Redox regulation of cell state and fate

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ABSTRACT
The failure in effective cancer treatment is thought to be attributed to a subpopulation of tumor cells with stem cell-like properties. These cancer stem cells (CSCs) are intimately linked to tumor initiation, heterogeneity, maintenance, recurrence and metastasis. Increasing evidence supports the view that a tight redox regulation is crucial for CSC proliferation, tumorigenicity, therapy resistance and metastasis in many cancer types. Since the distinct metabolic and epigenetic states of CSCs may influence ROS levels, and hence their malignancy, ROS modulating agents hold promise in their utility as anti-CSC agents that may improve the durability of current cancer treatments. This review will focus on (i) how ROS levels are regulated for CSCs to elicit their hallmark features; (ii) the link between ROS and metabolic plasticity of CSCs; and (iii) how ROS may interface with epigenetics that would enable CSCs to thrive in a stressful tumor microenvironment and survive therapeutic insults.

1. Redox homeostasis and signaling
Reactive oxygen species (ROS) is a collective term used to describe oxygen-containing, chemically reactive molecules [1]. The tight control of ROS generation and elimination is of paramount importance to normal and cancer cells since ROS (low to moderate levels)-mediated cell signaling can significantly impact a variety of cellular pathways, including cell growth, differentiation, survival and angiogenesis [2–4]. Interestingly, cancer cells and CSCs appear to have distinct redox profiles, with CSCs exhibiting redox patterns that are more similar to normal stem cells. We will briefly touch upon the basics of redox homeostasis and signaling, and refer the reader elsewhere for a comprehensive review of this topic [5–7].

1.1. ROS generation
The mitochondria is the primary endogenous source of ROS in mammalian cells as ROS is a by-product of oxidative phosphorylation (OXPHOS) [8]. The enzyme complexes of the electron transport chain, mainly complex I and complex III, leak free electrons which drive the mono-electronic O₂ reduction to superoxide (O₂•⁻), that is rapidly reduced by superoxide dismutases (SODs) to H₂O₂ (a non-radical ROS) [9,10]. Simultaneously, by the well-described Fenton reaction, Fe²⁺ and H₂O₂ can react with each other to yield -OH radicals [11]. The NADPH oxidase (NOX) family of membrane-bound enzymes represents another major endogenous source of ROS [12]. All members of the NOX family are able to drive the NADPH-dependent reduction of O₂ to O₂⁻ [12,13]. In addition to O₂⁻, NOX4, dual oxidase 1 (DUOX1) and DUOX2 generate regulated levels of H₂O₂ [14,15]. Other endogenous sources of ROS include enzymes such as oxidases (e.g. xanthine oxidase) and oxygenases (e.g. cytochrome P450), peroxisomal oxidative metabolism and oxidative protein folding in the endoplasmic reticulum [16–18]. Lastly, ROS is also produced by exogenous agents, including chemotherapy, radiation, heavy metals (or metal complexes), atmospheric pollutants, chemicals, drugs and xenobiotics [19,20].

The current paradigm is that cancer cells generate higher levels of ROS than normal cells due to the activation of oncogenes, inactivation of tumor suppressor genes, aberrant metabolism, mitochondrial malfunction, inflammation or genotoxic stress [3,21,22], and this is compensated for by a more robust antioxidant system (Fig. 1) [23,24]. Consequently, cancer cells have a lower buffering capacity against disruptions in ROS levels [23,25].

1.2. ROS scavenging
When ROS production exceeds the activity of antioxidant defense, oxidative stress ensues and this is associated with many disease states, including autoimmunity and cancer [5,11]. Optimal ROS scavenger...
systems are required to keep ROS levels in check and include enzymatic antioxidants such as SODs, catalases, thioredoxins, peroxiredoxins, glutathione peroxidases, p38-mitogen-activated protein kinases (MAPKs) and various sirtuins (SIRTs) [26–30]. Non-enzymatic antioxidants such as glutathione (GSH), vitamin C (ascorbate), vitamin E (tocopherols) and polyphenols, also act directly on oxidative agents [31].

1.3. Redox signaling

The deployment of ROS in cell signaling is known as redox signaling [32]. At redox-sensitive amino acid residues such as cysteine and methionine, ROS can oxidize cellular proteins to allosterically change their conformation and function [33,34]. With a longer half-life than other ROS agents, H2O2 acts as a second messenger for intracellular signaling through cysteine-based modifications [35], while other ROS agents, including O2•− and •OH, are more associated with cellular damage [7].

Redox sensors detect changes in ROS levels and initiate an appropriate cellular response that culminate in antioxidant responses, gene transcription, differentiation, cell growth, cell proliferation and apoptosis [33,36]. Several transcription factors have established roles in redox sensing, including members of the forkhead box O (FOXO) family, hypoxia inducible factors (HIFs), kelch-like ECH-associated protein 1 (KEAP1) with nuclear factor erythroid 2 (NRF2) and the p53 tumor suppressor. The direct and indirect effect of ROS on these molecules have been widely reported and reviewed [37–41]. Diverse enzyme families are also amenable to redox modulation, including kinases such as AKT kinases, MAPKs, ataxia-telangiectasia mutated (ATM), and mammalian target of rapamycin (mTOR), as well as phosphatases like phosphate and tensin homolog (PTEN) and SIRTs [25,33,41,42]. The downstream pathways of these enzymes are often involved in the mediation of ROS levels, for instance, through modulation of transcription factors (e.g FOXOs) [38,43].

2. Redox regulation in cancer stem cells

Cancer is a disease of heterogeneity at the genetic, phenotypic and functional levels. CSCs represent a subpopulation of cancer cells with robust self-renewal capacity, multipotency and tumorigenic potential, and contribute to tumor heterogeneity by perpetuating themselves and generating various differentiated progenitors (Fig. 2). The CSCs are widely associated with various clinical hallmark features of cancer, including therapy resistance, tumor recurrence, invasiveness and metastasis [44]. An emerging theme is that CSCs are not homogeneous even within the same tumor; rather, they may be heterogeneous in their cell cycle, metabolic and redox profiles, which may explain for the lack of a congruent correlation between ROS levels and CSC function from various studies. Here, we will summarize the findings that highlight the importance of redox regulation in supporting the hallmark features of CSC and attempt to crystallize a coherent view from these studies.
Robust self-renewal/proliferation and tumorigenic potential

CSCs are thought to possess robust self-renewal and tumorigenic potential, which is often assessed by the ability of CSCs to generate tumor xenographs (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the ROSlow, CD44+ CSCs are highly enriched in leukemia-initiating cells without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions. In T-cell acute lymphoblastic leukemia, the tumor xenograft (in vivo) or tumorspheres (in vitro) either with or without limiting dilutions.

In hepatocellular carcinoma (HCC), disulfiram treatment reduces CSC marker expression, tumorsphere formation and tumorigenicity in xenograft experiments in a ROS-p38 MAPK pathway-dependent manner [48]. These suggest that liver CSCs may prefer a lower ROS cellular environment. Indeed, liver CSCs reduce mitochondrialOXPHOS and ROS production through NANG0 that is in turn regulated by the Toll-like receptor 4 (TLR4)-E2F1 axis [49]. Notably, paraturin (an inducer of ROS) treatment or NANG0 silencing decreases tumor formation and suppression of JNK pathway [47]. In hepatocellular carcinoma (HCC), disulfiram treatment reduces CSC marker expression, tumorsphere formation and tumorigenicity in xenograft experiments in a ROS-p38 MAPK pathway-dependent manner. In hepatocellular carcinoma (HCC), disulfiram treatment reduces CSC marker expression, tumorsphere formation and tumorigenicity in xenograft experiments in a ROS-p38 MAPK pathway-dependent manner. 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In summary, the most compelling evidence is that ROS levels are lower in leukemia and liver CSCs when compared to non-CSCs, and this is required for the proliferation/self-renewal and tumorigenicity of CSCs. More rigorous experiments that take into account the potential cell cycle and redox heterogeneity of CSCs, as well as cancer subtype differences will be necessary to evaluate if ROS levels may directly impact CSC activity in other cancer types.

2.2. Therapy resistance

The role of dysregulated ROS levels in CSC therapy resistance is well supported in numerous studies. In AML, the ROSlow, quiescent leukemic cells exhibit CSC properties and overexpress B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein [54]. The inhibition of Bcl-2 increased mitochondrial ROS levels, decreased GSH levels and selectively eliminated therapy-resistant, quiescent CSCs. In colorectal cancer, ROSlow CSCs with low proteasome activity are enriched after radiation and chemotherapy treatment [55]. These CSCs overexpress EID3 which upon depletion overcomes therapy resistance of colorectal cancer cells. In HCC, chemotherapeutic drugs or radiation treatment invariably result in the enrichment of CSCs with a ROSlow profile that is accompanied with either the ability to reduce ROS-induced DNA damage after genotoxic insult, increased GSH levels or MAPK/Pi3K activation [56–58]. Importantly, the inhibition of CD13 (a liver CSC marker) or treatment of CD133+ CSCs with sulfasalazine (a potent xCT inhibitor) overcomes resistance to chemotherapeutic and radiation treatment by increasing ROS levels in the resistant cells [56,58,59]. In pancreatic adenocarcinoma, ROSlow CSC are also implicated in radioresistance [60,61]. Glutamine deprivation or the inhibition of non-canonical glutamine metabolism sensitizes pancreatic CSCs to radiation treatment in vitro and in tumor xenograft experiments via intracellular ROS accumulation [60].

Stem cell maintenance pathways can also contribute to therapy resistance. For example, radiation induces the expression of Jagged-1 and intracellular Notch-ICD in CD24+/low/CD44+ -enriched breast CSCs, indicating the activation of the developmental Notch1 signaling pathway [62]. These breast CSCs exhibited higher radioresistance and lower ROS levels (suggesting higher reactive species scavenger levels) than non–breast CSCs. Collectively, all the above studies converge to a
of extracellular matrix components [63]. An emerging view is that ROS modulate the CSC phenotype, including increased migratory capacity, in-vitro tumorsphere formation [77,81]. Luo et al. [64] later showed that knockdown of DUOX1 decreases ROS production and enhances stem-like properties and invasiveness of a gemcitabine-resistant pancreatic cell line, which suggested a metabolic switch of the OXPHOS-dependent CSCs.

In pancreatic cancer, 2-deoxy-D-glucose or H$_2$O$_2$ treatment enhances cytotoxicity of gemcitabine, and suppresses CSC (including expression of CSC markers) and EMT phenotypes (including EMT marker expression and migration) of a gemcitabine-resistant pancreatic cell line, which could be reversed by N-Acetyl cysteine (NAC) treatment [65]. In lung cancer, CD24low CSC express low levels of DUOX1 when compared to the CD24high non-CSC [66]. Silencing DUOX1 (which presumably decrease H$_2$O$_2$ levels) increases CSC frequency, mesenchymal gene expression, tumor invasiveness and resistance to tyrosine kinase inhibitors.

In HCC, the treatment of liver cancer cell lines with transforming growth factor-beta (TGF-$eta$) increases the expression of mesenchymal markers and CD13, and tumorigenicity [67]. Higher ROS levels and stem cell maintenance gene (BMI1 and Notch1) expression are reported in the CD13$^+$/N-cadherin$^+$ cells than the CD13$^+$/N-cadherin$^-$ cells, reinforcing the idea of CSC and hence ROS heterogeneity. In general, the majority of studies have revealed a trend implicating low levels of ROS and the EMT phenotype of CSCs.

### 3. Metabolism and redox in CSCs

The reciprocal crosstalk between redox balance and metabolism has been gaining attention due to their implications in malignant progression and therapy resistance in cancer [23]. Recent studies support the view that the metabolic state of CSCs differ between cancer types, subtypes of the same cancer and even cycling stages within the same tumor [44,54,68,69]. To add on to this complexity, the CSCs readily switch their metabolic profile according to their needs (i.e. metabolic plasticity) [68]. An interesting study by Sancho et al. [70] demonstrated metabolic heterogeneity within pancreatic CSCs by characterizing a pre-existing pro-glycolytic subpopulation of CSCs with enhanced metformin resistance. Treatment with metformin resulted in the expansion of the pro-glycolytic subpopulation, which suggested a metabolic switch of the OXPHOS-dependent CSCs.

In addition, secondary metabolic processes such as glutaminolysis, fatty acid oxidation (FAO) and one-carbon metabolism, can be activated in CSCs as additional means of energy generation, contributing to the complexity of CSC metabolism [6,71–75]. Chen et al. [49] demonstrated that NANOG contributes to HCC progression in mice by repressing OXPHOS activity and mitochondrial ROS generation, while activating FAO to support CSC self-renewal and drug resistance [49,76]. Notably, the restoration of OXPHOS activity and inhibition of FAO renders CSCs susceptible to sorafenib, highlighting a potential strategy to combat chemoresistance of HCC.

In basal-like breast CSCs, pro-glycolytic metabolic reprogramming decreases ROS production and enhances stem-like properties and in vitro tumour sphere formation [77]. Luo et al. [64] later showed that mesenchymal-like breast CSCs (M-BSCs) similarly prefer glycolysis and are sensitive to glycolysis inhibitor treatment. They overexpress several antioxidant enzymes such as mitochondrial SOD to counteract excessive ROS production.

### Table 1

Identified metabolic phenotypes for various cancers.

| Types of cancer | Metabolic processes involved | Effect on ROS levels and CSC state and fate | Ref. |
|-----------------|-----------------------------|--------------------------------------------|------|
| Breast CSCs     | Glycolysis                   | Low FBP1 expression in basal-like breast cancer promotes glycolysis while suppressing OXPHOS, thereby reducing ROS levels and maintaining CSC population. | [77,81] |
|                  | Glycolysis and OXPHOS       | Mesenchymal-like breast CSCs have enhanced glycolysis and require a low level of ROS to maintain their quiescent state. | [64] |
| Brain CSCs      | OXPHOS FAO                  | Induction of H$_2$O$_2$ and O$_2^\cdot$ generation in glioma stem cells occurred through electron transport chain activation. | [83] |
| Colon CSCs      | Glycolysis                   | The colon CSC secretome is enriched in proteins involved in glycolysis and gluconeogenesis, and have enhanced antioxidant networks, suggesting that the maintenance of low ROS levels contributes to their intrinsic drug resistance. | [86] |
| Leukemia CSCs   | OXPHOS                      | In acute myeloid leukemia, ROSlow CSCs are defined by quiescent cell cycle status, low energy production and Bcl-2 overexpression. However, these CSCs are paradoxically dependent on OXPHOS. Bcl-2 inhibition suppresses OXPHOS and increases mitochondrial ROS. | [54] |
| Liver CSCs      | Glycolysis and FAO           | In HCC, CSCs with repressed ROS generation have increased glycolysis and FAO accompanied by lower OXPHOS. | [49] |
| Ovarian CSCs    | OXPHOS                      | More stem-like CD44$^+$/CD117$^-$ ovarian CSCs contain higher levels of H$_2$O$_2$ than CD44$^+$/CD117$^+$ cells. The epithelial ovarian CSCs privilege OXPHOS and inhibition of the mitochondrial respiratory chain induces cell death. | [87] |
| Pancreatic CSCs | Glycolysis                   | In gemcitabine-resistant pancreatic CSCs, the up-regulation of glycolysis and maintenance of low ROS promotes stemness, EMT and therapeutic resistant phenotypes. | [65] |
|                 | Glutamine metabolism        | ROSlow CSCs are reliant on the non-canonical glutamine metabolic pathway and glutamine deprivation significantly inhibited CSC self-renewal and sensitizes CSC to irradiation. | [60] |

It is noteworthy that the maintenance of low ROS levels does not always correspond to a preference for glycolysis. Lagadinou et al. [54] demonstrated that leukemia CSCs have characteristically low levels of O$_2^\cdot$, but are surprisingly reliant on BCL-2-mediated OXPHOS for
survival and maintenance of a quiescent, stem-like state. A summary of how specific metabolic processes may influence CSC state and fate in a variety of cancer types is shown in Table 1. Notwithstanding the complexity of CSC metabolism and ROS regulation, this area clearly warrants further investigation as the modulation of CSC metabolism represents an attractive and actionable avenue for novel CSC therapeutics.

4. ROS, epigenetics and CSCs

The acquisition of a stem cell-like phenotype in cancer cells is often accompanied by the accumulation of driver mutations in a wide range
of epigenetic regulators, resulting in the uncontrolled self-renewal and repression of cellular differentiation of the resulting cells [88,89]. Indeed, epigenetic alterations can affect the expression of metabolism and antioxidant defense genes, deregulating ROS levels that are conducive for CSC growth. In basal-like breast cancer, the Snail-G9a-DNMT1 complex decreases ROS levels by repressing fructose-1,6-bisphosphatase transcription, increasing CSC-like characteristics [77]. In AML, TXNIP is downregulated due to PRC2-mediated gene silencing [90]. Disruption of PRC2, either by 3-Deazaneplanocin A (DZNep); a histone methyltransferase inhibitor) treatment or EZH2 knockdown, reactivates TXNIP, inhibits TXN activity, increases ROS and apoptosis of leukemia CSCs. Moreover, ROS can directly alter the expression and thus activity of DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs) or microRNAs (miRNA) [91–93], resulting in gene expression changes that may or may not be compatible with CSC function.

MiRNAs are an emerging class of epigenetic regulators which control gene expression at the post-transcriptional level through mRNA translation and stability [94–96]. They play critical roles in fine-tuning translation and stability [94–96]. They play critical roles in fine-tuning transcription, increasing CSC-like characteristics [77]. In AML, TXNIP is downregulated due to PRC2-mediated gene silencing [90]. Disruption of PRC2, either by 3-Deazaneplanocin A (DZNep); a histone methyltransferase inhibitor) treatment or EZH2 knockdown, reactivates TXNIP, inhibits TXN activity, increases ROS and apoptosis of leukemia CSCs. Moreover, ROS can directly alter the expression and thus activity of DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs) or microRNAs (miRNA) [91–93], resulting in gene expression changes that may or may not be compatible with CSC function.

5. Future outlook

According to the CSC model, stem cell-like cancer cells with the greatest proliferative and tumorigenic potential, reside at the apex of cellular hierarchy, leading to the promise that the eradication of CSCs should reduce cancer relapse and treatment failure. Given the emerging view that ROS levels can impact CSC stemness, metabolism and epigenome, ROS modulating agents may have efficacy in anti-CSC therapy. Indeed, there are a variety of strategies that exploit ROS perturbations, genome, ROS-modulating agents may have efficacy in anti-CSC therapy.

Indeed, there are a variety of strategies that exploit ROS perturbations, genome, ROS-modulating agents may have efficacy in anti-CSC therapy.

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