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Reactive oxygen species produced by altered tumor metabolism impacts cancer stem cell maintenance

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ABSTRACT

Controlling reactive oxygen species (ROS) at sustainable levels can drive multiple facets of tumor biology, including within the cancer stem cell (CSC) population. Tight regulation of ROS is one key component in CSCs that drives disease recurrence, cell signaling, and therapeutic resistance. While ROS are well-appreciated to need oxygen and are a product of oxidative phosphorylation, there are also important roles for ROS under hypoxia. As hypoxia promotes and sustains major stemness pathways, further consideration of ROS impacts on CSCs in the tumor microenvironment is important. Furthermore, glycolytic shifts that occur in cancer and may be promoted by hypoxia are associated with multiple mechanisms to mitigate oxidative stress. This altered metabolism provides survival advantages that sustain malignant features, such as proliferation and self-renewal, while producing the necessary antioxidants that reduce damage from oxidative stress. Finally, disease recurrence is believed to be attributed to therapy resistant CSCs which can be quiescent and have changes in redox status. Effective DNA damage response pathways and/or a slow-cycling state can protect CSCs from the genomic catastrophe induced by irradiation and genotoxic agents. This review will explore the delicate, yet complex, relationship between ROS and its pleiotropic role in modulating the CSC.

1. Introduction to cancer stem cells

Stem cells are a self-renewing population at the apex of tissue hierarchy due to their capacity for multilineage differentiation [1–3]. Their maintenance within tissues is tightly regulated for proper homeostasis and repair. Quiescence and proliferation are regulated through intrinsic and extrinsic mechanisms where quiescence (G_0) is a poised, slow to non-dividing cell cycle state that allows for survival and longevity to maintain tissue homeostasis [4,5]. Upon exiting G_0 into a proliferative state, stem cells can undergo symmetric or asymmetric division to respectively generate similar or different (for example one progenitor and one more differentiated cell) progeny. This cycling of quiescence to proliferation is tissue dependent as certain tissues need to be replenished more readily than others [5–7]. Since stem cells are plastic in nature, protecting this population from continual stimulation or long-term damage from environmental stimuli, such as oxidative stress, is critical for normal physiology. Although we will briefly consider redox regulation in normal stem cell homeostasis throughout this review, we will not be able to fully explore this particular topic since it extends beyond the scope of this review. In Chakrabarty and Chandel's review [8], they provided a current understanding of reactive oxygen species (ROS), particularly hydrogen peroxide and superoxide, and the importance of maintaining low endogenous ROS in order to maintain normal stem cell potency and self-renewal. Importantly, extrapolating key findings for oxidative stress in stem cell regulation can draw parallels to better understand cancer [8].

This fundamental understanding of stem cell biology is relevant to cancer as disease maintenance is postulated to involve a malignant stem cell-like population known as the cancer stem cell (CSC) [9–13]. The presence of a self-renewing population in cancer could contribute to explanations of disease dormancy, metastasis, and recurrence. Much like their normal stem cell counterparts, CSCs are thought to have some degree of hierarchical ordering, with a subset of stem cell marker positive cells being capable of symmetric or asymmetric division. CSC division could potentially maintain the CSC pool, generate more proliferative progenitors, and/or generate differentiated progeny. Unlike their normal stem cell counterparts, CSCs have the genetic alterations present in the tumor from which they are derived and can initiate tumors in immunocompromised mouse models (for this reason, they are also known as tumor initiating cells). While the CSC hierarchy may not

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be as well-ordered as in normal tissues due to plasticity, information from single cell RNA-sequencing will continue to assist with identifying CSC signatures and subsets [14–21]. Important for our discussion here, especially as ROS can have distinct effects depending on disease status, we are referring to CSCs that are present within an existing tumor. While it is true that stem cells can acquire mutations to initiate tumors, CSCs discussed here are not necessarily the origin of the disease. However, the ability of CSCs to maintain and propagate the disease in animal models has directed much interest in this population as we try to reassess therapeutic strategies.

1.1. Reactive oxygen species in cancer and potential roles for NRF2 in cancer stem cells

Aberrant redox states are present in cancer cells due to differences in production and scavenging of reactive species [22–24]. ROS are oxygen-containing molecules considered a metabolic consequence of cellular respiration with volatile, deleterious properties when in excess [23,25]. While ROS are comprised of several species, each driving different malignant features, our understanding of ROS has been relatively limited to a handful of species with prominent roles in signal transduction [23,26]. ROS include both non-radical and radical (containing a free, unpaired electron) subtypes, with hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) being among the most well characterized examples. Sources of cellular superoxide include activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and complexes I-III of the mitochondrial electron transport chain [27–31] [Fig. 1, figures generated in Biorender (https://biorender.com)]. However, endogenous ROS can be introduced by environmental factors, including radiation, drugs, toxins, and lifestyle [32]. Under normal physiological conditions, hydrogen peroxide is kept at low nanomolar concentrations with localized activity involving reversible oxidation of thiols within proteins. Redox-regulated thiols, including cysteine thiols affected by H_2O_2, alter protein structure and function to regulate global cellular processes such as the cell cycle, migration, and angiogenesis [23,24,33]. These same biological processes are critical in cancer where cellular adaptation to environmental stressors producing ROS provides a survival advantage.

Reactive species can serve as secondary messengers with controlled timing, location, and concentration [25]. ROS elevation, especially superoxide and its downstream product, hydrogen peroxide, is common in cancer cells and can be caused by and help to further promote altered cell signaling [23,24]. As an example using pathways known to be important for CSC maintenance [34–37], platelet-derived growth factor (PGDF) and epidermal growth factor (EGF) bind to their respective receptor tyrosine kinases (RTKs) and transient increases in hydrogen peroxide inhibit phosphatase activity that would revert RTK induction [38–43]. The induction of PDGF or EGF signaling leads to cell proliferation and increased nutrient uptake [44] (Fig. 1). Intuitively, the increased metabolism resulting from these processes leads to increased superoxide production from NOX and the mitochondrial electron transport chain [44,45]. Unfortunately, ROS overall also oxidizes, and thus inactivates, the phosphatases and tumor suppressors that reverse this process, allowing the growth signal to continue [24,46].

Several systems and enzymes are in place to control the increased ROS production, like superoxide, hydrogen peroxide and hydroxyl groups, that occur in response to the dynamic processes of metabolism. There is a diverse family of antioxidants that specialize in the breakdown of different reactive species, and antioxidant research in cancer has demonstrated their significance in disease maintenance and progression [47,48]. Throughout the cell, NADPH is frequently utilized for metabolic activities due to its high reducing potential [45,47]. Although NADPH is an excellent reducing agent, its subsequent oxidation by NOX produces superoxide radicals [49]. Superoxide can be reduced by

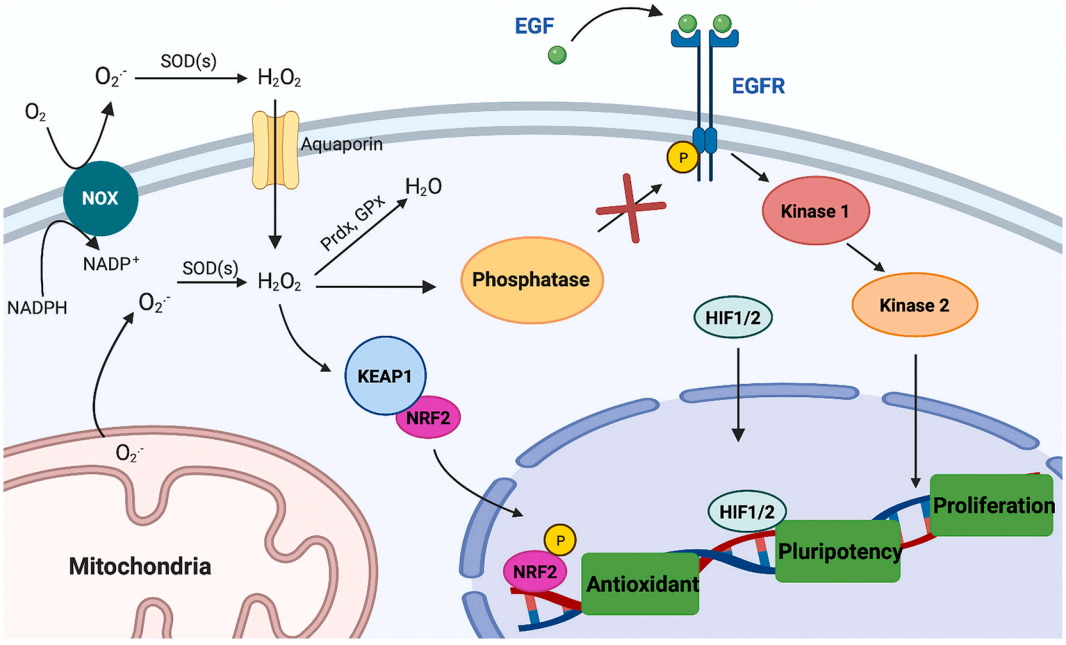

Fig. 1. The malignant role of reactive oxygen species (ROS).
ROS such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) are produced from various cellular processes, including NADPH oxidase (NOX) and oxidative phosphorylation in the mitochondria. Superoxide dismutase (SOD) converts $O_2^{\cdot-}$ into H_2O_2, which can then be used in protein functional group modification (not shown) or converted into H_2O by peroxiredoxin (Prdx) or glutathione (GPx). H_2O_2 also reversibly inactivates tyrosine phosphatases, which catalyze phosphate removal from cell membrane receptors, including epidermal growth factor receptor (EGFR). Growth-related genes and pathways are thus elevated, contributing to the uncontrolled proliferation that is characteristic of malignancy. Increased H_2O_2 leads to dissociation of nuclear factor erythroid 2-related factor 2 (NRF2) from Kelch-like ECH associated protein 1 (KEAP1), stabilizing NRF2 and allowing it to increase transcription of antioxidants. NRF2 and/or NRF2 target genes have been shown to be elevated in CSCs. Hypoxia inducible factors (HIF1α and HIF2α) can be stabilized by hypoxic conditions or ROS and translocate into the nucleus to activate HIF target genes. Hypoxia and HIF are important regulators of cancer cell growth, angiogenesis, metastasis and pluripotency.

superoxide dismutases (SODs) to H_2O_2, which is important in cell signaling pathways [23,33,47,50]. H_2O_2 can be further converted to water by peroxiredoxins (Prdxs), catalases, and glutathione peroxidases (GPxs, Fig. 1). GPxs are part of the glutathione antioxidant system which is a major regulator of cellular redox state, with glutathione (GSH) being at the millimolar level and thus the highest cellular non-protein thiol. In the GSH antioxidant system, GSH is used by GPX to reduce H2O2 and forms glutathione disulfide (GSSG). GSSG is then converted back to GSH by glutathione reductase, using NADPH. Thus, the GSH to GSSG ratio is a classic readout of redox status. Together, these pathways ensure that the signaling capabilities of hydrogen peroxide are preserved with careful regulation to prevent irreversible cellular damage amidst the abundance of ROS that is characteristic of malignancy.

One important mechanism through which antioxidants are upregulated to promote cancer survival [46,47] is through the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2). In the absence of oxidative stress, NRF2 is associated with cytoplasmic Kelch-like ECH-associated protein 1 (KEAP1), which mediates proteosomal degradation of NRF2 via the Cullin 3-based ubiquitin E3 ligase. Redox-active cysteines of KEAP1 detect hydrogen peroxide, leading to NRF2 stabilization [51]. NRF2 can then enter the nucleus and regulate transcription of genes containing antioxidant response elements (AREs) [23,52–55] (Fig. 1). The core consensus ARE, TGACnnnGC, has been identified in the promoters of many of genes that code for enzymes that assist with detoxification and eliminate reactive species [54,55]. NRF2 target genes in the human and/or mouse include the SODs, Prdxs, glutathione peroxidases, and catalases highlighted above. In addition, NRF2 regulates genes include those involved in glutathione biosynthesis [catalytic and regulatory subunits of the rate limiting enzyme glutamate-cysteine ligase (GCLC, GCLM) and glutathione reductase] and iron transport (Ferritin H, Ferritin L) as well as glutathione S-transferases, and thioredoxins, among others [55]. Several NRF2 target genes (including but not limited to GCLC, Prdx2, Ferritin L, and Ferritin H) were found to be elevated in the colon CSC secretome, suggesting NRF2 activation [56]. NRF2 was elevated in cervical CSCs [57] and promoted breast CSC maintenance as determined by mammosphere formation [58]. The NRF2 target gene Prdx1 was significantly elevated in glioblastoma CSCs where it was critical for protecting the cells from oxidative stress [59,60]. GTP cyclohydrolase I (GCH1), the rate limiting enzyme in the tetrahydrobiopterin pathway, was also reported to be a NRF2 target gene important for mitigating side effects of radiotherapy [61], and GCH1 was critical for hydrogen peroxide regulation in glioblastoma CSCs [62]. Furthermore, the metabolite, tetrahydrobiopterin, was demonstrated to act as a free radical-trapping antioxidant that protected lipid membranes from lipid peroxidation during hematological malignancies [63]. The stem cell marker Nestin was recently found in lung cancer to bind to KEAP1, leading to NRF2 stabilization and increased antioxidants [64]. Thus, as NRF2 is a key regulator of redox state, NRF2 inhibition is a potential strategy to target CSCs and improve the efficacy of ROS-inducing therapies, including radiation [65]. The viability of NRF2 knockout mice [66] supports the notion that there could be a therapeutic window for NRF2 targeting. However, the loss of NRF2 in the regulation of phase II detoxifying enzymes including glutathione S-transferases can lead to increased drug-induced toxicity or carcinogenesis [52,67,68]. The timing of NRF2 targeting for cancer treatments is, therefore, critical to consider [69].

2. Hypoxia/HIF regulation of stem cell maintenance and ROS mediated HIF stabilization

Regulation of stem cell fate, which is critical for tissue repair and homeostasis, is, in part, controlled by the niches in which they reside [70–72]. Hypoxia is a component of embryonic, hematopoietic, neural, and mesenchymal stem cell niches [71–78]. The importance of such low oxygen environments was elucidated in studies in developing embryos demonstrating that hypoxia increased self-renewal and viability, and these findings that were later observed in adult stem cells [78,79]. High oxygen tension was suggested to leave stem cells vulnerable to oxidative stress induced DNA damage, as early studies of mouse embryonic fibroblasts cultured at 20% or 3% oxygen demonstrated higher rates of senescence and the accumulation of mutations under normoxia [80]. This observation was corroborated in other tissue types, including hematopoietic stem cells, where oxygen rich conditions led to exhaustive proliferation, cell cycle arrest and apoptosis [78–84]. The exhaustive proliferation and induced senescence are related to increases in oxidative stress. Later studies in induced pluripotent stem cell (iPSC) research demonstrated additional roles for hypoxia in the promotion of the stem cell state: cellular reprogramming achieved by the transcription factors Oct 3/4, c-myc, klf4 and Sox2 [85] was increased under low oxygen tension [86,87]. Furthermore, cells differentiated from embryonic stem cells or iPSCs could revert to their stem-like state under hypoxia through induction Oct 3/4. Hypoxia also has known roles in regulating key stem cell transcription factors and prominent pathways, like NOTCH and WNT, which are determinant in cell fate and self-renewal [88]. Together, these data demonstrated critical roles of low oxygen tension in stem cell self-renewal and cell fate [87].

The specific pathway through which hypoxia regulates stem cell maintenance involves the hypoxia inducible factors (HIFs). HIFs are heterodimeric transcription factors that contain an α and β subunit [89–91]. Regulation of HIFs is through oxygen-dependent hydroxylation of HIF-alpha by prolyl hydroxylase domain proteins (PHD), which subsequently recruits von Hippel-Lindau (VHL) to ubiquitinate HIF-α for degradation under normoxia. Under hypoxia, PHD cannot hydroxylate HIF-α, leading to the accumulation of HIF-α that translocates to the nucleus to dimerize with HIF-β. Once dimerized, HIF binds to hypoxia response elements (HRE), which activate hypoxia response pathways that promote cell survival and self-renewal [90–92] (Fig. 2). HIF-1α and HIF-2α can be differentially expressed depending on the degree of hypoxia, but both the bind to the core consensus HRE G/A CGTG in the promoter of hypoxia response genes. However, adjacent binding elements as for ETS, MYC, STAT3, or USF2 can confer specificity [93–95]. As this implies, HIF-1α and HIF-2α have been found to have common and distinct roles, including in reprogramming and redox biology. For example, both HIF-1α and HIF-2α were important for the metabolic shifts that accompany reprogramming, but HIF-2α directly increased the

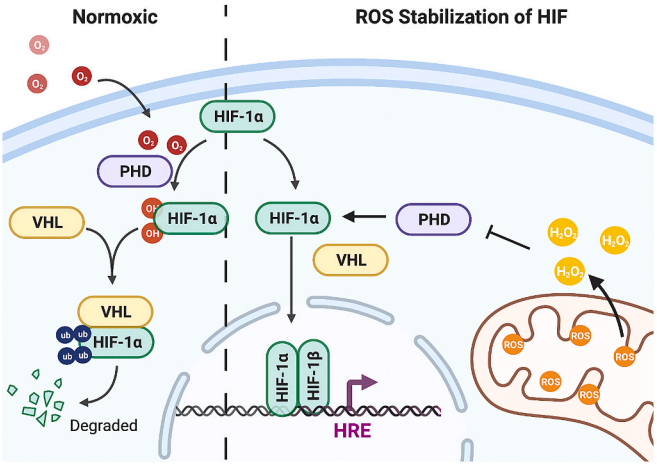

Fig. 2. ROS induced stabilization of HIF signaling.
Induction of hypoxia/HIF signaling is known to promote and maintain CSC phenotypes. Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase domain proteins (PHD) which targets HIF-1α for ubiquitination by von-Hippel Lindau (VHL) for proteasomal degradation. During malignancies, mitochondrial ROS production can be elevated. Hydrogen peroxide (H_2O_2) can stabilize HIF-1α, in the absence of hypoxia, to facilitate its translocation into the nucleus to dimerized, with HIF-1β. Once dimerized, HIF-1α and HIF-1β can bind to the hypoxia response element (HRE) to induce gene expression.

expression of Oct4 to regulate pluripotency [96,97]. Extrapolation from these iPSC studies provided insights into CSCs, which can also reside in low oxygen niches [78,98] and for which both hypoxia and HIFs are important for CSC maintenance [99,100]. Initial studies in brain tumors demonstrated a role for HIF-2α in regulating CSC self-renewal based, in part, on its preferential expression in CSCs [100]. As loss of HIF-1α or HIF-2α reduced stem cell maintenance in glioblastoma CSCs, the data suggest a central role for hypoxia in CSCs [100,101]. Indeed, hypoxia increased the percentage of glioma cells expressing stem cell markers, including in cultures initially depleted for CSCs [102–104]. A similar regulation of CSC state by HIFs has been noted in several cancer types where ablation of HIF impairs tumorigenic potential [98,101,103,105–107].

Although hypoxic conditions by definition have reduced oxygen tensions, ROS do play important roles in hypoxia [92,108,109]. Adaptation to hypoxic environments and metabolic shifts involve superoxide production by the mitochondrial electron transport chain, and ROS, particularly hydrogen peroxide, can regulate HIF signaling. ROS elevation, including during hypoxia, was demonstrated to occur within the mitochondria at complex III [29–31,110,111]. Superoxide produced from functional mitochondria, specifically at Q_0 of complex III, stabilized HIF-1α [112]. Stabilization required cytosolic ROS in the form of hydrogen peroxide, as glutathione peroxidase or catalase (but not SOD) elevation blocked HIF-1α accumulation in hypoxia [112–114]. Induction of mitochondrial superoxide and HIF-1α stabilization via hydrogen peroxide also resulted from loss of Regulated in Development and DNA damage response 1 (REDD1), which is induced by HIF and inhibits mammalian TOR complex 1 activity [115]. Conceptually, this demonstrated an important tumor suppressing mechanism (REDD1) that involved a feedback loop regulating HIF-1α via reducing mitochondrial superoxide and hydrogen peroxide, which suggests a pseudo-hypoxic state can occur in cancer in a redox dependent manner. Elevated levels of polyunsaturated fatty acids in hypoxia were also found to increase ROS [102], providing additional potential links between hypoxia/HIF and redox biology. Although the extent of hypoxia and ROS, particularly hydrogen peroxide and superoxide, interactions in CSCs has yet to be fully explored and is an area for future investigations, there is evidence that HIF regulation of glutathione synthesis impacts CSC maintenance. In breast cancer, GCLM transcription was HIF-1α, but not HIF-2α, dependent, with loss of GCLM or HIF-1α inhibiting glutathione synthesis and breast CSC enrichment post chemotherapy [116]. Thus, it is important to consider how hypoxia/HIF contribute to the redox state of CSCs and how the redox state can contribute to CSC molecular and biological signals in hypoxia.

Hypoxic mimetics include iron chelators that can inhibit ferroptosis, a form of iron-dependent cell death caused by elevated lipid peroxidation [117]. Free iron produces a hydroxyl radical via the Fenton reaction [118], and iron homeostasis regulates ROS generation via the balance of iron uptake, use, storage, and scavenging. In ovarian cancer, GPX4 inhibitors preferentially induce ferroptosis in Wnt receptor expressing ovarian CSCs [119]. Dichloroacetate, which inhibits pyruvate dehydrogenase kinase and increases oxidative phosphorylation, promoted iron storage and increased ferroptosis to decrease colorectal CSC maintenance [120]. While the induction of ferroptosis to target CSCs is being pursued [121], an alternate strategy of iron chelation has been proposed [122]. Indeed, the FDA-approved iron chelator deferiprone decreased breast CSC maintenance in association with a broad decrease in metabolism and an elevation of mitochondrial superoxide levels [123]. Ovarian CSCs also exhibited more sensitive reductions in cell growth induced by the iron chelator desferrioxamine [124]. Targeting a reliance on iron metabolism in CSCs is based on multiple studies indicating that mechanisms regulating iron homeostasis are altered [125]. For example, in glioblastoma CSCs, transferrin, an iron-binding and transporting molecule, and its receptor are upregulated to increase iron uptake. Ferritin, which is an iron storing protein made of a heavy (FTH1) and light (FTL) chain is also elevated in glioblastoma CSCs, indicating increased iron scavenging [126]. However, targeting FTH1 in an ovarian cancer line was associated with increased glucose utilization and increases in the CSC markers CD34, CD117, aldehyde dehydrogenase (ALDH), Nanog, and Oct4, but not CD133 [127]. Together, these data suggest the complexity of the interplay between oxygen tension, iron homeostasis, ROS, and metabolism that will affect our ability to effectively target CSCs.

3. Glycolysis in CSCs and mitigation of oxidative stress

Glycolysis contributes to malignancy [50,128–130] and is important for somatic reprogramming [131–135], indicating that metabolic shifts contribute to both cancer and stem cell states. Hypoxia in the tumor microenvironment (TME) increases glucose uptake and glycolytic activity, because many glycolysis pathway enzymes are HIF target genes. Cancer and other highly proliferative cells utilize aerobic glycolysis regardless of oxygen tension whereas non-neoplastic cells may shift from oxidative phosphorylation to anaerobic glycolysis depending on oxygen tension [50,128–130]. This glycolytic metabolic shift in cancer, called the Warburg effect, does not produce as much ATP as compared to oxidative phosphorylation, but ATP is produced at a higher rate [136,137]. In addition, glycolytic intermediates permit increased macromolecule biosynthesis that provides benefits for the growth of rapidly proliferating cells. Glycolytic intermediates are also shunted toward antioxidant production, providing a mechanism through which the altered metabolism of cancer mitigates oxidative damage by ROS [50,128,130]. Glycolytic intermediates entering the pentose phosphate pathway lead to increased NADPH and metabolic reprogramming activates the master antioxidant production regulator NRF2 [22–24,46]. Thus, metabolic shifts in cancer contribute to altered redox states [138].

Several studies demonstrate that CSCs rely on glycolytic metabolism with increased antioxidant pathways to regulate redox status. In non-small cell lung cancer (NSCLC), CSC maintenance was dependent upon lactate dehydrogenase A (LDHA) [139], an enzyme that inter-converts pyruvate and lactate in glycolysis. Genetic and/or pharmacologic targeting of LDHA in NSCLC CSCs reduced tumorsphere formation and tumorigenic potential [139]. Reduced CSC phenotypes with LDHA targeting were associated with decreased extracellular acidification rates and increased dependency on oxidative phosphorylation. Reduced glutathione synthesis with LDHA targeting was suggested to result in observed elevations of mitochondrial ROS as measured by 2′, 7′-dichlorofluorescin diacetate (DCFDA) [139]. Carbon tracing also demonstrated metabolites entering the Krebs cycle, which suggested, in part, mitochondrial sourced ROS.

LDHA and other glycolysis regulators were also decreased in glioblastoma CSCs cultured in low glucose and beta-hydroxybutyrate to mimic a ketogenic (low carbohydrate and high fat) diet [140]. The ketogenic diet, developed to treat epilepsy, has been shown to cause oxidative stress that resulted in increased NRF2 and increased GSH to GSSG ratios in animal models, due in part to increased GSH biosynthesis [141]. In agreement with these data, culture of glioblastoma CSCs in ketogenic diet-mimetic media increased mitochondrial ROS, which was measured by DCFDA and MitoTracker labeled cells, which were associated with a decrease in glucose uptake and glycolysis [140]. Changes in total ROS and metabolism correlated with reduced CSC phenotypes including expression of stem cell markers, neurosphere formation, and tumorigenic potential [140]. Additional studies supported a reliance of glioblastoma CSCs on glucose uptake and glycolysis for stem cell maintenance [142,143].

Breast cancer stem cells (BCSCs) also showed preference for glycolysis with increased activity of LDHA as well as glucose-6-phosphate dehydrogenase (G6PDH) and antioxidant including mitochondrial superoxide dismutase [144]. The upregulation of G6PDH further suggests shunting of glycolytic intermediates towards pentose phosphate pathway (PPP) which supports NADPH production for thioredoxin, glutathione and peroxiredoxin as a likely strategy for overcoming ROS

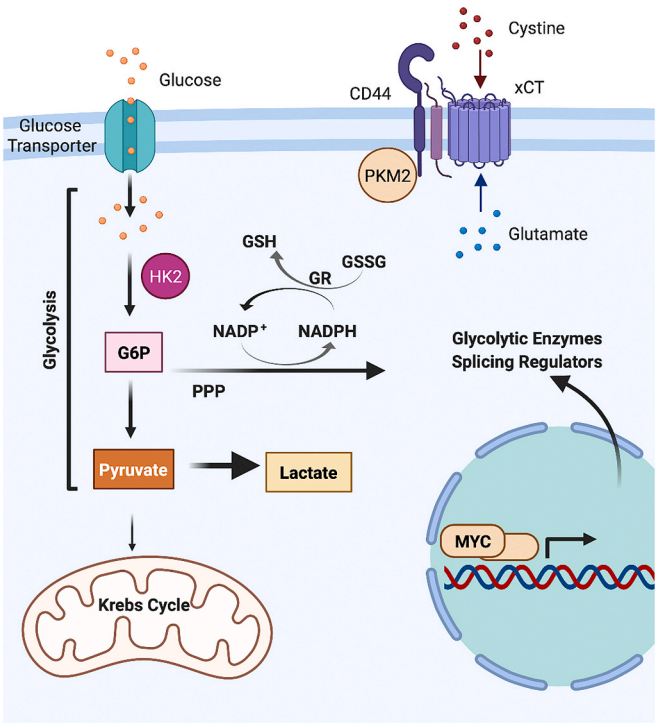

Fig. 3. Enhanced glycolysis in CSCs and control of oxidative stress.
The transcription factor MYC is an important regulator of CSC phenotypes and promotes the production of key glycolytic enzymes. MYC can also lead to alternative splicing of pyruvate kinase into its M2 isoform (PKM2) to further regulate metabolism. Glucose-6-phosphate (G6P) can shuttle into the Pentose Phosphate Pathway (PPP) for NADPH production to fuel antioxidant pathways, thus promoting a reductive environment for CSCs. Cluster of Differentiation 44 (CD44) is a common CSC marker with a role in enhancing glycolysis and interacts with PKM2. In addition, CD44 variant can regulate the cystine/glutamate antiporter system x_c via stabilization of the xCT subunit. Overall, glucose can be shuttled through glycolysis with reduced oxidative phosphorylation in the mitochondria and multiple mechanisms utilized to reduce overall ROS.

(Fig. 3). A similar bias toward glycolysis was observed in ovarian and colon CSCs [56,145]. Isotope tracing of ^{13}C revealed increased glycolysis and PPP activity in ovarian cancer spheroids with increases in the stem cell markers for ALDH1, CD34, Nanog and c-myc. Secretome characterization of colon CSCs by mass spectrometry and subsequent Ingenuity Pathway Analysis also indicated reliance on glycolysis and PPP [56]. The preferential utilization of glycolysis observed in various CSCs with shunting of glycolytic metabolites towards PPP provides reductive power for antioxidant systems [56,139,142,144,145]. These observations provide insights for therapeutic opportunities in targeting glycolytically dependent CSCs that can reduce ROS scavenging systems dependent on NADPH to sensitize cells to oxidative stress inducing agents [56,139,142,144,145].

Early studies in iPSCs established the relationship between stemness and metabolic reprogramming [131–135]. MYC, one of the core Yamanaka reprogramming transcription factors, is considered a master regulator of metabolic reprogramming [85,146]. Early studies utilizing chromatin immunoprecipitation techniques demonstrated MYC binding to key glycolysis enzymes and glucose transporter promoters [145–148]. Outside of its direct transcriptional role, MYC can further regulate metabolism via regulation alternative splicing of pyruvate kinase which has two isoforms, pyruvate kinase type M1 (PKM1) and PKM2 [146,149]. MYC increases transcription of splicing regulators to favor elevation of PKM2 (Fig. 3), shifting PKM2/PKM1 ratios and altering metabolism. Accumulation of glucose-6-phosphate (G6P) led to increased activity with PPP in lung cancer cells [150]. High PPP activity resulted in elevated NADPH levels that lowered oxidative stress induced by hydrogen peroxide or hypoxia. This bioenergetic strategy against superoxide and hydrogen peroxide could link PKM2 activity with LDHA in promoting metabolic reprogramming in NSCLC [139,150].

Cluster of differentiation 44, CD44, is a common transmembrane glycoprotein that is utilized as a cancer stem cell-like marker for several tumor types [151]. Different splice variants of CD44 demonstrate its link with regulating glycolysis, especially within hypoxic tumors and p53 deficient cells [151,152]. Within $CD44^+$ breast cancer cells, CD44 was found to enhance glycolysis through the c-Src/Akt pathway which activated lactate dehydrogenase via HIF-1α [153]. Ablation of CD44 ultimately led to reduced glucose uptake, a reduction in glycolytic genes and, intriguingly, resulted in a shift towards oxidative phosphorylation with higher viability observed compared to the non-targeted control under glucose starvation. Ultimately, this study demonstrated a metabolic link between $CD44^+$ breast cancer cells and glycolysis. In a different study, $CD44^+CD24^-$ breast CSCs displayed mesenchymal-like characteristics that were associated with quiescence [154]. Furthermore, these $CD44^+CD24^-$ breast CSCs displayed preferential utilization of glycolysis that resulted in overall lower basal ROS, as assessed by CellROX. Metabolic or oxidative stressors, like hydrogen peroxide or 2-deoxy-D-glucose (2-DG), shifted $CD44^+CD24^-$ breast CSCs towards oxidative phosphorylation, indicating the potential for metabolic plasticity. Between these two studies, breast cancer cells expressing CD44 utilize glycolysis as a strategy to maintain lower cellular ROS, thus demonstrating a link between stemness markers and metabolic characteristics in breast cancer [153,154]. In addition, CD44 interacts with PKM2 to regulate glycolysis in cells with loss p53 activity, where CD44 is important for regulating the flow of glycolytic intermediates into PPP to produce glutathione [152]. Furthermore, variant CD44 can stabilize a subunit of the cystine/glutamate antiporter system x_c^- which imports cystine important for glutathione synthesis [60]. Inhibition of system x_c^- with sulfasalazine and sorafenib induces oxidative stress driven by ferroptosis [155,156], and targeting system x_c^- also increased ROS and decreased colorectal CSC maintenance [157]. Thus, current markers used to identify CSCs highlight a correlation between stemness and metabolic pathways associated with reducing oxidative stress, providing valuable insight for therapeutic targeting.

4. Oxidative phosphorylation in CSCs and ROS

Hierarchical function of CSCs is thought to mirror normal stem cells, with CSCs potentially being metabolically biased towards glycolysis [131–135]. Initial studies to metabolically profile CSCs suggested a reliance on glycolysis, however there were noted discrepancies in translatability of *in vitro* studies [139,158–165]. This lack of congruency is likely attributed to the lack of environmentally relevant conditions, such as utilizing fresh patient samples or *in vivo* studies that would better preserve the metabolic characteristics of CSCs. Limited identification of CSCs in most solid tumors, likely marks a mixed population of CSCs including relatively quiescent cells and more proliferative progenitors thus observing mixed metabolic profiles. Indeed, recent studies have now indicated oxidative phosphorylation or mitochondrial metabolism as the preferred energy source for CSCs [132,158–165]. However, mixed energy utilization in CSCs indicates heterogeneity within this population or plasticity in their energy profile. For example, glioma CSCs were suggested to more readily use oxidative phosphorylation and have greater mitochondrial reserve capacity than their differentiated counterparts but shift towards glycolysis when needed to overcome metabolic stress [166].

Metabolic characterization of CSCs reveals heterogeneous fuel utilization depending on cancer type. Within breast CSCs, bioenergetics has been associated with cellular phenotypes with proliferative, epithelial-like or quiescent, mesenchymal-like cells [154]. Breast CSCs that are epithelial-like are characterized by their high expression of aldehyde dehydrogenase I (ALDH1) and reliance on oxidative phosphorylation with markedly higher superoxide. When mesenchymal-like breast CSCs

(discussed above) with low basal ROS exited quiescence and proliferated, more epithelial-like was observed with elevated mitochondrial superoxide as measured by MitoSOX and overall basal ROS indicated by CellROX. Under the epithelial-like state, there was an induction of ROS scavenging systems and increased stemness as indicated by elevation in NRF2, NOTCH signaling, and ALDH. This not only suggests plasticity within breast CSCs, but multiple strategies in manipulating their redox states. As a result, to target both populations of breast CSCs, there likely needs to be dual inhibition where targeting glycolysis forces BCSCs out of dormancy, and inhibition of thioredoxin and glutathione sensitizes epithelial breast CSCs to oxidants that it can no longer scavenge as effectively. In a different study, triple negative breast CSCs that co-express MYC and myeloid cell leukemia-1 (MCL-1), a Bcl-2 anti--apoptotic factor, were elevated post-chemotherapy treatment [159]. Together, MYC and MCL-1 enhanced oxidative phosphorylation by increasing mitochondrial biogenesis, resulting in increased mitochondrial membrane potential, oxygen consumption rate, elevated mitochondrial superoxide, and cytosolic hydrogen peroxide levels as measured by ROS-Glo and MitoSOX. The elevation in ROS was thought to induce stabilization of HIF-1α that ultimately led to increases in stem-associated characteristics like higher sphere formation, expression of ALDH, NANOG, and drug resistance.

In the case of pancreatic CSCs, a main source of ATP production was through oxidative phosphorylation and their limited metabolic plasticity was independent of *KRAS* status [160]. Low MYC expressing pancreatic CSCs expressed elevated PGCA1. High PGCA1 resulted in higher mitochondrial function, as marked by increased mitochondrial mass and membrane potential in addition to elevated expression of pluripotent markers. As a result, these cells displayed reduced glycolytic capacity, making them susceptible to mitochondrial inhibitors. However, the heterogenous pool of pancreatic CSCs included a less stem-like population with lower mitochondrial mass, thus leading to greater metabolic flexibility. These metabolically flexible pancreatic CSCs are glycolytically active and resistant to metformin treatment, an inhibitor of mitochondrial complex I. Additional studies in pancreatic ductal adenocarcinoma indicated that during the initial phases of dedifferentiation, there is higher glycolytic and PPP activity, likely attributing to the highly anabolic state of these cells [161]. Long-term culture in stem cell enriching conditions resulted in pancreatic CSCs shifting their metabolic profile towards oxidative phosphorylation and eventually entering quiescence. Interestingly, reversal of quiescence leads to reactivation of glycolysis, proliferation, and robust induction of PPP, demonstrating metabolic plasticity within these cells.

Hepatocellular CSCs and many other CSCs express NANOG, a well-known embryonic stem cell transcription factor [162]. In hepatocellular CSCs, increased NANOG expression resulted in repressed oxidative phosphorylation while converse results were observed with its silencing. Although oxidative phosphorylation was not the main energy source in hepatocellular CSCs, these cells heavily depended on mitochondria via fatty acid oxidation. Despite this dependency on mitochondrial metabolism, NANOG suppressed mitochondrial ROS, likely due to glutamine metabolism. The metabolic bias towards fatty acid oxidation was in turn, accompanied by chemoresistance phenotypes. Intriguingly, the leukemic stem cells (LSC) in chronic myeloid leukemia (CML) are drug resistant with high activity in fatty acid oxidation (FAO) [163]. As a result, these LSCs evade chemotherapy by escaping into adipose tissue, providing protection for LSCs. The advantage of FAO is producing NADH and acetyl-coenzyme A, which can reduce oxidative stress by inhibiting mitochondrial activity and therefore, induce quiescence [162–165]. Thus, preferential utilization of oxidative phosphorylation in LSCs provides a survival edge in reducing overall cellular ROS, superoxide, and energy demand, resulting in quiescence and longevity.

Overall, the documented reliance on oxidative phosphorylation in numerous CSC populations demonstrates metabolic similarities across multiple cancer types [158–165]. Higher mitochondrial activity is often paired with increases in mitochondrial superoxide and hydrogen peroxide which, in part, stabilizes HIF-1α and may mediate pluripotent pathways or chemoresistance. ROS scavenging pathways by NRF2, glutamate metabolism and glutathione were some strategies mentioned in this section to lower ROS, particularly superoxide, associated with oxidative phosphorylation. This display of enhanced mitochondrial respiration demonstrates metabolic heterogeneity within a tumor, where therapeutic strategies should consider inhibiting mitochondrial activity. However, due to the metabolic plasticity often seen in CSCs, dual targeting of mitochondrial respiration and glycolysis may be a more promising, but potentially more toxic, strategy for targeting CSC.

5. Importance of CSCs in therapeutic resistance

Therapeutic resistance and disease recurrence are a major setback in cancer treatment [167,168]. CSCs are believed to be a major obstacle in preventing complete remission in patients. The inherently therapy resistant CSCs are superiorly equipped to withstand or reverse the damaging effects of standard of care. Standard of care for many cancers comprises of surgery, genotoxic agents like chemotherapy and/or irradiation [168–171]. The administration of chemotherapy or irradiation can directly or indirectly cause oxidative stress. Irradiation mainly exerts lethality through the ionization of water which produces hydroxyl free radicals that damage DNA structures [169,170]. Similarly, anti-neoplastic agents can induce cell death by peroxyl radicals produced from lipid peroxidation [171]. Current standard of care with irradiation and anti-neoplastic agents are usually well suited for bulk tumor and highly proliferated cells: however, despite aggressive treatment, CSCs often escape therapy induced cell lethality [167,168]. Therapy resistance observed in CSCs is likely attributed to multiple factors: (1) high endogenous antioxidants and detoxification pathways prevent CSC toxicity, (2) enhanced DNA repair pathways reverse DNA damage that would otherwise lead to apoptosis, and/or (3) the slow cycling state protects more quiescent CSCs from the cytotoxic effects of chemotherapy and irradiation. Within this section, we will discuss the intrinsic characteristics of CSCs that allow this sub-population to persist after treatment.

6. High antioxidants and active DNA repair promote CSC therapeutic resistance

Irradiation and genotoxic agents are common clinical practices for treating cancer and are often effective against proliferative cells [168–172]. Irradiation can directly damage DNA or exert its toxic effects through the ionization of water, which forms free radicals causing DNA damage [169,170]. Failure to resolve DNA damage, such as double stranded breaks, should result in cancer cell death. Genotoxic agents are specific chemicals that also cause DNA damage and are cytotoxic [168, 171]. Although the mechanism of action is different, genotoxic agents have been documented to lethally elevate ROS levels through lipid peroxidation mediated pathways. CSCs escape therapeutic intervention, in part, by upregulating key antioxidant and detoxification pathways that neutralize ROS (Fig. 4). Such pathways were briefly mentioned earlier and have been reviewed elsewhere [167,173].

Elevations in antioxidants are linked with tumorigenesis and tumor progression and maintenance [22–24]. Early studies with the $CD44^{+}CD24^{-/low}Lin^{-}$ breast CSCs indicated low basal ROS and mitochondrial superoxide as measured by DCFDA and MitoSOX staining [174]. Furthermore, microarray analysis demonstrated the enrichment for antioxidant genes, thus contributing to the inherently low ROS phenotype. Pathways that lowered oxidative stress were related to an elevation in glutathione synthesis via increased expression of glutamate cysteine ligase and Forkhead Box O 1 (FoxO1). Enrichment of breast CSCs was observed after irradiation, demonstrating their radioresistant phenotype and the survival advantage from expressing low basal ROS. Importantly, pretreatment of breast CSCs with buthionine sulfoximine (BSO), which inhibits the synthesis of glutathione, resulted in

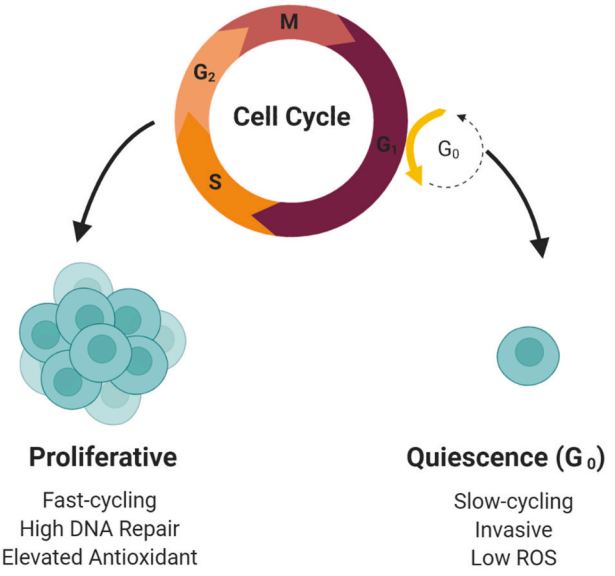

Fig. 4. Quiescence and CSC modes for therapeutic resistance.
Heterogenous cell cycling in CSCs may confer different characteristics for therapeutic resistance. Proliferative CSCs may upregulate antioxidants, thus preventing cell death induced by high oxidative stress during cancer treatment. Increased DNA repair activity resolves single-strand breaks, double-strand breaks, and replicative stress caused by irradiation, chemotherapy, or rapid proliferation. During quiescence, CSCs have low intercellular ROS. By not entering the cell cycle, G_0 CSCs could avoid cancer treatment induced death at cell cycle checkpoints. Quiescence is a transient state and CSCs may re-enter the cell cycle for cancer homeostasis.

sensitization of breast CSCs to irradiation. In glioblastoma, the $CD133^+$ CSC fraction is also inherently less sensitive to radiotherapy as demonstrated by increased percentages of $CD133^+$ cells post-irradiation [175]. This radioresistant phenotype can further be observed in breast, hepatocellular, lung CSCs [176–178]. ALDH, a common marker for CSCs, is also associated with low cellular ROS expression [179–184]. ALDH is known to reduce cellular stress induced by toxic aldehydes, especially post-therapy. High ALDH1 expression in breast CSCs, and several other tumor types, correlates with radioresistance. Thus, targeting ALDH or other elevated antioxidants or ROS scavengers could provide a therapeutic opportunity in targeting CSCs, including through increased radiosensitivity.

Glutamine metabolism is a process that supplies carbon and nitrogen sources that sustain the Krebs cycle, and its activation is driven, in part, by NRF2 [185,186]. In isocitrate dehydrogenase (IDH) mutant glioblastoma, the oncometabolite 2-hydroxyglutarate is produced instead of alpha-ketoglutarate [187,188]. IDH mutant cells are considered vulnerable to oxidative stress since the production of NADPH and alpha-ketoglutarate are limited [187–192]. As a result, IDH mutant glioblastoma CSCs are metabolically biased in producing antioxidants through glutaminase where the dual inhibition of glutathione and glutamate production lethally radiosensitizes CSCs [192]. Such examples of high antioxidant activity in CSCs are also observed in several other tumor types where markedly reduced ROS levels prove to be advantageous against scavenging ROS inducing therapies that are otherwise lethal [154,159,160,174,193–195].

Although high antioxidant levels prevent cellular death induced by ROS, unresolved DNA lesions or double stranded breaks can prove to be lethal if left unrepaired [170,172]. As a result, increased DNA repair helps to preserve the CSC genome during therapy [167,173]. In comparison to their differentiated counterparts, CSCs have been shown to have elevated DNA repair mechanisms, and lower levels of basal ROS can spare this population from cytotoxicity [154,159,160,174,193–195]. Such efficient DNA repair is notably observed in brain tumors where irradiation is a component of standard of care [196–198]. Glioblastoma CSCs are enriched for homologous recombination repair and non-homologous end joining repair pathways in comparison to non-malignant neural progenitor cells [197–201]. Elevations in PARP1 activity in glioma CSCs efficiently resolve single stranded breaks induced by oxidative stress thus contributing to therapeutic resistance [199,200]. Furthermore, tumors are highly proliferative cells and prone to replicative stress [202]. The higher expression of BRCA1 in glioblastoma protects the tumor from replicative stress where loss of BRCA1 reduces cell viability and increase incidences of double stranded breaks [201]. Loss of functional BRCA1 also sensitizes glioblastoma cells to PARP1 inhibitors, demonstrating an opportunity to introduce oxidatively lethal therapeutics.

7. Quiescence: a state of therapeutic evasion

As aforementioned, the effect of irradiation and genotoxic agents are more effective against proliferating cells since damaged DNA recognized during cell cycle checkpoints can lead to cell cycle arrest and apoptosis [168–172,202]. Although the quiescent state in normal and malignant stem cells remains poorly understood, this cellular state is believed to protect and maintain the longevity of the stem cell pool [12,203,204]. Under different stimuli, quiescent stem cells can reenter the cell cycle for tissue homeostasis, like hematopoietic, neural, hair follicle and muscle stem cells [4,5,12]. However, continual stimulation with ROS can lead to exhaustive proliferation and depletion of the stem cell pool [8,71–77]. Therefore, it is advantageous for normal stem cells to retain this slow-cycling, low endogenous ROS phenotype, in order to protect stem cells. Similarly, quiescence is believed to protect CSCs, as tumor recurrence or metastasis is observed in many cancers [12,167,173,203,204]. Identification of quiescent CSCs was first achieved in leukemia by utilizing label retaining dyes. Since the discovery of long-term leukemic stem cells, other quiescent CSCs were identified using similar label retaining dye methods for glioblastoma, breast, ovarian, pancreatic, liver and colon cancer [205–210].

In glioblastoma, different populations of CSCs have been defined by their cycling rate [206,211,212]. Slow-cycling glioblastoma cells identified based on retention of carboxyfluorescein succinimidyl ester fluorescent dye were enriched for CSC markers and tumorigenic potential [206]. Slow-cycling glioblastoma cells were more dependent upon oxidative phosphorylation and more closely resembled recurrent glioblastoma than fast cycling, highly proliferative glioblastoma cells that were more dependent upon glycolysis [211]. The slow-cycling glioblastoma cells were associated with invasion (Fig. 4) and increased chemoresistance [211], displaying these commonly associated CSC phenotypes. In another elegant study, a transgenic mouse model that expressed endogenous green fluorescent protein under the control of the promoter of Nestin, a marker for neural stem cells, labeled glioblastoma CSCs within the subventricular zone [212]. The promoter in this transgenic mouse also controlled thymidine kinase, so long-term treatment with temozolomide and ganciclovir led to a significant reduction in tumor and percentages of Nestin positive glioblastoma cells. These data suggested that there was a quiescent stem cell population in the glioblastoma mouse model responsible for sustaining long-term tumor growth and that progeny eventually faced exhaustive proliferation [212]. However, it is important to note that glioblastoma CSC therapeutic resistance can be impacted by changes in metabolism that may be independent of changes in proliferation. Oncostatin M increases oxidative phosphorylation in association with its receptor (OSMR) localizing to mitochondria and interacting with complex I [213]. Loss of OSMR in glioblastoma CSCs led to an increase in ROS post-radiotherapy and significantly increased apoptosis [213]. As OSM could still increase oxidative phosphorylation in post-mitotic neurons, mitochondrial OSMR may be a mechanism of radioresistance that is metabolism, but not necessarily cell cycle, dependent [213].

In colon cancer, slow cycling CSCs were recently identified as $Lgr5^+$

and additional studies suggest the importance of the $CD133^{+}CD44^{+}$ fraction [214,215]. $CD133^{+}CD44^{+}$ cells showed higher chemoresistance than $CD133^{-}CD44^{-}$ cells when treated with oxaliplatin or 5-flurouracil (5-FU), chemotherapy agents that can increase oxidative stress through lipid peroxidation and increased hydrogen peroxide levels [214–217]. Gene-set enrichment analysis of the chemoresistant colon CSC showed enrichment for antioxidant genes such as glutathione, thioredoxin, peroxiredoxin and superoxide dismutase. High antioxidant and ROS scavenging systems not only resulted in lower basal ROS in $CD133^{+}CD44^{+}$ colon CSCs but likely contributed to chemoresistance by preventing lethal elevations of ROS during drug treatment. An additional mechanism that protects quiescent colon cancer cells from increases in superoxide is through the transcription factor FOXO3a, which has been linked to stem cell maintenance [218]. FOXO3a protected quiescent colon cancer cells by upregulating manganese superoxide dismutase, which subsequently conferred protection from oxidative stress induced by glucose deprivation.

In the case of pancreatic adenocarcinoma, slow-cycling CSCs were attributed to stronger malignant and invasive features when compared to the bulk tumor cells, which were faster-cycling [207]. Furthermore, dormant hepatocellular carcinoma cells that express $CD13^{+}$ were identified as a G_0 subpopulation [219]. These cells were highly resistant against 5-FU or doxorubicin but also presented with low basal and mitochondrial superoxide, as measured by DCFDA and MitoSOX staining, when compared with $CD13^{-}$ liver cancer cells. It was found that within the $CD13^{+}$ population, there was an enrichment for the glutathione pathway that likely scavenged transient increases in ROS induced by irradiation or chemotherapy treatment. $CD13^{+}$ hepatocellular carcinoma cells remained after drug treatment with rapid recovery of DNA damage while the $CD13^{-}$ population was sensitive to treatment.

As just discussed, slow-cycling, quiescent cells seem to confer protection against oxidative stress [218,220]. This protection from oxidative stress likely drives therapeutic resistance against standard of care [167–171,173,220]. The effects of ROS-induced cellular death during cancer treatment is prominent in highly proliferative cells, likely due to different cellular ROS statuses within the cell cycle [220]. In asynchronous cancer cells, high cellular ROS, as measured by DCFDA, and cysteine oxidation were observed in the mitotic cell cycle phases. Damaged proteins and nucleotides were most prominent during mitosis and the G2 phase as indicated by higher ROS and 8-oxo-guanine adducts, an indicator of oxidative stress-induced DNA damage. The exact regulation of quiescence, the role of quiescence in CSC subsets, and the mechanisms regulating ROS levels in CSCs and in different cell cycle phases remain to be fully determined. However, the data suggest slow-cycling CSCs with low basal ROS and enhanced ROS scavenging systems (Fig. 4), as through glutathione or the NRF2 pathway, are critical to target for improved patient therapies [12,154,163,203,204,207,214,215]. Such observations may explain the ability of CSCs to evade therapeutic interventions that are best suited for highly proliferative cell populations.

8. Summary/conclusion

Within the last few years, we have gained valuable insights within the CSC field. The heterogeneous nature of CSCs, in addition to their plasticity, reveals a distinct, yet diverse, redox profile. This altered redox signature drives key pathways in sustaining stemness, proliferation and therapeutic resistance. Importantly, CSCs retain some similar features across tumor types and targeting key metabolic pathways, such as inhibiting glycolysis and mitochondrial function, may provide a therapeutic vulnerability that standard of care does not. Furthermore, investigation into antioxidant pathways being utilized in CSCs could identify mechanisms to render this population susceptible to chemotherapy and irradiation, a strategy that relies on inducing lethal levels of ROS, particularly superoxide and hydroxyl, or genomic stress. Overall, as the field progresses, a better characterization of the elusive CSC will provide us critical pieces in understanding tumor biology and disease evolution which will improve our treatment for cancer patients.

Additionally, rapidly growing solid tumors must adapt to hypoxia and consideration of how HIF signaling is modulated by ROS is important. Oxygen-level-sensitive HIFs enter the nucleus, whereby they activate multiple genes important for tumor growth: while increase in angiogenesis as via vascular endothelial growth factor (VEGF) that permits the recruitment of blood vessels for sustained tumor growth and metastasis are well known, the importance of these signals for CSC maintenance is increasingly recognized [221,222]. How ROS is the absence of hypoxia could impact HIF signaling is less well characterized. Consequently, therapeutic consideration for lethally depleting or elevating ROS to perturb vital cellular processes in cancer in the context of the tumor environment is important [23,47]. Such strategies may be extended for targeting CSCs, a population with distinct redox profiles [223]. The redox characterization of CSCs remains to be fully understood, but continued studies will reveal the biological necessities for ROS related processes.

Thus, the redox landscape within tumors is heterogenous, and this includes the CSC population [223]. These differences among CSCs suggest a diverse role for ROS in driving tumor biology, where redox changes are driven by varying environmental pressures.

Declaration of competing interest

We have no conflicts of interest to declare.

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