cell fate. Many times, neither their complete absence nor their full-on activation seemed beneficial to stemness. What also did become clear is that the induction of differentiation, loss of stemness or alteration of proliferative capacity repeatedly seems to be an undesired byproduct of the cell’s fight against oxidative stress and its attempt to return to a balanced redox state. It will be interesting to see if some stem cells will ultimately cycle between reduced and oxidized states, in cases where the initial trigger persists, and the kinetics of the differentiation event do not outpace the redox-cycling.

Acknowledgements

We apologize to the many authors whose wonderful work we could not cite here due to space constrains. NzN is funded by an RO1 from the National Institute of Dental and Craniofacial Research, grant no RO1DE025330. None of the authors have any conflict of interest to declare.

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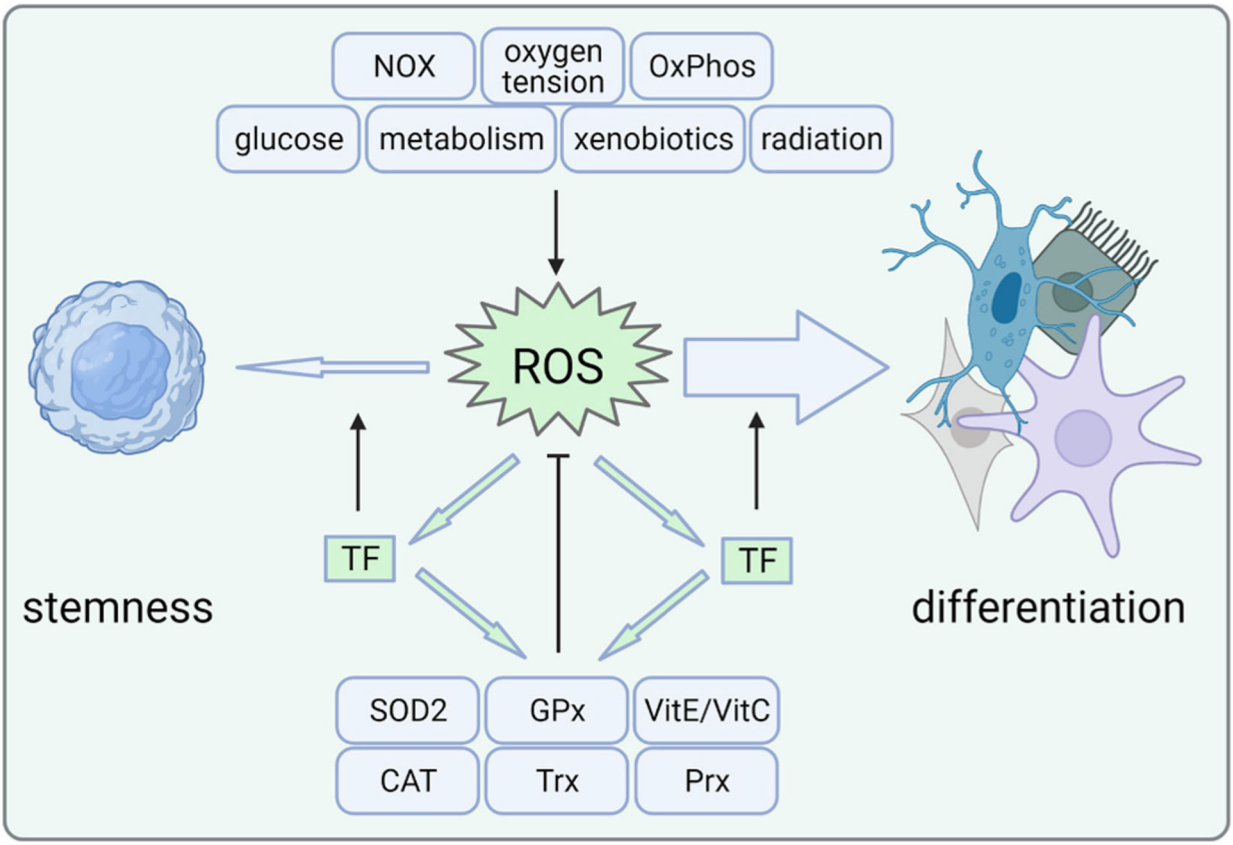

Fig. 1. ROS levels govern stemness and differentiation.

ROS can be generated by several endogenous processes or external insults. In a healthy steady-state, stem cells seem to possess numerous antioxidant mechanisms that balance their redox state including enzymes that act as redox sensors and non-enzymatic antioxidants. When there is an excess production of ROS relative to the cellular antioxidant defense, differentiation is initiated, possibly mediated by redox-sensitive transcription factors. These in turn may act to increase the cellular defense against oxidative stress, however may also modulate the expression of stemness-associated genes. CAT, catalase; GPx, glutathione peroxidase; NOX, NADPH oxidase; OxPhos, oxidative phosphorylation; Prx, periredoxin; ROS, reactive oxygen species; SOD, superoxide dismutase; TF, transcription factor; Trx, thioredoxin; Vit, vitamin. Created with BioRender.com.

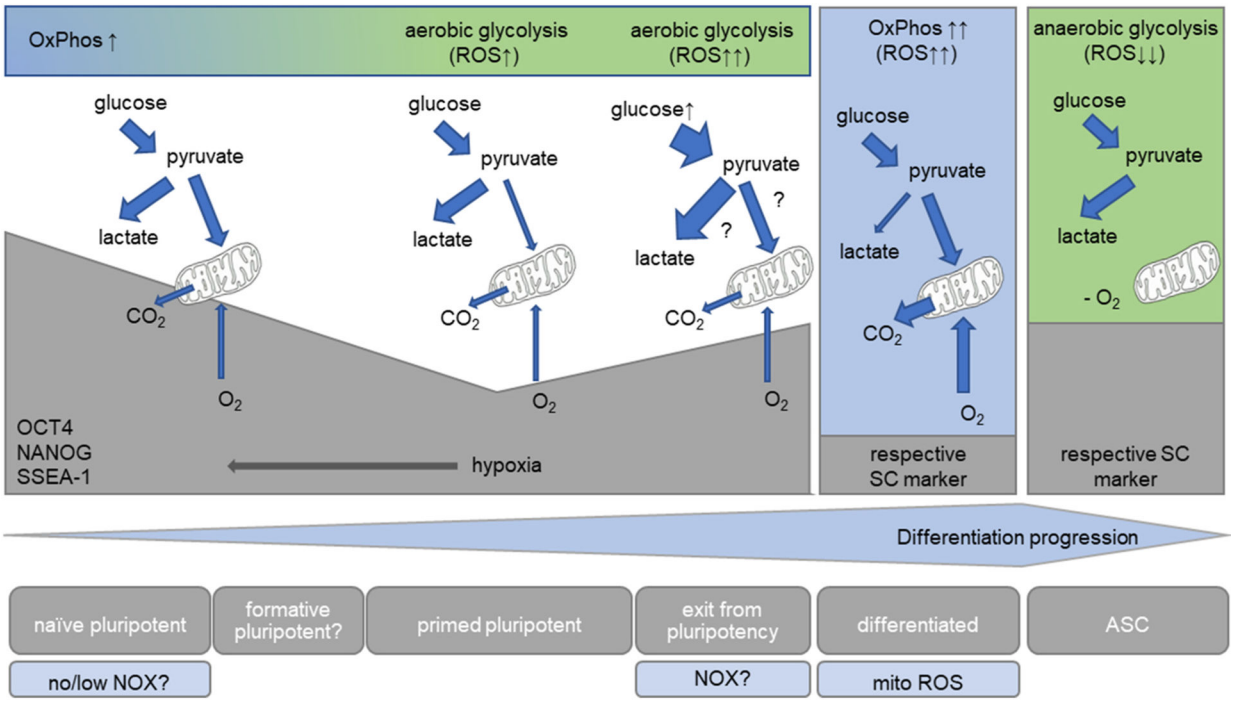

Fig. 2. Energy metabolism and stem cell fate.

Since they are located in hypoxic niches, ASCs conduct glycolysis in the absence of oxygen, which seems to contribute to their self-renewal and potency. In contrast, differentiated cells primarily use OxPhos, a process which generates mitochondrial ROS (by means outlined in the main text), suggesting that an increase in ROS may contribute to the loss of stemness and could be necessary for differentiation. Primed PSCs, in turn, have oxygen at their disposal, but still prefer glycolysis, which, compared to differentiated cells, keeps ROS levels low. This contrasts with naïve pluripotent cells: both mitochondrial respiration and glycolysis generate ATP. Due to the lower numbers of mitochondria found in PSCs compared to differentiated cells, ROS may originate from NADPH oxidases. In serum + LIF cultured murine ESCs, which represent a mixed population of primed and naïve pluripotent cells, glucose stress increases the available pyruvate, which either may be converted into lactate or fed into the mitochondria. The glucose-induced increase in $O2^{\bullet -}$ may thus either result from NOXs or might be mitochondria-mediated. Similar to ASCs, an increase in ROS seems associated with a deviation form the stem cell state. For details see the text. ASC, adult stem cell; NOX, NADPH oxidase; OxPhos, oxidative phosphorylation; ROS, reactive oxygen species; SC, stem cell.

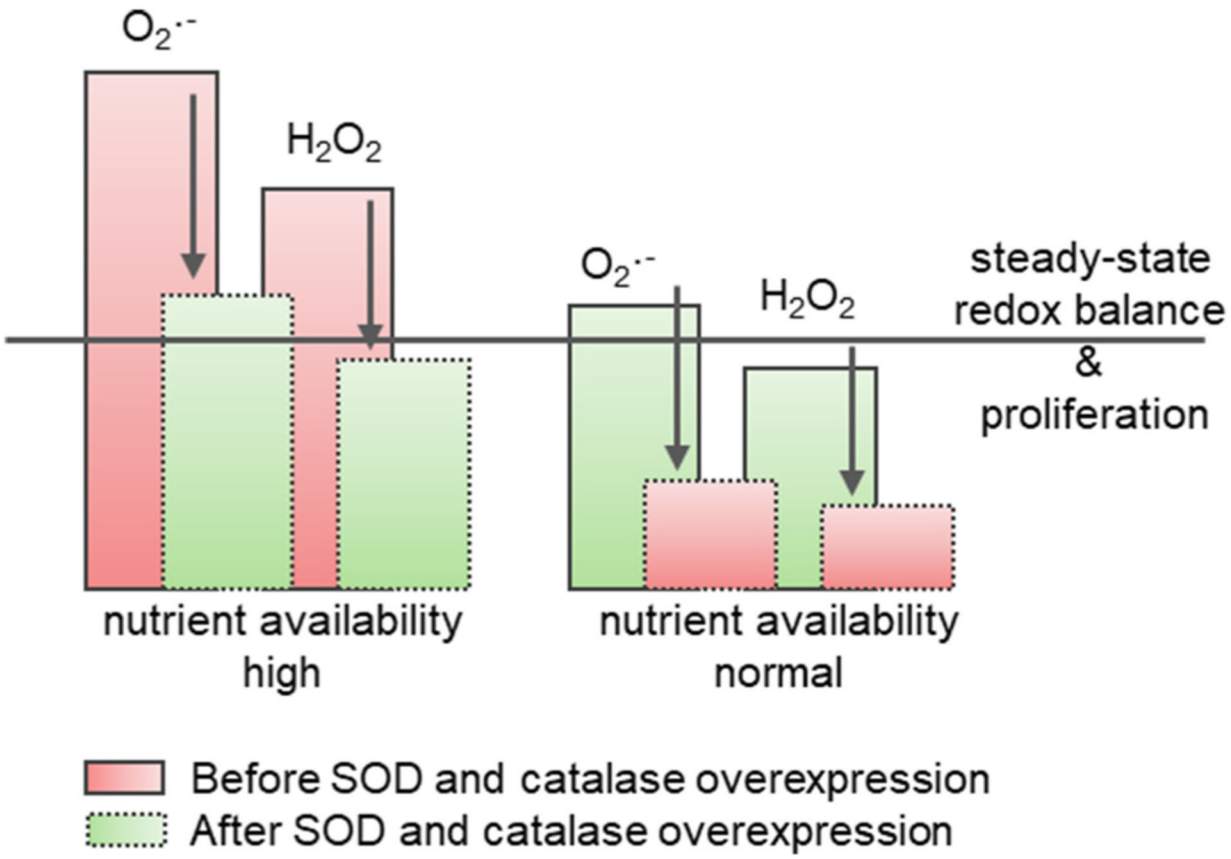

Fig. 3. Outcome of SOD and Catalase enhancement depends on cellular redox state.

In cells with high steady-state levels of ROS, either due to a specific cellular metabolism or trigger-induced, overexpression of *Sod* and *catalase* rescues redox-balance and reduces proliferation to healthy levels. In contrast, the reduction of proliferation stemming from their overexpression in cells with healthy redox-state negatively impacts survival.

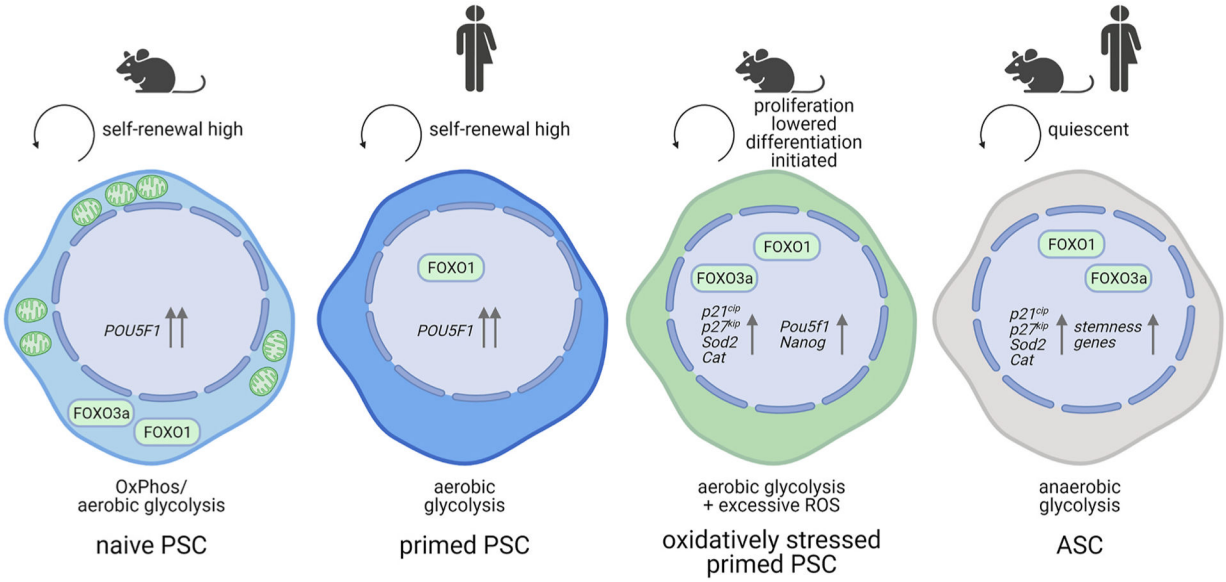

Fig. 4. Cellular redox state and FOXO activity status seem differentially regulated in PSCs versus ASCs.

Quiescent ASCs exhibit high FOXO1 and FOXO3a activity [190,192]. In turn, FOXO3a and FOXO1 are mostly cytoplasmic in murine ESCs selected for naiveté, but both shuttle to the nucleus when oxidatively stressed [106]. In primed hESCs, however, nuclear FOXO1, not FOXO3a, was shown to transactivate the OCT4 gene, *POU5F1*. ASC, adult stem cell; OxPhos, oxidative phosphorylation; PSC, pluripotent stem cell. Created with BioRender.com.