Targeting Cancer Stem Cell Redox Metabolism to Enhance Therapy Responses

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Abstract

Cancer has long been viewed as a disease of altered metabolism. Although it has long been
recognized that the majority of cancer cells display increased dependence on glycolysis, the
metabolism of "cancer stem-like cells" (CSCs) that drive tumor growth and metastasis is less well
characterized. In this chapter, we review the current state of knowledge of CSC metabolism with
an emphasis on the development of therapeutic strategies to exploit the metabolic vulnerabilities of
these cells. We outline emerging evidence indicating distinct metabolic pathways active in the
proliferative, epithelial- (E) and quiescent, mesenchymal-like (M) CSC states in triple negative
breast cancer (TNBC). These CSC states are characterized by their different redox potentials and
divergent sensitivities to inhibitors of glycolysis and redox metabolism. We highlight the roles of
two redox-regulated signaling pathways, HIF1α and NRF2, in regulating CSC epithelial-
mesenchymal plasticity during metabolic/oxidative stress, and discuss clinical strategies using
combinations of pro-oxidant based therapeutics simultaneously targeting E- and M-like CSCs. By
specifically targeting CSCs of both states, these strategies have the potential to increase the
therapeutic efficacy of traditional chemotherapy and radiation therapy.

Keywords

Cancer Stem Cells (CSCs); Triple Negative Breast Cancer; Reactive Oxygen Species (ROS);
Glycolysis; Oxidative phosphorylation (OXPHOS); HIF1α; NRF2; Thioredoxin and Glutathione
Antioxidant Pathways

1. Introduction

There is substantial evidence that many cancers, including breast cancer, are hierarchically
organized and driven by a small population of tumor cells displaying stem cell properties [1–
3]. These "cancer stem-like cells (CSCs)" or "tumor initiating cells (TICs)", which reside at
the apex of tumor heterogeneity, have the capacity for self-renewal, tumor initiation and

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generation of differentiated tumor progeny constituting the tumor bulk. In addition to their tumorigenic potential, CSCs are inherently resistant to traditional cancer therapies including chemotherapy and ionizing radiation, leading to treatment failure, metastases, and cancer relapse [4–10].

As discussed in several recent reviews, tumor cells including the small population of CSCs display remarkable genetic/epigenetic heterogeneity and cellular plasticity [11–13]. Adding another layer of complexity, recent studies demonstrate that BCSCs maintain the plasticity to transition between proliferative, epithelial-like (E) and quiescent, mesenchymal-like (M) states [14]. These distinct E- and M-like BCSCs exhibit discrete patterns of marker expression characterized by elevated aldehyde dehydrogenase (ALDH) activity and $CD24^-CD44^+$ expression respectively [14]. The transition of CSCs from the E- to M-like state closely resembles the epithelial-to-mesenchymal transition (EMT), which is associated with the acquisition of stem cell properties [15]. The equilibrium of these dynamic CSC states is regulated by the tumor microenvironment through mechanisms including, but not limited to, cytokine/chemokine signaling, genetic/epigenetic regulation of key transcription factors as well as growth factor receptors and microRNA/LncRNAs [13, 16, 17]. Most recently, we demonstrated that the dynamics of BCSCs in distinct E- and M-like states is tightly controlled by changing reduction-oxidation (redox) states induced by metabolic stressors [18]. In this chapter, we highlight the emerging knowledge regarding redox-regulated CSC plasticity, the underlying signaling mechanisms governing redox regulation of CSC state dynamics, and the different metabolic pathways contributing to their differential redox potentials as well as divergent responses to inhibitors of glycolysis and redox metabolism. We emphasize the implications of these findings for the development of novel combinatory therapeutic strategies to effectively target CSCs of distinct phenotypic states, and discuss how these strategies may enhance tumor responses to the conventional treatment approaches including chemo- and radiation therapies.

2. CSC plasticity as a mechanism conferring treatment resistance and metastatic relapse

Current cancer therapies are mainly aimed at reducing tumor mass by targeting rapidly proliferating bulk tumor cells. Although these therapeutic strategies are effective in reducing the size of tumors, they frequently fail to eradicate advanced tumors and are associated with metastatic relapse. The CSC hypothesis suggests that conventional antitumor strategies targeting rapidly proliferating cells may fail to target CSCs, which divide infrequently and are endowed with multiple mechanisms accounting for their therapeutic resistance[19]. Indeed, a large body of studies has indicated that BCSCs are resistant to ionizing radiation and chemotherapy [9, 20–22]. This has been demonstrated in cultured cancer cell lines, in primary mammary tumor cells [7, 23, 24], in patient derived tumor xenografts [9, 25, 26] as well as in the clinical setting of breast cancer patients undergoing neoadjuvant chemotherapy [8, 25].

The demonstration that BCSCs exist in a dynamic equilibrium of E- and M-like states [14] suggests that CSC epithelial-mesenchymal plasticity may contribute to therapeutic resistance

and metastatic relapse. The plasticity of CSCs allowing them to transition from a proliferating epithelial state to an invasive, mesenchymal state facilitates these cells escaping from traditional therapies and disseminating into the circulation and distant organs. Conversely, the plasticity allowing them to transition from an invasive, mesenchymal state to a proliferating epithelial state facilitates metastatic colonization. This suggests that CSCs are not a fixed population but rather a dynamic, metaplastic phenotypic state that is regulated by the tumor microenvironment (i.e., growth factor/inflammatory signaling, stromal-tumor interactions and metabolic reprograming). In agreement with this notion, published studies have shown that HER2 overexpression drives the self-renewal of ALDH^{+} E-BCSCs that are sensitive to the HER2 antibody trastuzumab [27]. Conversely, resistance to HER2 blockade is associated with an increase in CD24^{−}CD44^{+} M-BCSCs resulting from the activation of an IL6 driven inflammatory loop [28]. Moreover, in trastuzumab-resistant HER2^{+} breast cancer, a combinatory approach targeting IL6 receptor by tocilizumab and HER2 by trastuzumab synergistically abrogated tumor growth and metastases by eliminating both M- and E-BCSCs [28], providing a proof-of-concept that simultaneously targeting distinct CSC states may eradicate metastatic/drug-resistant breast cancer by eliminating the refractory CSC population. These studies also imply that therapeutic approaches targeting either state alone may not be sufficient to eliminate CSCs, since the targeted cell population could be rapidly regenerated by cells in alternative states. Thus, multiple CSC therapies attacking distinct forms of CSCs may be required to effectively eliminate these lethal seeds of cancer, leading to more effective therapies.

3. Cell redox as a key regulator of CSC plasticity

Cancer cells are characterized by increased levels of oxidative stress, generated by increased metabolic demands and oncogenic signaling [29, 30]. Many chemotherapeutic agents or ionizing radiation, by inducing oxidative stress, are able to elicit ROS-mediated cytotoxicity in bulk tumor cells which are more susceptible to second oxidative insults compared to their normal counterparts [31, 32]. However, ROS levels in the quiescent CSCs are significantly different as compared to the bulk of tumor cells [7]. Recently, we demonstrated that modulation of redox potential through co-inhibition of glycolysis and NRF2-mediated antioxidant responses is able to disrupt the state equilibrium of BCSCs, leading to terminal differentiation and apoptosis of both M- and E-BCSCs [18]. This provided a novel framework using pro-oxidant based therapeutics targeting metabolism of distinct CSC states to effectively eliminate the refractory CSC populations.

As is the case for their normal tissue counterparts, CSCs obtain energy principally through two different metabolic pathways: mitochondrial respiration, which depends on oxygen; and fermentation of glucose through aerobic glycolysis, which is oxygen-independent. CSCs appear to be capable of deriving energy from both sources. In the quiescent M state, they primarily rely on glycolysis; in the proliferative E state, they utilize oxygen to fuel mitochondrial oxidative phosphorylation (OXPHOS). Such dynamic utilization of distinct metabolic pathways depending on the tumor microenvironment is critical for cells in these CSC states to meet redox homeostasis and bioenergetics needs. Thus, the reactive oxygen species (ROS) generated from the mitochondrial OXPHOS is not merely a by-product of cancer metabolism, but rather serves as a key mediator regulating CSC plasticity.

3.1. ROS as a secondary messenger in cell signaling

Metabolic reactions utilizing molecular oxygen (O_2) produce reactive oxygen species (ROS) including superoxide (O_2^-, hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^.$). Although ROS are generated in various cellular compartments such as the peroxisomes and endoplasmic reticulum (ER), it is well recognized that mitochondrial OXPHOS serves the major source of ROS production, as ~2% of the oxygen consumed by the mitochondria is estimated to be reduced to form superoxide anion [29, 33, 34]. Mitochondrial ROS are not stable and rapidly dismutated by the manganese superoxide dismutase (MnSOD, SOD2) at the mitochondria or Cu/Zn-containing SOD (SOD1) in the cytosol into H_2O_2, which is permeable to the cell/nuclear membranes and further converted to $HO^.$ [35].

At high levels, ROS induce damage to various cellular components including DNA, proteins and lipids, leading to cell senescence, death or oncogenic transformation[29]. The extensive production of ROS during normal metabolism requires the activation of adequate antioxidant defenses to maintain redox homeostasis. Recent evidence has suggested that, although high levels of ROS are toxic, low-to-intermediate levels of ROS are crucial for cell development and homeostasis [36]. ROS act as important secondary massager to modulate a wide variety of signaling molecules including kinases, phosphatases, and transcription factors including JAK-STAT[37, 38], p38 MAPK/ERK/JNK[39–41], PI3K-AKT[42, 43], NF-kB[44–46], PKA[47], PKC[48, 49], NRF2/Keap1[50], Hippo-FOXO[51], etc., which in turn stimulate diverse cellular responses including cell survival, proliferation and differentiation[52, 53] and cell migration, adhesion and invasion[54, 55].

3.2. CSC radio-resistance is associated with low reactive oxygen species

The antitumor effects of both radiation and chemotherapy are mediated, at least partially, by the generation of ROS, which results in DNA damage and subsequent single-stranded and double-stranded breaks [56–58]. In neural stem cells (NSCs) and hematopoietic stem cells (HSCs), protection from oxidative stress is critical for the maintenance of their self-renewal [59–61], and mice deficient in ATM kinase or *FoxO1, FoxO3*, and *FoxO4* transcription factors exhibit elevated ROS levels in the HSC compartment, leading to rapid extinction of HSCs [60, 62, 63]. In parallel studies, Diehn et al. documented that human and mouse BCSCs, similar to their normal tissue counterparts, are characterized by low levels of ROS associated with increased expression of free radical scavenging systems that account for the radio-resistance of these cells[7]. This study suggests that strategies that block these antioxidant defenses may successfully target CSCs. Indeed, pharmacological depletion of ROS scavengers in BCSCs markedly decreases their clonogenicity resulting in radio-sensitization [7]. Accordingly, treatment of human acute myeloid leukemia (AML) with parthenolide, a naturally occurring compound that induces ROS, effectively targets AML stem and progenitor cells for apoptosis [64].

3.3. E- and M-like CSCs intrinsically differ in their redox states

As summarized above, there is compelling evidence that low levels of intracellular ROS are required for maintaining CSCs including hematopoietic stem cells (HSCs) [60–62] and mammary stem cells (MaSCs) [7]. Like their normal counterparts, the CD44+/CD24– mesenchymal BCSCs are characterized by low ROS [7]. Epigenetic silencing of the

gluconeogenic enzyme fructose-1,6-biphosphatase FBP1 by Snail facilitates increased glycolysis while suppressing OXPHOS to maintain low ROS levels in M-like BCSCs, promoting their self-renewal and an EMT-like CSC phenotype in TNBC [65].

The mammary epithelium is comprised of an outer layer of basal and an inner layer of luminal epithelial cells maintained by distinct basal and luminal stem cells that give rise to cells restricted to the basal and luminal lineages respectively during mammary gland development [66]. Interestingly, MaSCs located in the basal compartment (EpCAM$^{-/low}$MUC1$^-$CD49f$^+$CD90$^+$) are characterized by lower ROS levels while the luminal epithelial cells including the primitive luminal stem and/or progenitor cells (EpCAM$^+$MUC1$^+$CD49f$^+$CD90$^-$) are characterized by higher levels of ROS [67]. Moreover, this study also demonstrated that the primitive luminal stem cells/progenitor cells display increased mitochondria mass and greater rates of oxygen consumption, and that glutathione-dependent and -independent antioxidant defense mechanisms are active in the basal and luminal mammary epithelial compartments respectively [67]. These distinct luminal and basal stem cell populations identified in mammary epithelium are reflective of the ALDH$^+$ and CD24$^-$CD44$^+$ CSC states isolated in different subtypes of breast cancers [14].

The finding that basal and luminal MaSCs maintain different ROS levels and lineage-specific mechanisms of ROS control suggest that the E- and M-like BCSCs reflective of their respective luminal and basal stem cell lineages may inherit distinct ROS levels and metabolic pathways to control their respective redox states. Indeed, our recent studies in TNBC revealed that E-BCSCs and M-BCSCs display significantly different ROS levels and divergent responses to metabolic/oxidant stressors [18]. We further demonstrated that metabolic/oxidative stress generated by 2DG, H_2O_2 or hypoxia promotes the transition of ROS^{lo} M-BCSCs to a ROS^{hi} E-state, and this transition was reversed by N-acetylcysteine and mediated by activation of the AMPK-HIF1α axis [18]. These differential responses of E- and M-BCSCs to metabolic/oxidative stressors are linked to their distinct bioenergetics and redox metabolism, which is discussed in the following section.

4. Metabolic pathways of CSCs

For most slowly proliferating normal tissue cells, mitochondria serve as the main source of energy production through the tricarboxylic acid (TCA) cycle coupled with OXPHOS in the mitochondria membrane, which generates 36 ATP per molecule of glucose. Cancer cells, however, are characterized by high rate of proliferation and thus need to adapt their cellular metabolism to support increased proliferation by providing rapid ATP generation as well as biosynthesis of nucleotides, proteins and lipids to support tumor growth. Almost 100 years ago, Otto Warburg reported that cancer cells preferentially utilize glycolysis (which generates 2 ATP per unit of glucose) to generate copious amounts of lactate, regardless of the presence of oxygen[68]. Such an inefficient way of energy production, however, is necessary for generation of various metabolic intermediates important for maintaining tumor redox homeostasis and anabolic metabolism (macromolecular biosynthesis). Despite a glycolytic phenotype in the bulk of tumor cells, the metabolic pathways utilized by the small subset of CSCs are less well characterized. Currently there is no consensus on the

metabolism of CSCs and a growing body of evidence in different cancer types indicates that the metabolism of CSCs is context-dependent and reliant on glycolysis and/or OXPHOS.

4.1. Metabolic heterogeneity and plasticity of CSCs

It was originally hypothesized that CSCs display a glycolytic phenotype with similarities to the quiescent adult stem cells [69]. This hypothesis is supported by the findings that transition from somatic oxidative metabolism into pluripotency-dependent glycolysis facilitates nuclear reprogramming and formation of induced pluripotent stem cells (iPSCs) [70, 71]. In contrast, bioenergetics transition from glycolysis to OXPHOS occurs during differentiation of human embryonic stem cells (hESCs) [72]. Thus, metabolic reprograming is not merely associated with sternness, but also acts as a regulator of cell differentiation.

A number of studies in breast cancer supported the above notion by demonstrating that BCSCs enriched by sphere formation [73], CD24$^-$CD44$^+$[65, 73] or CD49f^{high}EpCAM^{low} [74] preferentially utilize glycolysis over mitochondrial OXPHOS compared to the differentiated cells from the same tumor. This glycolytic phenotype is also found in the CD133$^+$ hepatocellular CSCs [75] and the radio-resistant sphere-forming nasopharyngeal carcinoma cells [76]. Despite the studies suggesting a dependence of CSCs on glycolysis, other studies demonstrate that CSCs might also rely on oxidative metabolism. For example, quantitative proteomics studies of breast cancer cells revealed that key mitochondrial-related proteins involving in fatty acid β-oxidation and ketone metabolism as well as mitochondrial biogenesis were significantly upregulated in the mammospheres relative to the epithelial monolayers, suggesting that clonal expansion of BCSCs requires mitochondrial OXPHOS [77]. Indeed, XCT790, a well-established inhibitor of estrogen-related receptor a (ERRa), which functions as an essential cofactor of PGC-1α required for mitochondrial biogenesis [78, 79], suppresses BCSC activity by blocking several independent signaling pathways normally required for the self-renewal of CSCs, including Sonic hedgehog, TGFβ-SMAD, STAT3 and Wnt signaling [80]. This PGC-1α mediated mitochondrial biogenesis and OXPHOS is essential for the functional motility of breast cancer cells and metastasis [81]. The requirement of mitochondrial biogenesis for the self-renewal of CSCs is also supported by the findings that a number of antibiotics (i.e., Azithromycin and Tetracycline) targeting mitochondrial ribosomes effectively eradicate CSCs across multiple tumor types [82]. This functional link of mitochondria to the CSC phenotype was subsequently confirmed by the findings that proliferative ALDH$^+$ BCSCs display increased mitochondrial mass and activity, and that high mitochondrial mass identifies a sub-population of BCSCs that are chemo-resistant [83, 84]. A recent study further demonstrated that MYC and MCL1, two genes frequently amplified in TNBC, cooperatively promote mitochondrial OXPHOS, leading to ROS-mediated HIF1α stabilization and enrichment of ALDH$^+$ BCSCs [85].

The roles of mitochondrial oxidative metabolism in conferring therapeutic resistance by promoting the maintenance of CSCs have also been described in other malignancies. For example, the oncofetal insulin-like growth factor 2 mRNA-binding protein IMP2 in glioblastoma has been shown to control OXPHOS, which is crucial for preserving CD133$^+$ glioblastoma CSCs [86]. In acute myelogenous leukemia (AMF), the quiescent leukemia stem cells (FSCs) express high levels of BCF-2 and have a selective dependency on

oxidative respiration [87]. Furthermore, the BCF-2 inhibitors ABT-737 and ABT-263 selectively inhibit the quiescent FSCs by targeting OXPHOS [87]. Similarly, in chronic myeloid leukemia (CMF), the primitive CD34$^+$ leukemic stem cells (FSCs) rely on oxidative metabolism for their survival. Treatment with the combination of imatinib and tigecycline, an antibiotic inhibiting mitochondrial protein translation, selectively eradicates CML LSCs *in vitro* and in a xenograft model of human CML [88]. The antidiabetic drug metformin, which blocks mitochondrial function via inhibition of the ETC complex I, inhibits cellular transformation and selectively kills BCSCs by depleting the TCA cycle and glycolytic intermediates and suppressing nucleotide biosynthesis [89]. In pancreatic ductal adenocarcinoma (PDAC), metformin specifically targets CD133$^+$ CSCs, which display elevated expression of PGC-1α essential for mitochondrial biogenesis, OXPHOS functionality, and the self-renewal and tumorigenic potential of pancreatic CSCs [90]. Interestingly, metformin-resistant CSC clones in PDAC emerge due to an intermediate glycolytic/OXPHOS phenotype driven by increased expression of MYC, and genetic/pharmacological suppression of MYC restores metformin sensitivity in metformin-resistant pancreatic CSCs [90]. Thus, the balance of MYC/PGC-1α expression appears to determine the dynamic metabolic states (glycolysis versus OXPHOS) of pancreatic CSCs. This metabolic heterogeneity and plasticity of CSCs identified in various tumor types highlight that CSCs display metabolic plasticity enabling them to adapt to changing tumor microenvironments and nutrient availability.

4.2.   CSCs in distinct E and M states depend upon different metabolic pathways

As discussed in the previous section, the Warburg effect characterized by increased aerobic glycolysis in the bulk of tumor cells represents a striking metabolic difference between cancer and its normal tissue cells. However, despite the dependence of cancers on glycolysis, glycolytic inhibitors such as 2-Deoxyglucose (2DG) and Lonidamine exhibit little effect on solid tumor growth [91, 92]. Although the mechanisms accounting for the lack of sensitivity of cancer cells to glycolytic inhibition remain to be fully characterized, a recent study suggested that mTORCl-dependent metabolic rewiring underlies the escape of cancer cells to glycolytic addiction [93].

The phenotypic plasticity of BCSCs enabling them to transition between a quiescent mesenchymal state and proliferative epithelial state facilitates the capacity of BCSCs to initiate and grow primary tumors, to invade the basement membrane and tissue vasculature, and ultimately colonize distant organs to form clinically significant metastases [17, 19]. Despite the functional significance of this CSC plasticity in tumor development, treatment resistance and metastatic relapse, the cellular and molecular mechanisms regulating BCSC plasticity by the complex tumor microenvironment remain largely elusive. Currently, little is known about the metabolic pathways active in distinct BCSC states and how these CSC states evolve under various metabolic stressors such as glycolytic inhibition, nutrient deprivation, hypoxia, and xenobiotic/oxidant stress. These metabolic differences of M- and E-like CSCs may confer resistance to glycolytic inhibition and play critical roles for tumor cell survival and progression under stress. As CSCs exhibit a dynamic equilibrium of proliferative E- and quiescent M-like states coordinately driving tumor growth, metastasis and treatment resistance, elucidation of these metabolic differences will help to define and

exploit metabolic vulnerabilities of each CSC state, providing a conceptual framework to effectively target this critical tumor cell population.

To address these questions, we investigated how metabolic/oxidative stress modulates the state dynamics of BCSCs and identified markedly different responses of M- and E-BCSCs to oxidant stress that are closely linked to their distinct redox potentials. Specifically, metabolic/oxidative stress generated by 2DG, H_2O_2 or hypoxia, by activating AMPK-dependent HIF1α stabilization and NRF2-mediatted antioxidant responses, promotes the transition of BCSCs from a quiescent, ROS^{low} M-like state to a ROS^{high} proliferative E-state [18]. Interestingly, these divergent responses of M- and E-BCSCs to oxidative stress are closely associated with their different metabolic pathways. As depicted in Figure 1, the $CD24^-CD44^+$M-BCSCs and $ALDH^+$ E-BCSCs significantly differ in many aspects of cellular metabolism. Although both CSC states exhibit elevated glycolysis regulatory genes, the relatively quiescent M-BCSCs exhibit higher glycolytic rate in glucose-rich culturing conditions. In contrast, the proliferative E-BCSCs are endowed with robust expression of mitochondrial OXPHOS regulatory genes and exhibit highest metabolic plasticity for OXPHOS under glycolytic inhibited conditions. These proliferative E-BCSCs are also endowed with robust NRF2-mediated antioxidant responses, exemplified by highly elevated expression of a wide variety of antioxidant genes involved in drug transport and detoxification, NADPH production, TXN and GSH antioxidant pathways, etc. These metabolic differences of M- and E-BCSCs suggest that while glycolysis plays an important role in supporting the low redox states of M-BCSCs, both mitochondrial respiration and NRF2-mediated antioxidant defenses are important for E-BCSCs to maintain their proliferation and to prevent the accumulation of oxidative stress generated from mitochondrial OXPHOS.

As metabolic/oxidative stress produced by 2DG-mediated glycolytic inhibition facilitates the transition of quiescent, ROS^{low} M-BCSCs to a proliferative, ROS^{high} E-state susceptible to the blockade of NRF2 or its downstream thioredoxin (TXN) and glutathione (GSH) antioxidant pathways, we validated a combinatory treatment approach utilizing 2DG together with inhibitors of TXN (by AUR) and GSH (by BSO) antioxidant pathways. This combination treatment approach significantly suppressed tumor growth, tumor-initiating potential and metastasis by abrogating both M- and E-BCSCs in patient-derived xenograft (PDX) and systemic metastasis models of TNBC [18]. As 2DG is not an FDA-approved cancer therapeutic, our preclinical studies suggest a pro-oxidant based therapeutic approach utilizing standard-of-care radio-chemotherapy and antiangiogenic therapies (all of which generate oxidative stress) in combination with inhibitors of NRF2-mediated antioxidant responses to treat metastatic cancer (i.e., TNBC). As illustrated in Figure 2, this combinatory approach, by elevating oxidant stress in the ROS^{low} M-like CSCs, promotes the transition of quiescent M-BCSCs to a ROS^{high} E-state that is more susceptible to inhibition of NRF-mediated antioxidant pathways (i.e., NRF2 inhibitor Trig or inhibitors of TXN/GSH pathways such as AUR/BSO), leading to terminal differentiation and subsequent apoptosis of the both M- and E-like BCSCs. Thus, elucidating metabolic differences of distinct CSC states may provide a conceptual framework to effectively target CSCs in various tumor types, leading to enhanced treatment responses for traditional cancer therapies.

5. Redox-regulated signaling pathways modulating CSC phenotypic/ metabolic plasticity and therapeutic responses

Although a plethora of ROS-dependent signaling pathways including PTEN/PI3K/AKT/ mTOR [94, 95], JAK/STAT[37, 96], Notch [97–99], Hippo [51], ATM/p53 [100–102], Wnt [65, 103, 104] and NF-kB [46, 105–107] cascades have been implicated in regulating CSCs through modulation of cancer metabolism and redox homeostasis, our recent studies demonstrate a prominent role of ROS-induced HIF1α and NRF2, two redox-sensitive transcription factors, in regulation of BCSC plasticity during metabolic stress[18]. In this section, we focus on the roles of HIF1α and NRF2 in regulating CSC metabolism and phenotypic plasticity as well as the clinical implications of targeting these redox-sensitive signaling pathways to overcome therapeutic resistance.

5.1. Roles of HIF1α in regulation of redox and intermediary metabolism

Advanced cancers often display intra-tumor hypoxia, which promotes tumor metastasis and therapeutic resistance largely mediated by induction/stabilization of hypoxia-inducible factors (HIFs) [108, 109]. HIFs including HIF1 and HIF2 are transcription factors mediating hypoxic responses and HIF1 functions as a master regulator of glycolysis [110]. HIF1 has two subunits: HIF1α and HI FI β. HIF1 protein levels increase under hypoxic stress and decrease under normoxic conditions due to oxygen-dependent prolyl hydroxylation of HIF1α, which targets HIF1α for proteasomal degradation via the von Hippel-Lindau (VHL) ubiquitin ligase complex [111]. HIF1 promotes glycolysis through transcriptional activation of a number of glycolytic genes including glucose transporters (i.e., GLUT1 and GLUT3), hexokinases (i.e., HK1 and HK2), pyruvate kinase (PKM) and lactate dehydrogenase (LDHA). HIF1α also promotes the transcription of the *PDK1* gene encoding pyruvate dehydrogenase kinase I, which phosphorylates and inactivates pyruvate dehydrogenase (PDH), the first enzymatic entry into the TCA cycle [112, 113]. This HIF1α-mediated metabolic rewiring by increased influx to the glycolytic pathway and reduced influx to the TCA cycle helps to overcome elevated oxidant stress associated with hypoxia by blunting the production of mitochondrial ROS.

In addition to modifying metabolic influx to glycolytic pathway versus TCA cycle, HIF1α also mediates increased metabolic influx to the serine biosynthetic pathway and mitochondrial one carbon (folate cycle) metabolism. This facilitates NADPH and glutathione synthesis to reduce mitochondrial oxidant stress to promote BCSC maintenance and lung metastasis [114, 115]. This role of HIF1α in maintaining ROS homeostasis including redox balance at the mitochondria is critical for the maintenance of CSCs under metabolic stressful conditions (i.e., chemotherapy or antiangiogenic therapy). Indeed, when TNBCs were treated with paclitaxel or gemcitabine, induction of HIF activity including HIF1α and HIF2α led to increased ALDH$^+$ BCSCs and chemotherapy resistance, associated with increased secretion of IL-6 and IL-8 [116], two prominent inflammatory cytokines promoting BCSC activity [117, 118]. Subsequent studies identified a redox-mediated mechanism of HIF1α promoting a chemotherapy-induced BCSC phenotype [119].

The development of antiangiogenic agents was thought to be a major advance in cancer treatment. Several therapeutic agents targeting tumor neovascularization have been developed, including antibodies (i.e., bevacizumab) targeting vascular endothelial growth factor (VEGF) and receptor tyrosine kinase inhibitors (i.e., Sunitinib and Nintedanib) that block signaling mediated by the VEGF receptor. Despite the encouraging responses of some tumor types including colorectal [120], renal cell [121] and pancreatic neuroendocrine [122] carcinomas to antiangiogenic therapies, numerous other tumor types, particularly breast cancer [123–125], are poorly responsive to antiangiogenic regimens. Previous studies in our laboratory showed that that tumor hypoxia generated by anti-angiogenic agents enriches ALDH[+] E-BCSCs within the tumor hypoxic zones in a HIF1α dependent manner [126], suggesting that the induction of ALDH[+] E-BCSCs by standard antiangiogenic therapy may contribute to antiangiogenic resistance in breast cancer. Thus, targeting HIF1α-dependent induction of ALDH[+] BCSCs by chemo or antiangiogenic therapy may overcome chemotherapy or antiangiogenic resistance. In support of this idea, co-administration of HIF inhibitors such as digoxin together with standard chemotherapeutic (paclitaxel and gemcitabine) overcome chemo resistance and prevented tumor relapse in TNBC xenograft mice [116]. Similarly, concurrent administration of a novel HIF1α inhibitor CRLX101 (an investigational nanoparticle-drug conjugated with camptothecin) and bevacizumab led to decreased induction of both HIF1α and ALDH[+] CSCs in breast tumors leading to tumor regression and delayed tumor recurrence in preclinical mouse models [127].

5.2. Roles of NRF2 in regulation of redox and intermediary metabolism

The nuclear factor erythroid 2-related factor 2 (NRF2) is one of the most important transcription factors orchestrating intrinsic resistance to oxidative stress and adaptive antioxidant responses to various environmental stressors [50, 128]. NRF2 has been traditionally regarded as a tumor suppressor due to its roles as the main defense mechanism of the cell and a major regulator of cell survival. Yet, recent studies demonstrate that hyperactivation of the NRF2 pathway creates an environment that not only supports the survival of the normal cells, but also the growth and progression of malignant cells, protecting them against oxidative/xenobiotic stress, chemotherapeutic agents, and radiotherapy[129]. Accumulating evidence is emerging to demonstrate that NRF2 profoundly influences the metabolism of glucose, lipids, amino acids and nucleotides [128, 130]. Through regulation of redox and intermediary metabolism of tumor cells, NRF2 pathway serves as a driver of cancer progression, metastasis, and resistance to therapy.

5.2.1. Regulation of NRF2 activity—NRF2 belongs to the cap 'n' collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors equipped with 7 modular NRF2-ECH homology domains (Nehl-7), each of which preforming distinct functions to regulate NRF2 activity [128]. In unstressed conditions, NRF2 protein levels remain low in the cytoplasm, where it forms a complex through dimerization of its Neh2 domain with the Kelch-like ECH-associated protein 1 (Keap1), a NRF2 negative regulator that targets NRF2 for ubiquitination and proteasomal degradation [131, 132]. Electrophiles and oxidants inhibit Keap1-mediated proteasomal degradation of NRF2, thereby enabling NRF2 accumulation and nuclear translocation to initiate the transcription of a wide variety of antioxidant genes that facilitate cellular adaptation to stress[128]. Once activated, NRF2

hetero-dimerizes with the small MAF proteins to control the basal and inducible expression of over 200 genes that contain antioxidant response elements (AREs) in their regulatory regions [133]. Although NRF2 is principally regulated by Keap1, the Neh6 domain of NRF2 confers additional mechanism of stability control by recruiting β-transducin repeat-containing protein (β-TrCP), a substrate adaptor for the S-phase kinase-associated protein 1 (Skpl)–Cul1–Rbx1 core E3 complex. This negative regulation of NRF2 by β-TrCP is enhanced by glycogen synthase kinase 3 (GSK3) activities [134–136].

In addition to ubiquitination, NRF2 protein expression is subject to other forms of post-translational modifications including phosphorylation and acetylation, which allow modulation of NRF2 activity by fine-tuning its subcellular localization in the cytoplasm/nucleus. For example, activation of the AMP-activated protein kinase (AMPK), a master regulator of metabolism to maintain cellular bioenergetics during metabolic stress, increases NRF2 activity through phosphorylation of GSK3β, leading to the inhibition of GSK3β function in promoting NRF2 nuclear exclusion and degradation [137, 138]. AMPK is also capable of directly phosphorylating NRF2 at Ser550 to facilitate its nuclear localization [139]. In addition to the phosphorylation of NRF2, multiple lysine residues in the Nehl domain of NRF2 were shown to be acetylated by p300/CBP, which augments promoter-specific DNA binding of NRF2 to ARE sequences and induction of antioxidant responsive gene expression during stress [140].

NRF2 activity is also controlled by transcriptional regulation. For example, functional AREs in mouse NRF2 promoter region have been identified, which mediate the autoregulation of NRF2 through transcriptional activation, providing a positive feedback mechanism [141]. NRF2 activity can also be regulated by various polymorphisms in its promoter and protein coding regions and a single nucleotide polymorphism in the ARE-like sequences of human NRF2 promoter is associated with diminished NRF2 expression a single nucleotide polymorphism in the ARE-like sequences of human NRF2 promoter is associated with diminished NRF2 expression and increased susceptibility to lung cancer[142]. The NRF2 promoter also contains a NF-kB binding site, thereby enabling its transcriptional activation by inflammatory stimuli such as lipopolysaccharide [143]. This constitutively high NRF2 expression driven by NF-kB in human acute myeloid leukemia underlies its chemo-resistance [144].

5.2.2.   NRF2 in metabolic reprograming of cancer

Beyond its function as a master regulator of antioxidant responses, NRF2 has recently been recognized as a key transcription factor mediating metabolic reprogramming in cancer cells [128, 130, 145]. Through direct regulation of key metabolic genes or indirectly through crosstalk with other transcription factors, NRF2 serves as a major hub to modulate numerous enzymes in major metabolic pathways of carbohydrates, nucleic acids, lipids, and amino acids.

NRF2 and glucose metabolism—Metabolic reprogramming refers to a process that cancer cells utilize in order to rewire their metabolic pathways and energy production network to support their proliferative and/or invasive characteristics. This phenomenon was

first demonstrated in cancers by the Warburg effect of aerobic glycolysis observed in the 1920s [68]. Although glycolysis represents one of the remarkable features of cancer metabolic alternations, the underlying mechanisms driving this metabolic alteration and its influences on cancer development and progression are still not fully understood. Activation of NRF2 increases glucose uptake and diverts it to the pentose phosphate pathway (PPP) by promoting enzyme expression in the oxidative (including glucose-6-phosphate dehydrogenase, G6PD and 6-phosphogluconate dehydrogenase, PGD) and non-oxidative (including transketolase, TKT and transaldolase, TALDO1) arms of PPP [130, 146, 147]. In addition to G6PD and PGD, NRF2 also promotes the expression of two other NADPH synthetic enzymes ME1 and IDH1[146], suggesting a role of NRF2 in replenishing NADPH, the main reducing equivalent required for redox hemostasis and biosynthesis of lipids and nucleotides. This role of NRF2 in promoting nucleotide synthesis is supported by the findings that the expression of *de novo* purine synthesis enzymes, phosphoribosyl pyrophosphate amidotransferase (PPAT) and methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), was significantly decreased following knockdown of NRF2 in A549 cells (which is deficient of Keap1) [146]. The role of NRF2 in redirecting glycolytic intermediates into anabolic pathways to support tumor growth was further demonstrated in a recent study by showing that activation of NRF2 upregulates the expression of key serine biosynthesis enzymes including PHGDH, PS ATI and SHMT and diverts glycolytic intermediate 3-phosphoglycerate (3PG) to serine/glycine synthetic pathway, which augments glutathione and nucleotide production and confers poor prognosis in human non-small cell lung cancer (NSCLC) [148].

The folate cycle, through transfer of one carbon unit between tetrahydrofolate and its derivatives in the cytoplasm or mitochondria, produces metabolites essential for cell growth, including nucleotides, methionine and NADPH [149]. Interestingly, MTHFD1L, an enzyme critical for folate cycle maintenance[150], is transcriptionally activated by NRF2, which confers metabolic advantages in hepatocellular carcinoma [151]. Together with the study showing that HIF1α induced by chemotherapeutic agents promotes BCSCs and lung metastasis by redirecting glycolytic intermediates to serine synthesis and mitochondrial one carbon metabolism [115], these studies provide evidence for a role of NRF2 and HIF1α to confer metabolic advantages to cancer cells including CSCs through metabolic reprograming.

NRF2 and glutamine metabolism—In addition to glycolysis, many tumors fuel their cellular bioenergetics by degradation of glutamine through glutaminolysis, which is catalyzed by a NRF2 target gene glutaminase (*GLS*), thereby providing cancer cells with nitrogen for the biosynthesis of nucleotides and nonessential amino acids [128, 146]. The glutamate generated by GLS can be deaminated into α-ketoglutarate to fuel the TCA cycle and this step is enhanced by the NRF2 target gene *ME1*[152]. Alternatively, glutamate can be used to fuel glutathione biosynthesis, which is catalyzed by glutamate-cysteine ligase (with catalytic GCLC and regulatory GCLM subunits) and glutathione synthetase (GS), two enzymes also regulated NRF2[152]. Upregulated expression of GCLC and GCLM and ME1 by NRF2 has been shown to direct increased carbon flux from glutamine toward GSH biosynthesis and TCA cycle [146]. illustrating a role of NRF2 in rewiring glutamine

metabolism in cancer. Of note, the ability of NRF2 to promote cell proliferation through metabolic reprogramming (i.e., PPP shunting, NADPH production, nucleotide synthesis and glutamine metabolism) is augmented in the presence of active PI3K-Akt signaling [146]. suggesting a positive feedback mechanism between oncogenic pathways and NRF2 in driving the malignant phenotype.

NRF2 and lipid metabolism—As summarized in two recent reviews [128, 130]. NRF2 negatively regulates a variety of genes involved in fatty acid biosynthesis, desaturation and transport. In contrast, a number of lipases involved in the degradation of triglycerides/ phospholipids and enzymes involved in mitochondrial fatty acid β-oxidation (FAO) are positively regulated by NRF2. The FAO products including NADH and FADH2 counteract ROS accumulation to reduce oxidative stress and increase ATP production by serving as the electron carriers of the ETC complex [153]. Several studies have reported a function of FAO in the maintenance of hematopoietic stem cells (HSCs) and CD8+ memory T cells, which display some stem like characteristics [154–156].

Recent studies also implicate a role of FAO in driving a CSC-like phenotype and chemo-resistance. For instances, the promyelocytic leukemia (PML) gene expressed in breast cancer acts as both a negative regulator of PGC-1α acetylation and a potent activator of PPAR signaling and fatty acid oxidation, which promotes ATP production and inhibits anoikis, a property associated with CSCs and metastasis[157]. The expression of the oncogenic transcription factor MYC is elevated in many TNBCs, which drives dysregulated FAO activity [158]. Pharmacologic inhibition of FAO catastrophically decreased energy metabolism in MYC-overexpressing TNBC cells and blocked tumor growth in a MYC-driven transgenic TNBC model and a MYC-overexpressing TNBC patient-derived xenograft model [158]. The activation of JAK/STAT3 promotes M-like BCSC phenotype and tumor growth [159]. Inhibiting JAK/STAT3 blocks the self-renewal of M-like BCSCs and the expression of diverse lipid metabolic genes, including carnitine palmitoyltransferase IB (CPT1B), a rate-limiting enzyme of FAO pathway; and blocking FAO re-sensitize breast cancer cells to chemotherapy while reducing cancer sternness *in vivo* [160].

5.3. Roles of NRF2 and HIF1α in promoting CSC plasticity

Our recent studies demonstrated that NRF2 and HIF1α are critical for promoting an E-BCSC phenotype during metabolic/oxidative stress and suppression of NRF2 or HIF1α impedes the maintenance and clonal expansion of E-BCSCs [18]. As shown in Figure 3, three different mechanisms by which metabolic/oxidant stress induces an enhanced E-BCSC phenotype are identified: 1) conversion of M- to E-BCSCs, which is mediated by AMPK-dependent HIF1α stabilization, 2) activation of HIF1α-NOTCH self-renewal pathway, 3) activation of NRF2-mediated ALDH1A1/3 expression, which is independent of HIF1α.

Although our studies identify ROS-AMPK-HIF1α and NRF2 pathways in regulation of BCSC state dynamics, additional studies are necessary to determine how activation of AMPK regulates HIF1α stability during metabolic/oxidative stress. Interestingly, previous studies showed that NRF2 knockdown reduces HIF1α protein levels and, consequently, the expression of VEGF, PDGF, and angiopoietin in glioblastoma [161], suggesting that NRF2

mediates HIF1α stabilization induced by hypoxic stress. Therefore, it is likely that that enhanced NRF2 expression not only promotes the self-renewal/proliferation of E-BCSCs, but also mediates AMPK-dependent HIF1α stabilization required for facilitating M to E state transition of BCSCs under metabolic/oxidative stress. If so, NRF2 would serve as an important hub in facilitating BCSC M to E state transition as well as maintenance and proliferation of E-BCSCs under metabolic/oxidant stress (Figure 3). In addition, as the tumor microenvironment including nutrient/oxygen availability and tumor stroma constantly impacts tumor behavior, future studies to assess how these environmental factors affect BCSC metabolism and phenotypic plasticity *in vivo* will be critical.

One plausible mechanism of NRF2 and HIF1α mediating treatment resistance is through metabolic reprograming, which confers metabolic advantages in CSCs, especially E-like CSCs that express elevated levels of NRF2 and HIF1α. This idea is supported by our recent gene profiling analyses of E- and M-BCSCs isolated from two patient derived xenograft (PDX) models of TNBC, which identified differential expression patterns of glycolysis and PPP pathway genes in M- and E-BCSCs[18]. Specifically, a wide variety of glycolytic enzymes ranging from HK1 to LDHD were highly elevated in M- but less robustly in E-BCSCs. In contrast, various PPP enzyme genes, including G6PD, were highly upregulated in E- but less robustly in M-BCSCs [18]. This robust elevation of PPP genes in E- but not M-BCSCs suggests that elevated expression of NRF2 and/or HIF1α in E-BCSCs redirects glycolytic intermediates to PPP pathway, promoting NADPH production and nucleotide biosynthesis to support the propagation of E-BCSCs.

6. Concluding remarks

Recent findings that E- and M-like CSCs significantly differ in their redox states, metabolic pathways and sensitivities to metabolic/oxidant stress have identified a novel framework exploiting metabolic vulnerabilities of distinct CSC states [18]. As increased oxidant stress, through activation of HIF1α and NRF2 signaling, promotes the transition of quiescent M-CSCs to an E-like state that is more susceptible to the inhibition of redox metabolism especially NRF2-mediated antioxidant pathways [18], future pro-oxidant-based therapeutic strategies utilizing conventional cancer therapies (i.e., standard chemotherapeutic/antiangiogenic agents, radiation therapy) together with agents targeting NRF2-mediated antioxidant responses may prove to be efficient in overcoming resistance associated with traditional cancer therapies by targeting both M- and E-like CSCs.

In addition to traditional cancer therapies that are associated with toxicity to normal cells, pharmacological ascorbate (high doses of Vitamin C given via intravenous injection) has re-emerged as a non-toxic and easily implementable pro-oxidant which has the potential to increase treatment efficacy when combined with standard-of-care radio-chemotherapy [162–164]. Recent studies have demonstrated that oxidant-mediated disruption of iron metabolism causes the selective susceptibility of NSCLC and GBM cancer cells to pharmacological ascorbate [165], supporting a generalized mechanism for the use of pharmacological ascorbate in cancer therapy. As iron-dependent accumulation of lipid hydroperoxides can trigger ferroptosis, a regulated cell death nexus linking metabolism, redox biology and

diseases [166], future studies are necessary to determine if pharmacological ascorbate disrupts NRF2-regulated iron metabolism in CSCs to induce ferroptosis.

Considering the central roles of NRF2 in regulating antioxidant defenses and metabolism, future metabolic tracing studies dissecting how activation of NRF2 rewires the metabolism of CSCs to promote their phenotypic/metabolic plasticity and proliferation under metabolic/ oxidative stress are also required, which will not only validate NRF2 as a critical target to abrogate CSC plasticity and proliferation, but also identify potential new targets downstream of NRF2 in driving CSC self-renewal/proliferation and treatment resistance. These mechanistic studies may reveal novel therapeutic strategies to target the metabolic vulnerabilities of CSCs, thereby improving treatment efficacy.

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	E-BCSCs	M-BCSCs	Bulk tumor cells
ROS level	++	+/-	+++
Glycolysis	+	++	+/-
OXPHOS	++	+	+/-
NRF2 activity	high	low	low
2DG sensitivity	low	high	high
Sensitivity to NRF2 pathway inhibition	high	low	high

Figure 1. Metabolic differences of E- and M-BCSCs versus the bulk tumor cells.
The CD24$^-$CD44$^+$ M-BCSCs and ALDH$^+$E-BCSCs significant differ in many aspects of cellular metabolism as compared to the bulk tumor cells identified as ALDH$^-$CD24$^+$CD44$^-$.
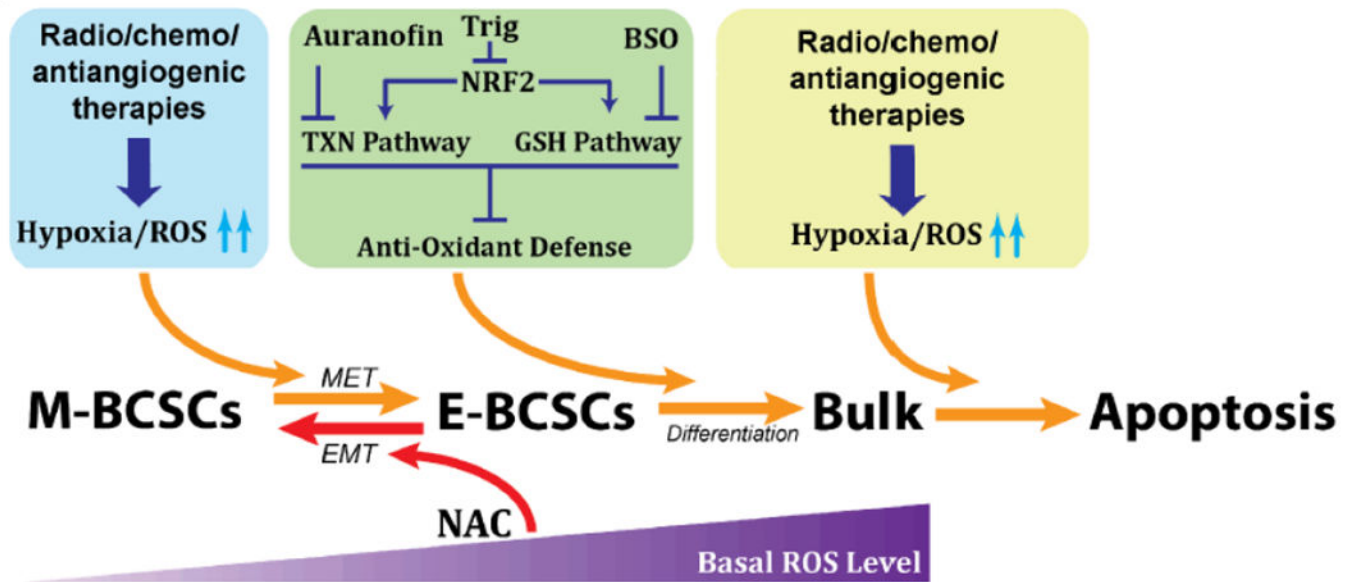
Figure 2. A pro-oxidant based therapeutic approach with potential to increase treatment responses for traditional cancer therapies.

Combination strategies utilizing traditional cancer therapies (i.e., standard radio-chemotherapies or anti-angiogenic therapies) in combination with inhibitors of NRF2-mediated antioxidant responses (i.e., NRF2 inhibitor Trig or inhibitors of TXN/GSH antioxidant pathways including AUR/BSO) enhance treatment responses by simultaneously targeting CSCs of distinct states.
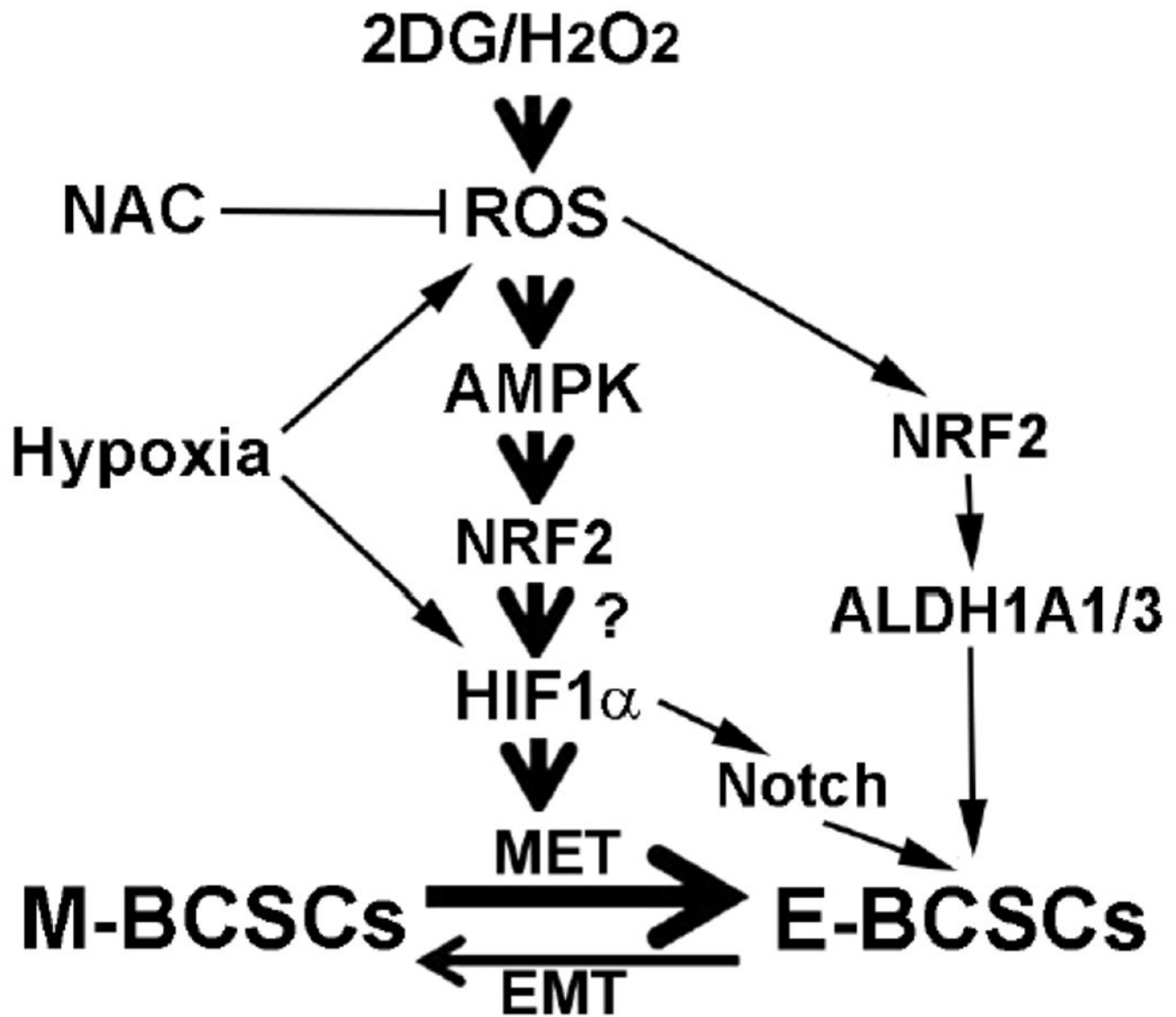
Figure 3. NRF2 acts as a central hub to facilitate the transition of BCSCs from the M to E state as well as the maintenance/proliferation of E-BCSCs under metabolic/oxidant stress.
The activation of NRF2 during metabolic/oxidant stress promotes CSC plasticity by mediating AMPK-dependent HIF1α stabilization, which is required for the conversion of M- to E-BCSCs. NRF2 activation also mediates the activation of HIF1α-NOTCH self-renewal pathway and the expression of NRF2 target genes ALDH1A1/3 to facilitate the propagation of E-BCSCs.