THERAPEUTIC TARGETING OF CANCER STEM-LIKE CELLS (CSC) – THE CURRENT STATE OF THE ART

EDITED BY: Cyril Corbet and Alexandre Prieur
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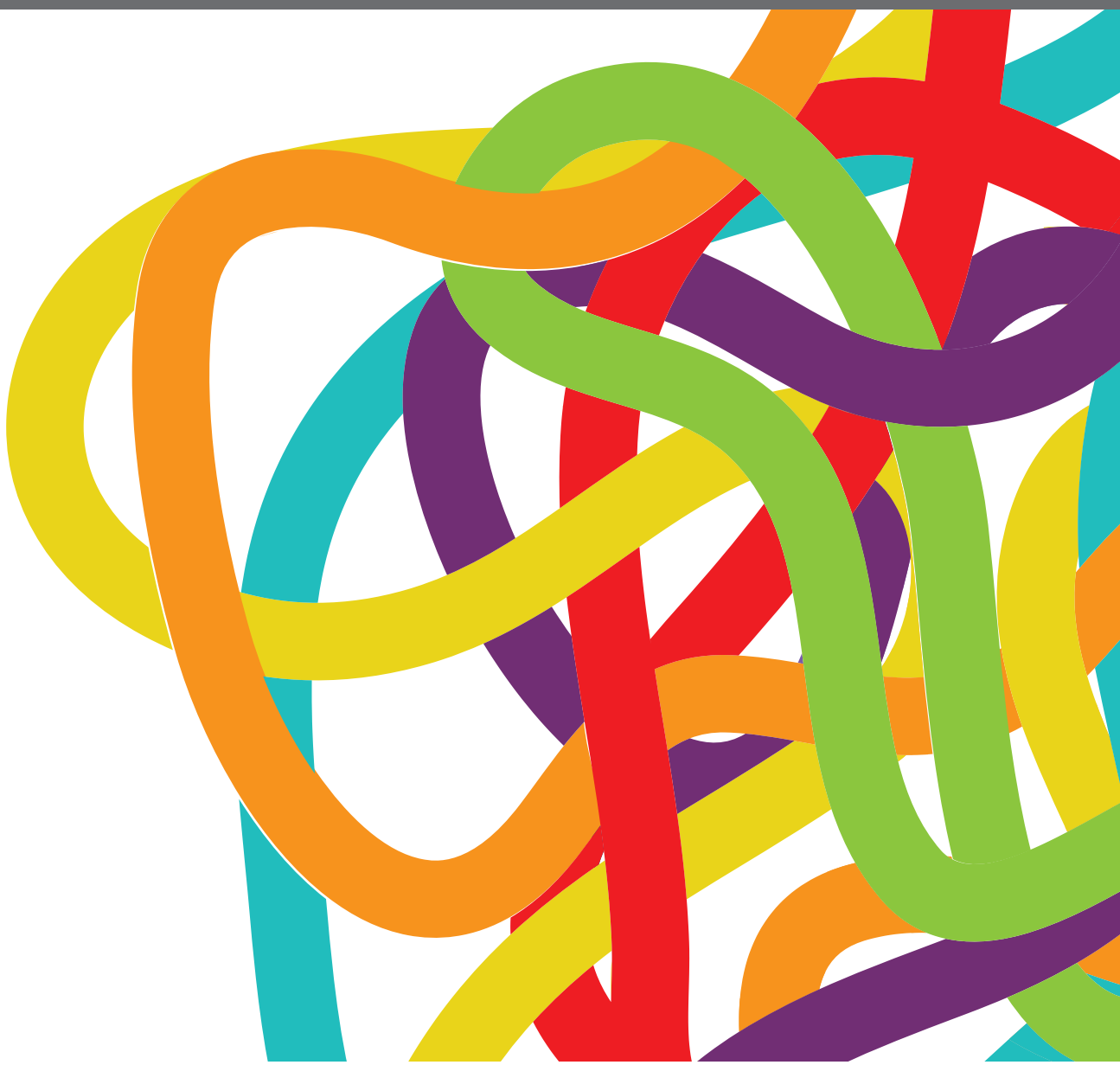

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THERAPEUTIC TARGETING OF CANCER STEM-LIKE CELLS (CSC) – THE CURRENT STATE OF THE ART

Topic Editors:
Cyril Corbet, Catholic University of Louvain, Belgium
Alexandre Prieur, ECS-Progastrin, Switzerland

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EDITORIAL
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Editorial: Therapeutic Targeting of Cancer Stem-Like Cells (CSC) – The Current State of the Art

Cyril Corbet[1]* *and Alexandre Prieur*[2]

[1] Pole of Pharmacology and Therapeutics (FATH), Institut de Recherche Expérimentale et Clinique, UCLouvain, Brussels, Belgium, [2] ECS-Progastrin Chemin de la Meuniere 12, Prilly, Switzerland

Keywords: cancer stem cells (CSC), therapeutic targeting, antibodies, transcription factors, small molecules

Editorial on the Research Topic

Therapeutic Targeting of Cancer Stem-Like Cells (CSC) – The Current State of the Art

Cancer stem-like cells (CSC) represent a small population of tumor cells that are thought to exhibit a tumor-initiating potential, as well as enhanced therapy-resistant and metastasis-forming capacities, thereby actively contributing to clinical relapse and poor prognosis in cancer patients. Although all these phenotypic properties make CSC targets of great interest in drug discovery, there are currently only few therapeutic approaches that have reached late stages of clinical development in oncology. This Special Topic of Frontiers in Oncology attempts to address some major concerns related to the therapeutic targeting of CSC: what are these cells, how may the specific permissive microenvironment (the so-called stem cell niche) be therapeutically exploited, and what are the emerging therapeutic avenues aiming to eradicate this specific tumor cell subpopulation.

The papers in this Special Topic can be categorized into three main parts. The first part highlights the influence of tumor microenvironment (TME) peculiarities on the emergence and/or maintenance of stem-like phenotypes and how this can be therapeutically integrated and exploited. This subject is broadly reviewed by Sun et al. and then also addressed by De Angelis et al. in the specific contexts of cell dormancy and therapy resistance. Chan et al. discuss the interplay between CSC and stromal cells, including cancer-associated fibroblasts and tumor-infiltrating mesenchymal stem cells (MSC). Avnet et al. also summarize the currently available preclinical models that help evaluate the functional interaction between MSC and cancer cells. Finally, Vander Linden and Corbet describe how tumor acidosis, a common hallmark of TME in solid tumors, may provide a permissive niche to shape more aggressive stem-like cancer cell phenotypes. These different articles also review new therapeutic options aiming to eradicate CSC by integrating and/or exploiting the TME niches in order to overcome therapy resistance and metastatic dissemination.

The second set of papers relates to the metabolic preferences of CSC and emerging metabolism-based therapeutic strategies that are currently in (pre)clinical testing for cancer treatment. Jagust et al. make an overview of metabolic pathways that support CSC phenotypes in different cancer types, while Garnier et al. discuss specifically about the metabolism of glioblastoma stem-like cells and its role in tumor progression and clinical relapse. Recalcati et al. report an increasing evidence for dysregulated iron homeostasis in cancer cells, with a special focus on liver CSC. These authors also discuss new therapeutic options aiming to manipulate iron metabolism for anti-tumor therapy. Another review article from Lucena-Cacace et al. reports the important role for nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD^+ salvage pathway, in the maintenance of a glioma cancer stem-like cell (GSC) population. They discuss how NAD^+ homeostasis supports metabolic and non-metabolic processes that contribute to a GSC phenotype. Finally, Han et al. report, in an research

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Edited and reviewed by:
Amit K. Tiwari,
University of Toledo, United States

Correspondence:
Cyril Corbet
cyril.corbet@uclouvain.be

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article, that radioresistant breast cancer (stem) cells rely on fatty acid metabolism to survive and grow, with carnitine palmitoyl transferases 1A and 2 as main actors and potential therapeutic targets.

The third and final part of this special issue is focused on new therapeutic avenues to target CSC populations. Marcucci et al. discuss the potential application of antibody-drug conjugates as tools for a selective eradication of CSC and how some limitations related to their use may be addressed. Roth et al. describe the roles of the renin-angiotensin system (RAS) in CSC biology and how RAS modulators may offer new therapeutic approaches to target CSC and reduce tumor growth. Finally, Civenni et al. review the influence of transcriptional regulators in the emergence and/or maintenance of a stem-like cell population in the context of prostate cancers, and describe current therapeutic strategies aiming to interfere with specific transcriptional programs and associated stem-like phenotypic changes.

AUTHOR CONTRIBUTIONS

CC has written the editorial. CC and AP have collected and edited the articles of the Research Topic.

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REVIEW
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Glioblastoma Stem-*Like* Cells, Metabolic Strategy to Kill a Challenging Target

Delphine Garnier[1], *Ophélie Renoult*[1], *Marie-Clotilde Alves-Guerra*[2,3], *François Paris*[1,4] and *Claire Pecqueur*[1,5]*

[1] CRCINA, INSERM CNRS, Université de Nantes, Nantes, France, [2] INSERM CNRS, Institut Cochin, Paris, France, [3] Université Paris Descartes, Sorbonne Paris Cité, Paris, France, [4] Institut de Cancérologie de l'Ouest - René Gauducheau, St Herblain, France, [5] LabEx IGO "Immunotherapy, Graft, Oncology", Nantes, France

Over the years, substantial evidence has definitively confirmed the existence of cancer stem-*like* cells within tumors such as Glioblastoma (GBM). The importance of Glioblastoma stem-*like* cells (GSCs) in tumor progression and relapse clearly highlights that cancer eradication requires killing of GSCs that are intrinsically resistant to conventional therapies as well as eradication of the non-GSCs cells since GSCs emergence relies on a dynamic process. The past decade of research highlights that metabolism is a significant player in tumor progression and actually might orchestrate it. The growing interest in cancer metabolism reprogrammation can lead to innovative approaches exploiting metabolic vulnerabilities of cancer cells. These approaches are challenging since they require overcoming the compensatory and adaptive responses of GSCs. In this review, we will summarize the current knowledge on GSCs with a particular focus on their metabolic complexity. We will also discuss potential approaches targeting GSCs metabolism to potentially improve clinical care.

Keywords: Glioblastoma, cancer stem cells, cancer metabolism, tumor microenvironment, cancer heterogeneity, cancer plasticity

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Edited by:
Cyril Corbet, Catholic University of Louvain, Belgium

Reviewed by:
Michael P. Lisanti, University of Salford, United Kingdom
Hiroaki Nagashima, Massachusetts General Hospital and Harvard Medical School, United States

***Correspondence:**
Claire Pecqueur
claire.pecqueur@univ-nantes.fr

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INTRODUCTION

Glioblastoma (GBM) is the most common primary brain tumor in adults, defined as a grade IV glioma according to the WHO (World Health Organization) classification of central nervous system tumors (1). GBM is characterized by a highly infiltrative nature within the surrounding brain parenchyma and a dismal prognosis despite aggressive treatments. Present GBM standard of care, as defined in the Stupp protocol, includes surgical tumor resection followed by radiotherapy with concomitant and adjuvant chemotherapy with Temozolomide. With this therapy, patient median survival is only 18 months with <5% of patients surviving over 5 years (2). This poor response rate can be explained by the almost inevitable GBM recurrence within a year of initial diagnosis in part due to the limitations of surgical resection given GBM propensity for infiltration, but also to an extensive tumoral heterogeneity resulting in a large range of variabilities in crucial biological responses like cell proliferation, invasion, and sensitivity to conventional treatments. At the genetic level, GBM display a highly mutated genome including loss, amplification, or mutation of EGFR (including expression of the constitutively active form EGFRvIII), PDGFRA, NF1, PTEN, RB1, and p53, resulting in the deregulation of many signaling pathways. Furthermore, epigenetic modifications are also well-characterized in GBM, especially on the O^6-methylguanine

methyltransferase (MGMT) gene, a DNA repair enzyme involved in the fixation of damages induced by alkylating agents such as Temozolomide. Inactivation of the MGMT enzyme, following its promoter hypermethylation, correlates to a better prognosis due to the resultant inability of the MGMT enzyme to remove alkyl groups from DNA (3). This heterogeneity becomes even more complex when therapy comes into the picture with the emergence of drug-resistant clones with highly mutable phenotypes (4–6).

At the cellular level, functional GBM heterogeneity can be explained by the existence of multiple cellular subpopulations of cancer cells. In particular, Glioblastoma stem-*like* cells (GSCs) display stem cell properties of self-renewal and multi-lineage differentiation. These cells generate cellular heterogeneity by establishing a differentiation hierarchy leading to a wide range of distinct cell types present in the tumor. Importantly, extensive studies have implicated these GSCs in GBM recurrence. Recently, an increased focus upon this GSCs subpopulation suggests that their eradication is definitively required in order to successfully treat GBM patients.

Normal stem cells are unique in their ability to self-renew, proliferate, and differentiate in various cell types. They are also characterized by poorly developed mitochondria and a strong glycolytic metabolism. Whereas, the metabolic alterations have been included as a hallmark of cancer cells, contradictory results have been reported for GSCs suggesting a metabolic flexibility. The aim of this review is to summarize and emphasize some of the key aspects of GSCs, with a particular focus on their dynamic emergence and metabolic plasticity. Given the obvious need for improvement of current therapies for GBM, we will also present data on how metabolic targeting might be exploited to eradicate GSCs and hopefully improve clinical outcomes.

GLIOBLASTOMA STEM-*LIKE* CELLS

Definition and Origin of Cancer Stem-*Like* Cells

The cancer stem-*like* cells (CSCs) concept was originally proposed to reconcile the complex phenotypic heterogeneity of tumors and the fact that only a few cancer cells are actually tumorigenic. CSCs possess the capacity to self-renew, initiate a tumor as well as the potential to differentiate to reconstitute the initial tumor mass, including its heterogeneity (7). An increasing amount of evidence based on preclinical and clinical studies demonstrates the importance of CSCs in tumor progression and relapse suggesting that cancer eradication requires killing of CSCs.

Since the CSCs concept emerged in the 1970's, the origin of these cells is still controversial with opposite models to explain their presence in tumors. The initial and traditional theory is based on a hierarchical and unidirectional model, where CSCs constitute a specific and rare subpopulation of cells that possess the unique capacity to repopulate and reconstitute tumor heterogeneity through symmetric self-renewal of the CSCs pool, and asymmetric divisions to generate differentiated cancer cells (8, 9). In this model, CSCs may have emerged after acquisition of mutations in normal neural stem cells. However, this model has been challenged by subsequent studies highlighting cancer cell plasticity occurring in tumors and giving rise to a new stochastic model based on clonal evolution (10–12). In this model, some tumor cells can progressively accumulate mutations and reacquire a self-renewal potential, forming several CSCs clones (13). Therefore, all the cells forming the tumor bulk have the potential to become CSCs through a dedifferentiation process, already underlining the complexity of their characterization

In conclusion, whereas the non-CSCs constitute the tumor bulk and the CSCs are involved in tumor relapse and metastasis, the hierarchy between CSCs and non-CSCs is definitively bi-directional and highly dynamic, adding further complexity to our understanding of the tumor.

Phenotypic Plasticity of Glioblastoma Stem-*Like* Cells

In Glioblastoma, GSCs were first identified by Singh et al., as a population of cells capable of initiating tumor growth *in vivo* (8). Like their normal counterparts the neural stem cells, GSCs exhibit self-renewing and multilineage differentiation into neurons, astrocytes, and oligodendrocytes, and even transdifferentiation abilities [review in (14)]. However, in contrast to neural stem cells, GSCs display the ability to initiate a tumor upon transplantation and to recapitulate its initial phenotype and heterogeneity. GSCs are highly resistant to chemotherapy (15, 16) and radiation (17), and have been involved in GBM tumorigenicity. Indeed, GSCs are slow-cycling, have the capacity to limit DNA lesions through strong and efficient DNA damage response, and prevent cytotoxicity through high drug efflux by ABC transporters. Recently, several studies have highlighted that GSCs may also be involved in the infiltrative nature of GBM (18–20). In particular, expression level of Wnt5a defines the infiltrative capacity of GBM cells, including in GSCs. In fact, its overexpression in GSCs confers an exacerbated invasive phenotype while its inhibition reduces their invasive potential both *in vitro* and *in vivo*.

In Glioblastoma, several studies demonstrated the bidirectional plasticity between GSCs and more differentiated GBM cells as a result of environmental factors. First, besides promoting the self-renewal of GSCs, hypoxia through HIF2α promotes a stem-like phenotype in the non-stem population by upregulating several stem cell factors, such as Oct4, Nanog and c-Myc (21). Second, as we described before, chemotherapy as well as radiation consistently increase the GSCs pool over time. In fact, therapeutic doses of Temozolomide trigger a phenotypic shift in the non-GSCs population to a GSCs state (22) while radiation increases tumorigenicity through reacquisition of stemness markers and stem-associated properties of GBM cells, in part via a survivin-dependent pathway (23). Recently, it has been suggested that Sox2, a well-known transcriptional factor involved in stemness maintenance, might be central in tumor cell plasticity by regulating dedifferentiation and acquisition of GSCs properties, through a transcriptional regulation of distinct genes set in differentiated tumor cells and GSCs (24). Hyperactivation of the tyrosine kinase c-Met, involved in the reprogramming of induced pluripotent stem (iPS) cells, also

induces GSCs reprogramming via a mechanism requiring Nanog (25). Finally, activation of Epithelial-to-Mesenchymal transition (EMT) enables the conversion of non-CSCs in CSCs through both intracellular and extracellular signaling pathways.

Besides genetic and cellular heterogeneities, The Cancer Genome Atlas (TCGA) has established a molecular classification of GBM using transcriptional profiling data of bulk tumor based on dominant genes expressed in each GBM subtype (26, 27). Three main GBM subtypes can be easily distinguished based on molecular signature, therapy responses and patient survival: the mesenchymal (MES), classical, and proneural (PN), (28). Notably, the MES subtype of GBM is associated with relatively poor prognosis compared with that of the other subtypes and shows resistance to conventional therapy. GSCs also display these molecular signatures with distinct activated signaling pathways, biological phenotypes, and residing niches (29–31). In agreement with the worst outcome, MES GSCs are more resistant to radiation and display more aggressive phenotypes *in vitro* and *in vivo* (31, 32). Recent studies have added a layer of complexity in this molecular classification by demonstrating that molecular subtypes are flexible and vary spatially and temporally within the same tumor. First, a study collecting spatially distinct tumor specimens from the same tumor as well as single-cell RNA-sequencing resolution revealed that a single tumor consists of a heterogeneous mixture of tumor cells from different subtypes, with one subtype usually being highly represented (33, 34). Second, the molecular subtype can evolve with time, microenvironment or stress, in particular toward a MES transdifferentiation from the other subtypes (35), in agreement with a higher frequency of the MES subtype at recurrence (33, 36). Furthermore, radiation of PN GSCs up-regulated mesenchymal-associated markers while down-regulating PN-associated markers. Collectively, these studies underlined the strong unstable nature of GBM, fully in agreement with its previous designation as Glioblastoma Multiform. Since the molecular patterns of GBM can partially explain clinical outcomes and predict responses to treatment, this classification should help understanding the GBM tumorigenesis and progression and provide diagnostic and prognostic information, as well as help the development of new therapeutic approaches. However, several clinical trials based on either targeted therapies for specific mutations or subtypes have been completed with no consistent changes in clinical activity [review in (37)].

Taken altogether, these studies underlie the great diversity of GSCs states suggesting that emergence of GSCs, and CSCs in general, should be consider as a phenotypic shift rather than a true dedifferentiation process.

Characterization and Isolation of GSCs

As we described previously, CSCs in general constitute a very rare population but one of the most important to target, for their unique ability to reconstitute the initial tumor and their strong resistance to therapy. The ability of all cells forming the tumor bulk to shift into GSCs already underlines the complexity of their characterization. The identification of GSCs is classically based on cell surface markers such as CD133 [review in (38)]. It has been shown that CD133 positive GSCs better survive radiation than CD133 negative cells and were able to give rise to a tumor in a xenograft model. However, some later works showed that CD133 negative cells were also able to initiate tumor growth. Furthermore, while both subtypes exhibit similar GSCs enrichments, PN GSCs clearly exhibit CD133 at their surface in contrast to MES GSCs that do not (39, 40). The sets of markers being used to identify GSCs are being constantly updated and includes Sox2, Olig2, Nestin, CD15/SSEA-1, CD44, integrin alpha6, L1CAM, as well as drug efflux transporters like ATP-binding cassette transporters (ABC) [review in (41)]. However, those markers are also expressed by normal stem cells. Furthermore, purified CD133 positive cells are able to re-establish the initial ratio of CD133 positive and CD133 negative cells (11, 12). This was true not only for CD133 marker but also for 15 commonly used CSCs markers including CD44, ABCB5, or cadherin. Finally, in line with CD133 expression, while PN and MES GSCs are able to self-renew both *in vitro* and *in vivo*, bioinformatic analyses have revealed distinct GSCs phenotypes for these two molecular subtypes into full and restricted stem-like phenotypes, respectively (42). Thus, despite many years of investigation, there is no consensus on an appropriate way to identify these cells. It is nowadays commonly accepted that the identification of GSCs and CSCs in general, requires the combination of several markers and also a functional demonstration of their stem cells features such as self-renewal and their ability to initiate tumor growth.

Because of the difficulty to precisely define GSCs specific markers, the isolation of this rare population of cells becomes very delicate. However, the development of relevant *in vitro* models is essential to better understand GSCs biology, to uncover potential vulnerabilities and to identify novel therapeutic targets. Culture methods, initially developed for neural stem cells, have been adapted to enrich primary GBM cultures in GSCs. Post-surgery GBM specimens are mechanically dissociated and can grow in two different phenotypes, each requiring specific media. They can grow as adherent monolayer cells in presence of serum, which represent differentiated GBM cells, or as free-floating tumorospheres enriched in GSCs when cultured in serum-free medium containing EGF and bFGF (43). Importantly, these two phenotypic states, mutually reversible, differ in proliferation rate, invasion, migration, and chemosensitivity. The validation for GSCs enrichments is based on the combinatorial expression of cell surface markers (CD133, CD44, CD15), intracellular stem cell markers (Nestin, Sox2) and most importantly key stem cells features such as self-renewal and tumor initiation. Self-renewal can be evaluated by limiting dilution assay or colony forming cell assay since one CSC is able to form a tumorosphere due to its self-renewal potential. Based on their high expression of drug efflux transporters, another way to determine GSCs enrichment in GBM cultures is to identify the cells which are able to exclude fluorescent dye, defined as the side population (44). High aldehyde dehydrogenase (ALDH) activity is also one feature of CSCs in general, including GSCs (45). Finally, the most rigorous criteria to establish the presence of GSCs in GBM cultures is to test their ability to initiate a tumor *in vivo*. Importantly, in contrast to commercially available cell lines, these primary GBM cultures enriched in GSCs reproduce the overall behavior of

GBM in patients, in particular their highly invasive feature. Thus, while not perfect, these *in vitro* GBM models present a unique opportunity to develop effective CSCs-directed therapies.

Interactions of GSCs With Their Microenvironment

Like normal stem cells, GSCs rely on a similar permissive tumor microenvironment (TME), also called the niche, to retain their exclusive abilities to self-renew and give rise to more differentiated progenitor cells. This niche includes many different cell types from stromal to immune cells with many reciprocal communications essential for GSCs maintenance, survival and proliferation, as well as TME recruitment, activation, programming, and persistence. Moreover, the CSCs niche also has a protective role by sheltering GSCs from diverse genotoxic insults contributing to their enhanced therapy resistance.

Cellular Components of the Niches

Mesenchymal stem cells (MSC) have remarkable tumor tropism that allows them to migrate toward and engraft specifically into the tumor sites, especially cells that have escaped the main tumor mass. MSC are multipotent progenitors that can differentiate into adipocytes, osteocytes, and chondrocytes. They are present in GBM, and more particularly in GSCs niches (46). The role of MSC in tumor progression is still debatable since other studies have shown an opposite effect with MSC inducing the inhibition of GBM tumor growth (47, 48). MSC provide supportive signals to GSCs, as indicated by the inverse correlation between glioma patients survival and the percentage of MSC (49, 50). Besides their homing capacity, they also stimulate proliferation, invasion, and tumorigenesis in GBM, in part via the cytokine IL-6 (51). Aside from a communication through direct cell-cell contact, GSCs can also exchange signals with MSC through Extracellular Vesicles (EV) [review in (52)]. EVs are membrane structures secreted by cells in the extracellular media and can transfer various information (DNA, miRNA, mRNA, proteins, lipids…) after their uptake by recipient cells. These EV contribute to tumor progression, either by reprogramming adjacent cells or by the modification of the supportive tumor microenvironment. Interestingly, the diversity of transcriptomic profiles observed in GBM subtypes is also found at the level of EV, contributing to maintain the GBM heterogeneity through the transfer of oncogenic signals and miRNA (53, 54). Moreover, MES and PN GSCs and their differentiated counterparts secrete different kinds of vesicles, and their uptake by recipient cells such that endothelial cells is also subtype-dependent (55). Thus, the use of EV as blood biomarkers recently received much attention as a fast and non-invasive way to detect and follow GBM tumors, identify their subtypes (55), or even as a marker of resistance acquisition to treatment (4). Indeed the EV content is the perfect reflection of the tumor phenotype as well as its microenvironment, and can easily be collected from a blood sample.

Although the brain has long been considered as immune-privileged due to the blood-brain barrier, recent studies have reported the existence of a direct communication between the CNS and the immune system. In Glioblastoma, the TME has been shown to be particularly immunosuppressive, such that tumor cells can escape from immune surveillance. Notably, GSCs have a lower immunogenicity and a higher suppressive activity compared to non-GSCs GBM cells, through several mechanisms: the inhibition of T cells proliferation (56), the proliferation of regulatory T cells (57) and the recruitment of myeloid-derived suppressor cells (MDSCs), via the secretion of macrophage migration inhibitory factor (MIF) or exosomes (58, 59). Additionally GSCs are involved in the tropism of immunosuppressive cells toward the tumor site including tumor-associated macrophages, through the secretion of numerous cytokines or growth factors, such as TGF-β1, SDF-1, or VEGF [review in (60)]. Interestingly GSCs seem to be more sensitive to Natural Killer (NK) cell toxicity, compared to their differentiated counterpart (61, 62). However, to prevent their recognition and elimination by cytotoxic NK cells, some GSCs, such as IDH-mutant GSCs, exhibit significantly lower NKG2D expression to prevent their recognition by NK cells (63). Inversely different soluble factors secreted by immune cells also play essential roles for the biology of GSCs, for example TGFβ secreted by myeloid cells is an inductor of EMT involved in GSCs emergence.

Oxygen Is a Critical Component of GSCs Niches

Oxygen concentration plays a fundamental role in stemness maintenance defining several GSCs niches, in particular the perivascular and the hypoxic niches (64, 65). The most frequently described GSCs niche is the perivascular one in which the vascular component plays a crucial role in stemness maintenance and survival as well as GSCs dissemination (66). Accordingly, orthotopic co-implantation of GSCs with endothelial cells increases the GSCs fraction in xenograft tumors. Besides the direct contact between GSCs and endothelial cells, soluble factors produced by these cells also enhance stemness markers (66, 67). Notch signaling, in part through NO, plays a critical role in both GSCs maintenance (68) and GSCs radioresistance (69). In return, GSCs secrete several cytokines or growth factors, such as SDF-1 or VEGF, to promote angiogenesis through endothelial cells proliferation and recruitment (70, 71). Upon particular stimuli, GSCs can transdifferentiate into endothelial cells or pericytes to directly contribute to the perivascular niche (14). Importantly, this transdifferentiation can also transfer to endothelial cells the capacity of resistance and genetic mutations (72). The communication between the perivascular niche and the GSCs is therefore a two-way exchange with benefits to both blood vessels and GSCs.

One hallmark of GBM tumors is the existence of hypoxic zones which have been shown to be enriched in GSCs (73). Indeed, hypoxia directly supports GSCs self-renewal as well as controls stem cell plasticity and non-GSCs reprogrammation through transcriptional regulation via activation of HIF1α, HIF2α, and the Notch pathways, epigenetic regulations and metabolic reprogramming [review in (21, 74)]. HIF2α also activates c-Myc, another key stem cell regulator. Hypoxia also causes the secretion by GSCs of several soluble factors such as TGF-β, an activator of EMT that favors the dedifferentiation of tumor cells, or VEGF and SDF-1 to promote angiogenesis by recruiting mesenchymal stem cells and myeloid cells (71). Other secreted factors induced by hypoxia also include CXCR4, the

glucose transporter 1 (GLUT1), Serpin B9, Oct4, lysyl oxidase (LOX), hypoxia inducible gene 2 (HIG2), all these factors being involved in the maintenance and proliferation of GSCs [(74, 75)].

These findings highlight the critical role of the TME in regulating the differentiation status of tumor cells and the plasticity of GSCs and non-GSCs hierarchy. Importantly, each subtype is associated with a particular environment, the MES GSCs being located in hypoxic niches while the PN GSCs are in perivascular niches.

METABOLIC PHENOTYPES OF GSCS

Dynamic changes also occur at the bioenergetic machinery level which strongly contributes to tumor heterogeneity. Indeed, the ability of the tumor mass to face the large need for both biomass precursors and ATP production required by intense tumor proliferation, mainly relies on the metabolic plasticity of cancer cells.

Altered Metabolism Is a Hallmark of All Cancer Cells

In normal cells, glucose homeostasis is reciprocally controlled by catalytic glycolysis/oxidative phosphorylation and the anabolic neogluconeogenesis pathway. In the catabolic reaction, glucose is converted to pyruvate which can be further metabolized to mitochondrial acetylCoA to fuel the Tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS), or to cytosolic lactate. Importantly, whereas OXPHOS is bioenergetically highly efficient with 36 molecules of ATP produced from one molecule of glucose, the direct conversion of glucose to lactate, usually occurring in absence of oxygen, produces only 2 molecules of ATP. Paradoxally, in 1924, Otto Warburg discovered that tumor cells use large amounts of glucose to produce lactate, even in the presence of oxygen (**Figure 1**). This counter intuitive energy generation pathway occurs in most cancer cells, independently of mitochondrial functional integrity. In fact, by increasing their glycolytic and anaplerotic fluxes, tumor cells cope with increased bioenergetic as well as biosynthetic needs. This finding resulted in the development of 2-[18F]-fluoro-2-deoxy-D-glucose positron emission tomography (PET) to detect glucose uptake and lactate production for tumor imaging. In 2011, altered energy metabolism has been integrated as a fundamental core hallmark of cancer cells (76).

Traditionally, metabolic reprogramming is viewed as driven by oncogenic gain-of-function events or loss of tumor-suppressors. For example, tumor suppressor gene p53, mutated in more than 50% of human cancers, including GBM, triggers glycolysis besides its key roles in genetic instability, tumor progression, and metastasis. Loss of PTEN leads to the constitutive activation of AKT1, which stimulates glucose uptake by enhancing GLUT4 expression and activating HK2 (77, 78). Finally, activation of c-MYC also induces glycolysis by inducing LDH-A and PDK1 expression facilitating the production of lactate (79). Interestingly, in the past 2 decades, it was shown that loss-of-function mutations of the TCA cycle enzymes succinate dehydrogenase and fumarate hydratase were implicated in the pathogenesis of paragangliomas, pheochromocytomas, and renal cell cancers (80). Notably, mutations in the genes encoding isocitrate dehydrogenase (IDH)-1 and IDH-2 have been described in 5% of *de novo* GBM and more than 90% of secondary GBM [review in (81)]. In fact, since 2016, the CNS WHO divided GBM in 2 main groups (1) IDH-wildtype (about 90 % of cases), which corresponds most frequently to *de novo* GBM and predominates in patients over 55 years of age, and (2) IDH-mutant (about 10 % of cases), corresponding to secondary Glioblastoma arising from lower grade diffuse glioma and preferentially arises in younger patients (1). Of note, in GBM, IDH mutants are consistently classified as PN. Tumor-derived IDH mutations disrupt their normal catalytic activity that converts isocitrate to α-ketoglutarate (α-KG) to a remarkable neomorphic activity that converts α-KG to D-2-hydroxyglutarate (D-2-HG), now referred to as an oncometabolite (82). Besides being involved in multiple metabolic pathways, α-KG is also a co-factor for several α-KG-dependent dioxygenases including prolylhydroxylases (PHD) involved in HIF stabilization, histone demethylases and the TET family dioxygenases involved in epigenetic modifications. Interestingly, D-2-HG that is structurally highly similar to α-KG, acts as a competitive inhibitor leading to dioxygenases inhibition and singular methylation profile.

Importantly, these mutations in genes encoding for important metabolic enzymes raised the possibility that under certain conditions, altered metabolism could be the cause and not the consequence of cancer transformation. In line with this challenging notion, several studies have evidenced a retrograde mitochondrial-to-nucleus signaling. For example, disruption of mitochondrial integrity generates singular nuclear genes expression profiles, including genes involved in metabolism (83). Thus, tumor cells might exhibit increased metabolic autonomy in maintaining an anabolic phenotype with oncogene and tumor-suppressor gene originated through evolution as components of metabolic regulation rather than cancer-driving mutations driving metabolic pathways (84). This notion would explain why cancer cells with different genetic alterations display similar metabolic phenotype whereas cancer cells with identical genetic alterations have different metabolism.

Metabolic Plasticity of GSCs

In contrast to the proliferating tumor mass, GSCs are slowly proliferating and reside in specific niches requiring constant metabolic adjustments in order to adapt to transient bioenergetic crisis caused by hypoxia or nutrients deprivation. While they also display metabolic alterations, the metabolism of GSCs have been shown to deeply influence their maintenance and survival.

GSCs Rely on Both Oxidative and Non-oxidative Glucose Metabolism

GSCs, as most CSCs, have been described as relying mostly on glycolysis. Glucose is uptaken by the cells through the glucose transporters (GLUT), in particular GLUT1 and GLUT3, and converted to pyruvate through several enzymatic reactions (**Figure 1**). Pyruvate represents a critical metabolic control point, as it can be converted to lactate by LDH-A or imported within the

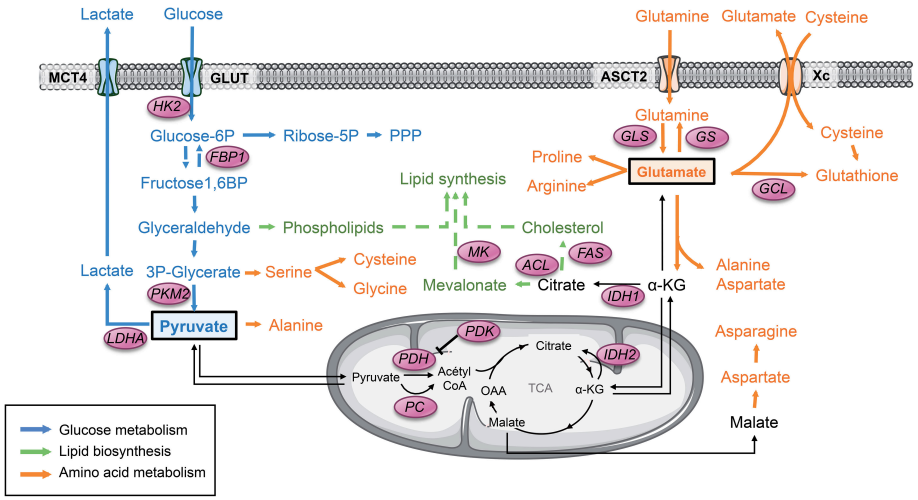

FIGURE 1 | Pyruvate and glutamate are 2 major metabolic hubs in GSCs Tumor cells usually display a strong glycolytic metabolism. Glucose is uptaken by glucose transporter GLUT and then converted to pyruvate through several enzymes. All along this pathway, the glycolytic products are diverted from this main metabolic road to fuel other biosynthetic pathways such as the PPP as well as lipids and amino acids biosynthesis. PKM2 plays a key role in this dynamic process through conformational modulation. Glycolytic pyruvate will then either be converted to lactate or fuel mitochondrial OXPHOS and the TCA cycle. Another key metabolite that can fuel the TCA is glutamate once converted to αKG. Glutamate is produced either by GLS from glutamine or from glucose. Glutamate is involved in several biosynthetic pathways including amino acids and lipids biosynthesis as well as mitochondrial anaplerosis. Glutamate is also involved in glutathione synthesis, directly and indirectly by providing cysteine to the cells. α-KG, α-ketoglutarate; FAS, Fatty acid synthase; FBP1, Fructose-1,6 bisphosphatase 1; GCL, glutamate-cysteine ligase; GLS, Glutaminase; GS, Glutamine synthetase; GLUT, Glucose transporter 1; HK2, Hexokinase 2; IDH, Isocitrate dehydrogenase; LDHA, Lactate dehydrogenase A; MCT, Monocarboxylase transporter; MK, Mevalonate kinase; OAA, Oxaloacetate; PC, Pyruvate carboxylase; PDH, Pyruvate dehydrogenase; PDK, Pyruvate dehydrogenase Kinase; PKM2, Pyruvate kinase M2; PPP, Pentose phosphate pathway; TCA, Tricarboxylic acid cycle.

mitochondria to be converted to acetyl coenzyme A (AcetylCoA) by pyruvate dehydrogenase (PDH) to fuel the TCA cycle. Activation of the transcription of both Pyruvate dehydrogenase kinase (PDK), which phosphorylates and inactivates the catalytic domain of PDH, and the Lactate dehydrogenase A (LDH-A), in particular through HIF-1, has been reported in GSCs. As a result, pyruvate is actively shunted away from the mitochondria and converted to lactate by LDH-A, corresponding to a non-oxidative glucose metabolism. However, Marin-Valencia et al. have elegantly demonstrated using 13C-nutrient labeling in orthotopic murine models that GSCs are not confined to non-oxidative glycolysis (85). Indeed, they show that pyruvate is converted to lactate but also channeled through PDH with a significant contribution of glucose carbons to the TCA cycle. Collectively, these results demonstrate that GSCs use glucose to produce energy and biosynthetic precursors through both non-oxidative and mitochondrial oxidative pathways. Since these tracers are not radioactive, this approach is feasible with GBM patients when tumor resection is planned in the course of the treatment. Importantly, similar metabolic profiles of both oxidative and non-oxidative glucose fates were observed in extracts of surgically-resected tumors obtained from GBM patients infused with 13C-Glucose (86).

Furthermore, depending on oxygen availability, a reciprocal metabolic switch has been reported between glycolysis and the pentose phosphate pathway (PPP), an alternative anabolic pathway to glycolysis, which produces ribose-5-phosphate and NADPH for nucleic acids and fatty acids synthesis in GSCs. Indeed, anabolic PPP is highly active in rapidly proliferating tumor cells but suppressed under hypoxia, switching to glycolysis (87). The isoform M2 of pyruvate kinase (PKM2) plays a critical role in this metabolic switch. In contrast to PKM1, which exists in a constitutively tetrameric active form, PKM2 exists under a dimeric inactive form and a tetrameric active form. The dimeric PKM2 results in the accumulation of upstream glycolytic intermediates, in particular fructose-1,6-phosphate (FBP), favoring their redistribution toward other biosynthetic pathways through the PPP. However, accumulation of FBP induces the association of dimeric forms into tetramers, which in turn leads to lactate production, until the level of FBP is reduced leading to the tetramer dissociation into dimers. Thus, the dynamic dimer:tetramer ratio of PKM2 determines whether carbons from glucose are converted into lactate via pyruvate or channeled into building block synthesis.

Mitochondrial Function Is Critical for GSCs Survival

While the above reported studies show that GSCs mainly rely on glycolysis, several other studies showed that these cells possess a preference for mitochondrial oxidative metabolism. In many tumor types including Glioblastoma, growing evidence has demonstrated that quiescent or slow-cycling CSCs are less glycolytic, consume less glucose, and produce less lactate, whereas they contain higher ATP levels than their differentiated cancer counterparts (88). Coupling between EMT and enhanced mitochondrial respiration has also been reported. Moreover, some CSCs have an increased mitochondrial mass and enhanced

oxygen consumption rates (89, 90). One striking difference with non-GSCs cancer cells is that GSCs lack mitochondrial reserve capacity suggesting that they fully oxidize substrates pool in contrast to differentiated cells exhibiting significant bioenergetic reserves.

In either case, mitochondrial function is critical and plays a crucial role in CSCs functions including stemness and drug resistance. Inhibition of PGC1α, the master regulator of mitochondrial biogenesis, reduces stemness properties of breast CSCs (91) while NANOG, a pluripotency gene, induces tumorigenesis through metabolic reprogramming to OXPHOS and fatty acid metabolism (92). Increased OXPHOS as well as PGC1α expression seems to be related to chemoresistance in CSCs (93–95). In fact, mitochondrial mass tracking using fluorescent probes has been described as a simple and efficient tool to identify CSCs (96). Thus, mitochondrial metabolism is critical for GSCs maintenance.

Glutamine Metabolism in GSCs

In addition to glucose, amino acids can also be channeled into the mitochondrial TCA, as their catabolism results in the production of TCA intermediates. Glutamine has an important role in cell growth and energy metabolism, and glutamine addiction has been proposed as a mark of GBM (97). Following the entry of glutamine into the cell via its transporter ASCT2 (or SLC1A5), the first step of glutamine catabolism is its conversion into glutamate through glutaminase (GLS) (**Figure 1**). Glutamate is a major metabolic hub: it can replenish the cells in lipid biosynthesis precursors and TCA intermediates through mitochondrial anaplerosis, generate *de novo* reduced glutathione, a major anti-oxidant molecule (98) either directly through its combination with cysteine by glutamate-cysteine ligase (GCL) or indirectly allowing cysteine import through cysteine/glutamate transporter Xc (or SLC7A11), or involved in the synthesis of non-essential amino acids, purine, and pyrimidine through aminotransferases (99). In human hematopoietic stem cells, glutamine availability modulates their differentiation either toward the erythroid or the myelomonocytic lineage (100). In GSCs, glutamine deprivation reduces cell proliferation, in particular by reducing mitochondrial anaplerosis and energy production (39). However, glutamine addiction has not been observed in all GSCs (39, 101). Indeed, GLS activity and glutamine anaplerosis is dispensable in GSCs expressing Glutamine synthetase (GS), which controls glutamine homeostasis by catalyzing the opposite reaction of GLS and is highly expressed in GSCs as compared to tumor cells. Based on these studies, we proposed a model where in GS-positive cells, glucose is used to synthetize glutamate that is subsequently converted into glutamine through GS to sustain nucleotides biosynthesis. In contrast, in GS-negative cells, or when GS was inhibited pharmacologically or molecularly, glutamine deprivation decreased cell proliferation as well as self-renewal mostly through reduced nucleotides biosynthesis (**Figure 2**). Importantly, glutamine-dependency is not observed in non-GSCs and is restricted to one particular GSCs subtype, the MES one.

Lipid Metabolism of GSCs

Lipid metabolism is another source of metabolic intermediates and energy for processes involved in cell transformation and tumor progression. Cancer cells can fulfill their avidity for lipids either by increasing exogenous lipid uptake or endogenous production through *de novo* synthesis (**Figure 1**). A major outcome of both glucose and glutamine metabolism is the production of citrate to produce biomass needs required for cell proliferation. After its import into mitochondria, glycolytic pyruvate is decarboxylated into acetylCoA, which will be condensed with the glutamine-derived TCA intermediate oxaloacetate (OAA) to generate citrate. Citrate can then be exported out of the mitochondria and cleaved by ATP citrate lyase (ACL) to regenerate OAA and acetylCoA. Interestingly, in absence of glutamine, some tumor cells generate both acetylCoA and OAA directly from glucose through the pyruvate carboxylase (PC) pathway (102). Glutamine can also contribute to acetyl-coA production in the absence of glucose. Usually, glutamine-derived glutamate is converted to the TCA cycle intermediate αKG mainly through transamination, which transfers nitrogen from glutamate to alanine or aspartate, since Glutamate Dehydrogenase (GDH) activity is usually inhibited in cancer cells (98). In absence of glucose, cells engage an alternative pathway of αKG production either through enhanced activity of GDH (103) or reductive carboxylation through reverse activity of isocitrate dehydrogenase (IDH) to generate isocitrate and subsequently citrate (104). This latter pathway has been demonstrated when mitochondria are defective (105) or under hypoxia (106, 107). Importantly, glutamine-reductive carboxylation shown to be of particular importance for sustaining cell proliferation has also been observed in GBM cells, including GSCs [(98), Oizel unpublished data].

Besides lipid anabolism, lipid catabolism seems critical for CSCs self-renewal. Indeed, some studies have shown that cancer stem cells can also rely on active fatty acid oxidation for their maintenance and function (108). For example, inhibition of fatty acid oxidation by etomoxir decreases viability and tumorosphere formation in breast cancer stem cells while it has no effect on non-stem cancer cells (109).

Finally, the mevalonate pathway is an essential metabolic pathway allowing the production of 5-carbon building blocks providing cells with bioactive molecules, in particular cholesterol and coenzyme Q as well as molecules involved in signal transduction, which have been shown to be crucial for different cellular processes, including cell proliferation, differentiation, and survival [review in (110)]. Upregulation of several enzymes upstream of the mevalonate pathway has been associated with CSCs enrichment at least in breast tumor cells (111).

In conclusion, GSCs dispose of numerous bioenergetic and biosynthetic redundant pathways in order to fulfill their requirement to sustain their survival, growth, and invasion. All GSCs are able to sustain the emergence of a primary tumor *in vivo*, independently of their predominant glycolytic or oxidative metabolism. Their metabolic flexibility allows their adaptation to harsh microenvironments, where nutrients or oxygen can be scarce. Importantly, in contrast to non-GSC, their metabolic dependency to OXPHOS sets

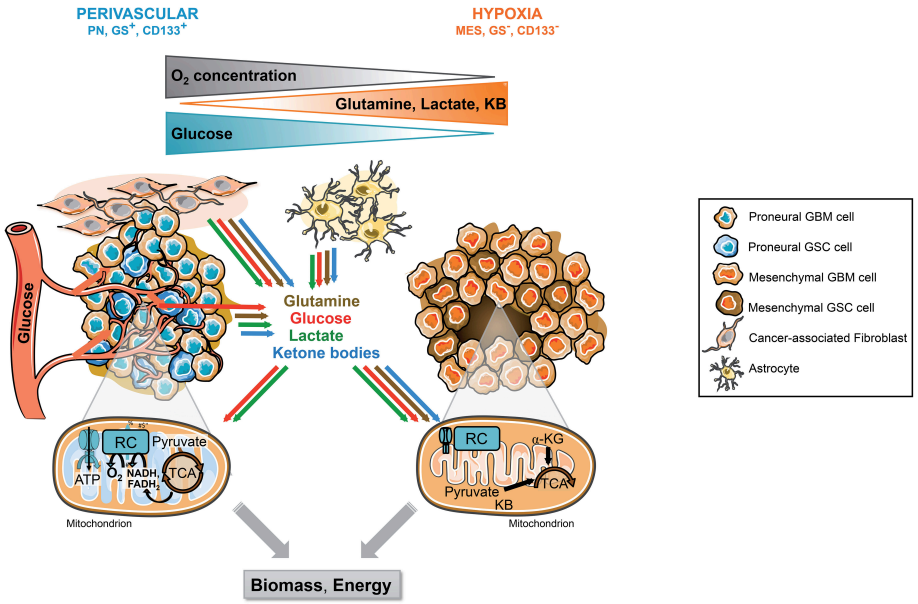

FIGURE 2 | Tumor microenvironment and molecular signature, 2 drivers of GSCs metabolic phenotype. GSCs reside in singular tumor microenvironment in GBM called the perivascular and the hypoxic niche. Each niche is associated with specific molecular signature and metabolic phenotype. The perivascular GSCs display a PN signature and a strong glycolytic metabolism mainly based on blood glucose. In these cells, glucose can be directly converted to lactate or oxidized within the mitochondria through OXPHOS. Furthermore, these cells highly express GS allowing the direct synthesis and secretion of glutamine within the TME. In contrast, hypoxic GSCs usually belong to the mesenchymal subtype and do not express GS. These cells are residing in a harsher environment display flexible metabolism fueled by lactate, ketone bodies, amino acids, including glutamine, released in the microenvironment by other cancer cells and/or stromal cells including astrocytes or cancer-associated fibroblasts. In absence of O2, these substrates supply a truncated TCA cycle allowing the generation of energy and biosynthetic precursors such as lipids or nucleic acids, as well as antioxidant glutathione synthesis. CAF, Cancer-associated fibroblast; GBM, Glioblastoma; GSCs, Glioblastoma stem-*like* cells, GS, Glutamine synthetase; αKG, Alpha-ketoglutarate; KB, Ketone bodies; MES, Mesenchymal; OXPHOS, Mitochondrial oxidative phosphorylation; PN, Proneural; TCA, Tricarboxylic acid cycle.

mitochondrial metabolism as potential therapeutic target to efficiently eradicate GSCs.

Drivers of GSCs Metabolic Phenotypes

Metabolic Phenotypes of GSCs Mirror Molecular Signature

Several studies have characterized the metabolic phenotype of the different subtypes of GSCs, either based on stemness expression markers such as CD133 expression or on the molecular signature (39, 112–117). Interestingly, all studies converge toward the proneural subtype, also characterized as CD133 positive or full stem-like phenotype, displaying a strong glycolytic metabolism (**Figure 2**). In contrast, the mesenchymal subtype, CD133 negative or restricted stem-like phenotype, displays higher metabolic flexibility with both glycolytic and oxidative metabolisms. In particular, as described previously, glutamine dependency relies on GS activity, which is only observed in MES GSCs. Furthermore, in contrast to PN GSCs, MES GSCs easily bypass targeted metabolic inhibition through a wide range of metabolic adaptation. Importantly, these findings highlight that first, different GSCs can have similar metabolic profile despite being derived from independant tumors with different driver mutations. Conversely GSCs derived from identical tumor can exhibit different metabolic features depending on tumor specimen localization or molecular signature. Second, the molecular signature correlates with metabolic flexibility and the spatial distribution of various TME, namely hypoxic and perivascular niches. For example, in agreement with they dominant glycolytic phenotype, the proneural subtype localized in tumor zones surrounded by functional vasculature will easily have access to oxygen and blood glucose. In contrast, the mesenchymal GSCs localized in hypoxic zone display higher metabolic flexibility allowing them to use a wide range of nutrients in order to sustain their survival and proliferation in a relatively poor microenvironment. Importantly, these various metabolic features of GSCs residing in different tumor areas allow the establishment of a metabolic cooperation between tumor cells.

Tumor Microenvironment Mitigates Metabolism of GSCs

GSCs are able to adapt their metabolism by displaying various metabolic and bioenergetic abilities depending on energy-rich nutrients or energy-producing mitochondrial metabolites. This is usually dictated by oxygen and nutrients supply from the TME either through the tumor vasculature or through metabolic cooperation between cells which is often not uniformly distributed across the tumor bulk. Several studies have shown the impact of the TME on CSCs metabolism, in particular in GBM. First, as we previously described, one remarkable characteristic

of GBM microenvironment is hypoxia due to aberrant tumor vasculature. Low oxygen conditions are mainly mediated by the transcription factors HIF, which regulate the expression of a large panel of genes involved in several key biological functions, including cell metabolism (118). In particular, HIF-1α activates the transcription of genes all along the glycolytic pathway, from the glucose transporters GLUT1 and GLUT3 to key glycolytic enzymes such as hexokinases (HK), PKM2, or LDH-A. Besides directly activating aerobic glycolysis, hypoxia also triggers the epithelial-mesenchymal transition (EMT), a characteristic of embryonic development playing critical roles during organogenesis. Accumulating evidences indicate that EMT is of paramount importance in a plethora of cancer-related events, including acquisition of both stem cell-like properties and mesenchymal characteristics (74). During tumor progression, EMT has been shown to increase the ability of cancer cells to invade and dissiminate, as described previously, to favor CSCs emergence. Among the transcription factors involved in EMT phenotype, Snail has been identified as a key repressor of Fructose1,6 biphosphatase (FBP1) expression in breast cancer stem cells (119). In this study, Snail silences FBP1 expression through methylation of its promoter, providing metabolic advantages to breast CSCs. Indeed, loss of FBP1 induces glycolysis, increased glucose uptake, macromolecules biosynthesis and a constitutively active form of PKM2.

Metabolic reprogramming can be accelerated by TME acidification (120). In fact, lactate is not only a metabolic waste for glycolytic cells but is also a metabolic fuel for oxidative cells (121). Indeed, in 2008 Sonveaux et al. suggested a symbiotic relationship among cancer cells in the TME where cancer cells distal to a blood vessel would be deprived of oxygen in contrast to cancer cells close to the blood vessel (**Figure 2**) (122). In this model, perivascular cells would spare glucose for hypoxic cells. Then, the distal hypoxic cells would convert glucose to lactate, which could then be imported into perivascular cells and converted to pyruvate for mitochondrial oxidation. This metabolic interplay between glycolytic and oxidative cells, called the "reverse Warburg effect," also occurs with the stromal compartment that surrounds the tumor. Indeed, the Lisanti group showed that stromal cells, in particular cancer-associated fibroblasts (CAFs), increase their aerobic glycolysis resulting from enhanced mitochondrial turnover and generate excessive lactate, pyruvate, and other ketones bodies, which are secreted into the intracellular space (123). These metabolites are then re-used by cancer cells for OXPHOS. Thus, the stromal cells feed the cancer cells with lactate and other metabolites and this metabolic symbiosis limits TME acidification. The utilization by cancer cells of the high-energy stromal metabolites pyruvate, lactate, and ketones may increase the transcriptional expression of gene profiles normally associated with stemness, including genes commonly upregulated in embryonic stem cells (121). Interestingly, hypoxic tumor cells through mitochondrial reductive carboxylation, independently of oxygen availability, can also use these metabolites. In the brain, glycolytic oligodendrocytes and astrocytes also export lactate through MCT1 and MCT4 (124). Furthermore, astrocytes, which have been shown to highly express GS, generate and secrete glutamine, which can then be used by GS negative tumor cells to support their growth (101).

Finally, CSCs communicate with the surrounding microenvironment by direct cell-cell interaction through gap junctions to execute coordinated programs required for growth, differentiation, and therapeutic response. In the past decade, several publications have reported an important role of tunneling nanotubes (TNT) in direct intercellular communication in GBM. TNT are long cytoplasmic bridges that enable long-range and direct communication, including metabolites, mitochondria and vesicles transfer, between connected cells (125). Mitochondria are the TNT cargos most widely studied so far. TNT-mediated mitochondria transfer was reported in GBM but also in many different cancer types, both between cancer cells, and between cancer cells and normal cells of the microenvironment such as MSC (126). A direct effect of the TNT-mediated transfer of mitochondria is the modification of the target cell energetic metabolism, with increased OXPHOS and ATP production (126–128). This has functional consequences for cancer cells as it enhances their proliferative and migratory properties as well as their capacity to develop resistance to therapeutic treatment.

METABOLIC TARGETING OF GSCs

GSC involved in GBM poor response to treatment and relapse represent the tumor cornerstone. With the identification of key metabolic features involved in their survival, growth, invading properties and self-renewal, one possible therapeutic strategy would be targeting the biochemical energetic reactions occurring in GSCs.

Inhibition of IDH-Mutant

Since IDH mutations are present in only 5% of *de novo* GBM while its occurrence increases to 95% in secondary GBM (129), targeting IDH-induced metabolic alteration might open new therapeutic avenues, at least in secondary GBM. As described previously, IDH mutations result in metabolic alteration, including the production of the oncometabolite D2-HG, epigenetic dysregulation via inhibition of αKG-dependent histone and DNA demethylases, and differentiation blockade. Several compounds inhibiting IDH-mutants have been developed and tested in anti-tumor therapy [see review in (130)]. The majority of these studies have been realized in another tumor type, the acute myeloid leukemia (AML), which has been shown to frequently harbor IDH-mutant. Several mouse models have been engineered to express IDH-mutant specifically in hematopoietic tissue leading to hematologic malignancy (131). Genetic knockdown or pharmacologic inhibition of IDH-mutant in these models led to a decrease in D2-HG production and tumor cell growth while inducing cellular differentiation. These encouraging preclinical results provided a proof-of-concept for a targeted treatment of IDH mutants in AML and clinical trials are underway for AML patients (130).

These compounds are now tested in GBM. However, they may not be as effective in all IDH-mutant tumors. Indeed, inhibitors of IDH-mutants delay growth and promote differentiation in some IDH-mutated GBM cells but not all (132, 133).

Furthermore, despite a robust ability to lower D2-HG in GBM cells and tumors, they are unable to reverse epigenetic deregulation. Nevertheless, the presence of IDH-mutant and/or high levels of D2-HG might introduce therapeutic vulnerability to different agents. For example, tumors harboring an IDH mutation display increased sensitivity to inhibition of oxidative mitochondrial metabolism (134), depletion of coenzyme NAD (133) and chemotherapy (135). Importantly, other studies have shown both *in vitro* and in patients that inhibiting mutant IDH could confer radiation resistance to some therapies or can antagonize the effects of radiation therapy in glioma (136, 137). Thus, whereas encouraging results have been observed for AML patients, IDH-mutant targeting in GBM definitively requires more investigations. In particular, long-term studies are definitively required to determine the impact of these compounds on tumor relapse in order to define whether these compounds also target cancer stem-*like* cells.

Metabolic Targeting Requires Multiple Bioenergetic Pathway Inhibitions

CSCs display highly plastic metabolic profile allowing them to fulfill bioenergetic requirements and this flexibility seems to be required for their survival (138). Since CSCs have been shown to be enriched in mitochondrial mass and relying heavily on OXPHOS, disrupting this pathway has become attractive as a therapeutic strategy (**Figure 3**). Accordingly, different classes of mitochondrial inhibitors have been reported to decrease stemness and invasion properties as well as increasing cell death. For example, a large number of retrospective clinical studies have revealed that Metformin, a first-line diabetes drug, is linked to cancer prevention [review in (139)]. In fact, Metformin directly inhibits mitochondrial complex I of the respiratory chain and OXPHOS activity (140). As a consequence, this drug induces cell death in cancer cells. However, this effect is observed only upon glucose deprivation. First, this study highlights the metabolic plasticity of cancer cells, which are able to survive to OXPHOS inhibition by Metformin when glucose is present. Similarly, in glutamine-addicted cells, GSCs easily switch from glutamine-based to glucose-based metabolism to sustain their survival (39). Second, to overcome their metabolic flexibilities, an efficient metabolic targeting will require the blockade of several metabolic pathways. Indeed, in preclinical cancer models, dual inhibition of both glycolysis and OXPHOS, respectively, using the glucose analog 2-DG and metformin, has been shown to effectively reduce tumor growth and dissemination (141). Similarly, phenformin, another biguanide drug, leads to cancer cell death through metabolic catastrophe only when combined with genetic disruption of MCT involved in cancer metabolic symbiosis (142).

Interestingly, since mitochondria evolved from bacteria, many classes of FDA-approved antibiotics, recently emerged as additional inhibitors of mitochondrial biogenesis and functionality (143, 144). For example, doxycycline, very well-tolerated in patients, could be re-purposed clinically as a "safe" mitochondrial inhibitor, targeting mitochondrial biogenesis in CSCs (145).

As described above, TME provides cancer cells and GSCs with various bioenergetic nutrients. At the molecular level, MCT play a key role in the metabolic symbiosis, whereby lactate produced by one cell type is made use of as a fuel by another one. Simultaneous inhibition of both MCT and OXPHOS might represent a candidate strategy for combinatorial metabolic targeting. At the cellular level, GSCs are known to closely interact with endothelial cells (66, 70). First, endothelial cells promote cancer cells stemness in GBM (146). Second, endothelial cells, and in particular radiation-resistant endothelial cells, can protect stem cells and tumor cells from radiation damage (147–149). Furthermore, we recently showed that radiation induced metabolic mitochondrial alteration in these radiation-resistant endothelial cells (150). Thus, disrupting the tumor-TME interaction that support GSCs survival is another approach to killing GSCs.

Metabolic Targeting to Sensitize GSCs to Radiation

Hypoxic niches protect cancer cells from radiation-induced killing since oxygen is a critical determinant of cell response to radiation (151). A certain degree of reoxygenation can be achieved in some tumors after radiation by a process where the surviving hypoxic tumor cells become better oxygenated due to the aerobic population being killed. However, this process is highly variable within tumors. The HIF-1α pathway enables tumor cells to survive by changing glucose metabolism toward a glycolytic phenotype, by inducing angiogenesis and by regulating pH balance and proliferation rate.

Differentiation of normal stem cells is associated with a metabolic shift from glycolysis to mitochondrial OXPHOS while iPS reprogramming is accompanied by the reverse modifications (152, 153). Since the glycolytic switch occurs before acquisition of pluripotent markers, mitigating metabolism to induce GSCs differentiation appears as an appealing therapeutic avenue. Importantly, cancer cells, as normal cells, are more sensitive to conventional treatments, including radiation, upon differentiation (154). Differentiation therapy has been exploited with Bone Morphogenetic Protein-4 (BMP4) treatment, to induce glial differentiation and reduce tumor growth in gliomas. Interestingly, after this treatment, GSCs are unable to form tumors after transplantation in series in immunocompromised animals (155). Collectively, these studies suggest a new treatment for GBM that would force the GSCs to enter differentiation, resulting in sensitization of GSCs to treatment and as a result the reduction of the tumor mass and relapse occurrence (**Figure 3**). In agreement with this idea, Dichloroacetate (DCA), an indirect PDH activator, leads to both GSCs differentiation as well as increased radiation sensitivity (156, 157). One report showed that DCA, already used in the clinical treatment of genetic mitochondrial diseases, may improve clinical outcome in some patients with GBM (158). Following this study, 4 phase 1 clinical trials have investigated the chronic safety of oral DCA doses in adults with recurrent malignant brain tumors or other solid tumors (159–161). However, whereas these clinical trials showed that DCA was generally well-tolerated despite a peripheral

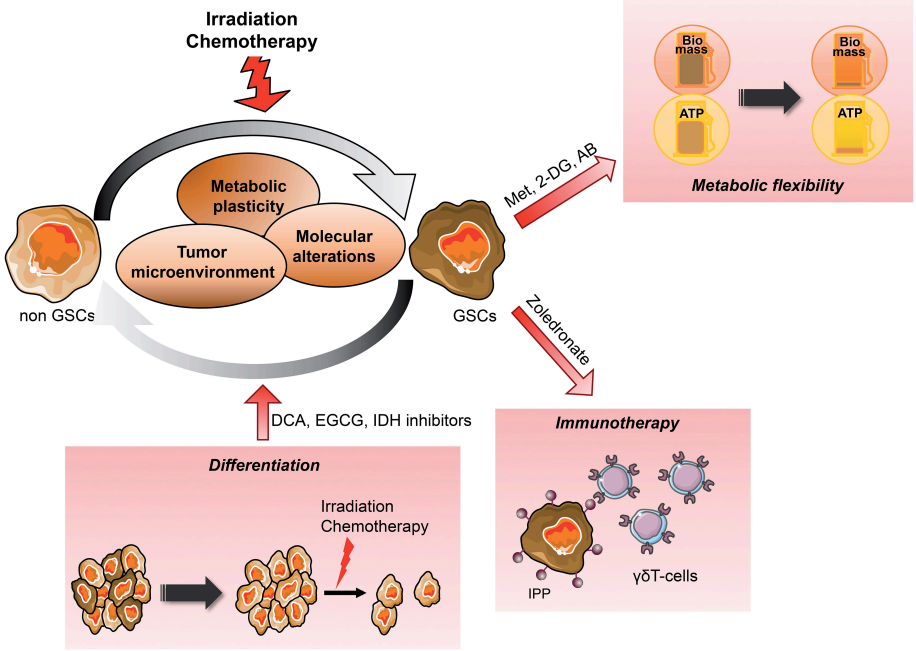

FIGURE 3 | Metabolic targeting of the dynamic emergence of GSCs from differentiated GBM GSCs emerge from differentiated tumor cells, which can reacquire stem cells properties such as self-renewal, multilineage differentiation and the ability to give rise to the initial heterogenous tumor *in vivo*. This dynamic differentiation/dedifferentiation balance is driven by the TME, molecular events as well as their metabolic phenotype. Thus, metabolic targeted therapy appears as a potential novel therapeutic avenue, in particular when combined to conventional treatment such as radiation therapy. Several angles can be taken to eradicate GSCs from the tumor, reducing their metabolic plasticity and/or metabolic cooperation with the TME, inducing their differentiation to sensitize them to treatment or increasing their recognition by immune effectors. AB, Antibiotics; CAF, Cancer-associated fibroblast; DCA, Dichloroacetate; 2-DG, 2-Deoxyglucose; EGCG, Epigallocatechine gallate; GSCs, Glioblastoma Stem-*like* cells; IPP, Isopentenyl pyrophosphate; Met, Metformin; MSC, Mesenchymal stem cell.

neuropathy in some patients, there was in general no strong indication for a relevant effect of DCA in tumor response.

Other metabolic inhibitors have been involved in GSCs differentiation. As described previously, the oncometabolite D2-HG, produced by IDH mutant, is also involved in cell differentiation (162). Furthermore, inhibitors of LDH decrease GBM cell proliferation, trigger cellular apoptosis and more importantly induce GSCs differentiation (163). EGCG, a bioactive polyphenol present in green tea and described to inhibit glutamine metabolism, has been shown to decrease stemness while increasing Temozolomide efficiency (39, 164). The effect of their use in combination with radiation has not been studied yet and would be worthy of further investigation.

Other studies have shown that metabolic targeting increased treatment efficiency, independently of GSCs differentiation. For example, modulation of mitochondrial metabolism influences radiation sensitivity both *in vitro* and in preclinical models (165). The glucose analog 2-DG, which causes a significant reduction of ATP levels by inhibiting glucose catabolism especially in cells with mitochondrial defects or under hypoxia (166), has entered numerous clinical trials and seems to potentiate the effect of radiotherapy in patients with brain tumors (167). Collectively, these studies suggest that targeting metabolism may offer benefit in GBM treatment, in particular in combination with radiation. However, further investigations are definitively required in particular in preclinical studies.

Metabolic Targeting to Improve Immunotherapy Efficacy

The immune cells being an important component of the tumor microenvironment, cancer immunotherapy has emerged as a powerful new therapeutic approach either by active, passive or adoptive immunotherapies [review in (168)] (**Figure 3**). Immunotherapy usually boosts antitumor immune responses either by adoptive T cells transfer, chimeric-antigen receptor T-cells, or monoclonal antibodies. For example, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the programmed cell death protein 1 pathway (PD-1/PD-L1) checkpoint inhibitors are currently arising as a novel strategy to fight cancer, including GBM. However, the highly immunosuppressive TME combined with low GBM cells immunogenicity limits immunotherapy efficacy. Furthermore, several recent reports have described a variety of "metabolic checkpoints" including glucose and amino acid depletion, hypoxia, high acidity and lactate accumulation, that impairs TIL ability to survive, proliferate and function (169). Interestingly, the immune checkpoint blockade favors T cell activation while inhibiting cancer cells proliferation through metabolic alterations. For example, blocking PD-1 and PD-L1 can reduce glycolysis level in cancer cells by inhibiting the mTOR pathway (170). As a consequence, tumor glucose uptake and lactate secretion decrease, restoring glucose availability in TME. Interestingly, increased glucose availability in TME in response to immune checkpoint inhibitors has been shown to improve T-cell

glycolysis and cytotoxic function in a melanoma murine model (171). However, other studies have shown that the upregulation of both tumor PD-L1 and CTLA-4 drives T cells exhaustion through metabolic alterations. Recent publications also underline the reprogrammation of the immunosuppressive TME through metabolic alterations of tumor cells. The best example is the overexpression of the C4-metabolite carrier UCP2 in melanoma cells triggering the engagement of anti-tumor immune responses following CXCL10 secretion in the TME (172). Thus, tumor metabolism modulates tumor immune evasion through nutrients availability for T cells and reprogrammation of the TME.

Besides leveraging the body's own immune system, immunotherapy strategy may involve adoptive T cell transfer which offers the potential to overcome one of the significant limitations associated with tumor patients who are often immune compromised. Importantly, in such settings, Vγ2Vδ2 T cells are particularly interesting since they are able to recognize and kill most tumor cells in a major histocompatibility complex (MHC)-unrestricted fashion and independently of the number of tumor mutations. Instead, Vγ2Vδ2 T cells respond to the presence of small isoprenoid metabolites, such as self isopentenyl pyrophosphate (IPP) in a process requiring the butyrophilin-3A1 protein, an immunoglobulin superfamily protein present on all normal and tumor cells. Accumulation of IPP in tumor cells can be achieved by inhibiting the Farnesyl diphosphate synthase (FDPS), an enzyme downstream of the mevalonate pathway, by aminobiphosphonate compounds such as zoledronate. The mevalonate metabolic pathway provides cells with bioactive molecules, in particular cholesterol and coenzyme Q as well as molecules involved in signal transduction, which have been shown to be crucial for different cellular processes, including cell proliferation, differentiation, and survival [review in (110)]. In some tumor types, upregulation of several enzymes upstream of this metabolic pathway has been associated with CSCs enrichment (111). Interestingly, we have recently shown that allogeneic transfer of human Vγ9Vδ2 T cells in orthotopic murine models of human GBM enriched in GSCs leads to the efficient elimination of cancer cells, including GSCs (173). These results are in agreement with previous pre-clinical studies showing that statins could be used as anticancer agents in Glioblastoma (174, 175). Interestingly, asides from metabolic targeting, this paper also raised the idea that allogeneic transfer of human Vγ9Vδ2 could be potentiated with the combinatorial use of monoclonal GD2-antibody. Whereas, GD2 is also expressed in gliomas and in some normal structures of the brain, the monoclonal *O*-acetyl-GD2 antibody recognizes GSCs and more importantly is tumor specific (176). Thus testing the combinatorial effect of the monoclonal *O*-acetyl-GD2 antibody with adoptive transfer of human Vγ9Vδ2 should be investigated.

The Limits of GSCs Metabolic Targeting

Several drug targeting metabolic pathways have been investigated in human randomized trials after the promising results obtained *in vitro* and in preclinical models. The majority of these metabolic inhibitors has been well-tolerated and do not interfere with normal cellular metabolism in a clinically meaningful manner. Unfortunately, few of these drugs have shown encouraging results in patients to date. *In vitro* studies provide a solid platform to include metabolism as a definite hallmark of cancers and to consider the metabolic profile of CSCs as a relevant therapeutic target. However, one difficulty is to gain integral information on which bioenergetic and biosynthetic reactions of GSCs are key players in tumor progression and/or response to therapy, in particular in light of the inter- and intra-tumor heterogeneity. Furthermore, contradictory metabolic phenotypes have been obtained between *in vitro* studies and *in vivo* studies. For example, ovarian CSCs display a strong oxidative metabolism *in vitro* while being highly glycolytic *in vivo* (177). Thus, while *in vitro* models are definitively improving to better recapitulate the TME, the lack of a relevant TME remains one of the main pitfalls in studying CSCs *in vitro*. Indeed, in this review, we provided numerous evidences of the importance of the tumor niche in driving CSCs bioenergetic and biosynthetic deregulation.

One concern about metabolic targeting is that the adaptation of metabolic pathways in conjunction with the use of alternative nutrients could overcome the targeted metabolic inhibition in GSCs. Indeed, it is likely that in many cases in which glycolysis is inhibited, cells will respond by increasing OXPHOS. One way to circumvent this compensation is to combine several metabolic inhibitors. The exploitation of the metabolic reprogramming to selectively target tumor cells may also be limited by the existence of multiple isoforms of the enzymes and the fact that small-molecule inhibitors may not distinguish between the predominant isoform expressed by cancer cells and the isoforms expressed by normal cells. Developing small-molecule inhibitors that preferentially inhibit the cancer-specific isoform could be challenging.

Metabolic targeting has minimal efficacy by itself and tumor cells may develop mechanisms to adapt and resist to metabolic inhibitors. In contrast, combination therapy seems more promising. Indeed, radiation targets highly proliferating tumor cells while sparing low-proliferating GSCs. Thus, targeting GSCs metabolism appears as new therapeutic strategies to successfully eradicate them. Some metabolic inhibitors such as inhibitors of glucose transporters, hexokinase or PKM2 agents have been clinically disappointing with no beneficial effect in GBM patients, alone or combined with radiation therapy either because of toxicity, poor tumor penetrance, or lack of efficacy *per se* (178). However, phase I/II clinical trials have shown that 2-DG administered orally was well-tolerated and triggers a moderate increase in the survival of GBM patient treated with radiation therapy, with a significant improvement in the quality of life and better protection of normal brain tissue (167). A phase III multi-centric trial to evaluate the efficacy of the combined treatment is in progress. In this regard, the use of metabolic targeting as an adjuvant therapy to increase the efficacy of conventional treatment might be a better strategy.

CONCLUSION

The crucial role played by GSCs in tumor initiation, progression, recurrence, and resistance to therapy indicates that new therapeutic strategies require the eradication of this population. The inter- and intra-tumoral heterogeneities combined with the

variable patterns of vascularization and nutrients availabilities might directly draw the metabolic landscape of Glioblastoma rather than oncogenic genetic events. Whereas, GSCs metabolic plasticity represents a major challenge in the design of efficient therapies, tumor metabolic targeting holds great therapeutic potential in improving cancer treatment as shown by encouraging results observed using IDH mutant inhibitors in AML. In Glioblastoma, we believe that metabolic targeting has to be used in combination with standard anticancer reagents such as radiotherapy. Thus, while a well-defined metabolic portrait of GSCs still needs to be depicted to fully exploit GSCs metabolic vulnerabilities, in particular how to prevent their metabolic flexibilities and to define the best therapeutic window combining metabolic inhibition, immunotherapy and radiotherapy, we believe that GSCs metabolic targeting holds great therapeutic potential as an adjuvant therapy.

AUTHOR CONTRIBUTIONS

DG and CP: manuscript writing. OR: figures drawing. M-CA-G and FP: scientific discussions and extensive proofreading.

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Iron Metabolism in Liver Cancer Stem Cells

*Stefania Recalcati[1], Margherita Correnti[2], Elena Gammella[1], Chiara Raggi[2,3], Pietro Invernizzi[4] and Gaetano Cairo[1]**

[1] Department of Biomedical Sciences for Health, University of Milan, Milan, Italy, [2] Humanitas Clinical and Research Center, IRCCS, Rozzano, Italy, [3] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy, [4] Division of Gastroenterology, Department of Medicine and Surgery, Center for Autoimmune Liver Diseases, San Gerardo Hospital, University of Milano–Bicocca, Monza, Italy

Cancer stem cells (CSC) which have been identified in several tumors, including liver cancer, represent a particular subpopulation of tumor cells characterized by properties similar to those of adult stem cells. Importantly, CSC are resistant to standard therapies, thereby leading to metastatic dissemination and tumor relapse. Given the increasing evidence that iron homeostasis is deregulated in cancer, here we describe the iron homeostasis alterations in cancer cells, particularly in liver CSC. We also discuss two paradoxically opposite iron manipulation-strategies for tumor therapy based either on iron chelation or iron overload-mediated oxidant production leading to ferroptosis. A better understanding of iron metabolism modifications occurring in hepatic tumors and particularly in liver CSC cells may offer new therapeutic options for this cancer, which is characterized by increasing incidence and unfavorable prognosis.

Keywords: iron, liver cancer, cancer stem cells, ferroptosis, chelators

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***Correspondence:**
Gaetano Cairo
gaetano.cairo@unimi.it

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INTRODUCTION

The phenotypical and functional heterogeneity of cells within tumors can be explained by both the conventional mechanism centered on clonal evolution and a model based on the presence of cancer stem cells (CSC) which over the last decade received support by increasing experimental evidence. CSC have similar properties to adult stem cells, such as the ability for unlimited self-renewal and differentiation and are believed to be a major source of cancer initiation and progression, thus resulting in a heterogeneous tumor cell progeny (1–3). Moreover, CSC are characterized by drug resistance, an element allowing tumors to survive therapies. In fact, the relatively quiescent CSC can escape cell death after standard chemotherapy treatments, which preferentially eliminate rapidly proliferating cells; as a consequence, the remaining CSC may lead to cancer relapse and metastasis, whose treatment is more complex and often unsatisfactory. Notably, it is now also realized that some of the alterations of iron homeostasis that have recently emerged as key factors in cancer growth and progression are present also in CSC [reviewed in (4)]. In this Review, we discuss current knowledge of the role of iron as a key factor in cancer development, particularly in liver and hepatic CSC, and we also address iron-centered therapeutic approaches.

PRIMARY LIVER CANCER AND CANCER STEM CELLS

Primary liver cancer (PLC) is the fifth most common cancer worldwide and the second most frequent leading cause of cancer-related mortality and its incidence and mortality are increasingat a fast rate, especially in western countries (5, 6). Hepatocellular carcinoma (HCC) and

cholangiocarcinoma (CCA) represent the two major forms of PLC, and account for ∼ 90 and 5% of all primary liver tumors, respectively (5, 6).

HCC arises from malignant transformation of hepatocytes and is often associated with known risk factors, such as excessive alcohol intake, infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), aflatoxin B1 intake, fatty infiltration, autoimmune liver diseases and alterations of iron metabolism leading to hepatic iron accumulation like hemochromatosis (5, 7). Unlike the HCV-related HCCs, the incidence of HCC linked to the metabolic syndrome is increasing, principally due to obesity epidemic continuous rising (8).

On the other hand, CCA arise from neoplastic transformation of intra- and extra-hepatic biliary epithelial cells (cholangiocytes) and the frequency of its established risk factors mostly differs depending on geographic area (9). For example, infection with liver flukes (*Opisthorchis viverrini* and *Clonorchis sinensis*) is a common risk factor for CCA development in Southeast Asia (6, 10). Conversely, primary sclerosing cholangitis (PSC) is the most common predisposing condition for CCA in the western countries (6). HBV or HCV infection and cirrhosis have been also proposed as potential etiologies of CCA, shared with HCC (6, 7).

While liver transplantation, surgical resection and locoregional therapies are possible curative options at early phases, the majority of PLC patients unfortunately present advanced stages of the disease, for which treatments are very limited and the prognosis remains unfavorable (5, 11).

Like most other solid tumors, PLC are characterized by an extensive clinical and pathobiological heterogeneity in terms of cellular morphologies as well as genetic and epigenetic landscape (12–14). Such intra-tumor heterogeneity may reflect the presence of different clonal subpopulations exhibiting differential sensitivity to drugs (7, 12, 13). In this respect, the recent advent of the CSC hypothesis has added a new level of complexity in understanding PLC heterogeneity and drug resistance. According to this model, CSC are thought to drive tumor growth and progression, as well as tumor metastasis, recurrence and therapy resistance, representing the unique unit of selection within the tumor (2, 15–17). Interestingly, a new "CSC plasticity model" has been proposed, further increasing the complexity of tumor biology. According to this theory, the different tumor cell subpopulations are highly plastic and dynamic, continuously switching between non-CSC and CSC phenotypes, depending on various intrinsic and extrinsic stimuli (18).

While the CSC existence has been confirmed in HCC (19–22) and recently also in CCA (23, 24), no consensus has yet been reached regarding the origin of hepatic CSC. In addition to the classical idea that CSC originate from normal liver resident stem cells (1), it is now become accepted that CSC may originate also from more committed progenitor cells and mature differentiated tumor cells through a reprogramming process (11, 25, 26). These considerations go hand in hand with the unsettled debate about the true nature of the PLC cell-of-origin, about which a consensus has not been reached, yet.

Another open question concerns the identification of a common recognized method for isolation and subsequent characterization of liver CSC. During the last years several attempts have been made to obtain a cell population enriched in liver CSC using a variety of techniques. The antigenic approach, which is one of the first methods used to isolate CSC, relies on surface CSC markers detection, including CD133, CD44, OV6, CD90, EpCAM, CD13, CD24, CD47 (27). However, the antigenic approach has several shortcomings, such as lack of clearly defined surface markers specific for individual tumor type, such as PLC, and the fact that different cancer cell populations with tumor-initiating activity can be isolated using different markers within a tumor type (18, 28). Therefore, none of the proposed markers is specific for liver CSC and universally expressed in all liver CSC (29). In addition, the surface marker expression can diverge depending on the specific context (28). In addition to the classical antigenic approach, there are several assays based on CSC functional properties, including Side Population (SP) analysis, Aldefluor assay and tumor-sphere formation assay (14, 24, 27). All these functional techniques are based on different key CSC features: the first one on the typical drug resistance of CSC (30, 31), the second one on the measurement of aldehyde dehydrogenase activity (32) and the last one on long-term self-renewal capability of CSC (21, 24, 33). Possibly, a combinatorial strategy might be a valid alternative to isolate a better-defined PLC stem-like subset, but the gold-standard approach to evaluate CSC tumorigenic potential remains the *in vivo* approach based on xenotransplantation in immune-deficient mice (14, 24, 27).

IRON AND LIVER CANCER

Iron is an essential component of living organisms, as it is necessary for cellular metabolism, replication and growth. However, excess iron can facilitate the generation of the most reactive and toxic forms of oxidants through the Fenton reaction (34); therefore, iron levels are carefully kept within an optimal range at both systemic and cellular levels (**Figure 1**). The major players in maintaining cellular iron homeostasis are the transferrin receptor (TfR1) that internalizes transferrin-bound iron, ferroportin (Fpn), the only cellular iron exporter, and ferritin that stores excess iron (35) (**Figure 2**). A number of epidemiological studies indicate a positive association between cancer and high body iron content in the general population (36). Since the liver is the organ where excess iron accumulates (37) and plays an important role in maintaining iron homeostasis, a large body of evidence from human, animal, and *in vitro* studies supports the positive relation between increased body iron stores and the risk of liver cancer. In fact, HCC is the prevalent tumor found in hemochromatosis patients (38).

Studies investigating cancer risk in subjects undergoing blood transfusion or phlebotomy suggest that iron excess is not merely associated with cancer but plays an active role in carcinogenesis. The biological basis of the association between iron and cancer is double-face as it probably rests in both oxidative stress-mediated DNA damage and availability of the metal to support fast growth (39). Iron may therefore play a role both as an initiator in an early phase and, once malignant change has occurred, as a promoter

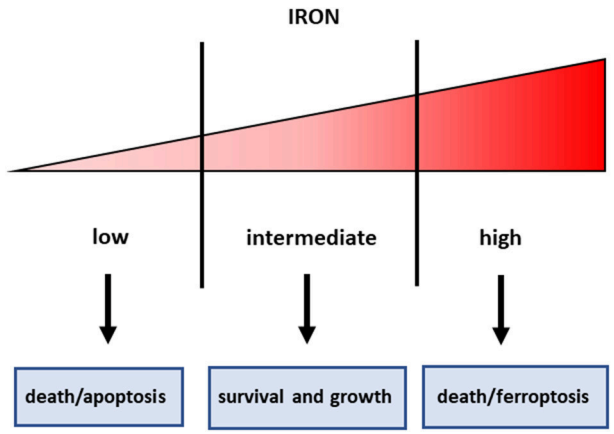

FIGURE 1 | Iron threshold concept. Certain iron levels are required for cell survival and homeostasis, but iron concentrations too low lead to apoptotic cell death, whereas excess iron equally triggers cell death.

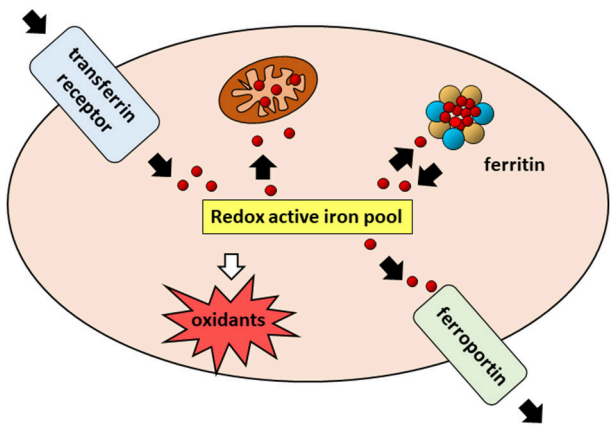

FIGURE 2 | Cellular iron pathways in a nutshell. Transferrin bound iron, internalized through endocytosis of the transferrin receptor (TfR1), enters a pool of redox-active iron whose concentration is kept under control by mechanisms ensuring that the iron which is not used for biochemical processes, particularly in mitochondria, is either safely stored in cytoplasmic ferritin or exported by ferroportin.

that allows the transformed cell to fully express its potential of unrestricted growth.

In addition, recent studies showed that both systemic and cellular iron metabolism is altered in tumors (40). In general, given the high iron needs of tumor cells to sustain cell proliferation, the alterations of iron trafficking in cancer cells lead to iron acquisition. To this purpose, cancer cells usually increase iron uptake, for example by up-regulating TfR1, decrease iron release by inhibiting Fpn, or both. Several studies have demonstrated that these alterations of cellular iron metabolism are directly dependent on the action of oncogenes and tumor suppressors (39). Notably, the "iron addiction" of tumors was confirmed by the analysis of different cell lines using a novel method (41), which showed that cancer cells had significantly increased redox-active iron pools compared to non-tumorigenic cells. The role of iron in cancer is not related only to the "iron seeking" phenotype of most cancer cells. In fact, iron levels can modulate apoptosis in multiple ways, for example by affecting the alternative splicing of Fas/CD95 transcripts between the pro-apoptotic and anti-apoptotic isoforms (42). Moreover, the p53 pathway that regulates cell cycle and apoptosis interacts with iron metabolism in a complicated crosstalk that remains to be completely explained (43). As an example of opposing observations regarding the involvement of iron and p53 in the pathogenesis of HCC, it has been shown that exposure to iron down-regulated MDM2, the ubiquitin ligase which leads to degradation of p53 (44), whereas another study found decreased p53 protein levels in the liver of iron overloaded mice (45).

Iron metabolism has been investigated in rodent models of hepatic carcinogenesis as well as in regenerating liver, which represents an excellent example of controlled liver proliferation and hence a powerful model system to get insights into the processes leading to hepatocarcinogenesis. Similarly to other types of growing cells, increased expression of TfR1 has been found in rat liver preneoplastic nodules and HCC (46, 47), as well as in regenerating liver cells (48), probably in order to insure sufficient iron to sustain cell proliferation. Recently, a study investigating iron metabolism gene expression and prognostic features of HCC found that TfR1 are more expressed in HCC than in surrounding liver and correlate with poorer prognosis (49). Researches also addressed the role of iron in CCA, a severe liver tumor with limited therapeutic possibilities, concluding that high expression of TfR1, with consequent iron uptake, contributes to CCA progression and poorer clinical outcomes (50). Accordingly, we showed that elevated iron content is a negative prognostic factor in CCA patients (51).

However, recent studies indicated that altered expression of proteins of iron metabolism like TfR1 in tumor cells is not only a system to acquire more iron but may impinge on tumor growth in an iron-independent manner. In fact, in line with the demonstrated interaction of TfR1 with ligands other than transferrin (Tf) (52), it appears that, in addition to its role in iron uptake, TfR1 activates signaling pathways and has a role in apoptosis, a key process in cancer development. For example, TfR1, by activating JNK upon phosphorylation by Src, impairs apoptosis and thereby increases breast cancer cell survival (53). In addition, the interaction of TfR1 with Tf may also have roles which are different from iron uptake but are still important for tumor cell growth; in fact TfR1 appears to be implicated in epithelial mesenchymal transition (EMT) (54), which is an important process for cancer progression, and metastatic growth (55).

Over the last years, a number of studies have shown that the levels of Fpn are reduced in several cancer cells compared to their nonmalignant counterparts, so that Fpn downregulation appears as a common strategy that a variety of cancer cells adopt to enhance intracellular iron availability (39). Interestingly, Fpn expression is a strong and independent predictor of prognosis in different tumor types (39). Dysregulation of the hepcidin/Fpn axis may also play a relevant role in liver tumors. Recently, it has been shown that hypermethylation of specific sequences

in the promoter region of the gene coding for hepcidin, the liver hormone that regulates iron homeostasis by inhibiting Fpn-mediated iron export, results in transcriptional downregulation of hepcidin expression in HCC (56). A similar effect was found in a model of rat liver carcinogenesis, in which the downregulation of hepcidin and the consequent increase of Fpn-mediated iron release may underlie the decreased intracellular levels of iron in preneoplastic foci (57). This could be a specific feature of hepatic cancer, as Fpn is usually repressed in cancer cells and low iron levels are not attributed to increased iron export but to higher consumption. However, we found significantly reduced Fpn mRNA levels in tumor samples from CCA patients compared to matched surrounding liver, suggesting that also in PLC elevated iron content is a negative prognostic factor (51).

The reprogramming of iron metabolism in tumors comprises the repression of the iron storage protein ferritin, as a mean to increase iron availability for the high requirements of cancer cell (58). Conversely, a tumor-suppressive role for ferritin has been shown in several types of cancer, such as breast and colorectal cancer. Ferritin expression appears to be directly modulated by both oncogenes, which down-regulate ferritin, and tumor suppressors, which induce ferritin expression [reviewed in Torti (39)]. Accordingly, a recent study highlighted a new mechanism based on a complex miRNA network by which oncogenic miRNAs inhibit the expression of H ferritin and its pseudogenes, thus leading to prostate cancer growth (59). Ferritin expression in liver cancer has been investigated by a number of studies in rodent models of hepatocarcinogenesis and in human hepatomas (60), but a coherent picture has not emerged, probably as the result of different experimental approaches and models, but also because of the multiple mechanisms of regulation of this protein, which is affected by iron status, differentiation, growth rate, inflammation, etc. (61). Therefore, whether increased ferritin levels in HCC patients merely reflect increased accumulation of iron, which is the actual carcinogen, or play an active role in malignant transformation is still unknown.

LIVER CSC AND IRON

Over the last years, several investigations found increased iron content in CSC of several types of tumors and also showed that altered iron trafficking is functional to the role of CSC in tumor growth. Indeed, variations of iron levels in CSC were achieved by specific modulation of the expression of genes of iron metabolism; in particular, TfR1-dependent iron uptake is induced whereas Fpn-mediated iron export is down-modulated. In most studies, the enhanced iron content was mirrored by high levels of the iron storage protein ferritin (4, 62).

Support to the idea that higher iron levels have a functional role in CSC formation and the maintenance of stemness was provided by evidence that iron chelation inhibited tumor spheres (a proxy of CSC) formation in several types of cancers (4). Additionally, manipulation of iron levels modulated the expression of typical stemness markers (4). Of course, it is also conceivable that alterations of iron homeostasis induced by genes related to CSC, such as Myc-mediated inhibition of ferritin expression (63), cooperate in order to disrupt iron homeostasis in CSC.

Notably, *in vivo* studies showing higher tumorigenic capacity of iron-rich tumor spheres in mouse xenograft tumor models confirmed the role of iron (64–66). Moreover, poorer prognosis for human tumors with altered expression of iron proteins in CSC is a common finding (51, 64, 66–68).

As it regards the role of iron in liver CSC, knowledge is limited. We recently showed that the regulation of iron homeostasis is profoundly different if CCA cell lines are cultured under conditions inducing the formation of tumor spheres, as compared to CCA cells growing in monolayers. In particular, we found high H ferritin levels and TfR1 expression accompanied by diminished Fpn transcription, a pattern leading to elevated iron content. Moreover, this finding was mirrored by data showing a trend toward shorter survival in CCA patients with high expression of ferritin and hepcidin (51).

IRON AND CANCER THERAPY

In consideration of the role of iron in cancer, and particularly in CSC that are resistant to radio and chemotherapy, manipulation of iron levels appears a promising therapeutic strategy. Given the double-edged sword property of iron in controlling cell fate (**Figure 1**), both iron chelation (in order to starve tumor cells for this essential micronutrient) and iron overload (in order to exploit iron toxicity) have been proposed for cancer treatment, but there are still several concerns for the use of both strategies. In fact, the threshold above which iron levels are no longer supportive of growth but become toxic is not well-defined, even though recent findings indicated that the functional iron concentration that allows *in vitro* cell proliferation is very low (i.e., in the nanomolar range) (69). Iron chelation has been used in several types of cancer, including HCC (70). However, since desferrioxamine, an iron chelator in use since the nineties, has a rather poor bioavailability, limited success in treating cancer has been obtained with iron chelators so far, though more recently developed oral iron chelators like deferasirox showed some effect in leukemia patients (71). The major mechanisms by which sequestration of intracellular iron by classical iron chelators targets tumor cells are:(i) inhibition of the iron-containing ribonucleotide reductase, the rate-limiting enzyme for DNA synthesis, (ii) cyclin dependent kinases-mediated induction of cell cycle arrest, iii) activation of metastasis and tumor suppressor genes, such as NDRG1 and p53, respectively. Moreover, recent data indicate that chelators can also suppress cancer by inhibiting the EMT, a key characteristic of CSC (72). Since the prolyl hydroxylases controlling the levels of the hypoxia inducible factors (HIF) are iron dependent enzymes, iron chelators induce HIF and its numerous target genes (73). We have shown that HIF-1 is involved in the protective effect exerted by the iron chelator dexrazoxane against anthracycline cardiotoxicity (74). Therefore, one may legitimately wonder whether iron chelators may have a similar effect in cancer cells, thus limiting the therapeutic effect of anticancer drugs.

A new class of iron chelators, such as the thiosemicarbazone Dp44m, which were reported to inhibit the proliferation of cancer cell lines *in vitro* by inducing the expression of p21, a cyclin-dependent kinase inhibitor involved in cell cycle arrest (75), appear promising. Opposite to conventional iron chelators like desferrioxamine, Dp44m is a tridentate ligand that forms redox active iron complexes leading to increased oxidant levels and cytotoxicity (76). These compounds also limit the growth of tumor xenografts in nude mice and have entered clinical trials, but their effect on CSC has not been tested. However, considering the well-known drug resistance of CSC, it is worth to mention that lysosomal-targeted Dp44m prevents the sequestration of a chemotherapeutic anthracycline like doxorubicin in lysosomes, which is triggered by the stressful environment of the tumor (77). Through this mechanism, which allows doxorubicin to exert its toxic effects in the nucleus and the mitochondria, this chelator may thus favor the action of anticancer drugs. In the same line, a recent study showed that a novel iron chelator, DpdtC (di-2-pyridylketone hydrazone dithiocarbamate) can induce lysosomal oxidant production and growth inhibition of HCC cell lines (78).

The use of these compounds represents therefore an approach similar to that relying on the toxic side of iron for killing cancer cells (see below). This strategy gained momentum after the discovery of ferroptosis, a form on non-apoptotic cell death caused by iron-dependent production and accumulation of reactive and toxic hydroperoxides (79). Iron plays a dual role in ferroptosis, as iron on the one hand can promote Fenton chemistry and on the other hand stimulate the activity of the iron-dependent enzyme lipoxygenases that contribute to ferroptosis by degrading polyunsaturated fatty-acid-containing phospholipids (80). Malignant cells generate high levels of oxidants as by-products of the biosynthesis of macromolecules and must balance iron uptake for proliferation with the risk of generating oxidative stress (81). Since most ferroptosis-inducing agents have limited use *in vivo* due to low bioavailability (82), iron may thus have potential to trigger ferroptotic cell death in cancer, as high iron concentrations may push malignant cells beyond the breaking point of oxidative stress tolerance (34). Interestingly, it has been shown that iron is required to induce ferroptosis also in drug-resistant "persister" cancer cells, thus showing therapeutic promise to eliminate this pool of tumor cells characterized by non-mutational drug-tolerance (83).

Alternatively, iron may potentiate the effect of anticancer drugs like the multikinase inhibitor sorafenib, which is used for treating HCC. In fact, the evidence that iron chelation protected HCC cells against iron-dependent oxidative stress caused by sorafenib indicates that ferroptosis can represent an inhibitory mechanism of the growth of liver cancer cells (84). A further indication of the role of iron and ferroptosis in PLC was provided by a study showing that ferritin induction protects HCC cells from ferroptosis (85).

Triggering ferroptosis appears a promising approach also to attack CSC, which represent a negative factor for cancer outcome (86). Ovarian CSC showed higher sensitivity to ferroptosis than non-tumorigenic ovarian stem cells (66) and a recent study showed that in breast CSC exposure to salinomycin and its derivatives leads to lysosomal iron accumulation, oxidants production and ferroptosis (87). Similarly, increased intracellular iron levels provided by ferritin degradation can lead glioblastoma cells to ferroptosis (64). However, a number of recent studies have shown that CSC are iron-rich and iron-dependent (4); therefore, enhancing cellular iron may not always be an effective strategy to eliminate CSC selectively. Moreover, in general, low levels of oxidants have been reported in CSC, making difficult the approach based on targeting iron-dependent, oxidant related pathways in CSC. In fact, we found that sphere-forming CCA cells, in spite of higher levels of oxidants and iron, were more resistant to the ferroptosis inducer erastin than their counterpart growing as monolayer (51). It seems therefore that, before recommending the manipulation of iron homeostasis as a therapeutic tool for targeting tumors and the use of iron supplementation to promote ferroptosis of cancer cells, additional studies are needed to understand the role of iron in the pathways controlling cell death, as iron can possibly promote CSC cell growth, thereby affecting the survival of cancer patients.

It should be also kept in mind that HCC almost always develop in the context of chronic liver disease characterized by persistent damage and inflammation, which can be further stimulated by iron supplementation.

TARGETING IRON TO CANCER CELLS

Administration of massive quantities of iron successfully killed multiple myeloma cells (88), though these cells, which secrete large amounts of disulfide-rich immunoglobulins and are thus a source of oxidants (89), may be particularly sensitive to iron-dependent oxidative stress. Iron may also mediate the effect of high i.v. doses of vitamin C that were reported to kill liver CSC specifically both *in vitro* and *in vivo* by promoting oxidant production (90). This paradoxical effect of a recognized antioxidant may be explained by the strong reducing properties of ascorbic acid which, at pharmacological concentrations (>1 mM), maintains iron in the highly reactive ferrous form, thereby increasing, instead of preventing, oxidative stress and cell death.

How to load cancer cells, in particular CSC, with iron *in vivo*, possibly in a specific way ? Oral iron is poorly adsorbed and its uptake is subjected to a strict feed-back regulation (91), therefore, even recurring to novel nanoparticulate ferritin core mimetics (92), it does not appear a promising approach. Parenteral iron preparations, such as dextran iron, have higher efficacy but relatively poor safety due to hypersensitivity reactions. However, new formulations, such as iron gluconate and iron sucrose, do not present toxicity issues and two iv iron compounds prepared with new pharmaceutical technologies are currently approved for the treatment of iron deficiency anemia (93) and could be used in cancer patients. These iron complexes are endocytosed and processed by macrophages within the reticuloendothelial system, mainly in the liver, spleen and bone marrow, but the precise mechanism of recognition and internalization is not fully defined (94). Inside the macrophages, iron is released from the iron–carbohydrate complex in acidic endo-lysosomes through a mechanism incompletely understood

and subsequently transported to the cytoplasm, where it can be stored in ferritin or exported into the bloodstream by Fpn.

An approach alternative to exogenous iron administration is to impair safe intracellular iron storage, for example by triggering lysosomal ferritin degradation. Indeed, treatment of breast CSC with salinomycin resulted in increased ferritin degradation in lysosomes; the iron released then facilitated oxidants production and ferroptosis (87). An analogous release of catalytic ferrous iron from ferritin led mesothelioma cells to death after exposure to non-thermal plasma, which produces hydroxyl radicals (95). Similarly, artesunate, by enhancing lysosomal ferritin degradation, was able to induce cell death in HCC cell lines (96). Notably, regulated autophagic degradation of ferritin (ferritinophagy) contributes to ferroptosis and was found to occur in primary human hepatic stellate cells obtained from liver tissue of advanced fibrotic patients with HCC, thereby alleviating liver fibrosis (97). However, ferritin is not only, or not always, a source of iron for Fenton chemistry. Iron storage inside ferritin is a protective stratagem against iron-mediated oxidative injury (34, 61) and also mitochondrial ferritin shields this important organelle from oxidants damage (98). The relevance of this function in cancer has been shown by a study reporting that high ferritin expression in myeloma cells is directly related to increased resistance to oxidants generated by exposure to the proteasome inhibitor bortezomib (88). A similar effect was found in HCC cells in which oxidative stress mediated induction of ferritin protected from ferroptosis (85).

The alterations of iron homeostasis seem to involve not only cancer cells, but also other cell types of the tumor microenvironment, particularly macrophages. In response to signals in the tumor microenvironment, tumor-associated macrophages (TAM), which favor tumor growth and progression, often become similar to M2 polarized macrophages endowed with anti-inflammatory activity, which display a gene expression profile characterized by active iron uptake and release and ferritin repression (99, 100). Therefore, this kind of iron metabolism in TAM macrophages might promote tumor growth by providing iron to adjacent tumor cells (101). Notably, also CCA CSC prime TAM toward a tumor-promoting phenotype, although iron metabolism has not been explored in this setting (24). However, it should be noted that the TAM population may be heterogeneous (102), as it has been found that in one type of murine prostate cancer, but not in another model of prostate cancer, some TAM contain iron aggregates typical of iron storing macrophages (103, 104) and in ovarian cancer TAM presented a prevalence of M1 phenotype (105). In this case, TAM, by sequestering iron, may limit its availability to cancer cells, thus impairing tumor growth. On the other hand, accumulation of an excess of exogenously administered iron, in the same way as an excess of heme iron in hemorrhagic tumor regions (106), may induce a switch of TAM toward the M1 antitumor phenotype, which is associated with the most favorable prognosis, as recently confirmed by extensive immunogenomic analysis of thousands of diverse tumor types (102).

It should be noted that in malignancies induced (or accompanied) by constant damage and chronic inflammation like HCC, two factors can further impinge on iron trafficking: on the one hand, TAM are more M1-like (107) and could restrict iron availability in the microenvironment and exert toxicity against malignant cells; on the other hand, the high hepcidin levels caused by inflammation may weaken Fpn-mediated iron release from macrophages, thus contrasting the iron-donating activity of TAM. These considerations are at odd with the correlation between high hepcidin levels and tumor progression in breast cancer patients (108, 109) and poorer prognosis in CCA (51). However, hepcidin may interact with Fpn expressed by both TAM and cancer cells; moreover, other iron transporters like lipocalin2 (110) may be involved.

While the role of iron in TAM, which seems clearly context-dependent, remains to be fully clarified, iron handling by TAM may have therapeutic implications. In fact, a seemingly promising approach relies on the use of iron oxide nanoparticles, a type of nanocarriers used for cancer targeted drug delivery, which are internalized by macrophages, including TAM. In line with *in vitro* data showing that superparamagnetic iron oxides induce a macrophage shift from the M2 to the M1 subtype (111), a recent study showed that iron oxide nanoparticles inhibited tumor growth indirectly by inducing M1 polarization. Highly increased iron levels in TAM resulted in oxidants production and cancer cell apoptosis (112). While the specific targeting of some species of drug delivering nanoparticles to tumors relies on the higher permeability of leaky blood vessels inside the cancerous mass, thanks to the magnetic properties of iron oxide nanoparticles, a localized external magnetic field can be used to guide these nanoparticles to tumors, thus achieving an improved therapeutic response and reducing side effects.

CONCLUSIONS

There is growing evidence that iron homeostasis is dysregulated in cancer, including PLC, and over the past few years also insights into the key role of iron in CSC have emerged. CSC show alterations of iron metabolism leading to a phenotype characterized by elevated cellular iron content, so that the expression of their typical features, such as stemness, is inhibited by iron chelators, thus suggesting the use of these compounds for CSC-targeted therapy. On the other hand, most recent therapeutic approaches seem aimed at exploiting the capacity of excess iron to induce ferroptotic cell death in cancer cells. However, given the involvement of iron in many important pathophysiological settings, it should be considered that we need to better understand how manipulation of iron levels to contrast tumor growth may interfere with iron homeostasis in healthy tissues or worsen conditions accompanying cancer, such as inflammation or anemia. Moreover, unfortunately, the mechanism(s) underlying the redox regulation in CSC are still not fully understood, as indicated by the higher resistance of CCA CSC to ferroptosis despite a higher basal oxidative stress condition (51).

Despite the still limited understanding of many processes, the increasing recognition of the importance of iron in cancer biology offers new chances to unravel tumor pathogenesis and thus develop more effective iron-centered therapeutic strategies against liver cancer.

AUTHOR CONTRIBUTIONS

MC, SR, and GC wrote the manuscript. CR, EG, and PI made significant revisions to the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Therapeutic Targeting of Cancer Stem Cells: Integrating and Exploiting the Acidic Niche

*Catherine Vander Linden and Cyril Corbet**

Pole of Pharmacology and Therapeutics (FATH), Institut de Recherche Expérimentale et Clinique, UCLouvain, Brussels, Belgium

Cancer stem cells (CSC) or tumor-initiating cells represent a small subpopulation of cells within the tumor bulk that share features with somatic stem cells, such as self-renewal and pluripotency. From a clinical point of view, CSC are thought to be the main drivers of tumor relapse in patients by supporting treatment resistance and dissemination to distant organs. Both genome instability and microenvironment-driven selection support tumor heterogeneity and enable the emergence of resistant cells with stem-like properties, when therapy is applied. Besides hypoxia and nutrient deprivation, acidosis is another selection barrier in the tumor microenvironment (TME) which provides a permissive niche to shape more aggressive and fitter cancer cell phenotypes. This review describes our current knowledge about the influence of the "acidic niche" on the stem-like phenotypic features of cancer cells. In addition, we briefly survey new therapeutic options that may help eradicate CSC by integrating and/or exploiting the acidic niche, and thereby contribute to prevent the occurrence of therapy resistance as well as metastatic dissemination.

Keywords: cancer stem cell, acidosis, niche, microenvironment, drug resistance, metabolism, metastasis, dormancy

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Lido Calorini,
Università degli Studi di Firenze, Italy

***Correspondence:**
Cyril Corbet
cyril.corbet@uclouvain.be

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INTRODUCTION

Despite a broader arsenal of (targeted) therapies, prognosis is still very poor for several types of cancer. At present, most patients with advanced cancers die because tumor cells have a remarkable capacity to develop drug resistance, through both genetic and non-genetic mechanisms (1). Current therapeutic failures are thought to originate, at least partly, from the Darwinian nature of cancer according to which, both genetic alterations and highly selective local microenvironments (the so-called niches) help to develop tumor cell adaptive phenotypes to sustain malignant progression (2, 3). Indeed, while current clinical protocols aim to eradicate the tumor as quickly as possible [i.e., maximum-tolerated dose (MTD) strategies], they often lead to therapeutic failure due to the occurrence of tumor relapse and dissemination of cancer cells to distant organs, after an initial tumor response or the lack of effectiveness at the outset. This alarming observation is thought to arise from two neglected evolutionary concepts. First, phenotypic heterogeneity within a tumor makes it very likely that resistant cells are present before therapy regardless of the cancer genetic landscape (i.e., *de novo* drug resistance). Second, MTD-based therapy promotes the growth of resistant populations *via* the clonal selection of cancer cells with adapted phenotypes and elimination of all potentially competing populations (the so-called "competitive release") (4).

Cancer stem cells (CSC), also referred to as tumor-initiating cells, have been thought to actively contribute to the so-called "minimal residual disease" which is a small population of cancer cells

that survive drug treatment and re-initiate the malignant disease, with poor outcome, even some years later (**Figure 1**) (5, 6). Within the tumor mass, CSC are typically dormant (i.e., non- or slow-proliferating) but they have also the capacity to proliferate either for their maintenance (self-renewal) or for the generation of progenitor tumor cells (clonal tumor initiation and long-term repopulation) (**Figure 1**) (7). CSC are located in specific niches, determined by tumor microenvironment (TME) peculiarities, that enable them to be phenotypically better adapted and more prone to regain fitness (i.e., ability to survive and proliferate in a given environment) than other cancer cell populations within the tumor bulk (8, 9). Moreover, these niches are thought to help protect CSC from the immune system, resist conventional treatments by reducing their proliferation state and/or evading apoptosis, and facilitate their metastatic potential (9–11). Since most of the normal stem cell populations (e.g., hematopoietic, mesenchymal, and neural stem cells) are located in hypoxic niches, how hypoxia contributes to the maintenance and/or emergence of the CSC phenotype has been extensively studied and reviewed over the years (12–14). Moreover, the role of stromal cells (e.g., cancer-associated fibroblasts, adipocytes, endothelial cells, or immune cells), as cellular components of specific CSC-supportive niches, has been also reported elsewhere (15–18). In this review, we describe how acidosis, another hallmark of TME, may act as a permissive niche for adaptive stem-like cancer cell phenotypes. We also discuss the contribution of the acidic niche to tumor initiation and progression, as well as to therapy resistance and metastatic dissemination. This review finally explores potential therapeutic strategies that may help eradicate CSC by integrating and/or exploiting the acidosis-induced phenotypic alterations.

Acidosis and CSC-Related Phenotypic Features

Glycolysis, Mitochondrial Respiration, and Tumor Acidosis

Acidosis is now considered as a hallmark of the microenvironment in solid tumors with mean values of extracellular pH (pHe) ranging from 6.2 to 6.8 (19, 20). Although initially described as a strict consequence of the exacerbated glycolysis in tumor cells and the disorganized tumor vasculature, accumulation of H^+ ions in the TME also results from the mitochondrial respiration-derived CO_2 hydration (**Figure 2**) (21, 22). Direct measurements of both intratumoral pO_2 and pH have indeed revealed a spatial heterogeneity as well as an imperfect overlapping of hypoxia and acidosis gradients, with the existence of acidic areas that are also well-oxygenated (23, 24). Other studies have also shown that glycolysis-impaired or LDH-deficient tumor cell lines still have the ability to acidify the extracellular environment *in vivo* (25–27). More recently, Hulikova et al. (28) reported a role for stromal cells in the venting of hypoxia-induced acidosis, with gap junction-mediated connections that enable the cell-to-cell shuttling of cancer cell-derived H^+ ions and their venting at far distance from the hypoxic regions.

Tumor Acidosis and CSC-Related Gene Reprogramming

Although the effects of acid exposure on stem cell phenotype have been under controversy (29), there are now several lines of evidence for the role of tumor acidosis in the emergence and/or maintenance of CSC phenotypic features (e.g., slow-proliferating state, invasive capacities, and therapy resistance) that may participate to the minimal residual disease and the long-term clinical dormancy/relapse (30, 31) (**Figure 2**). Nevertheless, the straightforward contribution of a transcriptional acidosis-responsive element that could mediate gene reprogramming has not been reported so far. Several studies have however identified the hypoxia-inducible factor 2α (HIF2α) as a master regulator of gene expression in cancer cells, under acidic conditions (32–35) (**Figure 3**). Besides an increase of HIF2α abundance, acidosis also enhances its transcriptional activity through the activation of NAD^+-dependent histone deacetylases sirtuins 1 and 6 (SIRT1/6) (32, 36), thereby leading to the deacetylation of lysine residues in the HIF2α regulatory amino-terminal transactivation domain (N-TAD) region (32, 37). Another study has shown that highly acidic conditions (pH 5.8–6.2) can trigger nucleolar sequestration of the von Hippel-Lindau (VHL) tumor suppressor protein and subsequent HIF2α stabilization (35) (**Figure 3**). However, a recent report has observed that VHL-deficient renal carcinoma cells are still responsive to acidosis with an increase in HIF2α levels, and that the acidic pH-induced stabilization of HIF2α is mediated by the HSP90 chaperone protein (33). Acidic pH, under normoxia, was also found to induce L-2-hydroxyglutarate (L-2HG) production through several mechanisms including the activation of lactate dehydrogenase A and malate dehydrogenase 2 enzymes, the inhibition of the mitochondrial L-2HG removal enzyme L-2HG dehydrogenase and the stimulation of the reverse reaction of isocitrate dehydrogenase (carboxylation of α-ketoglutarate to isocitrate), thereby leading to stabilization of HIF-1α (38, 39). Although 2HG-mediated epigenetic changes have been thought to support a stem-like cell state (40–42), the direct implication of an acidosis/2HG/HIF-2α signaling axis in cancer stem cell biology remains to be determined (**Figure 3**). These data are however reminiscent of the occurrence of lactic acidosis in some 2-HG aciduric patients (43) and it could be hypothesized that acidotic episodes may induce 2HG accumulation.

In glioma, where HIF2α is now considered as a marker of CSC (44), acidic conditions were documented to increase both the expression of a panel of glioma stem cells (GSC)-associated genes, including *POU5F1* (*OCT4*), *OLIG2*, and *NANOG*, independently of a restricted O_2 availability (34), and the fraction of cells positive for the GSC markers CD133 and CD15 (33). Acidosis also promotes production of angiogenic factors such as VEGF and IL-8 in GSC that favor tumor growth through paracrine effects (34). Acidosis has been also correlated to stem cells through the role of mesenchymal stem cells (MSCs) within the tumor stroma. MSCs grown in acidic pH express a higher level of transforming growth factor-β (TGFβ) that induces an epithelial-to-mesenchymal transition (EMT)-like phenotype in melanoma cells (45). Acidosis-exposed MSCs also stimulate

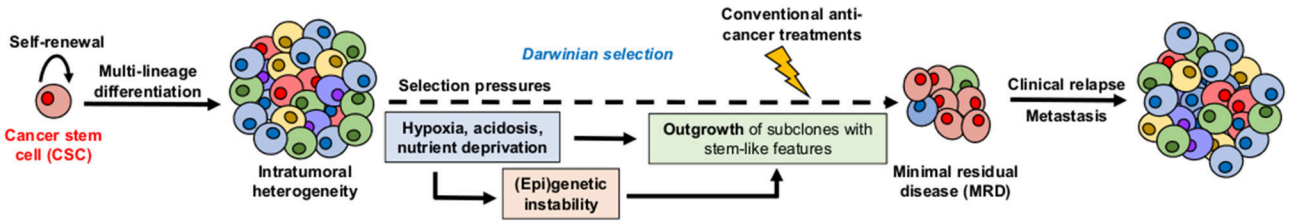

FIGURE 1 | Hypothetical model for the role of cancer stem cells (CSC) and microenvironmental selection pressure in clinical relapse. CSC display both self-renewal capacity and multi-lineage differentiation potential, leading to intratumoral heterogeneity. Local TME peculiarities such as hypoxia, acidosis, and nutrient deprivation act as high selection pressures for adaptive stem-like phenotypes that participate to therapy resistance, minimal residual disease, and long-term clinical relapse.

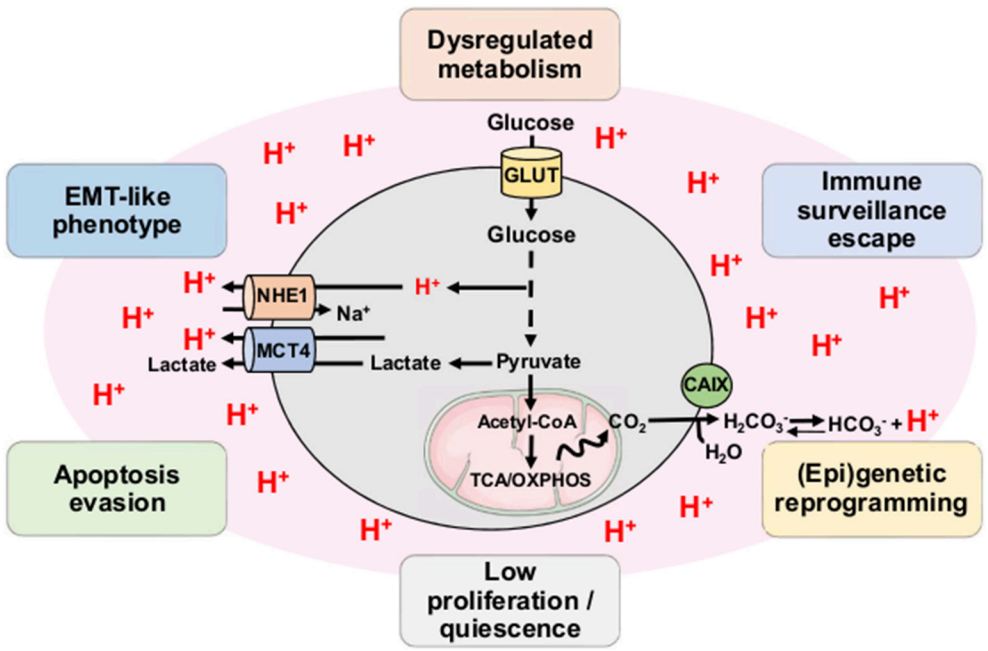

FIGURE 2 | Tumor acidosis influences and maintains CSC-related phenotypic features. Both exacerbated glycolysis and mitochondrial respiration-derived CO_2 hydration in tumor account for production of H^+ ions and subsequent environment acidification. Tumor acidosis contributes to the emergence and/or maintenance of stem-like phenotypic features such as dysregulated metabolism, immune surveillance escape, (epi)genetic reprogramming, low proliferation, apoptosis evasion, and EMT-like phenotype. CAIX, carbonic anhydrase IX; MCT4, monocarboxylate transporter 4; NHE1, sodium-hydrogen antiporter 1; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle.

the invasive and clonogenic capacities of osteosarcoma (OS) cells *via* the secretion of a variety of factors, including colony-stimulating factor 2 (CSF2, also referred to as GM-CSF), CSF3 (also known as G-CSF), bone morphogenetic protein 2 (BMP2), and interleukins 6 and 8 (IL6 and IL8) (46). MSCs, under acidic pH conditions, can also promote a stem cell phenotype in OS, by enhancing the sphere formation capacity and chemoresistance, *via* the induction of octamer-binding protein 4 (OCT4) (46). Finally, some studies have shown that cancer cell exposure to acidic conditions was associated with changes in the epigenetic landscape, including histone acetylation levels (36, 47), as well as a reprogramming of the genome-wide transcriptome (48, 49). Further investigations are however needed to study in depth the influence of the acidic niche on CSC (epi)genetic pattern, in other cancer types, but also in preclinical *in vivo* models.

Tumor Acidosis and Multidrug Resistance Phenotype

As stated above, CSC are resistant to anti-cancer treatments and they support long-term cancer cell survival and tumor relapse in patients. Acidosis has been directly correlated with drug resistance since it can reduce the passive permeability of weak base chemotherapeutic agents (e.g., doxorubicin, paclitaxel, mitoxantrone) by increasing their protonation state (the so-called "ion trapping" phenomenon) (50) (**Figure 4**). Several studies have indeed shown that neutralization of tumor-derived acid with systemic buffers (e.g., sodium bicarbonate, imidazoles, and lysine) (51) or the reversal of the pH gradient with proton pump inhibitors (e.g., omeprazole, esomeprazole) (52–54) can restore the sensitivity of cancer cells to chemotherapeutic drugs, such as doxorubicin.

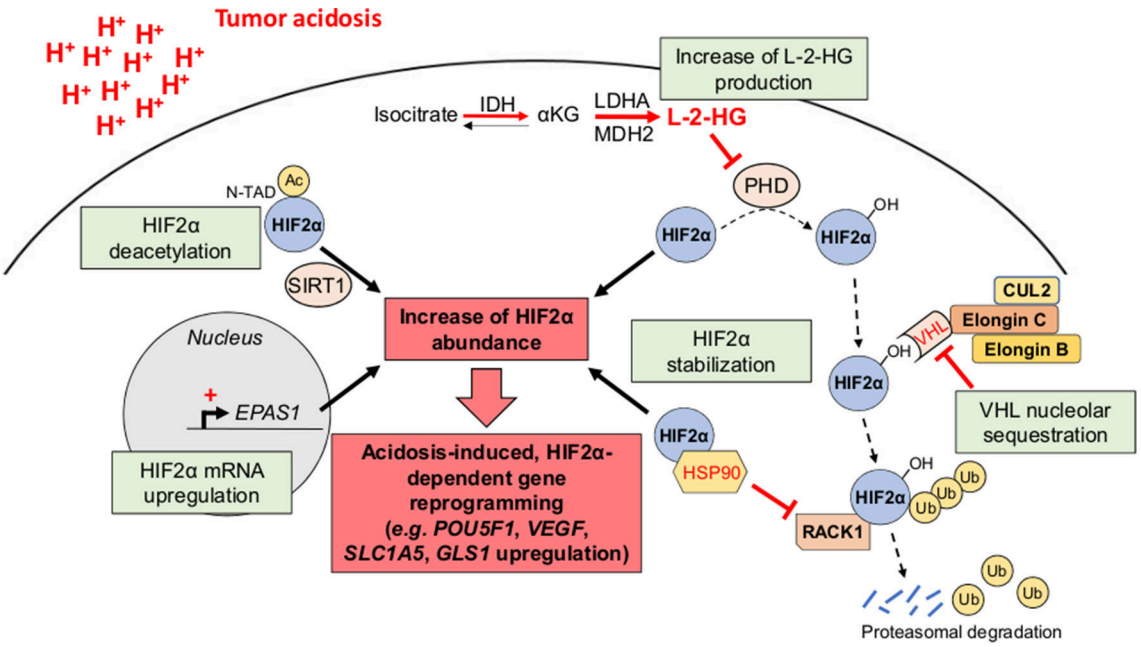

FIGURE 3 | HIF2α as a transcriptional regulator of acidosis-induced gene reprogramming. Tumor acidosis leads to an increase of HIF2α abundance either by directly inducing *EPAS1* gene transcription or by promoting HIF2α protein stabilization. For the latter, several mechanisms have been proposed, including the nucleolar sequestration of the von Hippel-Lindau (VHL) protein, the competitive binding of the chaperone heat shock protein 90 (HSP90) instead of the receptor of activated protein kinase C (RACK1), and an increased L-2-hydroxyglutarate (L-2-HG) production, thereby inhibiting HIF2α hydroxylation and subsequent proteasomal degradation. Besides its stabilization, acidosis also enhances HIF2α transcriptional activity through sirtuin-mediated deacetylation of lysine residues in its regulatory amino-terminal transactivation domain (N-TAD). αKG, α-ketoglutarate; Ac, acetyl group; CUL2, cullin-2; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; MDH2, malate dehydrogenase 2; PHD, prolyl hydroxylase; Ub, ubiquitin.

Besides this direct effect on the physico-chemical nature of anti-cancer drugs, acidosis can also promote a resistance-sustaining phenotype in cancer cells through different mechanisms. Indeed, while conventional treatments such as chemotherapies and/or radiation therapy are usually designed to eradicate highly proliferative cells, acidosis has been reported to reduce the proliferation status of cancer cells, that in some conditions even become relatively dormant (quiescent). Several studies have shown that cancer cells, exposed to acute acidic conditions, exhibit a low-proliferating phenotype as a consequence of a non-permissive intracellular acidification, an increased activity of the metabolic stress sensor AMP-activated protein kinase (AMPK) and a reduction of the multi-component mechanistic target of rapamycin complex 1 (mTORC1) signaling (**Figure 4**) (55–58). Another study reported that acidic conditions triggered a reduced proliferation state and high resistance to apoptosis in *BRAF*^{V600E} mutant melanoma cells (59). Acidosis-mediated melanoma cell phenotype was also associated with an acquired resistance to vemurafenib, a BRAF inhibitor, that could be overcome by treatment with everolimus, an inhibitor of mTOR activity (59).

Acidosis can also increase drug efflux capacities, both in *in vitro* and *in vivo* cancer models, through the upregulation and activation of membrane transporters such as the ATP-binding cassette protein ABCG2 (60), and the P-glycoprotein (P-gp) (61–64). For the latter, acidosis-induced chemotherapy resistance is mainly mediated through p38 signaling and can be reversed by treatment with verapamil, a P-gp inhibitor (61, 63). Another mechanism reported to mediate acidosis-induced therapy resistance is the unfolded protein response (UPR) pathway. Indeed, acidic conditions can trigger endoplasmic reticulum stress, thereby resulting in UPR activation and overexpression of the glucose-regulated protein 78 (GRP78) chaperone that contributes to chemotherapy-induced cell death resistance (65–67) (**Figure 4**). Finally, autophagy has also been described as an adaptive survival mechanism for cancer cells under acidosis, in particular through the enhanced expression of autophagy-related protein 5 (ATG5) (68, 69). Although an increased autophagic flux has already been associated with chemotherapy resistance in a variety of cancers (70), Avnet et al. (52) have reported that acidosis-induced doxycycline resistance in OS cells is not supported by autophagy since ATG5 gene silencing cannot restore drug sensitivity. These observations suggest that acidosis-driven drug resistant phenotype might be tumor type-dependent and/or supported by a variety of mechanisms that are redundant and have therefore the ability to compensate for the inhibition of one of them.

Tumor Acidosis and Immune Escape

Besides their ability to resist conventional treatments, CSC needs also to evade immune surveillance to support cellular dormancy and long-term clinical relapse. Acidic pHe conditions have been reported to decrease T cell proliferation and their capacity

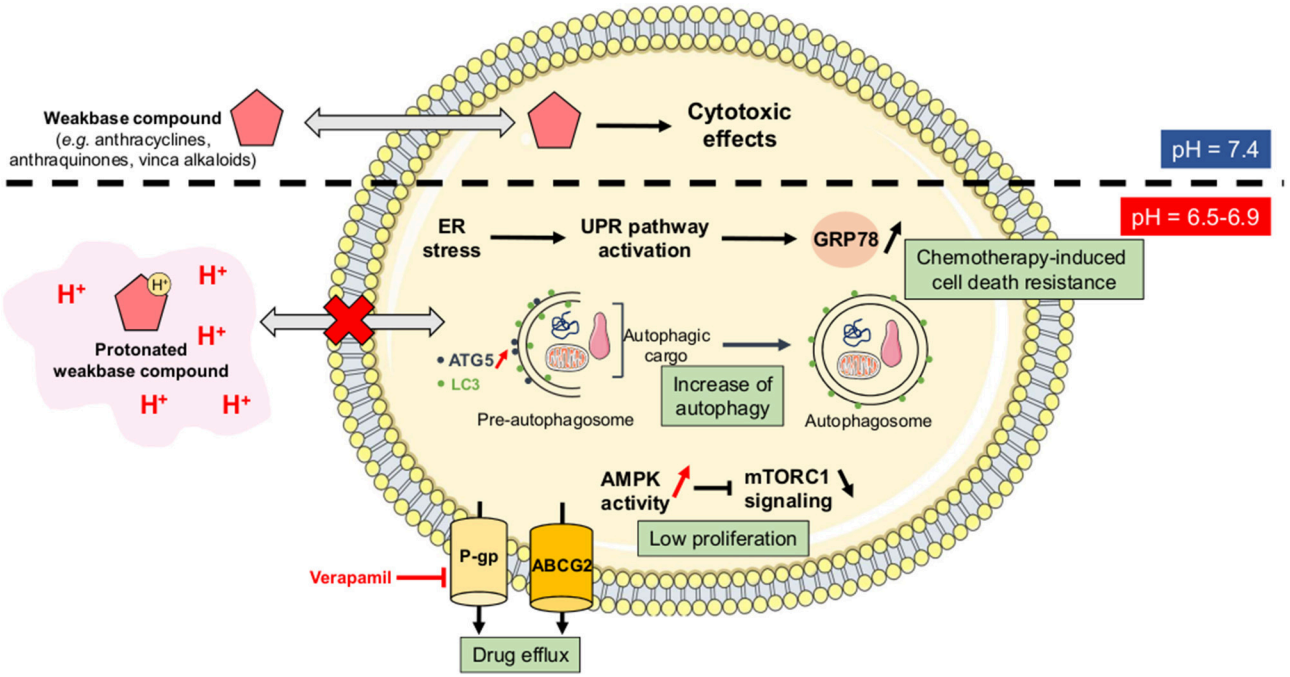

FIGURE 4 | Ion trapping phenomenon and acidosis-induced multidrug resistance. Under acidic conditions, several weak base chemotherapeutic agents become positively charged species and therefore lose their ability to diffuse passively across cellular membranes (the so-called "ion trapping phenomenon"). Tumor acidosis also directly promotes a resistance-sustaining cell phenotype through several mechanisms including the upregulation of glucose-related protein 78 (GRP78) or Bip, an increased autophagic flux, a lower proliferation rate, and an increase of drug efflux capacities *via* the P-glycoprotein (Pgp) and the ATP-binding cassette protein ABCG2. AMPK, AMP-activated protein kinase; ATG5, autophagy-related protein 5; ER, endoplasmic reticulum; LC3, microtubule-associated protein light chain 3; mTORC1, mammalian target of rapamycin complex 1; UPR, unfolded protein response.

to produce a variety of cytokines, including interleukin-2 (IL-2), interferon-γ (IFN-γ), granzyme B and perforin, in a dose-dependent manner (71). Tumor acidosis also impairs immune system functions by reducing dendritic cell maturation (72), monocyte-derived tumor necrosis factor (TNF) secretion (73), and natural killer (NK) activity (74). Indeed, tumor-derived H^+ and/or lactate accumulation, in the extracellular compartment, supports the suppressive effect on T cell function by inhibiting the glycolytic pathway within T cells (71, 73). Moreover, inhibition of the transcription factor nuclear factor of activated T cells (NFAT) has been proposed to mediate the blockade of IFNγ production in T cells and NK cells, upon intracellular accumulation of H^+ and lactate (75). The same authors also proposed a direct role of LDHA for lactate generation and the subsequent inhibition of tumor surveillance by T and NK cells (75). Mouse melanomas with reduced H^+ and lactate generation (upon LDHA genetic knockdown) actually exhibit a lower growth rate than control tumors and show an increased infiltration of IFNγ-producing T and NK cells (75). Importantly, this effect was lost when LDHA-knockdown tumors were grown either in immunodeficient *Rag2*$^{-/-}\gamma c^{-/-}$ mice or in *Ifng*$^{-/-}$ mice. Another study has revealed that phosphoenolpyruvate (PEP), a glycolytic intermediate, can act as a metabolic checkpoint to sense glucose availability and modulate a Ca^{2+}-NFAT signaling, such that a decrease of PEP intracellular concentration triggers a T cell anergy (76). A recent study also reported that extracellular acidification, within melanoma tumors, can be sensed by tumor-associated macrophages (TAMs), resulting in macrophage polarization and promotion of tumor growth (77). Mechanistically, a macrophage G-protein-coupled receptor (GCPR) can sense tumor acidification and leads to expression, by macrophages, of the inducible cyclic AMP early repressor (ICER), a transcriptional repressor that mediates the functional polarization into TAMs, which support tumor growth (77). Neutralization of tumor acidity with sodium bicarbonate (78), or with proton pump inhibitors (79) helps to improve the response to antitumor immunotherapy by restoring T cell cytolytic activity and cytokine secretion together with an increased tumor lymphocyte infiltration in mouse models but also in human cancer patients.

Tumor Acidosis and Metabolic Rewiring

Current controversy about the metabolic characteristics of CSC, described as either strictly glycolytic (80, 81) or instead dependent on mitochondrial metabolism (82, 83) may simply reflect their adaptability upon microenvironmental fluctuations. Here below, we will strictly focus on the current understanding of the influence of a low pH on cancer cell metabolism in an attempt to delineate the anticipated interplay between stemness and metabolism in the acidic TME niche.

Indeed, while cancer cells can use a variety of substrates to fulfill their need in energy and/or biosynthetic precursors (84, 85), we have recently documented, by using tumor cell lines chronically adapted to acidosis, a metabolic shift toward

a preferential use of glutamine to the detriment of glucose utilization (32). HIF2α was found to drive glutamine metabolism by increasing expression of the glutamine transporter ASC-like Na^+-dependent neutral amino acid transporter 2 (ASCT2) and glutaminase 1 (GLS1) (32). On the contrary, HIF1α activity and expression are reduced under chronic acidosis, thereby decreasing the expression of several target genes, including the glucose transporter GLUT1 and the monocarboxylate transporter 4 (MCT4) (32). Another study has also reported that several breast cancer cell lines, exposed to acute acidic conditions (24 h), show an increased glutaminolysis and redirection of glucose toward the oxidative branch of the pentose phosphate pathway (PPP), *via* a p53-dependent induction of glucose-6-phosphate dehydrogenase (G6PD), and glutaminase GLS2 expression (56). These metabolic changes certainly support an antioxidant response of acidosis-exposed cancer cells by increasing NADPH production and may have yet a broader impact considering how glycolysis inhibition may lead to various defects in protein glycosylation (86).

Besides changes in glutamine and glucose metabolism, tumor acidosis has also been related to profound alterations in lipid metabolism (**Figure 5**). Indeed, acidosis-induced reductive carboxylation of glutamine-derived α-ketoglutarate was reported as a source of acetyl-CoA from citrate to neo-synthesize fatty acids (36). Acetate was identified as another source of acetyl-CoA for fatty acid synthesis, under acidic conditions, in response to activation of sterol regulatory element-binding protein 2 (SREBP2) and subsequent upregulation of acyl-CoA synthetase short-chain family member 2 (ACSS2) (87). Importantly, fatty acid oxidation (FAO) is also stimulated in acidosis-exposed cancer cells (36, 56). This apparent juxtaposition of mitochondrial FA catabolism and cytosolic FA synthesis is rendered possible through a sirtuin-mediated histone deacetylation of the *ACACB* gene, encoding the mitochondrion-associated acetyl-CoA carboxylase 2 (ACC2) enzyme that normally prevents the degradation of neo-synthesized fatty acids in healthy tissues (**Figure 5**) (36).

Mild acidosis can also change mitochondrial morphology to preserve efficient ATP production regardless of O_2 levels (88); these data are supportive of the concept of an acidic niche that shapes cancer cells toward an OXPHOS-dependent metabolic phenotype. Interestingly, compelling evidence indicates that cancer stem-like cells, including therapy-resistant tumor cells, mostly rely on mitochondrial respiration and OXPHOS for growth (82, 83, 89, 90). Moreover, a recent study reported the isolation and characterization of a new distinct subpopulation of proliferating CSC, called "energetic" CSC, showing a significantly increased oxidative metabolism and mitochondrial mass, as well as a strict reliance on OXPHOS when grown in 3D anchorage-independent conditions (91). All these studies position an elevated mitochondrial metabolism as an important phenotypic adaptation for cancer stem-like cells and expand on the anticancer potential of mitochondrial biogenesis inhibitors, such as doxycycline or tigecycline (92).

More precisely, the role of FA metabolism, together with the concept of an adipose tissue niche, has been reported to support tumor growth and resistance to chemotherapy (18, 93, 94). Indeed, some investigators have documented that cancer cells transiently exposed to low pH conditions may accumulate neutral lipids into lipid droplets (LD) (95, 96). More recently, Menard et al. (97) reported that acute exposure of cancer cells to acidosis increases the uptake of lipoproteins, in a heparan sulfate proteoglycan (HSPG)-dependent manner, which are then accumulated into LD. This LD-loaded phenotype is associated with enhanced spheroid-forming capacity *in vitro* and metastatic potential *in vivo*; pharmacological or genetic targeting of HSPG could fully reverse these effects (97). This acidosis-induced LD-loaded phenotype is reminiscent of the accumulation of neutral lipids observed in colorectal CSC populations (**Figure 5**) (98–100). High levels of LD were actually found as a distinctive mark of CSC in colorectal cancer, as revealed by label-free Raman spectroscopy, and they correlated with CSC markers such as CD133 and Wnt pathway activity (98). Finally, an elegant study revealed that increased lipid desaturation, *via* the activity of the stearoyl-CoA desaturase 1 (SCD1) enzyme, is essential to stem-like characteristics in ovarian cancer cells (94). Indeed, the authors have shown that ovarian CSC ($ALDH^+/CD133^+$) have a higher ratio of unsaturated to saturated fatty acids, and this ratio is essential for the cells to retain stemness. Further investigations are however needed to address whether acidic conditions in the TME also induce similar changes of the lipid profile in cancer (stem) cells.

Acidosis-Based Therapeutic Strategies to Tackle CSC Compartment

Therapeutic Strategies to Directly Manipulate/Exploit Extracellular pH

Utilization of systemic buffers, such as sodium bicarbonate, imidazoles and lysine, was proposed several years ago as an obvious strategy to directly neutralize the tumor-derived acid and hamper tumor cell aggressiveness (101–106). Importantly, all these studies actually showed that oral administration of such buffers reduces the metastatic dissemination of cancer cells in animal models without affecting primary tumor growth. Many groups have already documented that acidosis could facilitate migration/invasion of cancer cells *in vitro* as well as metastasis formation *in vivo via* the activation of proteases (101, 107, 108), the secretion of pro-angiogenic factors (109) or the promotion of an EMT-like phenotype (110, 111). Further investigations are however needed to address whether interfering with tumor acidification is directly correlated with a decrease in stem-like cell population. As stated above, several studies have shown that buffer therapy (51) can restore the sensitivity of cancer cells to chemotherapeutic drugs, such as doxorubicin. There is however no direct evidence for a straightforward modulation of acidosis-induced cancer cell phenotype (vs. changes of the physico-chemical properties of the drug) by systemic buffer administration.

Because of their relative small proportion into the tumor bulk and their phenotypic features strongly associated with the local microenvironment peculiarities, CSCs are indeed inherently difficult to isolate and to maintain in culture, making almost unfeasible a direct CSC-selective screening of small molecules.

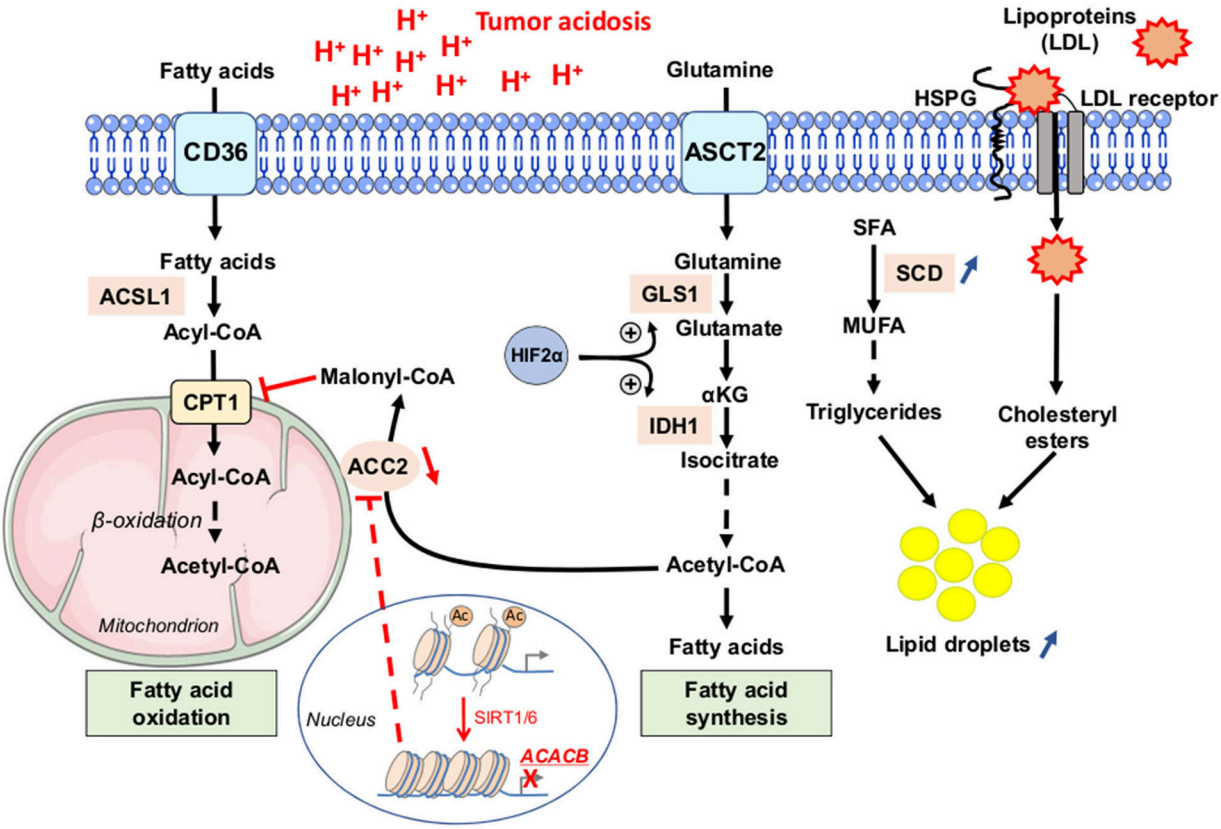

FIGURE 5 | Fatty acid metabolism dysregulation as a phenotypic feature of cancer cells under acidic conditions. Tumor cells, exposed to low pH conditions, exhibit profound alterations in lipid metabolism, with a concomitance of fatty acid oxidation (FAO) in mitochondria and synthesis from glutamine (FAS) in the cytoplasm rendered possible through a sirtuin 1 and 6 (SIRT1/6)-mediated downregulation of the acetyl-CoA carboxylase 2 (ACC2). Saturated fatty acids (SFA) can be actively transformed by stearoyl-CoA desaturase enzyme (SCD) into mono-unsaturated FA (MUFA), and then into triglycerides. Acidosis-exposed tumor cells also increase lipoprotein uptake in a heparan sulfate proteoglycan (HSPG)-dependent manner. Increased accumulation of neutral lipids (i.e., triglycerides or LDL particle-derived cholesteryl esters) into lipid droplets is observed in cancer cells under acidic pH conditions. Ac, acetyl group; ACSL1, long-chain fatty acid CoA ligase 1; ASCT2, alanine serine cysteine-preferring transporter 2; CD36, cluster of differentiation 36; CPT1, carnitine palmitoyl transferase 1; GLS1, glutaminase 1; IDH1, isocitrate dehydrogenase 1; LDL, low-density lipoprotein.

This obstacle has prompted the artificial induction of EMT to produce cells displaying CSC-like characteristics suitable for high-throughput phenotypic screening (112–114). Salinomycin, an ionophore antibiotic, was identified as a selective agent against experimentally-induced CSCs (113, 115). Interestingly, salinomycin-induced cytotoxic effects were enhanced under conditions of transient and chronic acidosis, with in particular an inhibition of autophagic flux in breast CSC-like cells (116).

Tumor acidosis can also be exploited in order to selectively deliver anti-cancer drugs (117). Over the years, a variety of pH-sensitive nano-systems, such as peptides, micelles, liposomes, nanoparticles and polymersomes, have been synthesized, as extensively reviewed elsewhere (118–120). Nevertheless, only few studies have reported the use of such nano-scale carriers, responding to an acidic pH, for the selective targeting of CSC. A pH-responsive prodrug (PEG-modified doxorubicin) has for instance been co-delivered with SN38, an active metabolite of irinotecan, to eradicate both breast CSC and non-CSC populations (121). Such stable nanomedicine with pH sensitivity enhances drug accumulation at the tumor site, thereby leading to a potent tumor growth inhibition, while reducing chemotherapy-induced adverse effects (121). Finally, pH low-insertion peptides (pHLIP) have recently emerged as new modalities for tumor-specific drug delivery, but also for tumor imaging (122). These water-soluble membrane peptides undergo pH-dependent folding that triggers insertion across the cell membrane (123, 124). A pHLIP can directly translocate cargo molecules (attached to its C-terminal tail) through cell membranes without binding to cell surface receptors or pore formation. Although systemic administration of pHLIP has been used for the translocation of a variety of molecules, including chemotherapeutic drugs such as paclitaxel and doxorubicin (125, 126), antimicrobial peptides (127), polar membrane-impermeable peptides (e.g., phalloidin and other toxins) (128, 129) and peptide nucleic acid antimiRs (130), none study has investigated a specific targeting of CSC-like tumor cells with pHLIP-conjugated anti-cancer drugs. pHLIP grafted with agents known to interfere with CSC phenotypic features could be particularly suited to selectively kill this small cell subpopulation.

Therapeutic Targets and Modalities to Exploit Acidosis-Induced Phenotypic Alterations

As stated above, tumor acidosis induces several CSC-like phenotypic features that could be directly targeted for a therapeutic purpose. Among them, acidosis-mediated metabolic rewiring has a huge potential to be genetically or pharmacologically targeted since many enzymes/transporters that sustain cancer cell growth under low pH conditions are known (see above). Indeed, systemic administration of chitosan-based nanoparticles loaded with siRNA targeting two key transporters of energy fuels for acidosis-adapted cancer cells, namely the lactate/acetate transporter MCT1 and the glutamine transporter ASCT2, could lead to significant *in vivo* antitumor effects (131). Moreover, *in vitro* and *in vivo* experiments revealed that acidosis accounts for a net increase in tumor sensitivity to BPTES, an inhibitor glutaminase GLS1 (32).

Dysregulated fatty acid metabolism is another critical determinant of acidosis-adapted cancer cell growth, with the simultaneous occurrence of FAO and FAS pathways. Inhibition of mitochondrial transport of acyl-CoA, *via* the blockade of carnitine palmitoyltransferase 1 (CPT1) activity with etomoxir, showed a selective growth inhibitory effect in acidosis-adapted cancer cells (36). This is in adequation with the important role of FAO to support tumor proliferation and survival in a wide panel of tumors, including triple-negative breast cancer, glioma, leukemia, and colon (132). It is noteworthy that some compounds of interest, able to interfere with FAO, are currently under clinical development or already in use (perhexiline, trimetazidine, ranolazine) for the treatment of cardiovascular diseases; the anticancer potential of these molecules could therefore be rapidly evaluated in clinical trials.

Finally, acidosis-induced (epi)genetic reprogramming is another feature that might be targeted to eradicate stem-like cancer cells. Dual inhibition of SIRT1/6, with EX-527 compound, could for instance trigger selective growth inhibition of acidosis-adapted cancer cells (32). This effect was indeed associated with the re-expression of ACC2 enzyme that prevents the concomitant occurrence of FA oxidation and synthesis in acidosis-adapted cancer cells (36). Another study documented that human osteosarcoma cells were more sensitive to the inhibitor of histone deacetylases MC 1742 under acidosis than under neutral pH (47). Of note, this compound was reported by others to suppress proliferation and induce apoptosis of CSC in the same cancer type (133). In view of the central role of HIF-2α signaling under acidosis (see above), the use of recently developed HIF2α-selective inhibitors, PT2399 and PT2385 (134, 135), also appears as a promising therapeutic approach to selectively kill cancer (stem) cells exposed to acidic conditions.

CONCLUDING REMARKS

Frequent occurrence of tumor relapse is a major limitation for the cure of many patients, and that despite major improvements in prevention, diagnosis, and treatments. It is now acknowledged that local microenvironmental conditions select stem-like cancer cell phenotypes that dictate therapy resistance and re-initiation of the disease at the primary site but also into distant organs after metastatic dissemination. Recent findings reviewed here point to acidosis as one of the major selection barriers in the TME forcing the outgrowth of adaptive fitter phenotypes, when therapy is applied. While hypoxia has been reported as a CSC-permissive niche for many years, effects of acidosis by itself on CSC-related features were investigated more recently, upon the compelling evidence that oxygen and pH gradients were not perfectly overlapping in tumors. The reliance of CSC on the acidic niche is mediated by several mechanisms, including gene reprogramming, metabolic rewiring, apoptosis evasion and immune surveillance escape. Because a low pHe is a common feature of most solid tumors (vs. healthy tissues), there is an obvious interest to identify new therapeutic modalities that aim to take advantage of acidosis to selectively deliver anti-cancer drugs into tumors and eradicate resistance-sustaining cell populations such as CSC.

Since environment-mediated phenotype of cancer (stem) cells evolves *de facto* with time and tumor development, relevant pre-clinical, experimentally tractable, models as well as innovative approaches are needed to explore the intimate relationship between TME (in particular acidosis), cancer cell phenotypic adaptations (e.g., metabolic preferences) and drug response. Indeed, despite the strong evidence supporting the CSC model in a variety of cancers, it is critical to acknowledge major limitations associated with the poor reliability of CSC identification based on cell-surface markers expression and the lack of direct evidence about their *in vivo* existence. Future challenges to tackle the contribution of CSC in tumor relapse and to evaluate their clinical significance during drug resistance, minimal residual disease and metastatic dissemination rely therefore on the capacity to better integrate and exploit the microenvironment-driven phenotypic changes (e.g., dormant, mesenchymal-like state), including specific metabolic alterations (e.g., dysregulated FA metabolism, OXPHOS dependence) in order to propose novel CSC-targeting therapeutic modalities.

AUTHOR CONTRIBUTIONS

CV and CC contributed to conception and design of the figures and the manuscript. CC developed the study concept, obtained funding, and wrote the final version of the manuscript. All authors wrote sections, revised, read, and approved the submitted version.

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REVIEW
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Metabolism-Based Therapeutic Strategies Targeting Cancer Stem Cells

*Petra Jagust[1], Beatriz de Luxán-Delgado[1], Beatriz Parejo-Alonso[2] and Patricia Sancho[1,2]**

[1] Centre for Stem Cells in Cancer and Ageing, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom, [2] Traslational Research Unit, Hospital Universitario Miguel Servet, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Cancer heterogeneity constitutes the major source of disease progression and therapy failure. Tumors comprise functionally diverse subpopulations, with cancer stem cells (CSCs) as the source of this heterogeneity. Since these cells bear *in vivo* tumorigenicity and metastatic potential, survive chemotherapy and drive relapse, its elimination may be the only way to achieve long-term survival in patients. Thanks to the great advances in the field over the last few years, we know now that cellular metabolism and stemness are highly intertwined in normal development and cancer. Indeed, CSCs show distinct metabolic features as compared with their more differentiated progenies, though their dominant metabolic phenotype varies across tumor entities, patients and even subclones within a tumor. Following initial works focused on glucose metabolism, current studies have unveiled particularities of CSC metabolism in terms of redox state, lipid metabolism and use of alternative fuels, such as amino acids or ketone bodies. In this review, we describe the different metabolic phenotypes attributed to CSCs with special focus on metabolism-based therapeutic strategies tested in preclinical and clinical settings.

Keywords: cancer stem cells, metabolism, mitochondria, oxidative phosphorylation, lipid metabolism, redox regulation

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***Correspondence:**
Patricia Sancho
psancho@iisaragon.es

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Abbreviations: α-ADD, alpha-aminoadipate; 2DG, 2-deoxy-D-glucose; AML, acute myeloid leukemia; ALDH1, aldehyde dehydrogenase 1; ATO, arsenic trioxide; BET, bromodomain and extra-terminal motif; BSO, buthionine sulfoximide; CAFs, cancer associated fibroblast; CML, chronic myeloid leukemia; CSCs, cancer stem cells; DHA, docosahexaenoic acid; DOX, doxorubicin; DQA, dequalinium; DRP-1, dynamin-related protein 1; EGCG, epigallocatechin gallate; EPA, eicosapentaenoic acid; ERRα, estrogen-related receptor alpha; EMT, epithelial-mesenchymal transition; ETC, electron transport chain; FAs, fatty acids; FAO, fatty acid oxidation; FASN, fatty synthase; FOXO, forkhead box; G6P, glucose-6-phosphate; GPx, glutathione peroxidase; HBP, hexosamine biosynthetic pathway; LDs, lipid droplets; LPA, lysophosphatidic acid; MAO-B, monoamine oxidase-B inhibitor; MCT 1/2, monocarboxylate transporter 1 and 2; Mdivi-1, mitochondrial division inhibitor; MPP, mitochondria penetrating peptide; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin; MTS, mitochondria targeting sequences; MUFAs, monounsaturated fatty acids; NER, nucleotide excision repair; NRF2, nuclear factor erythroid 2–related factor 2; OxPhos, oxidative phosphorylation; PDAC, pancreatic ductal adenocarcinoma; PDX, patient-derived xenograft; PEITCs, phenethyl isothiocyanates; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PHGDH, phosphoglycerate dehydrogenase; PI3K, phosphoinositide 3-kinase; PPP, pentose phosphate pathway; ROS, reactive oxygen species; S1P, sphingosine-1-P; SOD, superoxide dismutase; SREBP1, sterol regulatory element-binding protein 1; SCD-1, stearoyl-CoA desaturase; TCA, tricarboxylic acid; TICs, tumor initiating cells; TNBC, triple negative breast cancer; TPP, triphenylphosphonium.

INTRODUCTION

Cancer is a highly heterogeneous disease, not only in terms of variability among patients, but also within a single tumor. This heterogeneity constitutes the main cause for therapy resistance and cancer progression in some patients (Hanahan and Weinberg, 2011). We can find different levels of intratumor heterogeneity. First, a tumor is comprised of multiple genotypes, which belong to distinct subclones with diverse features, which may include differential morphology and/or functionality. Additionally, tumors (and the subclones within) are formed of a functionally heterogeneous cell population, where a particular subset of tumor cells have the ability to initiate and propagate tumor growth, survive chemotherapy and drive relapse (Phillips et al., 2006; Diehn et al., 2009; Labelle et al., 2011; Hu et al., 2012; Rhim et al., 2012; Touil et al., 2014; Li et al., 2015b). These cells, the so-called cancer stem cells (CSCs), have self-renewal capacity, and can give rise to a differentiated progeny, leading to the production of all cell types present within a tumor, thereby generating tumor heterogeneity through a differentiation hierarchy (Reya et al., 2001). This distinct population was initially identified in leukemia, but was also found in solid tumors, such as breast, lung, prostate, colon, brain, head and neck, liver, as well as in pancreatic ductal adenocarcinoma (PDAC) (Hermann et al., 2007; Castellanos et al., 2013; Du et al., 2017). Finally, non-cancer cells present in the tumor microenvironment constitute a third level of heterogeneity, since they can directly affect cancer cell plasticity and functionality (Malanchi and Huelsken, 2009; Batlle and Clevers, 2017).

Cancer stem cells can be originated either from a mutation of a normal stem cell or from differentiated cells acquiring stem-like abilities (Reya et al., 2001). Indeed, numerous studies have found an abnormal activation of stem cell regulatory genes and pathways in the CSCs population, such as c-MYC, Bmi-1, Hedgehog, Notch and Wnt (Somervaille et al., 2009; Sancho et al., 2015; Wang et al., 2016). Apart from the well-known developmental pathways such as Wnt, Hedgehog or Jagged, metabolic traits have recently been involved in governing the function of stem cells. Indeed, although stem cells are primarily glycolytic, acquisition of certain metabolic plasticity, together with an increase in oxidative metabolism, primes them for maturation and supports their lineage differentiation (Cho et al., 2006; Chen et al., 2008; Simsek et al., 2010). A parallel mechanism was also postulated for CSCs in different tumors (Dong et al., 2013; Shen et al., 2015; Chen C.L. et al., 2016). However, recent data indicate that CSCs may mainly depend on oxidative metabolism (Sancho et al., 2015). In any case, reported metabolic differences between CSCs and progenies introduce another source of heterogeneity within tumors: metabolic heterogeneity. The latter can be further amplified, since different CSCs subclones can bear different metabolic phenotypes (Gammon et al., 2013) as a result of genetic or microenvironmental factors (Guha et al., 2014; Raj et al., 2015; Sancho et al., 2016).

CANCER (STEM) CELL METABOLISM

One of the main cancer characteristics is uncontrolled growth and cell division. To support the abnormal survival and growth, cancer cells need to increase nutrient uptake to supply biosynthesis pathways (Vander Heiden et al., 2009; Kamphorst et al., 2015; Hensley et al., 2016). To achieve that, cancer cells usually modulate the activity of different metabolic pathways in order to produce metabolic precursors to satisfy energetic and anabolic demands, and maintain redox balance (Vazquez et al., 2016). Due to the crucial contribution of diverse metabolic pathways to malignant transformation and tumor progression, metabolic reprogramming recently became one of the cancer hallmarks (Hanahan and Weinberg, 2011).

Aerobic Glycolysis

The best example of reprogrammed metabolism in cancer is aerobic glycolysis (De Berardinis and Chandel, 2016): fast-proliferating tumor cells increase their glucose uptake to produce lactate in the presence of oxygen. This cancer hallmark was discovered by Otto Warburg and, thus, named the Warburg effect (Warburg et al., 1927; Warburg, 1956; Shim et al., 1998; Vander Heiden et al., 2009; Cairns et al., 2011). Glycolysis intermediates are used in diverse reactions to support high proliferation rates. For example, glucose-6-phosphate (G6P) can be used in the pentose phosphate pathway (PPP) to produce NADPH (Horton, 2002; Porporato et al., 2011; Doherty et al., 2014; Liberti and Locasale, 2016) or generate ribose groups, necessary for the synthesis of nucleotides (Lane and Fan, 2015; Vazquez et al., 2016). Alternatively, glycolytic intermediates can be used for anabolic reactions of glycogen or lipid synthesis (Gatenby and Gillies, 2004; Kroemer and Pouyssegur, 2008).

Glycolysis also facilitates survival and fast adaptation to the typically hypoxic tumor environment avoiding toxic Reactive Oxygen Species (ROS) accumulation through both low ROS production and increased detoxification systems (Brand and Hermfisse, 1997; Cairns et al., 2011; Doherty et al., 2014; Liberti and Locasale, 2016). Moreover, favoring glycolysis may bring other advantages to tumor cells, such as creating an acidic environment that can help invasion and suppress the immune response (Fischer et al., 2007; Swietach et al., 2007).

Even though aerobic glycolysis is quite inefficient in terms of ATP production, the rate of glucose uptake can be significantly elevated in cancer cells, resulting in ATP production to levels usually achievable with oxidative phosphorylation (OxPhos) (Liberti and Locasale, 2016). Additionally, although it was originally postulated that aerobic glycolysis is irreversible for tumor cells after cell division (Zu and Guppy, 2004; Jose et al., 2011; Porporato et al., 2011), it is well accepted nowadays that most cancers still produce ATP via OxPhos and modulate the contribution of both pathways in response to environmental factors or in different phases of the cell cycle (Smolková et al., 2011; De Berardinis and Chandel, 2016).

Importantly, glycolytic metabolism supports stemness in normal stem cells and CSCs of several cancer types (Folmes et al., 2011) (**Table 1**). Indeed, recent pieces of evidence demonstrate the involvement of oncogenes and pluripotency transcription

factors, such as MYC, p53, K-Ras, HIF1α, NANOG, MEIS1, Wnt or OCT4 in the metabolic reprogramming from oxidative-dependent metabolism to a glucose dependence in many types of cancer (reviewed in Gabay et al., 2014; Alptekin et al., 2017; Deshmukh et al., 2018). Different studies support the glycolysis dependence of CSCs in diverse types of cancer, such as in radioresistant nasopharyngeal carcinoma spheres with high expression of stage-specific embryonic antigen (SSEA) -3 and -4 compared to parental cells (Shen et al., 2015), $CD133^+$ human hepatocellular carcinoma cells and mouse models (Song et al., 2015; Chen C.L. et al., 2016), $ALDH^+$ (aldehyde dehydrogenase) non-small lung carcinoma cells and side population (SP) cells from human colon cancer (Liu et al., 2014).

On the other hand, enhanced glycolysis in CSCs could also constitute a secondary response to maintain energy balance, since reduction of mitochondrial metabolism seems to be essential for full stemness in some cancer types, such as osteosarcoma or glioblastoma (Zhou et al., 2011; Yuan et al., 2013; Palorini et al., 2014). Indeed, the downregulation of mitochondrial genes was associated with enhanced increased expression of genes related to epithelial-mesenchymal transition (EMT) usually linked to stemness (Gaude and Frezza, 2016). Importantly, such inverse relationship was functionally proven in embryonal carcinoma cells derived from teratocarcinomas, since the stimulation of mitochondrial function induced cell differentiation and loss of pluripotency (Vega-Naredo et al., 2014). In fact, the occurrence of this metabolic switch, not the final glycolytic phenotype, seems to be key for early state of tumorigenesis and acquisition of stemness-related properties in human mammospheres and brain CSCs in a mouse model of primitive neuroectodermal tumors (Dong et al., 2013; Ciavardelli et al., 2014; Malchenko et al., 2018).

Mitochondrial Metabolism

Mitochondria play a key role in eukaryotic cells coordinating energy production and distribution through OxPhos based on oxygen and substrate availability, although other important metabolic reactions such as fatty acid oxidation (FAO), glutaminolysis, or reductive carboxylation in cells with damaged mitochondria also take place in these organelles. Mitochondrial tricarboxylic acid (TCA) cycle is primarily fueled by acetyl-CoA produced by glycolysis (from pyruvate) or FAO. Alternatively, in highly glycolytic cells, such as Ras-mutant cells, glutamine can be the driving force for OxPhos (Fan et al., 2013) through its conversion to α-ketoglutarate and oxaloacetate, that can be then used for fatty acids (FAs) and nucleotide synthesis (Gaglio et al., 2011). Electron donors produced in the TCA cycle are used by the electron transport chain (ETC) to create a proton motive force to synthesize ATP by the complex V.

As opposed to what we summarized in the previous section, recent literature described OxPhos as the main source of energy in CSCs from a number of cancer types (**Table 2**). This has been convincingly shown for ROS^{low} quiescent $CD34^+$ leukemia CSCs (Lagadinou et al., 2013), lung spheroids and $CD133^+$ PDAC cells (Ye et al., 2011; Sancho et al., 2015), as well as CSCs-enriched spheroids form ovarian, cervical and papillary thyroid carcinoma that displayed a reprogrammed metabolism through TCA cycle (Sato et al., 2016; Caria et al., 2018). Since mitochondrial metabolism coupled to OxPhos constitutes a much more efficient

TABLE 1 | Stem-like cells with glycolytic metabolism for various cancer types (in chronological order).

METABOLIC PHENOTYPE: GLYCOLYSIS				
Cancer type	**Model of study**	**CSCs/Tumor cells**	**Methods**	**References**
Glioblastoma	*In vivo* (xenograft) and *in vitro*	Neurospheres	Clark-type oxygen electrode	Zhou et al., 2011
Glioblastoma	*In vitro*	Neurospheres	Gene expression analysis	Goidts et al., 2012
Breast cancer	*In vitro*	Bulk of tumoral cells	Isotope tracing and seahorse	Dong et al., 2013
Glioblastoma	*In vitro*	Neurospheres	Clark-type oxygen electrode	Yuan et al., 2013
Ovarian cancer	*In vivo* (xenograft) and *in vitro*	Spheres	Isotope tracing and seahorse	Anderson et al., 2014
Breast cancer	*In vitro*	Spheres	Proteomics and targeted metabolomics	Ciavardelli et al., 2014
Ovarian cancer	*In vitro*	Spheres	Isotope tracing combined with spectrometry	Liao et al., 2014
Lung cancer	*In vitro*	SP	Clark-type oxygen electrode	Liu et al., 2014
Colorectal cancer	*In vitro*	SP	Clark-type oxygen electrode	Liu et al., 2014
Osteosarcoma	*In vitro*	3AB−OS CSC−like line	Seahorse	Palorini et al., 2014
Teratocarcinomas	*In vitro*	P19SCs	Clark-type oxygen electrode	Vega-Naredo et al., 2014
Nasopharyngeal carcinoma	*In vitro*	Sphere-derived cells	Seahorse	Shen et al., 2015
Hepatocellular carcinoma	*In vitro*	$CD133^+$cells	Seahorse	Song et al., 2015
Lung cancer	*In vitro*	Spheres	Glucose uptake, glutamine, glutamate and NAD+/NADH determination	Deshmukh et al., 2018
Breast cancer	*In vitro*	Spheres	Glucose uptake, glutamine, glutamate and NAD+/NADH determination	Deshmukh et al., 2018
Brain cancer	*In vitro*	Tumor cell lines with BTIC features	Seahorse	Malchenko et al., 2018

TABLE 2 | Stem-like cells with OxPhos metabolism for various cancer types (in chronological order).

METABOLIC PHENOTYPE: OxPhos				
Cancer type	**Model of study**	**CSC/Tumor cells**	**Methods**	**References**
Lung cancer	*In vivo* (xenograft) and *in vitro*	Secondary spheres	Clark-type oxygen electrode	Ye et al., 2011
Glioblastoma	*In vitro*	Gliomaspheres	Seahorse	Janiszewska et al., 2012
Leukemia stem cells	*In vitro*	$CD34^+$ cells	Seahorse	Lagadinou et al., 2013
PDAC	*In vivo* (inducible mouse model of mutated KRAS2) and *in vitro*	Spheres	Isotope tracing, metabolomics and seahorse	Viale et al., 2014
Breast cancer	*In vitro*	Spheres	Label-free quantitative proteomics	Lamb et al., 2015b
PDAC	*In vivo* (xenograft) and *in vitro*	$CD133^+$ cells and spheres $CD44^+CD133^+$	Seahorse	Sancho et al., 2015
Ovarian cancer	*In vitro*	Spheres	Metabolomics	Sato et al., 2016
Papillary Thyroid Carcinoma	*In vitro*	Thyrospheres	GCMS	Caria et al., 2018

energy process, CSCs relying on OxPhos would theoretically make a better use of limited nutrients, which is an important advantage to survive in nutritionally poor environments. Indeed, mitochondria-dependent $CD44^+CD117^+$ ovarian CSCs and $CD133^+$ PDAC CSCs showed enhanced resistance to glucose or glutamine deprivation compared to their differentiated counterparts (Pasto et al., 2014; Sancho et al., 2015). On the other hand, a variety of metabolites released by stromal cells can be used by OxPhos-dependent cells to fuel the TCA cycle, conferring them with increased adaptability to the changing conditions of the tumor microenvironment (Anderson et al., 2014). The best known example is lactate uptake from hypoxic tumor cells or cancer-associated fibroblasts (CAFs) via monocarboxylate transporter 1 and 2 (MCT1 and MCT2) in a process known as reverse Warburg effect (Kroemer and Pouyssegur, 2008; Sonveaux et al., 2008; Porporato et al., 2011; Rattigan et al., 2012). Moreover, pancreatic stellate cells release alanine to fuel the TCA cycle and subsequent biosynthetic pathways in pancreatic cancer cells (Sousa et al., 2016). Additionally, recent evidence indicate that microvesicles found in the tumor microenvironment contain several metabolites, including aminoacids, lipids and TCA cycle intermediates to fuel central metabolism of oxidative tumor cells and, consequently, tumor growth (Santi et al., 2015; Zhao et al., 2016). In this sense, stromal cells would play a key role in tumor progression supporting OxPhos-dependent CSCs proliferation and survival in nutrient-deprived environments.

Even though Warburg hypothesized that mitochondrial respiration defects are responsible for cancer cells shifting to glycolysis, it is known today that cancer cells still retain mitochondrial functions and that, in fact, a significant amount of ATP is produced through OxPhos (Tang et al., 2011; Kang et al., 2014; Zong et al., 2016). Indeed, ATP from OxPhos proved to be important for cell movements and invasive/metastatic abilities of cancer (stem) cells (Yu et al., 2017), suggesting that mitochondria contributes to cytoskeletal alterations (Caino et al., 2015). Moreover, OxPhos activation in metastatic breast cancer models is crucial to escape from the metabolic dormancy derived from hormonal therapy (Sansone et al., 2016). Interestingly, OxPhos activation is caused by the horizontal transfer of mitochondrial DNA (mtDNA) in exosomes from CAFs to dormant CSCs, providing a possible mechanism to development of resistance to hormonal therapy and highlighting metabolic interaction between CSCs and their niche (Sansone et al., 2017).

Beyond energy production, mitochondria are involved in controlling cellular redox rate, ROS generation, calcium buffering and the synthesis of intermediate molecules, such as acetyl-CoA and pyrimidines. Additionally, mitochondria have a crucial role in apoptosis initiation through activation of the membrane permeability transport pore, and release of cytochrome C (Wallace, 2012). Furthermore, mitochondria may contribute to malignant transformation and tumor progression through increased ROS production by the ETC (Ishikawa et al., 2008; Liou et al., 2016), abnormal accumulation of specific mitochondrial oncometabolites modifying epigenetic signals (Sciacovelli et al., 2013), and functional deficits in apoptosis (Tomiyama et al., 2006; Izzo et al., 2016). For all these reasons, mitochondrial biogenesis is essential for survival and propagation of CSCs regardless of their metabolic phenotype (Bonuccelli et al., 2010; De Luca et al., 2015; De Francesco et al., 2018). In fact, mitochondrial biogenesis may be a primary driver of stemness since its inhibition efficiently eliminated hypoxic spheroids in breast cancer (Lamb et al., 2015b,c; De Francesco et al., 2017).

The mechanisms driving mitochondrial biogenesis and OxPhos in CSCs described above have not been fully characterized yet, although some studies shed some light on this matter. In fact, findings in glioblastoma spheroids demonstrated the role of the oncofetal insulin-like growth factor 2 mRNA-binding protein 2 (Imp2) in the regulation of OxPhos, and mitochondrial biogenesis and structure (Janiszewska et al., 2012). Interestingly, the metabolic profile and plasticity of PDAC $CD133^+$ cells rely on the balance between the MYC-driven glycolysis and the main regulator of the mitochondrial biogenesis peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Sancho et al., 2015). In this study, differentiated PDAC cells exhibited an overexpression of MYC that counteracted stemness maintenance through a negative regulation of PGC-1α. These results apparently contradict the role of MYC as driver of stemness via glycolysis

previously exposed, which may be due to a cell context-dependent modulation of stemness/differentiation. On the other hand, PGC-1α overexpression can also lead to different outcomes depending on the cellular context in BRAF driven melanomas: increased PGC-1α expression in primary tumors after BRAF inhibition with vemurafenib causes OxPhos addiction associated with poor patient prognosis (Haq et al., 2013), while it impaired growth rate and invasive abilities in metastatic settings (Luo et al., 2016).

Redox Regulation

It is well known that oncogenic transformation (Trachootham et al., 2009; Zhou et al., 2014), dysfunctional mitochondria (Kudryavtseva et al., 2016) and altered cell signaling (Tachibana et al., 2008; Raza et al., 2017) induce ROS accumulation in cancer cells, further promoting tumorigenesis and mutagenesis. However, due to the potential deleterious effects of ROS, a powerful antioxidant machinery formed of both enzymatic and non-enzymatic antioxidants is often found in cancers (Obrador et al., 2002; Townsend and Tew, 2003; Arnold et al., 2004; Estrela et al., 2006; Valko et al., 2006; Du et al., 2013; Harris et al., 2015; Raza et al., 2017).

Increasing evidence suggests an important role of ROS and redox signaling for CSCs functionality. It was known that quiescent stem cells reside in a low ROS niche that supports their stemness characteristics, like self-renewal capacity. On the other hand, increased ROS content promote stem cell proliferation and differentiation (Ito et al., 2006; Jang and Sharkis, 2007; Naka et al., 2008; Owusu-Ansah and Banerjee, 2009; Yahata et al., 2011; Bigarella et al., 2014). Only recently, it was shown that CSCs share the same redox-related properties (Diehn et al., 2009; Kobayashi and Suda, 2012; Yuan et al., 2015): murine $CD44^{+}CD24^{-/low}Lin^{-}$ and human $Thy1^{+}CD24^{+}Lin^{-}$ breast CSCs (Diehn et al., 2009; Luo et al., 2018), $CD44^{high}$ gastrointestinal cell lines (Ishimoto et al., 2011), tumorigenic ROS^{low} from head and neck carcinoma cell lines (Chang et al., 2018), human or murine $CD133^{+}$ glioblastoma cells from cell lines and tumors and chronic myeloid leukemia (CML) $CD34^{+}$ CSCs (Zhao et al., 2009; Qiang et al., 2012; Zhu et al., 2013) maintain low levels of intracellular ROS coupled to enhanced antioxidant capacity. Apart from stemness maintenance, bearing high antioxidant capacity grants CSCs resistance to ROS inducers, such as chemo and radiotherapy (Diehn et al., 2009; Izumiya et al., 2012; Wang et al., 2017b).

Redox balance can also be achieved through the regulation of ROS-dependent signaling pathways and redox-sensitive transcription factors, such as c-MYC, HIF1α, p53, NF-κB, AP-1, and the master regulator of antioxidant response, nuclear factor erythroid 2–related factor 2 (NRF2) (Chandel et al., 2000; Kamata et al., 2005; Soriano et al., 2009; Liou and Storz, 2010; Boyer-Guittaut et al., 2014; Jiang et al., 2014; Raza et al., 2017). In fact, CSCs regulate ROS levels via antioxidant transcription factors, such as NRF2 or FOXO (Diehn et al., 2009; Zhu et al., 2013; Wu T. et al., 2015; Ryoo et al., 2016; Chang et al., 2018; Luo et al., 2018). Most of these factors affect redox homeostasis by direct or indirect modulation of cellular metabolism. Indeed, NRF2 upregulation in different cancer types (Mitsuishi et al., 2012; Abdul-Aziz et al., 2015; Kim and Keum, 2016; Rocha et al., 2016; Milkovic et al., 2017) influences the switch between anabolic/catabolic glucose metabolism (Mitsuishi et al., 2012; Wallace, 2012; Heiss et al., 2013; Hawkins et al., 2016). In addition, the proto-oncogene c-MYC controls both cellular metabolism and redox homeostasis by increasing glycolysis (Ellwood-Yen et al., 2003; Kim et al., 2007; Wang et al., 2008; Miller et al., 2012; He et al., 2015; Davis-Yadley et al., 2016; Massihnia et al., 2017), and regulating glutamine metabolism (Anderton et al., 2017). Interestigly, both mechanisms could be interconnected, since c-MYC binds to the NRF2 promoter (Levy and Forman, 2010).

Importantly, most of the main signaling pathways governing CSCs functionality are regulated by ROS signaling. That is the case of stemness-regulatory pathways, such as Wnt and Notch (Takubo et al., 2010; Qiang et al., 2012; Paul et al., 2014), or key signaling nodes important for cell survival and growth, such as PTEN (Xia et al., 2013), PI3K (Le Belle et al., 2011), AKT (Zhou et al., 2007; Dey-Guha et al., 2011), ATM (Ito et al., 2004; Yalcin et al., 2008), STAT3 (Qiang et al., 2012; Zhang et al., 2016), and mammalian target of rapamycin (mTOR) (Dubrovska et al., 2009) and their downstream targets. Moreover, those pathways further modulate ROS production/detoxification in a positive feedback loop by activating redox-sensitive transcription factors (Miyamoto et al., 2007; Tothova et al., 2007; Dubrovska et al., 2009; Yeo et al., 2013; Zhang et al., 2016).

Lipid Metabolism

Besides the classical metabolic reprogramming related to glucose, alterations in diverse aspects of lipid metabolism are increasingly gaining attention as determinants of cancer, including CSCs function. In fact, highly proliferating cells require increased amounts of the cell membrane's main components: lipids and cholesterol. In that cellular location, lipids function as either membrane building blocks or signaling transduction modifiers, since membrane lipid composition modulates protein recruitment and interaction (lipid rafts) (Rysman et al., 2010; Staubach and Hanisch, 2011). In this sense, several reports indicate that CSCs accumulate unsaturated lipids, such as monounsaturated FAs (MUFAs), the precursors of several plasma membrane lipids. In fact, lipid desaturation, mainly via the enzyme stearoyl-CoA desaturase (SCD-1), plays essential functions controlling self-renewal and tumorigenicity in different cancer models (Noto et al., 2013, 2017; Lai et al., 2017; Li F. et al., 2017), possibly through the activation of stemness-related pathways, such as Wnt signaling (Lai et al., 2017). Additionally, differences in plasma membrane lipid composition between CSCs and their differentiated counterparts have been reported, which can be potentially used to identify CSCs. Indeed, even though CSCs may present an overall decrease in glycosphingolipids as described for the glioblastoma CSC-like cell line GSC11 (He et al., 2010), the expression of specific gangliosides, such as GD2 and GD3, identified cells with increased self-renewal capacity and tumorigenicity in breast cancer (Battula et al., 2012; Liang et al., 2013).

Lipids and cholesterol in tumors are either scavenged from exogenous sources or synthesized *de novo* through FA synthase

(FASN) or the mevalonate pathway, respectively (Beloribi-Djefaflia et al., 2016). Thus, different reports suggest that elevated *de novo* synthesis of lipids and cholesterol contribute to CSCs properties and survival. In fact, the expression of sterol regulatory element-binding protein 1 (SREBP1), master controller of *de novo* lipogenesis, is increased in $CD24^{-}CD44^{+}ESA^{+}$ cells from a ductal carcinoma *in situ* cell line as well as mammospheres and melanospheres (Pandey et al., 2013; Corominas-Faja et al., 2014; Giampietri et al., 2017). This transcription factor may be involved in resistance to hypoxia and nutrient scarce environments, as suggested for glioblastoma sphere-derived cells (Lewis et al., 2015). Moreover, *de novo* lipogenesis from glycolytic intermediates or acetate via FASN is critical for *in vitro* self-renewal (Corominas-Faja et al., 2014; Yasumoto et al., 2016), and tumor relapse and metastatic dissemination after withdrawal of anti-angiogenic treatment (Sounni et al., 2014). In the same line of evidence, the activation of the mevalonate pathway is important for self-renewal and tumor formation in breast and pancreatic cancer, as well as glioblastoma (Ginestier et al., 2012; Brandi et al., 2017; Wang et al., 2017a).

Although *de novo* synthesis has traditionally been considered the preferred source of FAs for tumor cells (Ookhtens et al., 1984), recent reports highlight the crucial role of FAs uptake via CD36 or FA binding proteins (Hale et al., 2014; Pascual et al., 2016). The same is also true for cholesterol uptake within lipoproteins (Guillaumond et al., 2015). Indeed, lipid uptake, either via lipoprotein receptors or CD36, favors proliferation of glioma $CD133^{+}$ cells (Hale et al., 2014) and label-retaining/$CD44^{+}$ cells from squamous cell carcinoma (Pascual et al., 2016). Interestingly, increased lipid uptake points to the crucial role of microenvironment supporting cancer (stem) cell functions: tumor-activated adipocytes provide FAs to support leukemia $CD34^{+}$ cells growth, survival and chemoresistance (Ye et al., 2016; Shafat et al., 2017) as well as omental metastasis from ovarian cancer (Nieman et al., 2011).

Fatty acids require covalent modification by CoA by fatty acyl-CoA synthetases to enter the bioactive pool of FAs. Afterward, they will be further esterified to form triacylglycerols or sterol esters and stored in lipid droplets (LDs). Importantly, recent reports correlate accumulation of LDs or stored cholesteryl-ester with tumor progression and aggressiveness (Yue et al., 2014; Guillaumond et al., 2015). In fact, activated and stored lipids play a crucial role supporting tumorigenicity of CSCs *in vivo*, as demonstrated in cells derived from neurospheres from glioblastoma and $ALDH^{+}$ $CD133^{+}$ ovarian cancer cells (Sun et al., 2014; Menard et al., 2016; Li J. et al., 2017). This may be a reflect of adaption to the harsh conditions found in the tumor microenvironment, since those lipids can be mobilized upon metabolic stress, providing ATP via FAO to ensure survival (Maan et al., 2018). Importantly, increased lipid storage in LDs may constitute a useful CSCs marker, as demonstrated in colorectal (CRC) and ovarian cancer (Tirinato et al., 2015; Li J. et al., 2017).

Activated FAs are not only incorporated into membranes or storage, but also used as substrate to synthesize signaling lipids or energy production in FAO. Although FAO is considered the main energy source in non-glycolytic tumors (Liu et al., 2010; Caro et al., 2012), a high activity of this pathway has been reported for aggressive tumor cells and CSCs, especially in nutrient scarce environments (**Table 3**) (Carracedo et al., 2013; Kamphorst et al., 2013; Daniëls et al., 2014; Pasto et al., 2014). In fact, ATP production and survival of matrix-deprived epithelial cells depend on FAO (Schafer et al., 2009; Carracedo et al., 2012), a metabolic process that also sustains the self-renewal capacity in both leukemia-initiating $CFSE^{high}CD34^{+}$ cells and hematopoietic long-term culture initiating cells (Samudio et al., 2010; Ito et al., 2012). Besides its well-known role in energy production, FAs metabolism via mitochondrial FAO regulates multiple functions of CSCs. Indeed, FAO contributes to pluripotency maintenance and chemoresistance (Wang T. et al., 2018), mainly by reducing ROS production (Lee et al., 2015; Chen et al., 2016) and may sustain metastatic properties of sphere-derived cells (Aguilar et al., 2016).

Finally, lipids can also regulate CSCs functionality in terms of self-renewal and tumorigenic abilities through their function as second messengers in signal transduction pathways, thus becoming potential therapeutic targets. Indeed, sphingolipids, such as sphingosine-1-P (S1P), eicosanoids, such as prostaglandin E2 or glycerophospholipids, such as lysophosphatidic acid (LPA), have been reported to increase CSCs proliferation and *in vivo* tumorigenicity, activating self-renewal and survival signaling pathways (Notch, AKT, NF-kB) in $ALDH1^{+}$ from breast cancer, label-retaining cells in bladder cancer, $CD133^{+}CD44^{+}$ cells in CRC and sphere-derived cells from ovarian cancer (Hirata et al., 2015; Kurtova et al., 2015; Wang et al., 2015; Seo et al., 2016).

Alternative Fuels

Cancer cells require the use of amino acids for their heightened metabolic needs. Indeed, one of the most important metabolic pathways for cancer cells is that related to glutamine (Wise and Thompson, 2010), since it is an important substrate for DNA and fatty acid synthesis, as well as anaplerosis of the TCA cycle. Indeed, glutamine addiction has become a hallmark of glycolytic tumors, especially those with increased c-MYC expression (Deberardinis and Cheng, 2010; Wise and Thompson, 2010; Korangath et al., 2015). In addition, glutamine is related to glutathione synthesis, well known for its powerful antioxidant ability and some other biological activities (Todorova et al., 2004; Son et al., 2013). Although OxPhos-dependent pancreatic $CD133^{+}$ CSCs are resistant to glutamine deprivation (Sancho et al., 2015), evidence of the involvement of glutamine metabolism in the maintenance of the stem-like SP phenotype has been provided in lung and pancreatic cancer by a β-catenin/redox-mediated mechanism (Liao et al., 2017). In fact, glutamine deprivation in pancreatic cancer cell lines inhibited their self-renewal capacity, reduced their stemness gene signature and increased sensitivity to radiotherapy (Li D. et al., 2015). Additionally, aminoacid metabolism, especially glutamine, is increased in acute myeloid leukemia (AML) ROS^{low} CSCs to fuel OxPhos and favor survival (Jones et al., 2018). Interestingly, leukemia CSCs may obtain their glutamine supply from neighbor stromal cells, as described for bone marrow adipocytes

supporting cancer cells growth after asparaginase treatment in high-risk leukemia patients (Ehsanipour et al., 2013).

Apart from glutamine, the metabolism of amino acids, such as lysine or serine may also support CSCs features. Indeed, CRC $CD110^+$ tumor-initiating cells (TICs) are rich in enzymes implicated in both lysine transport and catabolism, which activates β-catenin-dependent Wnt signaling, ultimately promoting self-renewal and metastasis (Wu Z. et al., 2015). Additionally, accumulation of alpha-aminoadipate, an intermediate of lysine catabolism, on brain TICs correlates with poor survival rate of glioblastoma patients, representing a marker of tumor aggressiveness (Rosi et al., 2015). On the other hand, recent data demonstrate that phosphoglycerate dehydrogenase (PHGDH), catalyzing the first step of the serine biosynthesis, maintains self-renewal and tumorigenicity of lung, breast and brain $CD133^{high}$ sphere-forming cells in a mechanism involving pluripotency gene expression and redox balance (Samanta et al., 2016; Sharif et al., 2018). Finally, endogenous tryptophan derivatives, such as Kyn (kynurenine) and ITE (2-(10H-indole-30- carbonyl)-thiazole-4-carboxylic acid methyl ester), may play opposite roles on cancer progression and stemness, regulating OCT4 expression through aryl hydrocarbon receptor (AhR) modulation: accumulation of the low-affinity AhR agonist Kyn in the tumor microenvironment favor carcinogenesis, whereas

TABLE 3 | Stem-like cells using alternative metabolism for various cancer types (in chronological order).

METABOLIC PHENOTYPE: OTHERS					
Cancer type	**Metabolic phenotype**	**Model of study**	**CSC/Tumor cells**	**Methods**	**References**
Breast cancer	FAO	*In vitro*	Detached tumor cells	Isotope tracing	Schafer et al., 2009
Breast cancer	Ketone bodies	*In vivo* (xenograft)		3-OH-butirate effects on tumor growth, migration and angiogenesis	Bonuccelli et al., 2010
Hepatic cancer	Glutamine	*In vitro*	Bulk of tumor cells	BD Oxygen Biosensor System	Hu et al., 2010
Leukemia-initiating cells	FAO	*In vivo* (xenograft) *In vitro*	Bulk of tumor cells	Clark-type oxygen electrode	Samudio et al., 2010
Hepatic cancer	Glutamine	*In vitro*	Bulk of tumor cells	Glutathione, glutamate and glutamine	Suzuki et al., 2010
Breast cancer	FAO	*In vitro*	Detached tumor cells	Isotope tracing	Carracedo et al., 2012
Leukemia-initiating cells	FAO	*In vivo*	$CD150^+CD48^-$ $CD41^-Flt3^-CD34^-$KSL cells sorted from $Pml^{+/+}$ or $Pml^{-/-}$mice	Isotope tracing and seahorse	Ito et al., 2012
Glioblastoma	PPP	*In vitro*	Gliomaspheres	Isotope tracing	Kathagen et al., 2013
Colorectal cancer	Glycolysis, TCA cycle, and cysteine/methionine metabolism	*In vitro*	$CD133^+$ cells	Metabolomics	Chen et al., 2014
Ovarian Cancer	OXPHOS and PPP	*In vivo* (xenografts) *In vitro*	$CD44^+CD117^+$ cells	Flow cytometry	Pasto et al., 2014
PDAC	Glutamine (non-canonical pathway of glutamine metabolism)	*In vivo* (xenografts) *In vitro*	Spheres	Gene expression and enzymatic assays	Li D. et al., 2015
Colorectal cancer	Lysine catabolism	*In vivo* (xenografts) *In vitro*	$CD110^+$	Transcriptomics	Wu Z. et al., 2015
Hepatocellular carcinoma	Glycolysis and FAO in sh-Nanog-TICs	*In vitro*	$CD133^+CD49f^+CD45^-$	Isotope tracing and metabolomics	Chen C.L. et al., 2016
Breast cancer	PPP	*In vitro*	Mammospheres and $ALDH^+$ cells	Glucose consumption, lactate, NADPH and G6P	Debeb et al., 2016
Cervical cancer	TCA	*In vitro*	Spheres	Metabolomics	Sato et al., 2016
Breast cancer	Mitochondrial biogenesis and FAO	*In vitro*	Mammospheres	Seahorse and label-free semi-quantitative proteomics	De Francesco et al., 2017
Pancreatic cancer	Glutamine	*In vitro*	ABCG2 high	ATP, NADP+/NADPH and glutathione	J Liao et al., 2017
Breast cancer	Ketone bodies	*In vitro*	Mammospheres	Seahorse	Ozsvari et al., 2017
Brain cancer	Purine metabolism	*In vivo* (xenograft) and *In vitro*	Brain TICs	Metabolomics	Wang et al., 2017c

the high-affinity AhR agonist ITE promotes its binding to the OCT4 promoter to suppress its transcription and, consequently, inducing cell differentiation in U87 glioblastoma neurospheres (Cheng et al., 2015).

Ketone bodies can also work as fuel to promote tumor growth and play a role in CSCs activity. Reports on breast cancer showed the role of ketone bodies increasing the expression of stemness-related genes, driving recurrence and metastasis, thus related to decreased patient survival (Bonuccelli et al., 2010; Martinez-Outschoorn and Lisanti, 2014).

The PPP has also come up as an alternative way to generate energy in CSCs. For instance, glioblastoma stem-like cells are remarkably metabolically flexibles, switching their metabolism depending on oxygen levels fluctuations: from high levels of PPP activity linked to active proliferation under acute oxygenation, to a glucose-dependent phenotype under hypoxia, when cell migration is stimulated (Kathagen et al., 2013). Additionally, Debeb et al. (2016) described that PPP inhibitors reduced the stemness-related markers in node-positive invasive breast carcinoma and a high rate in PPP activity was also reported in combination with an OxPhos-dependent phenotype in $CD44^{+}CD117^{+}$ CSCs from epithelial ovarian cancer (Pasto et al., 2014).

Overproduction of hyaluronan, a component of the extracellular microenvironment, supports self-renewal in human head and neck squamous cell carcinoma HSC-3 cells (Bourguignon et al., 2012) and dedifferentiation in breast cancer cells (Chanmee et al., 2014). Using metabolomic approaches, Chanmee et al. (2016) later described that increased hyaluronan production leads to a HIF1α-induced metabolic reprogramming toward glycolysis, thus creating a positive feedback loop through the hexosamine biosynthetic pathway (HBP). Interestingly, HBP inhibition considerably reduced the content of $CD44^{high}CD24^{low}$ cells and mammosphere-forming capacity.

Finally, purine metabolism has also been described to regulate stemness-related properties. Indeed, upregulation of MYC-mediated *de novo* purine synthesis maintained self-renewal, proliferation and tumor forming capacity in brain TICs, and was associated with poor prognosis in glioblastoma patients (Wang et al., 2017c).

Considerations on the Metabolic Heterogeneity of CSCs

As inferred from the information above, CSCs display a plethora of metabolic phenotypes diverging from the classical OxPhos/Warburg phenotypes (**Table 3**). However, such diversity cannot be completely attributed to the intrinsic heterogeneity of cancer, since conflicting data can be often found in the literature even for the same tumor entity.

The main source of reported disparities is the utilized model systems. On the one hand, the term CSC tend to be loosely used and include models as dissimilar as established cell lines grown as spheroids in 3D and sorted cells from human tumors expressing one or several surface markers. In fact, although resistance to anoikis and the ability to grow in anchorage-independent conditions are well-accepted characteristics of stem-like cells, the percentage of *bona-fide* CSCs within a spheroid may be as low as 1%. On the other hand, established cell lines are usually clonal and have been passaged *in vitro* for dozens or even hundreds of times: resemblance with the genetic and phenotypic heterogeneity found in tumors barely exists. Even when considering sorted cells from fresh tumors, we need to bear in mind that most surface markers are not completely reliable and may be lost or modified in sample preparation: in fact, trypsinization time may greatly affect expression of these markers. Most importantly, most *in vitro* studies are carried out in artificial metabolic conditions (e.g., high glucose and oxygen) lacking microenvironmental components of the CSC niche. In fact, tumor niche can support metabolic alterations in CSCs (Mateo et al., 2014; Ye et al., 2016) via signaling and metabolic crosstalk.

Interestingly, even with these limitations, different groups have found phenotypically diverse CSCs subpopulations coexisting in the same *in vitro* or *in vivo* conditions (Diehn et al., 2009; Sancho et al., 2015; Luo et al., 2018). For instance, although most pancreatic CSCs are dependent on OxPhos, a pre-existing subpopulation of $CD133^{+}$ resistant to mitochondrial inhibition, due to their increased metabolic plasticity, was detected (Sancho et al., 2015). Importantly, differences in metabolism may be associated to functional diversity inside the CSCs population. Indeed, metabolic heterogeneity, mainly in terms of redox state, has been associated to differences in stemness, as well as chemo and radioresistance (Diehn et al., 2009; Wang et al., 2013; Sancho et al., 2015; Ye et al., 2016). Additionally, several reports link CSCs with enhanced metastatic abilities to specific metabolic traits. Indeed, reduced mitochondrial DNA and function contribute to the acquisition of a metastatic phenotype in spheroid-forming breast cancer cells (Guha et al., 2014). Moreover, alterations in redox balance leading to NRF2 activation mediate a phenotypic switch from glycolytic mesenchymal-like to OxPhos-dependent epithelial-like breast CSCs (Luo et al., 2018). In addition to breast cancer, $CD44^{+}ESA^{low}$ cells with increased metastatic potential (upon EMT), linked to low ROS levels, as compared to their non-EMT counterparts, have been described for oral and skin carcinomas, as well as prostate cancer (Gammon et al., 2013; Aguilar et al., 2016). On the contrary, metastasis-initiating cells in melanoma bear redox stress, as inferred from their elevated ROS and reduced glutathione content (Porporato et al., 2014; Piskounova et al., 2015).

THERAPEUTIC TARGETING OF CSCs METABOLISM

Considering the involvement of CSCs in chemoresistance, tumor relapse and metastasis, there is a pressing need in cancer therapy to find new strategies to eradicate this aggressive cell population. As summarized in the previous section, the distinct metabolic features of CSCs, compared to non-CSCs, constitute a significant opportunity to targeting specifically the CSCs component of tumors and eradicate the tumor bulk.

Mitochondrial Metabolism

As mentioned above, mitochondria play a key role for CSCs functionality regardless of their dominant metabolic phenotype, suggesting that targeting mitochondrial metabolism may be the most effective therapeutic strategy for their elimination. Noteworthy, highly glycolytic cells with mutations in the TCA cycle or ETC still require functional mitochondria for the generation of metabolites from glutamine via reductive carboxylation (Mullen et al., 2011). For those reasons, pharmacological approaches designed to target different aspects of mitochondrial function for cancer treatment are currently under intense investigation in preclinical and clinical studies (**Figure 1**).

Targeting OxPhos

Inhibition of mitochondrial respiration by compounds blocking ETC complexes is one of the most studied metabolism-based strategies for cancer treatment. In fact, tumor cells in nutrient-deprived environments or displaying limited metabolic plasticity, as described for some gliomaspheres and PDAC $CD133^+$ cells (Janiszewska et al., 2012; Sancho et al., 2015), have restricted ability to cope with decreased mitochondrial ATP production. ETC inhibitors also target glycolytic CSCs, such as $CD44^+CD24^{low}$ cells in breast cancer or SP cells in nasopharyngeal carcinoma (Vazquez-Martin et al., 2010; Shen et al., 2013), highlighting the importance of ETC for coupled ATP production, avoiding electron loss in the form of ROS.

One of the most studied ETC inhibitors in the context of CSCs targeting is the antidiabetic drug metformin. Reported antitumoral effects of this drug relate to both systemic glucose decrease, and direct cancer cell targeting via ETC complex I inhibition (Wheaton et al., 2014). Although metformin shows cytostatic properties at low concentration, it induces apoptosis specifically in PDAC $CD133^+$ cells and $CD44^{high}$ $CD24^{low}$ mammospheres (Iliopoulos et al., 2011; Sancho et al., 2015), which, at least for PDAC $CD133^+$ cells, is attributable to their dependency on mitochondrial metabolism. Although metformin clinical testing for pancreatic cancer treatment showed no improvement in patient survival rate (Kordes et al., 2015; Reni et al., 2016), positive clinical data has been reported for breast, endometrial and prostate cancer. On the other hand, the development of resistance to metformin monotherapy *in vivo* suggests that the design of combinatory treatments (Sancho et al., 2015; Roh et al., 2017) or the use of stronger mitochondrial inhibitors may be needed. This could be the case of the ETC complex I inhibitor phenformin, which is more

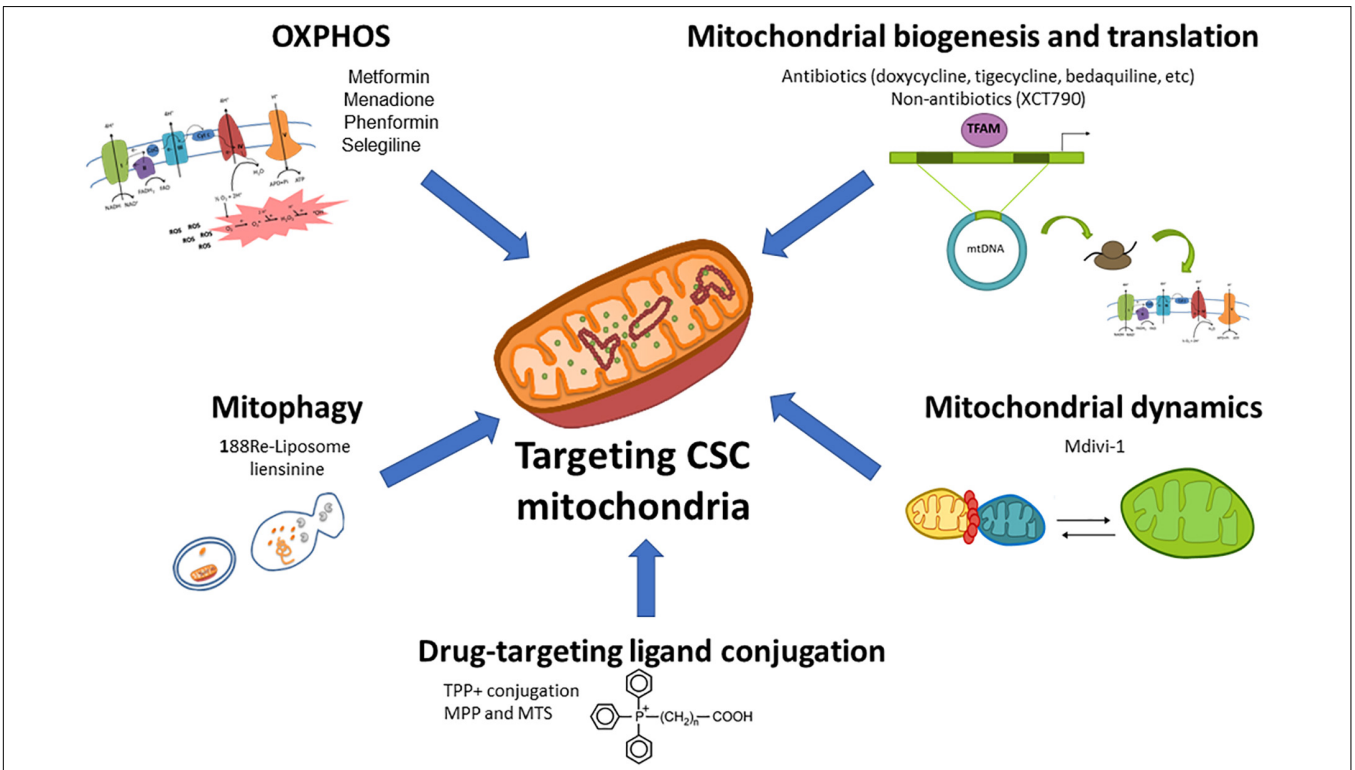

FIGURE 1 | Therapeutic targeting of mitochondrial metabolism in CSCs. Different aspects of the mitochondrial metabolism can be approached to target CSCs: (1) oxidative phosphorylation (OxPhos) can be impaired by ETC inhibitors such as the antidiabetic drugs metformin or phenformin, the reactive oxygen species (ROS) inductor and complex I inhibitor menadione, or the anti-Parkinson compound selegiline; (2) Mitochondrial biogenesis and translation can be targeted by FDA-approved antibiotics such as doxycycline, tigecycline, bedaquiline among others, or non-antibiotic inhibitors; (3) Mitochondrial dynamics can be disrupted by the mitochondrial division inhibitor Mdivi-1; (4) The blockage of mitophagy, an essential mitochondrial quality control system, with nanomedicines such as 188Re-liposome or the inhibitor liensinine may affect CSCs functions; (5) The use of nanocarriers (lipophilic cations, peptides and nanoparticles) conjugated with chemotherapeutics and small drugs may be used for a selective delivery of drugs in mitochondria.

efficiently delivered to mitochondria (Petrachi et al., 2017) and when combined with the ALDH inhibitor gossypol, suppresses stemness, invasiveness and cell viability in glioblastoma (Park et al., 2018). Additionally, menadione, with dual mechanism of action combining complex I inhibition and ROS induction, prevents the development of resistance (Sancho et al., 2015).

Following metformin's relative success, a great effort has been put on the drug repurposing for CSCs targeting in cancer treatment. In this sense, several known FDA-approved antibiotics target the ETC at different levels and have proven to selectively decrease CSC content. This is the case of antimycin A, a powerful complex III inhibitor that decreased lung spheroids (Yeh et al., 2013); the antituberculosis agent bedaquiline (complex V inhibitor) that targeted mammospheres (Fiorillo et al., 2016); oligomycin (another complex V inhibitor), which showed drastic synergistic effects suppressing cell growth and motility in glioblastoma cell lines when combined with 2-deoxy-D-glucose (2DG) (Kennedy et al., 2013); and niclosamide, an antihelmintic with ETC uncoupling properties, that inhibited TICs from ovarian and breast cancers (Yo et al., 2012; Wang et al., 2013). Similarly, numerous studies suggest the efficacy of the OxPhos inhibitor salinomycin for CSCs targeting *in vitro* and *in vivo* in diverse cancer types (Naujokat and Steinhart, 2012 and references therein). In this last case, however, the final antitumoral effect may be the result of a combination of factors, since salinomycin also interferes with ABC transporters or Wnt signaling (Naujokat and Steinhart, 2012). Besides antibiotics, the agent L-deprenyl (also known as Selegiline), a monoamine oxidase-B (MAO-B) inhibitor typically used for the treatment of Parkinson's disease, was found to exert antimitochondrial activity and cause apoptotic cell death in AML CSCs through the reduction of ETC and glycolysis-related gene expression, independently of MAO-B inhibition (Ryu et al., 2018).

Considering the therapeutic potential of OxPhos inhibition, compound discovery is currently taking place in order to identify new selective molecules with adequate *in vivo* properties. As an example, the compound VLX600 showed cytotoxicity in colon cancer spheroid-derived cells both *in vivo* and *in vitro*, by directly inhibiting ETC complexes in metabolically compromised microenvironments (Zhang et al., 2014).

Targeting Mitochondrial Translation and Biogenesis

As commented above, several FDA-approved antibiotics can disrupt mitochondrial function. Apart from direct OxPhos inhibition, certain widely prescribed antibiotics target either mitochondrial translation or biogenesis as an "off-target" effect (Lamb et al., 2015c), inhibiting self-renewal ability of multiple tumor types (Lamb et al., 2015a,b). For instance, the use of a tetracycline such as doxycycline induced apoptosis in pancreatic cancer cell lines and human cervical carcinoma tumorspheres (Son et al., 2009; Yang et al., 2015), while azithromycin (erythromycin family) demonstrated to inhibit the self-renewal capacity of PDAC spheroids (Lamb et al., 2015c). On the other hand, the use of the antimicrobial tigecycline selectively killed leukemia $CD34^{+}CD38^{-}$ cells without affecting normal hematopoietic cells through the inhibition of mitochondrial translation (Skrtic et al., 2011).

However, although this research line shows promising results, continuous treatment with antibiotics for cancer therapy may be ineffective (Esner et al., 2017). Indeed, long-term desensitization was reported for human metastatic breast cancer cells treated with different antibiotic classes including streptomycin, tetracycline, kanamycin, G418-geneticin (aminoglycoside), puromycine (aminonucleoside) and blasticidine (Esner et al., 2017). Nonetheless, the design of novel combinatory treatments or stronger derivatives could overcome this setback for future application in the clinical setting, taking advantage of the well-known safety profile of antibiotics.

On the other hand, non-antibiotic inhibitors of mitochondrial biogenesis are already available: XCT790, a specific inhibitor of the estrogen-related receptor alpha (ERRα)/PGC-1α, signaling pathway (responsible for mitochondrial biogenesis), inhibited breast $CD44^{+/high}CD24^{-/low}$ TICs and mammosphere survival and propagation by reducing OxPhos. These effects were prevented or reversed by stimulating mitochondrial biogenesis with the mitochondrial fuel acetyl-L-carnitine (ALCAR9) (De Luca et al., 2015).

Targeting Mitochondrial Dynamics

Several types of cancer show downregulation of mitochondrial fusion proteins (Zhang et al., 2013; Chen and Chan, 2017) or upregulation of fission proteins (Rehman et al., 2012; Kashatus et al., 2015; Wieder et al., 2015; Zhang et al., 2017). In fact, increased mitochondrial fragmentation has also been involved in malignancy, promoting tumor migration and invasion in breast cancer (Zhao et al., 2013). On the other hand, mitochondrial dynamics seem to regulate proliferation and survival of CSCs, similar to what is known in embryonic stem cells (Chen and Chan, 2017). Indeed, dynamin-related protein 1 (DRP1)-dependent fission regulates mitochondrial distribution in asymmetrical division, ensuring maximal mitochondrial fitness in the daughter stem cell (Katajisto et al., 2015).

Currently, the only available pharmacological strategy to target mitochondrial dynamics is the DRP1 inhibitor mdivi-1. On the one hand, mdivi-1 or DRP-1 knockdown reduced proliferation and induced apoptosis in lung cancer cells, whose mitochondria were in a situation of constant fission *in vitro* and *in vivo* (Rehman et al., 2012). This inhibitor also attenuates lung cancer and mesothelioma proliferation when combined with the MET inhibitor MGCD516 (Wang J. et al., 2018). Importantly, mdivi-1 reduced tumorsphere-forming ability of breast, lung, and melanoma cancer cell lines (Peiris-Pagès et al., 2018) and reduced tumorigenicity of brain TICs *in vitro* and *in vivo* (Xie et al., 2015).

Targeting Mitophagy

Mitophagy is an essential quality control system to selectively remove damaged, non-functional or unnecessary mitochondria in cells. However, its involvement in cancer is controversial, since contradictory reports on the role of this process in tumorigenesis have been published (Chourasia et al., 2015a).

On the one hand, the mitophagy promoter Parkin is frequently deleted in many cancer types (Cesari et al., 2003). In addition, defective mitophagy caused by BNip3 loss/inhibition promote invasion and metastasis in breast, pancreatic or

CRC (Okami et al., 2004; Chourasia et al., 2015b; Li et al., 2018). Moreover, the induction of mitophagy and mitoptosis by salinomycin treatment led to decreased mitochondrial mass and ATP depletion in prostate cancer and breast cancer $CD44^{high}CD24^{low}$ cells triggering a cytotoxic effect specific to tumor cells without damaging normal fibroblasts (Jangamreddy et al., 2013).

On the other hand, mitophagy can be triggered as a stress response against nutrient deprivation or hypoxia, promoting cell survival and tumorigenesis in hostile environments and contributing to drug resistance in human CRC $CD133^{+}CD44^{+}$ cells (Jangamreddy et al., 2013; Yan et al., 2017). In agreement with this notion, mitophagy is upregulated in esophageal squamous cell carcinoma undergoing EMT (Whelan et al., 2017). Accordingly, the use of mitophagy blockers, such as the nanomedicine 188Re-Liposome or the inhibitor liensinine, reversed drug resistance in ovarian cancer cells *in vitro* (Chang et al., 2017) and in breast cancer xenografts *in vivo* (Zhou et al., 2015), respectively. Additionally, the alkaloid matrine induced mitochondrial dysfunction and apoptosis by inhibiting mitophagy in HepG2 hepatoblastoma cells (Wei et al., 2018). Interestingly, a link between autophagy and mitochondrial respiration has recently been reported: the novel autophagy inhibitor aumitin blocks complex I activity (Robke et al., 2018), while pharmacologic or genetic inhibition of complex I inhibitor impairs autophagy (Thomas et al., 2018).

Mitochondrial Drug Delivery

In order to ensure an efficient and selective delivery in mitochondria, different small compounds or chemotherapeutic drugs can be conjugated with nanocarriers, including lipophilic cations, peptides and nanoparticles, with preferential accumulation in mitochondria.

One of the most studied strategies involves the conjugation of small compounds with delocalized lipophilic cations, such as triphenylphosphonium (TPP), dequalinium or rhodamine 123, that possess both lipophilicity and a positive charge, and accumulate in the mitochondrial matrix (Murphy, 2008). Importantly, as OxPhos-dependent CSCs show an elevated mitochondrial membrane potential ($\Delta\Psi m$), indicative of increased activity (Sancho et al., 2015), conjugated compounds will be delivered primarily to these cells. For example, MitoChromanol (vitamin E analog) or Gamitrinib (chaperone inhibitor) combined with TPP inhibit OxPhos and ATP production selectively in cancer cells (Chae et al., 2012; Cheng et al., 2013).

Commonly used chemotherapeutic agents can also be joined with lipophilic cations to exert their therapeutic action in the mitochondria and improve their effect. For example, doxorubicin (DOX) combined with TPP showed enhanced toxicity against DOX-resistant MDA-MB-453 breast cancer cells, even though TPP-DOX was as toxic as free DOX in wild type cells (Han et al., 2014). Interestingly, DOX fused with TPP-conjugated chitosan nanoparticles exhibited higher cytotoxicity than free doxorubicin in A549 and Hela cells (Hou et al., 2017). Additionally, the development of a cisplatin prodrug combined with TPP showed promising results treating cisplatin-resistant, aggressive cancers, such as neuroblastoma, since its delivery into the mitochondrial matrix circumvents the nucleotide excision repair pathway present in the nucleus (Marrache et al., 2014). Moreover, the union of paclitaxel with TPP also resulted in enhanced antitumor effects in Hela and in mouse mammary carcinoma cells (4T1) *in vitro* and *in vivo* (Biswas et al., 2012).

An alternative delivery strategy tested for chemotherapeutic agents involved their conjugation with mitochondria-penetrating peptide (MPP) or mitochondria-targeting sequences (MTS), which act independently of mitochondrial potential. This approach directs their activity toward mtDNA, thus promoting drug selectivity for cancer cells with reduced mtDNA integrity, while their stable mitochondrial location prevents the acquisition of resistance due to drug efflux (Chamberlain et al., 2013). As an example, cisplatin linked to MMP overcomes tolerance in cisplatin-resistant ovarian cancer 2780/CP70 cells (Marrache et al., 2014).

Finally, the combination of the antibiotic salinomycin with reduced graphene oxide-silver nanocomposites synergistically enhanced the activity of either compound alone, leading to mitochondrial dysfunction and selectively killing human ovarian $CD133^{+}$ cells (Choi et al., 2018).

Targeting Redox Homeostasis

It is well established that intracellular ROS accumulation induces cancer cell death, a strategy widely used in the clinics associated to classical chemo and radiotherapy. However, recent evidence suggests that this approach may not be effective against CSCs, due to their increased antioxidant potential (Diehn et al., 2009; Ishimoto et al., 2011; Yuan et al., 2015). Moreover, ROS can be a double-edged sword, since they may promote CSCs survival and invasive abilities acting as signaling molecules (Luo et al., 2018).

As previously mentioned, CSCs are characterized by a finely regulated redox metabolism (Le Belle et al., 2011; Paul et al., 2014; Chang et al., 2018), where glutathione plays an essential role to maintain stemness characteristics (Diehn et al., 2009; Ishimoto et al., 2011). For that reason, increasing oxidative stress by blocking glutathione synthesis could represent a novel therapeutic strategy for eliminating CSCs population and diminishing tumor growth (Diehn et al., 2009; Rodman et al., 2016). Thus far, buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis, has proven (Marengo et al., 2008) to be very effective in decreasing clonogenicity and enhancing response of CSCs to radiotherapy *in vitro* and *in vivo* (Diehn et al., 2009; Boivin et al., 2011; Rodman et al., 2016). Due to its importance for glutathione biosynthesis, especially in glutamine-addicted cancer cells, deprivation of glutamine increased oxidative stress and reduced SP cells in non-small lung and pancreatic cancer cell lines (Liao et al., 2017). Glutamine deprivation also inhibited metastatic potential of cancer cells, one of the main characteristics of CSCs (Wang et al., 2010).

Besides glutathione, strategies aimed at inhibiting cellular antioxidants are currently applied with relative success, mostly improving response to conventional therapies (**Figure 2**). For example, treatment with auranofin, a thioredoxin reductase inhibitor, increased the sensitivity of human breast CSCs to radiotherapy (Rodman et al., 2016), while a synergistic reduction

of the CD44v9$^+$ cells content was achieved by inhibiting glutathione-S-transferase and thioredoxin reductase in patient-derived xenograft (PDX) models of CRC (Tanaka et al., 2016). Arsenic trioxide (ATO), an FDA-approved drug for acute promyelocytic leukemia that increases ROS content and depletes superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Li et al., 2006), proved to reduce CSCs content in different cancer types (Ding et al., 2014; Li et al., 2015a; Chang et al., 2016; Bell et al., 2018). Moreover, the synergistic effect of ATO with glutathione depletion could present a novel treatment for cancers unresponsive to ATO treatment alone (Miller, 2002; Davison et al., 2003; Bhalla et al., 2009; Matulis et al., 2012). The anti-alcohol addiction drug disulfiram has been widely used as anticancer agent since it can increase oxidative stress by blocking SOD activation (Calderon-Aparicio et al., 2015) and inhibiting NRF2 (Xu et al., 2017). In studies with breast cancer cell lines *in vitro* and *in vivo*, disulfiram not only diminished mammosphere formation (Yip et al., 2011; Kim et al., 2017) and reduced CD44$^+$CD24$^-$ and CD49f$^+$CD24$^+$ subpopulations, but also managed to reverse paclitaxel and cisplatin resistance of triple-negative breast cancer (TNBC) (Liu et al., 2013). Moreover, disulfiram proved to diminish the ALDH1$^+$ population from

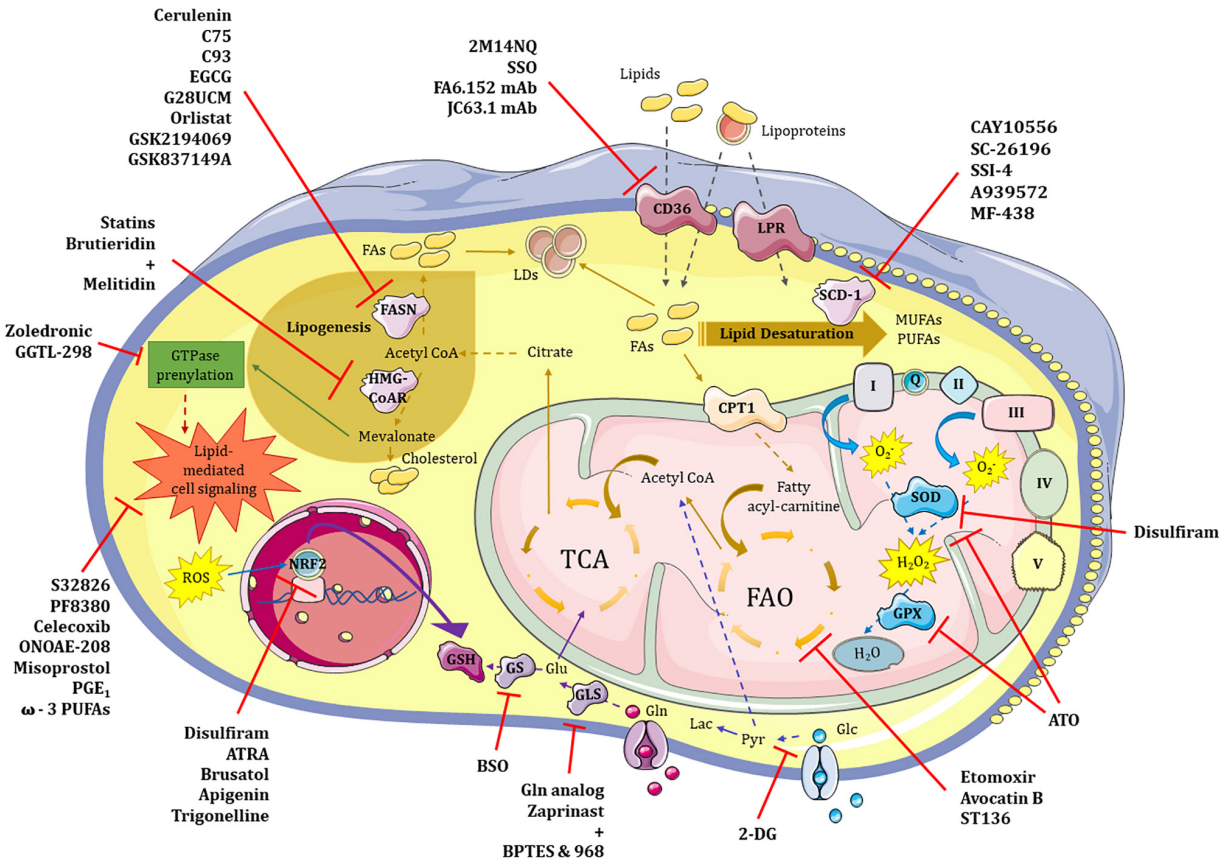

FIGURE 2 | Therapeutic targeting of glycolysis, lipid and redox metabolism in CSCs. Metabolic pathways such those involving glucose, lipids and redox balance are potentially targetable in CSCs. (1) *Glycolysis*. 2-DG represent the most promising therapeutic approach to neutralize highly glycolytic CSCs in combination treatments. (2) *Lipid metabolism*. 2M14NQ, SSO and the monoclonal antibodies FA6.152 and JC63.1 can block CD36 activity; substances like Etomoxir, Avocatin B or ST136 block fatty acid oxidation (FAO) in the mitochondria; FASN can be inhibited by drugs such as Cerulenin, C75, C93, EGCG, G28UCM, Orlistat, GSK2194069 or GSK837149A; while HMG-CoAR enzyme may be inhibited by either Statins or the combination of Brutieridin plus Melitidin; GTPase prenylation pathway in which mevalonate is involved can be targeted by both Zoledronic acid and GGTL-298; and different steps of the lipid-mediated cell signaling may be blocked with molecules such as S32826, PF8380, Celecoxib, ONOAE-208, Misoprostol, PGE_1 and ω-3 PUFAs; finally, targeting of the main enzyme of lipid desaturation route, SCD-1, can be achieved by CAY10556, SC-26196, SSI-4, A939572 or MF-438. (3) *Redox metabolism*. Antioxidant features of CSCs may be inhibited at different levels including SOD and GPX proteins with Disulfiram and/or ATO, respectively; ROS-induced NRF2 activity can be neutralized by Disulfiram, ATRA, Brusatol, Apigenin and Trigonelline; finally, glutathione synthesis may be inhibited either directly or indirectly by blocking GS or GLS enzymes with BSO or a glutamine analog, and a mixture of Zaprinast with BPTES or 968 compounds, respectively. 2-DG – 2-deoxy-D-glucose, Pyr – pyruvate, LDs – lipid droplets, LPR – lipoprotein receptor, FAs – fatty acids, FASN – fatty acid synthetase, HMG-CoAR – 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, SCD-1 – stearoyl-CoA desaturase, MUFAs – monounsaturated fatty acids, PUFAs – polyunsaturated fatty acids, FAO – fatty acid oxidation, TCA – tricarbolxylic acid cycle, CPT1 – carnitine palmitoyltransferase I, GTPase – guanosin triphosphatase, I/Q/II/III/IV/V – complexes of the electron transport chain, O_2^- – superoxide anion, H_2O_2 – oxygen peroxide, SOD – superoxide dismutase, GPX – glutathione peroxidase, ROS – reactive oxygen species, NRF2 – nuclear factor erythroid 2–related factor 2, GSH – glutathione, Glu – glutamate, Gln – glutamine, GS – glutathione synthase, GLS – glutaminase, 2M14NQ – 2-methylthio-1,4-naphtoquinone, SSO – sulfosuccinimidyl oleate, mAb – monoclonal antibody, EGCG – epigallocatechin gallate, ATRA – all-trans retinoic acid, BSO – L-buthionine-S,R-sulfoximine, ATO – arsenic trioxide.

non-small cell lung cancer cell lines (Liu et al., 2016) and $CD34^+CD38^+$ cells in AML cell lines and primary samples *in vitro* and *in vivo* (Xu et al., 2017). Now entering phase III in clinical trials, disulfiram may present a potential adjuvant therapy for cancer treatment, although it is highly unstable in blood. For that reason, disulfiram-containing nanoparticles have also been developed. Even though these nanoparticles proved to increase disulfiram blood levels (Song et al., 2016), further studies are needed to establish its full *in vivo* antioxidant and biological properties.

Increased NRF2 levels turned out to play a role in CSCs survival and chemoresistance (Kwak et al., 2001; Zhu et al., 2013; Ryoo et al., 2016), representing another potential target for eradicating CSCs. All-trans retinoic acid (ATRA) blocked NRF2 activation, diminishing self-renewal and tumorigenic capacity of $ALDH1^+$ lung cancer cells (Moreb et al., 2004) and ovarian cancer cell lines (Kim D. et al., 2018). Moreover, brusatol, which decreases NRF2 protein levels, was demonstrated to inhibit mammospheres formation and increase the sensitivity of human breast CSCs to Taxol (Wu T. et al., 2015). Similarly, the natural flavonoid apigenin (Kim et al., 2016; Erdogan et al., 2017) or the alkaloid trigonelline (Arlt et al., 2013; Roh et al., 2017), which inhibits NRF2 at transcriptional and translational level, can sensitize CSCs toward chemotherapeutic drugs. On the other hand, the combination of ROS inducers and NRF2 (or downstream targets) inhibition could represent a potential strategy for CSCs elimination (Diehn et al., 2009; Ishimoto et al., 2011; Kwak et al., 2016; Liu et al., 2016; Kim et al., 2017; Kim D. et al., 2018; Luo et al., 2018).

Paradoxically, some natural antioxidants that can increase NRF2 expression levels have also shown therapeutic potential. The NRF2-inducer sulforaphane, a dietary component from broccoli, inhibited self-renewal capacity of $CD44^+$ $LDH1^+$ pancreatic (Rausch et al., 2010) and $ALDH1^+$ breast cancer cells *in vivo* and *in vitro* (Burnett et al., 2017). Curcumin, an active ingredient of turmeric, diminished self-renewal capacity of $CD44^+$ $EpCAM^+$ pancreatic cancer cells *in vitro* and *in vivo* (Bao et al., 2012) and reduced proliferation and mammosphere formation of $ALDH1^+$ breast cancer cells (Kakarala et al., 2010). Additionally, resveratrol, oleanane triterpenoid or carnosol also proved to active and increase NRF2 expression which could have a positive effect on diminishing the CSCs population (Probst et al., 2015a,b; Sancho et al., 2015; Giacomelli et al., 2017). Treatment with naturally occurring antioxidants, such as vitamin C or phenethyl isothiocyanates (PEITCs), found in broccoli or Brussel sprouts, also diminished self-renewal capacity and clonogenicity of NCCIT human embryonic carcinoma and human colon cancer cell lines, and reduced $CD133^+$, $EpCAM^+$ and $OV6^+$ cells while inhibiting tumorspheres formation and growth of hepatocellular carcinoma cell lines and PDX models *in vivo* (Yun et al., 2017; Lv et al., 2018). However, even though natural antioxidants could represent an exciting strategy in anticancer therapy, clinical trials thus far showed no positive effect on patient survival. Indeed, published data highlighted the lack of specificity of antioxidant treatments. This fact, together with the possible contribution of antioxidants to stemness maintenance and cancer development, weakens the translation potential of this approach.

Targeting Lipid Metabolism

Lipid metabolism has become an interesting target in order to design new anti-CSC strategies, and a number of compounds have been tested during the last years (**Figure 2**).

Lipid Desaturation

Over the last few years, several SCD-1 inhibitors have demonstrated their effectiveness in different preclinical *in vitro* and *in vivo* models of cancer, by specifically targeting stemness-related properties. Indeed, the inhibitors CAY10556 and SC-26196 reduced stem cell-related markers and signaling pathways by downregulating Hedgehog and Notch expression in ovarian $ALDH^+CD133^+$ cells (Li J. et al., 2017). Interestingly, this led to the inhibition of sphere formation *in vitro* and tumorigenicity *in vivo*, with no effect on differentiated cells, suggesting the selectivity of this approach. In the same line of evidence, inhibition of SCD-1 with the compounds SSI-4 or A939572 modulates endoplasmic reticulum-stress-mediated differentiation in liver chemoresistant hepatospheres, sensitizing resistant PDXs to sorafenib treatment with low side toxicity *in vivo* (Ma et al., 2017). In parallel, effects of the inhibitor A939572 in $CD133^+CD49f^+$ liver CSCs have also been linked to Wnt-mediated self-renewal and *in vivo* tumorigenicity (Lai et al., 2017). Finally, MF-438 treatment induced anoikis in lung $ALDH1^+$ cells, decreasing self-renewal and pluripotency markers expression (Pisanu et al., 2017). Interestingly, these *in vitro* effects translated into reduced tumorigenic potential and reversion of chemoresistance *in vivo*.

Lipogenesis

Given the important involvement of the enzyme FASN in numerous tumor types, a number of inhibitors have been designed and/or tested in diverse cancer models: cerulenin, C75, C93, epigallocatechin gallate (EGCG), G28UCM, orlistat, GSK2194069 and GSK837149A. In fact, cerulenin treatment prevents proliferation *in vitro* of pancreatic spheres (Brandi et al., 2017) and neurospheres established from glioma patients (Yasumoto et al., 2016) CSCs. On the other hand, C75 at non-cytotoxic concentrations significantly reduced self-renewal in $HER2^+$ breast cancer cells (Corominas-Faja et al., 2017). However, it is important to highlight the critical selectivity and toxicity issues found for FASN inhibitors *in vivo*, which have compromised their translation to clinical trials. Only the inhibitor TVB-2640 is being currently tested in clinical trials for $HER2^+$ advanced breast cancer, high grade astrocytoma and colon cancer (NCT03179904, NCT03032484, NCT02980029, respectively).

Cholesterol Synthesis

Cholesterol synthesis through the mevalonate pathway can be inhibited by statins, for which the molecular target is the enzyme 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMG-COAR). In fact, treatment with different statins decreased self-renewal and CSCs content in breast (Ginestier et al.,

2012) and nasopharyngeal (Peng et al., 2017) carcinomas. Interestingly, similar effects were detected in $CD133^+$ brain TICs (Wang et al., 2017a) where overexpression of mevalonate pathway genes was controlled by MYC, highlighting the variety of metabolic pathways controlled by the oncogene. However, anti-CSCs effects of statins could also be related to inhibition of cellular signaling via small GTPases (e.g., Rho and Rac), since they require prenylation using mevalonate pathway intermediates. In fact, impaired self-renewal ability achieved with simvastatin treatment in breast tumorspheres was recapitulated by zoledronic acid and GGTI-298, inhibitors of the prenylation pathway (Ginestier et al., 2012). Moreover, a mixture of brutieridin and melitidin (natural products derived from bergamot with statin-like properties) impaired breast $ALDH1^+$ CSCs proliferation inhibiting both FAO and Rho-related signaling pathways (Fiorillo et al., 2018).

Lipid Uptake

Strategies targeting lipid uptake are mainly designed to inhibit the transporter CD36, by either pharmacological inhibition or blocking antibodies. CD36 blockade with 2-methylthio-1,4-naphtoquinone decreases self-renewal ability and induces apoptosis in glioblastoma $CD133^+$ (Hale et al., 2014). Another CD36 inhibitory compound, sulfosuccinimidyl oleate, decreases chemoresistant leukemic stem cells (Ye et al., 2016). Interestingly, CD36-neutralizing antibodies against either all known functions of CD36 (FA6.152) or the ones reported to block active FA and lipoprotein uptake (JC63.1) induced lipotoxicity in label-retaining/$CD44^+$ metastasis-initiating cells, thus, inhibiting metastasis initiation and progression in oral squamous cell carcinoma, with no reported toxicity *in vivo* (Pascual et al., 2016).

FAO

Fatty acid oxidation inhibition with etomoxir has been studied in preclinical *in vitro* and *in vivo* cancer models. Indeed, etomoxir treatment inhibits mammosphere formation and tumor growth *in vivo* in TNBC tumors bearing high MYC expression (Camarda et al., 2016). In addition, etomoxir treatment sensitizes hepatocarcinoma $CD133^+CD49f^+$ CSCs to standard chemotherapy with sorafenib (Chen C.L. et al., 2016). Moreover, etomoxir decreases the number of quiescent leukemia CSCs in AML patients and, combined with the BCl-2 inhibitor ABT-737, substantially decreases tumor burden (Samudio et al., 2010). However, etomoxir treatment induces normal hematopoietic stem cell exhaustion invalidating this compound for further clinical studies (Ito et al., 2012). Interestingly, alternative FAO inhibitors with higher selectivity for malignant cells are under investigation currently. For example, avocatin B is a lipid that accumulates in mitochondria inhibiting FAO and targets AML cells and leukemia $CD34^+$ CSCs with no effect on hematopoietic stem cells (Lee et al., 2014, 2015). Additionally, the compound ST136 showed antileukemic activity with no effect on normal $CD34^+$ stem cells (Ricciardi et al., 2015).

Lipid-Mediated Signaling

As stated in the previous section, lipid-mediated signaling plays an important role in cancer and, specifically, in CSCs functions. For that reason, several therapeutic approaches, including inhibitors and indirect modulation via dietary supplements, have been studied over the last few years. For example, several stemness-related functions of ovarian spheroid-derived cells from cell lines and primary cells from patients were dependent on LPA synthesis. Thus, inhibition of the LPA-producing enzyme autotaxin with the small molecules S32826 or PF8380 decreased tumorigenicity and chemoresistance *in vivo* (Seo et al., 2016). Interestingly, inhibition of LPA production not only affects cancer cells, but could also play an important role modulating the immune system and supporting tumor progression. Indeed, LPA induces the differentiation of monocytes into macrophages and favors the activation of CAFs phenotype (Ray and Rai, 2017; Radhakrishnan et al., 2019).

The most studied lipid mediator class in relation to CSCs is prostaglandins. Indeed, treatment of Apc^{Min}/þ mice with celecoxib, the prostaglandin-endoperoxide synthase 2 selective inhibitor, or the EP4 receptor (prostaglandin receptor) antagonist ONOAE-208 resulted in a reduction of tumor $CD133^+CD44^+$ cells and tumor burden (Wang et al., 2015). Importantly, celecoxib inhibited CSCs content and the number of liver metastatic tumors upon orthotopic injection of patient-derived CRC into NSG mice. Additionally, celecoxib impaired chemoresistance in bladder carcinomas, suggesting its utility as adjuvant therapy (Kurtova et al., 2015). On the contrary, activation of EP4 with the FDA-approved agonist misoprostol or PGE_1 reduced $CD34^+$ cells in a xenograft model of chronic myelogenous leukemia (CML) (Li F. et al., 2017), suggesting a context-dependent effect of prostaglandins in stemness.

On the other hand, preclinical and human observational studies suggest that dietary omega-3 polyunsaturated fatty acids (ω-3 PUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), decrease CRC risk and may be effective as adjuvant treatment of advanced CRC. Indeed, EPA and DHA reduced the $CD133^+$ content or stem properties in two different *in vitro* studies using CRC cell lines (De Carlo et al., 2013; Yang et al., 2013). Interestingly, EPA alone or in combination with chemotherapy decreased sphere-forming ability and suppressed tumor growth, likely through inhibition of proinflammatory metabolites in mice (Vasudevan et al., 2014). Importantly, studies in other tumor entities also suggest an anti-CSCs effect of ω-3 PUFAs besides CRC. Indeed, EPA and DHA supplementation also reduced proliferation and induced toxicity in breast tumorspheres, likely through alteration of the prostaglandin profile (Erickson and Hubbard, 2010). In addition, a metabolite derived from EPA eradicated leukemia $M34^+Kit^+Sca1^+$ CSCs in PDXs of CML (Hegde et al., 2011).

Targeting Metabolism of Alternative Fuels

As mentioned in the previous section, CSCs may utilize a number of different substrates, such as amino acids and ketone bodies, in order to support self-renewal and tumorigenicity. For that reason, diverse compounds which target the metabolism of these alternative fuels are currently under investigation.

On the one hand, the use of a glutamine analog reduced 20 times tumor growth and inhibited metastasis in the VM-M3

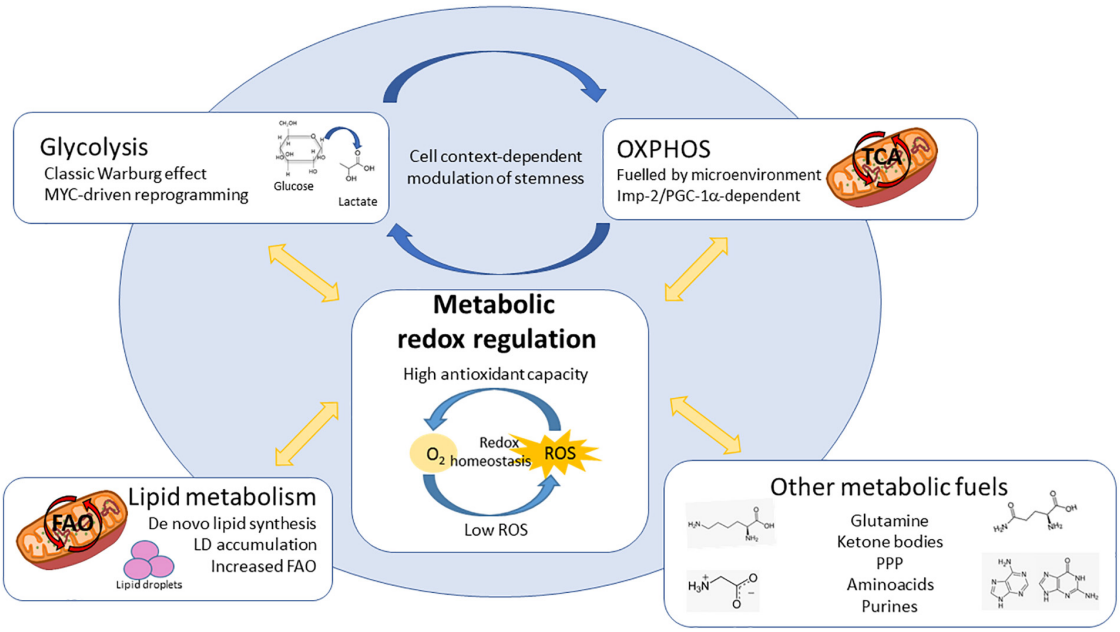

FIGURE 3 | Redox involvement in the different metabolic dependencies described for CSCs. CSCs bear diverse metabolic dependencies in a tumor and context-dependent manner: (1) Aerobic glycolysis, controlled by MYC; (2) OxPhos, fuelled by different microenvironmental substrates and controlled by Imp2 or PGC-1α; (3) Lipid metabolism, increasing either fatty acid synthesis and storage in lipid droplets (LDs) or utilization via mitochondrial FAO; (4) CSCs can be dependent on alternative substrates and pathways such as aminoacids, ketone bodies, PPP or purines. Interestingly, the metabolic phenotypes described for CSCs ensure the maintenance of cellular redox state. Keeping redox balance is crucial for CSCs in order to maintain their stemness characteristics, differentiation ability and resistance to chemo and radiotherapy, constituting one of the most important vulnerabilities independently of their origin or cellular context. ROS – reactive oxygen species, OxPhos – oxidative phosphorylation, LDs – lipid droplets, PPP – pentose phosphate pathway, FAO – fatty acid oxidation, TCA – tricarboxylic acid cycle.

murine tumor model of systemic metastasis, when compared with non-treated mice (Shelton et al., 2010). Interestingly, the anti-asthma compound Zaprinast was identified as a novel glutaminase inhibitor that, together with BPTES and 968, inhibited clonogenicity of pancreatic cancer cells in response to radiation (Elhammali et al., 2014).

Interestingly, Ozsvari et al. (2017) unveiled the potential anti-CSCs activity of a novel class of compounds denominated "mitoketoscins." These compounds block the active site of the enzymes involved in the recycling of ketone bodies into acetyl-CoA (OXCT1 and ACAT1), leading to inhibition of the CSCs activity and propagation in breast cancer spheroids. However, considering the sometimes contradictory results of diverse studies on the antitumor effects of ketogenic diet (high-fat/low-carbohydrate intake) (Vidali et al., 2015; Weber et al., 2018), the anti-tumor effect of these inhibitors would need to be carefully tested as dependency on ketone bodies strongly varies across tumor entities and specific genotypes.

Combination Treatments: Targeting Glycolysis

Considering the great intratumoral metabolic heterogeneity and plasticity found in tumors, mitochondrial inhibitors as single agents will unlikely become an effective therapy for cancer treatment. In fact, combination treatments, where two or more metabolic pathways are inhibited simultaneously, would block relapse and development of resistances. For instance, a dual inhibition of the main metabolic pathway together with its main escape mechanism will completely erase CSCs within the tumor. This has been reported for the combinations of metformin with either the bromodomain and extraterminal motif (BET) inhibitor JQ-1 in pancreatic cancer (Sancho et al., 2015) or PI3K inhibition for ovarian cancer (Li et al., 2012), which blocks OxPhos and indirectly inhibits glycolysis simultaneously.

In fact, direct glycolysis inhibition for cancer treatment has been studied intensively in preclinical and clinical settings over the last few years, although with low success rates. On the one hand, glucose transport inhibitors, such as silybin/silibinin [tested in a phase I/II clinical trials for prostate cancer and advanced hepatocellular carcinoma (Flaig et al., 2006)], phloretin, WZB117 and fasentin caused important side effects, since GLUT transporters are present in all the cells of the organism. Similarly, inhibition of glycolytic enzymes, such as hexokinase II with lonidamine, has been tested in several types of cancers, including breast, lung and ovarian cancer (Gadducci et al., 1994; De Lena et al., 1997; De Marinis et al., 1999; Berruti et al., 2002). However, there was no significant improvement in overall survival and many cases presented with elevated toxicity. Additionally, the glucose analog 2-DG was shown to be a promising agent in preclinical studies (Maschek et al., 2004; Coleman et al., 2008). In fact, it has been recently tested in phase II/III of a clinical trial for prostate cancer (NCT00633087),

although no results are available, since the trial was terminated due to the slow accrual.

Apart from direct inhibition, targeting tumor drivers affecting cellular metabolism might hinder glycolysis. For example, KRAS mutation is present in more than 90% of pancreatic cancer cases (Bailey et al., 2016) and controls both tumorigenesis and metabolic reprogramming (Ying et al., 2012; Son et al., 2013; Liou et al., 2016). In fact, KRAS drives glycolysis and the diversion of glycolysis intermediates into the non-oxidative branch of PPP, essential for the synthesis of nucleic acids (Yun et al., 2009; Ying et al., 2012; Blum and Kloog, 2014). However, even though small molecule inhibitors of KRAS proved to be promising in preclinical studies (Xie et al., 2017; Zeng et al., 2017), targeting KRAS or its downstream pathways showed no effect in overall survival and overall response rate in pancreatic cancer patients (Kindler et al., 2012; Infante et al., 2014; Chung et al., 2017).

Alternatively, c-MYC is another essential driver of tumorigenesis and glycolysis in cancer (Miller et al., 2012; Lin et al., 2013; He et al., 2015). For that reason, different compounds targeting MYC are currently undergoing clinical trials. Noteworthy, inhibitors of BET proteins directly downregulate MYC expression and suppress tumor growth *in vivo* (Delmore et al., 2011; Mazur et al., 2015; Garcia et al., 2016). Importantly, since MYC suppression blocks development of resistance to mitochondrial inhibitors (Sancho et al., 2015; Kim J.H. et al., 2018), combinatory approaches using this strategy can represent a promising anticancer therapy.

CONCLUDING REMARKS

Over the last few years, a huge collective effort to decipher metabolic reprogramming occurring in cancer has taken place. Technical advances have allowed the determination of the great metabolic heterogeneity, not only among individuals suffering from one type of cancer, but within a single tumor. Collectively, present literature indicates that both metabolic and redox state diversity define CSCs phenotype and fate, determining response to therapy. Importantly, most of the metabolic dependencies described for CSCs in diverse tumor entities have the tight control of redox state as a common factor (**Figure 3**), unveiling an important vulnerability that could provide new therapeutic opportunities. Other factors, such as tumor metabolic heterogeneity, microenvironmental cues or a cross-talk through metabolic and redox signaling between CSCs and cancer cells or stromal components (Riemann et al., 2011; Chen X. et al., 2016; Chang et al., 2018; Luo et al., 2018) can play an additional role in cancer progression and chemoresistance. Therefore, current knowledge suggests that carefully designed therapies, which target metabolically diverse populations and consider the tumor microenvironment may be crucial in order to develop more effective metabolism-focused treatment strategies.

AUTHOR CONTRIBUTIONS

PJ, BdL-D, and BP-A designed the figures. PS developed the study concept, obtained funding and wrote the final version of the manuscript. All authors contributed to conception and design of the manuscript, wrote sections, revised, read and approved the submitted version.

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REVIEW
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Antibody-Drug Conjugates (ADC) Against Cancer Stem-Like Cells (CSC)—Is There Still Room for Optimism?

Fabrizio Marcucci[1]*, *Carmelo Antonio Caserta*[2], *Elisabetta Romeo*[2] and *Cristiano Rumio*[1]

[1] Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy, [2] Fondazione per la Medicina Solidale, Reggio Calabria, Italy

Cancer stem-like cells (CSC) represent a subpopulation of tumor cells with peculiar functionalities that distinguish them from the bulk of tumor cells, most notably their tumor-initiating potential and drug resistance. Given these properties, it appears logical that CSCs have become an important target for many pharma companies. Antibody-drug conjugates (ADC) have emerged over the last decade as one of the most promising new tools for the selective ablation of tumor cells. Three ADCs have already received regulatory approval and many others are in different phases of clinical development. Not surprisingly, also a considerable number of anti-CSC ADCs have been described in the literature and some of these have entered clinical development. Several of these ADCs, however, have yielded disappointing results in clinical studies. This is similar to the results obtained with other anti-CSC drug candidates, including native antibodies, that have been investigated in the clinic. In this article we review the anti-CSC ADCs that have been described in the literature and, in the following, we discuss reasons that may underlie the failures in clinical trials that have been observed. Possible reasons relate to the biology of CSCs themselves, including their heterogeneity, the lack of strictly CSC-specific markers, and the capacity to interconvert between CSCs and non-CSCs; second, inherent limitations of some classes of cytotoxins that have been used for the construction of ADCs; third, the inadequacy of animal models in predicting efficacy in humans. We conclude suggesting some possibilities to address these limitations.

Keywords: cancer stem cell, epithelial-mesenchymal transition, resistance, antibody-drug conjugate, resting, proliferating

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***Correspondence:**
Fabrizio Marcucci
fabmarcu@gmail.com.

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CANCER STEM-LIKE CELLS (CSC), A TUMOR CELL SUBPOPULATION WITH PECULIAR PROPERTIES

CSCs are carcinoma cells that self-renew and give rise to differentiated tumor cells. CSCs by themselves, however, can arise from differentiated tumor cells when these cells undergo an epithelial-mesenchymal transition (EMT) (1). EMT involves changes that lead to loss of cell-cell adhesion and cell polarity, with acquisition of migratory and invasive properties (2). EMT encompasses a continuum of states from a fully epithelial to a fully mesenchymal phenotype, passing through intermediate, hybrid states (3). Interestingly, it has recently been shown that

acquisition of tumor-initiating potential is one of the earliest functions gained during EMT, while other functions, like invasiveness and metastatic potential are acquired during later stages (4). These results reinforce the close relationship between EMT and CSCs (1).

In addition to their tumor-initiating potential, CSCs possess also other functions that they share with EMT tumor cells, most notably drug resistance (5). Drug resistance implies that tumor cells survive drug treatment and become enriched in the tumor cell population. In fact, one key assay to ascertain the *in vivo* efficacy of anti-CSC compounds is to test the number of tumor cells that are required in order to initiate tumor growth in animal models before and after drug treatment (6).

Considerable efforts have been devoted to the phenotypic characterization of CSCs, in particular the identification of markers that distinguish CSCs from normal stem cells and the bulk of differentiated tumor cells. Overall, it has been difficult to define CSCs on the basis of their phenotypic profile (5). Thus, a large number of cell surface molecules that are expressed on CSCs have been identified; CD44, CD47, CD33, CD133, CXC chemokine receptor (CXCR) 4, and CD26 are some of these markers. Most of them, however, are not CSC-specific and in some cases are even ubiquitously expressed (e.g., CD44, CD47) (7). Some markers have a more restricted expression and/or are overexpressed on CSCs; these have been used as targets for ADCs, as will be discussed in the following.

The plasticity of CSCs is reflected also by the large number of signaling pathways that are involved in the induction and maintenance of CSCs. Given the functional relationship between CSCs and normal stem cells, the role of signaling pathways involved in the physiology of normal stem cells, such as WNT, Notch, and Hedgehog (Hh), has been investigated with particular attention (8).

Eventually, also post-transcriptional regulation contributes to the homeostasis and functions of CSCs. These include RNA modifications, RNA-binding proteins, mircoRNAs and long non-coding RNAs (9).

As regards the generation of CSCs from differentiated tumor cells, similarly to cells that undergo an EMT, tumor-initiating potential can be acquired when one of three different events occur. First, in response to stressors from the tumor microenvironment like hypoxia, low pH, immune responses, mechanical stress, and antitumor drugs (10, 11). Second, stressor-promoted epigenetic changes that induce heritable effects allowing retention of the mesenchymal state even when the stressors are no longer present (12, 13). Third, stimulus-independent activation of signaling pathways, owing to activating mutations or overexpression of pathway components (14, 15). Intuitively, these events are not mutually exclusive and may differ quantitatively and qualitatively in different tumors and, over time, even within the same tumor. Moreover, some of these events (e.g., stressor-induced responses) can be reversible and, consequently, CSCs can revert back to a differentiated phenotype, as already referred to above. Vice versa, tumor cells that have regained an epithelial and a non-CSC phenotype can undergo a *de novo* switch toward a more mesenchymal tumor-initiating phenotype, even after drug-induced depletion of CSCs. As such, depletion of CSCs is by no means a conclusive effect but, rather, a transient elimination of tumor cells engaged in the replenishment of a tumor cell population of epithelial phenotype.

ANTIBODY-DRUG CONJUGATES (ADC), TOOLS FOR THE SELECTIVE ELIMINATION OF TUMOR CELLS

ADCs comprise a monoclonal antibody (mAb) against a tumor-associated antigen, a covalent linker, and a cytotoxic payload (16). **Figure 1** gives a schematic view of an ADC and its individual components as will be discussed in the following. In most cases, ADCs are internalized upon binding to the cognate antigen and the cytotoxic payload is released, causing cell death. The targeted delivery of cytotoxins to tumor cells allows for the maximum efficacy and minimal toxicity.

The mAb should recognize an antigen expressed on the largest possible fraction of tumor cells and the smallest possible fraction of normal cells (17). With the exception of hematological malignancies, there is no known antigen that is homogeneously expressed on all tumor cells because the tumor cell population is, in itself, heterogeneous and composed, to varying degrees, of antigen-positive or strongly positive (preferably the vast majority) and antigen-negative or weakly positive tumor cells. Incidentally, the existence of phenotypic tumor cell heterogeneity justifies the aim of generating anti-CSC ADCs, because this rests on the assumption that CSCs display an antigenic profile that differs qualitatively and/or quantitatively from that of non-CSCs and, thus, may escape cytotoxicity induced by ADCs that target non-CSCs. MAbs that are currently used for the engineering of ADCs are of human origin or are humanized murine antibodies (18, 19) in order to minimize antigenicity and the induction of anti-drug antibodies.

The second component, the linker, is important for the stability of the ADC. It should be sufficiently stable to negate systemic release of the cytotoxic payload, but sufficiently labile to allow intracellular release, in most cases within the lysosomal compartment. Dipeptide linkers like valine-citrulline are typical examples of linkers that are cleaved with good selectivity within the lysosomal compartment (20). However, for some ADCs also non-cleavable linkers are used and, in this case, the cytotoxic payload is released as an amino acid conjugate upon degradation of the antibody. These linkers can be used if the drug-linker-amino acid residue conjugate retains drug activity

Abbreviations: ADC, antibody-drug conjugate; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CSC, cancer-stem like cell; DAR, drug-antibody ratio; DLL, Delta-like ligand; EFNA4, Eph-A4; EMT, epithelial-mesenchymal transition; Eph, Ephrin receptor; FLT3, fms-like tyrosine kinase 3; GO, gemtuzumab ozogamicin; HER2, human epidermal growth factor receptor 2; IL, interleukin; LRG5, leucine-rich repeat-containing; G protein-coupled receptor 5; LSC, leukemia stem cell; mAb, monoclonal antibody; MMA, monomethyl auristatin; mTOR, mechanistic target of rapamycin; NCAM, neural cell adhesion molecule; NSCLC, non-small cell lung cancer; PBD, pyrrolobenzodiazepine; PDX, patient-derived xenograft; PI3K, phosphoinositide 3-kinase; PTK, protein tyrosine kinase; SCLC, small cell lung cancer; SOC, standard-of-care; SPDB, N-succinimidyl 4-(2-pyridylothio)butyrate; TNBC, triple-negative breast cancer.

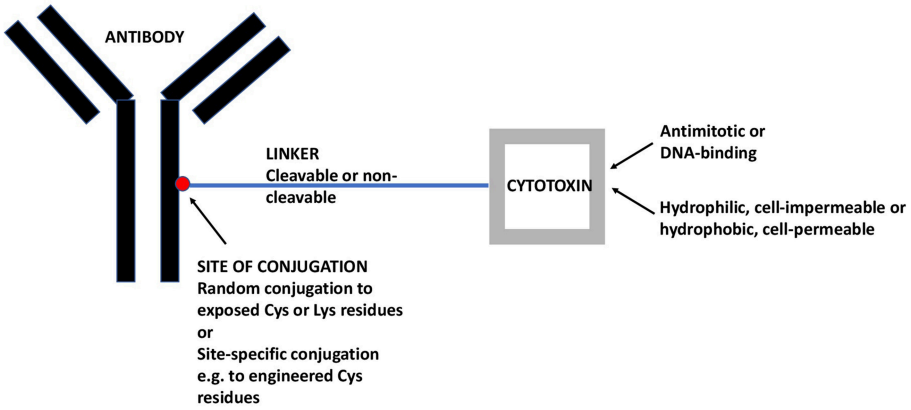

FIGURE 1 | A Schematic View of ADCs and its Individual Components. The mAb targets a tumor-associated antigen, in the present case an antigen that is preferentially expressed on CSCs. The linker may be cleavable (e.g., acid-sensitive or dipeptide) or non-cleavable (e.g., maleimidocaproyl). The cytotoxin may be an antimitotic drug, active only on proliferating cells or a DNA-binding drug, active also on quiescent cells. Moreover, the cytotoxin may be hydrophilic and act only within the internalizing cell or it may be hydrophobic and act also on nearby cells, whether antigen-positive or –negative (so-called bystander effect). ADC, antibody-drug conjugate; CSC, cancer stem-like cell; mAb, monoclonal antibody.

(21). Ado-trastuzumab emtansine is an example of an ADC with a non-cleavable linker yielding a lysine-linker-cytotoxic (DM1) complex (22).

Cytotoxins used for ADC synthesis are highly potent because of the limited number of payloads that each individual antibody molecule can carry. Most ADC payloads belong to two mechanistic classes. The first are antimitotic, tubulin-binding cytotoxins like auristatins and maytansines. The second are DNA-binding, cell cycle-independent cytotoxins like calicheamicins and pyrrolobenzodiazepines (PBD).

Once released, a cytotoxin may be cell-impermeable [e.g., more hydrophilic cytotoxins like monomethyl auristatin (MMA) F] and exert its cytotoxic effect exclusively on a single cell, or it may penetrate the cell membrane and exert bystander killing also on nearby cells (e.g., more lipophilic cytotoxins like MMAE) (23), whether or not these cells are positive for the target antigen. This potential advantage of cell-permeable cytotoxins must be weighed against the possibility of enhanced systemic toxicity due to back-flow of the liberated cytotoxin into the systemic circulation.

Until recently, most ADCs were generated by random conjugation of the linker to available cysteine or lysine residues on the antibody. This approach leads to ADC mixtures with different drug-antibody ratios (DAR) with the individual components having distinct properties that may lead to suboptimal *in vivo* efficacy of the final mixture. In recent years, site-specific conjugation methods have been developed, which yield ADCs with defined DARs. These ADCs have been shown to possess larger therapeutic windows and to be better tolerated than randomly-conjugated ADCs (24, 25).

An important question to answer in the present context is as to why an ADC should be preferred over a native mAb as an anti-CSC agent. The question is not trivial since also native antibodies are endowed with cytotoxic potential. In fact, mechanisms like antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity or antibody-dependent cellular phagocytosis contribute, to varying degrees, to the efficacy of an antitumor mAb (26). The answer lies in the greater antitumor efficacy that ADCs have demonstrated *in vivo* compared to native, equivalent mAbs (27) and the efficacy that ADCs, like ado-trastuzumab emtansine (T-DM1), have shown in tumors that were *ab initio* unresponsive or had become unresponsive to the native equivalent (trastuzumab) (28). It is to note, however, that the cytotoxic potential of the native mAb has been shown to contribute to the overall efficacy of ADCs (29).

ANTI-CSC ADCS THAT HAVE BEEN DESCRIBED IN THE LITERATURE

In the following we will discuss individual anti-CSC ADCs that have been reported in the scientific literature. In some instances, more than one ADC has been described against an individual target and we will briefly address the properties of each of these conjugates. We will first discuss those ADCs that target markers expressed on CSCs. In a following section we will discuss antitumor ADCs that were shown to have anti-CSC activity at a later stage.

ADCs Against Markers Expressed on CSC

Anti-delta-like Ligand (DLL) 3 ADC

This target is of particular interest in these days, because the development of an ADC against DLL3, rovalpituzumab tesirine, which had reached phase III clinical studies, has failed to show benefit in third-line small-cell lung cancer (SCLC) (30).

Rovalpituzumab tesirine had been constructed for the targeting of CSCs of high-grade pulmonary endocrine tumors (31). These tumors include SCLC and large cell neuroendocrine carcinoma, highly malignant neoplasms with few and inefficacious therapeutic options (32). CSCs of SCLC are thought to arise from normal pulmonary neuroendocrine cells,

the portion of the diffuse neuroendocrine system found in the respiratory epithelium.

DLL3 is a ligand of the mammalian Notch family that localizes to the Golgi apparatus without being able to activate Notch signaling like other Notch ligands. Rather, DLL3 appears to inhibit Notch pathway activation by interacting with Notch and the Notch ligand DLL1, thereby preventing their localization to the cell surface (33). Notch activation in neuroendocrine tumors has been shown to suppress tumor growth (34). Expression data showed that DLL3 mRNA is overexpressed in primary SCLC tumors, SCLC patient-derived xenografts (PDX), conventional SCLC cell lines, and large-cell neuroendocrine carcinoma PDXs, whereas mRNA expression in normal tissues appears limited primarily to the brain (31). Moreover, in contrast to normal cells, DLL3 was detectable at the surface of neuroendocrine tumor cells.

On the basis of these observations, DLL3 was selected as a target for the construction of an ADC. The ADC, SC16LD6.5 (later rovalpituzumab tesirine) is composed of a humanized anti-DLL3 mAb, conjugated to a PBD dimer. The payload was conjugated to cysteine residues on the mAb via a valine-alanine dipeptide, with a mean DAR of 2. SC16LD6.5 induced durable tumor regression in multiple PDX models after a single course of therapy and in a manner independent of their sensitivity to standard-of-care (SOC) chemotherapy. Lack of tumor recurrence was shown being due to depletion of $DLL3^+$ CSCs. In contrast, SOC chemotherapy did not reduce the frequency of CSCs nor provided durable responses in spite of efficacious tumor cell debulking. DLL3, however, was not CSC-specific since its expression was seen throughout the tumor, with most cells expressing it to some degree. For this reason, the rapid tumor debulking seen with SC16LD6.5 was likely due to DLL3 expression on most tumor cells. Eventually, SC16LD6.5 was efficacious also in a chemorefractory tumor model suggesting that patients with tumors resistant to SOC will be responsive to this conjugate. Of note, administration of the unconjugated anti-DLL3 mAb, even at high doses, or the equivalent dose of free PBD dimer had little or no effect on tumor growth, thereby supporting the superiority of the antitumor effects of the ADC.

Rovalpituzumab tesirine has entered numerous clinical studies and has progressed until phase III in patients with advanced SCLC after disease progression following SOC chemotherapy protocols (**Table 1**). Other clinical trials are earlier stage in SCLC alone (NCT02674568, NCT02819999, NCT03319940, NCT02874664) or in SCLC and other advanced solid tumors expressing cell surface DLL3 (NCT02709889). As referred to in the beginning of this section, rovalpituzumab tesirine failed to show benefit in one of the SCLC studies.

Anti-protein Tyrosine Kinase 7 (PTK7) ADC

PTK7 has been identified as a CSC marker and potential target for ADCs (35). It is a conserved member of the pseudokinase family of receptor protein tyrosine kinases. The lack of kinase activity is the consequence of substitutions at residues in the kinase domain. Oncogenic functions of PTK7, including resistance to chemotherapy, have been reported for various carcinomas and acute myeloid leukemia (AML) (49). PTK7 was shown to be overexpressed in tumors vs. normal tissues, and enriched in CSCs of different tumor types. PTK7 expression within each tumor is heterogeneous, and the extent of heterogeneity varies from tumor to tumor. PTK7 staining was also observed in some normal tissues, including esophagus, urinary bladder, kidney, mammary gland, lung, ovary, uterus, and digestive tract (35). The expression profile of PTK7 prompted the generation an anti-PTK7 ADC (35). The choice of an ADC was also dictated by the lack of catalytic function of PTK7, making it an unsuitable target for inhibitor antibodies or small molecules.

The anti-PTK7 ADC that was constructed, PF-06647020, comprises a humanized anti-PTK7 mAb, a cleavable dipeptide (valine-citrulline) linker, and Aur0101, an auristatin microtubule inhibitor. PF-06647020 induced sustained regressions in PDX models and reduced the frequency of CSCs. In addition to CSC depletion, PF-06647020 may also have additional antitumor mechanisms of action, including angiogenesis inhibition and stimulation of immune cells. These activities may be facilitated by the bystander effect of the membrane-permeable hydrophobic auristatin payload. Importantly, despite the expression of PTK7 in certain normal tissues, no target-dependent toxicities were observed in monkeys, possibly because microtubule inhibitors require high antigen expression and actively cycling cells to exert a cytotoxic effect (50).

Two clinical studies with PTK7-ADC/PF-06647020 are currently ongoing: a safety study of the combination gedatolisib plus PF-06647020 for metastatic triple-negative breast cancer (TNBC) (NCT03243331), and a phase I study of PF-06647020 in adult patients with advanced solid tumors (NCT02222922).

Anti-Eph-A4 (EFNA4) ADC

Ephrin receptors (Eph) are the largest family of receptor tyrosine kinases in the human genome and, together with their ligands, have been implicated in the development of breast cancer. Consequently, numerous therapeutics, mostly tyrosine kinase inhibitors are being actively developed to inhibit the function of this ligand/receptor family. A significant limitation of currently developed drug candidates is represented by the vast functional redundancy of this family, while general inhibition of ephrin receptors is toxic (51). In contrast, ADCs prescind from the functional role of ligand/receptor pairs and exert cytotoxic effects only on those target-expressing cells that internalize them.

MRNA expression of one of these receptors, *EFNA4*, was found to be elevated in CSCs compared to non-tumorigenic cells and normal tissues. Protein analysis of normal organs, primary breast tumor specimens and TNBC PDX tumor models demonstrated that EFNA4 was elevated in TNBC vs. normal tissues and other subtypes of breast cancer (36). This expression pattern suggested the possibility of targeting EFNA4 with an ADC.

In order to target EFNA4, the ADC PF-06647263 was constructed. It is composed of an anti-EFNA4-specific, humanized mAb, a hydrazone linker and a calicheamicin payload. Conjugation was to lysine residues of the antibody and yielded an average DAR of 4.6. Calicheamicin was selected as payload because of the presence, within the CSC population, of quiescent as well as cycling cells (67). Quiescent cells are resistant

TABLE 1 | Anti-CSC ADCs.

Target: ADC name	Linker: Cleavable/uncleavable	Cytotoxin: Antimitotic, DNA binder	Developmental stage Stage, ClinicalTrials.gov identifiers, results if available	References
ADCs AGAINST MARKERS EXPRESSED ON CSC				
DLL3: rovalpituzumab tesirine	Cleavable (dipeptide)	DNA binder (PBD dimer)	Phase III, NCT03061812, vs. topotecan in DLL3$^+$ advanced or metastatic SCLC at first disease progression after platinum chemotherapy Phase III, NCT03334487, evaluating the safety for third-line and later treatment of relapsed or refractory SCLC Phase II, NCT02674568, as third-line and later treatment for subjects with relapsed or refractory DLL3$^+$ SCLC Other phase I/II studies in patients with DLL3$^+$ SCLC (NCT02819999, NCT02874664) or SCLC and other solid tumors (NCT02709889)	(30, 31)
Protein tyrosine kinase 7 (PTK7): PF-06647020	Cleavable (dipeptide)	Antimitotic (Aur0101)	Safety study, NCT03243331: with gedatolisib in TNBC Phase I, NCT02222922: in adult patients with advanced solid tumors	(35)
Ephrin-A4 (EFNA4): PF-06647263	Cleavable (hydrazone)	DNA binder (calicheamicin)	Phase I, NCT02078752: in patients with advanced solid tumors	(36)
IL-3 receptor α chain (CD123): SGN-123	Cleavable (dipeptide)	DNA binder (PBD dimer)	Phase I, NCT02848248, in AML patients. Study terminated, presumably no longer in active development.	(37)
5T4: PF-06263507	Non-cleavable (maleimidocaproyl)	Antimitotic (MMAF)	Phase I, NCT01891669, no objective responses were observed	(38, 39)
5T4: MEDI-0641	Cleavable (dipeptide)	DNA binder (PBD dimer) Antimitotic (tubulysin)	Not reported	(40, 41)
5T4: H6-DM4	Cleavable (SPDB)	Antimitotic (DM4)	Not reported	(42)
LGR5	Cleavable (dipeptide) Non-cleavable (malemidopropionyl)	Antimitotic (MMAE) Antimitotic (MMAE)	Not reported Not reported	(43)
LGR5	Cleavable (dipeptide) Cleavable (acid-sensitive)	Antimitotic (MMAE) DNA binder (PNU159682)	Not reported Not reported	(44)
ANTITUMOR ADCs THAT WERE SHOWN TO HAVE ANTI-CSC ACTIVITY AT A LATER STAGE				
HER2: T-DM1, ado-trastuzumab emtansine	Non-cleavable	Anti-mitotic (DM1)	FDA-approved for the treatment of HER2-positive metastatic breast cancer.	(45)
CD33: gemtuzumab ozogamicin	Cleavable (hydrazone)	DNA binder (calicheamicin)	FDA approval in 2000 for the treatment of AML. Voluntarily withdrawn in 2010 due to safety concerns. Recently reapproved.	(46)
NCAM (CD56): lovortuzumab mertansine	Cleavable	Anti-mitotic (DM1)	It was in development as antitumor agent, not specifically as anti-CSC agent. Development now halted due to disappointing results in lung cancer patients	(47, 48)

ADC, antibody-drug conjugate; AML, acute myeloid leukemia; CSC, cancer stem-like cells; DLL3, Delta-like ligand 3; EFNA4, Ephrin-A4; FDA, Food and Drug administration; HER2, human epidermal growth factor receptor 2; LGR5, leucine-rich repeat-containing, G protein-coupled receptor 5; MMAE, monomethyl auristatin E; NCAM, neural cell-adhesion molecule; PBD, pyrrolobenzodiazepine; PTK7, protein tyrosine kinase 7; SCLC, small-cell lung cancer.

to antimitotic, cell-cycle-dependent microtubule inhibitors like auristatins and maytansines. Calicheamicins, on the other hand, are DNA-binding drugs that are cytotoxic independently of the cell cycle status.

PF-06647263 induced significant tumor regression in TNBC xenografts. The most robust responses were observed in non-claudin low TNBCs, with complete responses observed in several cases. This result correlates with the increased EFNA4 expression observed in this breast cancer subtype. EFNA4 expression was also elevated on a subset of ovarian cancers and PF-06647263 induced sustained regression also on xenografts of these tumors.

A phase I study has been performed with PF-06647263 in patients with advanced solid tumors (NCT02078752). The study has been completed and no other studies are currently ongoing.

Of note, also another anti-Eph ADC has been reported in the literature (MEDI-547) (52). This ADC targets EFNA A2 and showed substantial activity in *in vivo* models of endometrial carcinoma. However, no evidence was brought that this ADC has some preferential activity on CSCs. A clinical study with this

ADC has evidenced early and serious adverse events and has led to the discontinuation of its development (53).

An Anti-CD123 ADC

CD123 is the α chain of the interleukin (IL)-3 receptor. Upon binding of IL-3, CD123 heterodimerizes with the β subunit of the IL-3 receptor and gives rise to intracellular signals promoting cell survival and proliferation (54). CD123 is expressed on the surface of myeloblasts and leukemia stem cells (LSC, the equivalent of CSCs for hematologic malignancies) of AML patients (55, 56). These cells have been associated with chemotherapy resistance, persistence of minimal residue disease and unfavorable prognosis (57, 58). CD123 is expressed at very low levels or is absent from normal hematopoietic stem cells, thereby offering the possibility of targeting AML stem cells while sparing normal stem cells (59).

The generation and preclinical investigation of an anti-CD123 ADC has recently been reported (37). This ADC, dubbed SGN-CD123A, is composed of a humanized anti-CD123 mAb with engineered cysteines for site-specific conjugation, a valine-alanine dipeptide linker and a PBD dimer payload. *In vitro*, SGN-CD123A had potent cytotoxic effects on most $CD123^+$ AML cells lines and primary samples from AML patients. SGN-CD123A was highly active in various leukemia models and led to eradication in a disseminated AML model. SGN-CD123A was also tested in combination with the fms-like tyrosine kinase 3 (FLT3) inhibitor quizartinib. FLT3 inhibitors are in development for FLT3-mutated AML patients, but responding patients invariably develop resistance. SGN-CD123A enhanced the activity of quizartinib in FLT3-mutated xenograft models.

A phase I clinical trial has been performed with SGN-CD123A in AML patients (NCT02848248). This study, however, has been terminated, and on the company's website this product is not mentioned.

Anti-5T4 ADCs

5T4, or trophoblast glycoprotein, is a 72-kDa, N-glycosylated transmembrane protein. The extracellular domain contains leucine-rich repeats, which are commonly associated with protein-protein interactions. 5T4 is expressed in normal progenitor cells during embryonic development where it functions in EMT and cell migration (60). It is an oncofetal antigen with high expression in many types of carcinomas and low expression in normal tissues. 5T4 has been found to be overexpressed in CSCs in non-small cell lung cancer (NSCLC) and other cancers (61) compared to differentiated tumor cells. 5T4-overexpressing cells also show increased expression of EMT markers and have increased tumor-initiating potential (61). Moreover, as expected for EMT tumor cells and CSCs, 5T4 overexpression is associated with advanced-stage disease, drug resistance and worse prognosis in several solid tumors (62–64).

Several ADCs targeting 5T4 have been described in the literature. The first, PF-06263507, comprises a humanized anti-5T4 mAb linked to the tubulin inhibitor MMAF via a non-cleavable maleimidocaproyl linker (38). MMAF preferentially acts on proliferating cells due to its antimitotic mechanism of action. This ADC was very potent in several tumor models inducing long-term regressions with low doses. In a NSCLC xenograft model the ADC reduced CSC frequency. In safety studies in primates it was safe and had a half-life of 5 days.

In the following, PF-06263507 was tested in combination with the dual phosphoinositide 3-kinase (PI3K)/mechanistic target of rapamycin (mTOR) catalytic site inhibitor PF-384 or taxanes (65). *In vitro*, PF-06263507 or untargeted auristatins displayed strong synergistic or additive activity when combined with PF-384 or taxanes, respectively. These synergistic/additive activities were not due to the individual components of the combination acting on different tumor cell subpopulations (e.g., CSC and non-CSC cells) but, rather, to amplification of the effects of this ADC on translational components by PF-384 or the simultaneous binding of MMAF and taxanes to distinct binding sites on microtubules, respectively. In human breast and lung cancer xenografts, combination therapy with PF-06263507 + PF-384 or PF-06263507 + paclitaxel yielded enhanced antitumor effects with longer survival as compared with monotherapies.

The potential of PF-06263507 for the treatment of hematological malignancies was also investigated (66), given the finding that 5T4 was overexpressed on minimal residual acute lymphoblastic leukemia (ALL) cells. PF-06263507 significantly improved survival in mice engrafted with $5T4^+$ patient-derived ALL cells, and even more so in combination with chemotherapy or dexamethasone.

PF-06263507 has been investigated in a phase I clinical trial (NCT01891669) and the results of this study, where no objective responses were observed, have been reported (39).

In another approach, anti-5T4 ADCs were constructed by site-specifically conjugating a human anti-5T4 mAb via a valine-alanine dipeptide linker with payloads having different mechanisms of action: a PBD dimer or the microtubule destabilizing tubulysin (40). *In vivo* experiments in xenograft models of different carcinoma types showed that the ADC conjugated with a PBD payload, MEDI-0641, elicited more durable antitumor responses and inhibited more potently the growth of $5T4^+$ CSCs *in vivo* than the tubulysin conjugate. This result is consistent with the knowledge that CSCs comprise subpopulations of proliferating and quiescent cells (67) and that a DNA binder like a PBD is cytotoxic also on quiescent cells, while a tubulin binder like tubulysin acts only on the proliferating CSC subpopulation. Moreover, MEDI-0641 was cytotoxic on both CSC and non-CSC tumor cells, leading to depletion of both compartments. This result implies that 5T4 is pan-tumor cell marker, whether or not overexpressed by CSCs. In rats, MEDI-0641 had excellent *in vivo* stability and an acceptable safety profile.

MEDI-0641 was then tested on cells and xenografts of head and neck squamous cell carcinoma (HNSCC) (41). *In vitro*, it caused a significant depletion of CSCs. *In vivo*, in three patient-derived xenograft models of HNSCC, a single administration of MEDI-0641 caused long-lasting tumor regression, which was likely due to depletion of both CSCs and non-CSCs. In the three models, MEDI-0641 caused either a complete elimination of tumor-initiating cells or a significant reduction. Moreover, a single dose of MEDI-0641 prevented tumor recurrence when used in a neoadjuvant setting prior to surgery.

MEDI-0641 does not seem to have yet entered clinical development as it is not mentioned neither under clinicaltrials.gov nor in the company's website.

A third anti-5T4 ADC has been described very recently (42). This ADC, H6-DM4, is composed of a chimeric anti-5T4 mAb linked to an antimitotic cytotoxin, the maytansinoid DM4, through a cleavable N-succinimidyl 4-(2-pyridylothio)butyrate (SPDB) linker sensitive to intracellular reducing conditions with liberation of a lipophilic adduct, S-methyl-DM4, that can exert a bystander effect. H6-DM4 was cytotoxic against a panel of gastrointestinal cancer cell lines, including colorectal CSCs and colorectal cancer cells resistant to platinum compounds. CSCs were found to express higher levels of 5T4 than non-CSCs. 5T4 eradicated established gastrointestinal tumor xenografts in the low mg/kg range without observable toxicity. Tumor cell lines expressing higher levels of 5T4 were more sensitive to the effects of the conjugate *in vivo*, suggesting that the expression level of 5T4 could represent a predictive marker for patient selection.

Anti-leucine-rich Repeat-Containing, G Protein-Coupled Receptor 5 (LGR5) ADCs

LGR5 is a marker of adult stem cells in several epithelial tissues. In particular, $LGR5^+$ crypt cells in the gastrointestinal tract give rise to all differentiated cell types within intestinal epithelia, suggesting that it represents the stem cell of the small intestine and colon (68). LGR5 is overexpressed and gives rise to multiple cell types within gastrointestinal tumors (68, 69). These observations suggest a close relationship between normal adult gastrointestinal stem cells and CSCs of gastrointestinal tumors. LGR5 overexpression correlates with higher incidence of metastasis, drug resistance, and poor patient survival (70, 71). Given its overexpression and rapid, constitutive internalization independently of ligand binding (72), LGR5 was considered a good target for ADCs.

Two ADCs were constructed by conjugating an anti-LGR5 mAb to the membrane-permeable, antimitotic agent MMAE via a cleavable valine-citrulline linker or a non-cleavable malemidopropionyl linker (43). Both ADCs bound LGR5 with similar affinity and were rapidly internalized by gastrointestinal cancer cells. Cytotoxicity was induced in LGR5-overexpressing cancer cells, but not in LGR5-negative cells or cell lines where *LGR5* had been knocked down. However, the ADC with the cleavable linker was 10- to 20-fold more potent at killing these cells, probably due to a bystander effect (73). In fact, the ADC with the non-cleavable linker gives rise to charged metabolites (e.g., amino acid-linker-cytotoxin) that are membrane-impermeable. The ADC with the cleavable linker eradicated tumors and prevented recurrence in a xenograft model of colon cancer. Interestingly, these experiments yielded evidence of an interconversion between $LGR5^+$ and $LGR5^-$ CSCs that drove tumor regrowth after treatment with the ADC.

Another group has described the generation and testing of two other anti-LGR5 ADCs (44). The first is composed of a humanized anti-LGR5 mAb conjugated to MMAE through a cleavable valine-citrulline linker, via the cysteines that normally form the interchain disulfides of the mAb. Thus, this conjugate is very similar to the one described by Gong et al. (43). The second conjugate, NMS818, is composed of the same mAb connected, via an engineered cysteine on the antibody heavy chain and an acid-sensitive linker, to the C-14 hydroxyl of the DNA-binding, topoisomerase-inhibiting anthracycline PNU159682.

In vivo experiments showed the ADC anti-LGR5-MMAE to be efficacious without affecting homeostatic epithelia or any other tissues known to express LGR5. On the other hand, the ADC NMS818 showed target-dependent toxicities consistent with the known expression patterns of LGR5. The lack of gut toxicity with anti-LGR5-MMAE may possibly be due to the fact that the elimination of intestinal $LGR5^+$ cells is well-tolerated. On the other hand, NMS818 target-dependent toxicity observed in the intestine may be attributable to the combined elimination of target-expressing LGR5 cells and bystander cells. In fact, both ADCs release a membrane-permeable drug after internalization that could exert a bystander effect on neighboring cells. However, the free drug released from NMS818 is 10- to 100-fold more potent on dividing cells, including normal $LGR5^+$ cells, than MMAE. This could explain the greater toxicity of anti-NMS818 than anti-LGR5-MMAE.

Antitumor efficacy was observed both in xenografts as well as in genetically engineered mouse models of colon cancer, and both in tumors with uniformly high expression of LGR5 as well in tumors with heterogeneous and low expression of LGR5, the latter reflecting more closely the situation found in human tumors. Importantly, changes in tumor size were not immediately apparent, but became evident with long-term treatment, suggesting that depletion of CSCs takes longer to manifest as compared to the targeting of non-CSCs.

While there are clinical trials ongoing with a monospecific and a bispecific anti-LGR5 mAb, no trials are currently reported with one of the ADCs described here.

Antitumor ADCs That Were Shown to Have Anti-CSC Activity at a Later Stage

Anti-human Epidermal Growth Factor Receptor 2 (HER2) ADC

HER2 is a transmembrane receptor tyrosine kinase that mediates several functions like growth, differentiation and survival in malignant and normal breast epithelial cells. Breast cancers overexpressing HER2 have an aggressive clinical phenotype, increased disease recurrence and unfavorable prognosis. Moreover, HER2 is overexpressed on breast CSCs, even on those subtypes that are not classified as $HER2^+$ (74).

HER2 is the target of two antibody-based compounds that have gained approval: the first if the mAb trastuzumab (75), the second is the ADC ado-trastuzumab emtansine, which is composed of trastuzumab conjugated through a non-cleavable linker to the antimitotic drug maytansine DM1 (27). Clinical use of these compounds is for $HER2^+$ breast cancer, with HER2 overexpression on the bulk of tumor cells, independently of their co-expression on CSCs.

The knowledge that CSCs overexpress HER2, whether or not the bulk of tumor cells are $HER2^+$, led to investigate the effect of T-DM1 on CSCs (45). For this purpose, primary tumor cells and breast cancer cell lines were treated with T-DM1. The results

showed that breast CSCs with the $CD44^{high}CD24^{low}HER2^{low}$ phenotype were very efficient in internalizing T-DM1 and highly sensitive to it. This caused the depletion of breast CSCs at concentrations of T-DM1 that did not affect the bulk of tumor cells. Moreover, colony formation was also efficiently suppressed and EMT-mediated induction of stem cell-like properties was prevented in differentiated tumor cells. Importantly, the unconjugated antibody, trastuzumab, did not have these effects, pointing to a direct effect of the payload-induced cytotoxicity in depleting CSCs.

Anti-CD33 ADC

Gemtuzumab ozogamicin (GO) is an anti-CD33 ADC composed of a humanized anti-CD33 mAb linked to the cytotoxin calicheamicin via a hydrazone linker. GO has a tormented history. It received US Food and Drug Administration approval in 2000 for $CD33^+$ AML, but was voluntarily withdrawn in 2010 due to safety concerns. Later, in a meta-analysis of patient data from clinical trials, it was found that the combination of lower-dose GO and induction chemotherapy reduced the risk of relapse and improved the relapse-free survival and overall survival in adult AML patients with favorable cytogenetics (76). These results led, recently, to the reapproval of GO.

Induction chemotherapy based on daunorubicin and cytarabine was investigated in combination with GO in patient-derived xenograft AML models (46). The separate treatments reduced AML burden but left significant chemoresidual disease. Chemoresistant cells displayed markers of LSCs and showed greater ability to self-renewal than bulk leukemic cells. Interestingly, CD33 was coexpressed in the chemoresistant cells. Combination treatment, on the other hand, was highly effective in eliminating nearly all AML burden, extended overall survival and more effectively eliminated chemoresistant LSCs.

Anti-neural Cell Adhesion Molecule (NCAM, CD56) ADC

Wilms' tumor is the most frequent tumor of the genitourinary tract in children. It displays a triphasic histology: cell lineages similar to those observed during kidney development, undifferentiated blastema and stromal and epithelial derivatives (e.g., immature tubules and glomeruloid bodies). Evidence for the existence of CSCs in human Wilms' tumor was obtained in *in vitro* cultures derived from primary tumors. In these experiments it was found that $NCAM^+$ cells of blastema phenotype had enhanced capacity to expand and differentiate into mature renal-like cell types, showing that they were greatly enriched for CSCs (77). They could be further enriched by aldehyde dehydrogenase activity and overexpression of other stemness genes and showed preferential expression of Akt and strong reduction of the miR-200 family, a miRNA family involved in the down-regulation of EMT and maintenance of an epithelial phenotype (78).

In order deplete $NCAM^+$ cells in Wilms' tumors an ADC was used that had already been constructed and developed, lovortuzumab mertansine (47). This ADC is composed of a humanized anti-NCAM mAb, lovortuzumab, linked via a cleavable disulfide linker to the maytansinoid DM1, mertansine. *In vitro*, it inhibited the stemness properties of Wilms' tumor cell cultures that varied in the extent of NCAM expression. Results suggested also that EMT promoted the acquisition of a CSC phenotype generating highly tumorigenic cancer cells with a mesenchymal phenotype. *In vivo*, the ADC eradicated, at low doses, Wilms' tumors bearing high NCAM expression, while higher doses were required for Wilms' tumors with lower NCAM expression.

The clinical development of this ADC has recently been halted due to disappointing results in a clinical study in lung cancer patients (48).

A CRITICAL APPRAISAL OF ANTI-CSC ADCS

ADCs against CSCs, similarly to other anti-CSC compounds, have raised considerable hopes as regards their therapeutic efficacy. Recently, however, one of the most advanced ADCs of this class, the anti-DLL3 ADC rovalpituzumab tesirine, has yielded disappointing results in a clinical trial (NCT02674568) in patients with SCLC (30). This is despite encouraging results in an initial phase I clinical trial in the same indication (79). This failure parallels other pitfalls with drug candidates targeting CSCs, including other ADCs. The question now arises whether these approaches, including the ADC approach against CSCs, have entered a dead-end street or if there is room for envisioning a new start based on a better understanding of the reasons underlying these failures. In the following we will list some of the reasons that appear to us as the most likely ones.

The first aspect relates to the biology of CSCs. CSCs are themselves a heterogeneous population that can be grossly divided in two subpopulations: a proliferating and a quiescent subpopulation. There are strong indications that the quiescent subpopulation is in an autophagic state (67). Importantly, there are also evidences that these two subpopulations may occupy different niches within tumor tissues (80). The location of a tumor cell within a tumor is crucially important for their sensitivity to drugs, including antibodies and ADCs (81). This implies that, depending on their location, different CSC subpopulations may be differently sensitive to the same drug.

The existence of proliferating and quiescent CSC subpopulations implies also that ADCs carrying cell cycle-independent drugs like DNA binders have an advantage over cell cycle-dependent drugs like tubulin binders and may lead to a more complete elimination of CSCs because of their potential to delete both proliferating as well as quiescent cells. If, however, quiescent CSCs are in an autophagic state, and there is considerable evidence in favor of this possibility (reviewed in 40), then there is another dark side that we should consider because our knowledge on this aspect and the possible consequences is almost nil. In fact, we don't know whether the internalization and intracellular trafficking in autophagic cells follows the same kinetics and routes as that of the non-autophagic counterparts. Recent evidence suggests, indeed, that there are differences (82).

This implies that these differences may cause in autophagic CSCs to a release of the active drug that is less efficient that in proliferating CSCs.

Another limitation of the ADCs that are currently developed is that none of them targets a marker that is strictly CSC-specific. As already discussed before, these targets are also expressed, albeit to a lesser degree, on the bulk of tumor cells. This is notwithstanding the lack of reactivity of these ADCs for normal stem cells and the lack or limited reactivity for normal tissues. At this point one is led to ask whether these ADCs are truly anti-CSC ADCs or anti-tumor cell ADCs that embrace a tumor cell population that includes also CSCs. This is not necessarily a disadvantage, but then one has to evaluate the overall antitumor activity of the ADC in order to predict its efficacy and not just the anti-CSC activity.

One of the most important, yet disregarded aspects of CSC biology lies in the capacity of CSCs and non-CSCs to interconvert. This aspect has already been briefly addressed in the first section of this article. Nevertheless, it is important, at this point, to underscore the consequences that this may have on the whole tumor cell population and on CSCs. We have discussed that the conversion from non-CSC to CSCs can be driven by stressors in the tumor microenvironment, including hypoxia, mechanical stress, chemotherapy etc. These stressors may show inter- and even intratumoral variability depending on the geographical conditions of the tumor microenvironment. This implies that the fraction of CSCs may greatly vary from one tumor to the other and even within individual tumors. Such great variability has been documented in several instances (83, 84) and, in some cases, one out of four tumor cells have been shown to display properties of CSCs (85). It is clear that in these cases the boundaries between non-CSCs and CSCs become very blurred. The capacity of CSCs and non-CSCs to interconvert represents a substantial difference compared to normal stem cells. In fact, normal stem cells reside at the top of a pyramid where they can self-renew or give rise to a more differentiated progeny (86). This implies a unidirectional process, whereas the capacity of CSCs and non-CSCs to interconvert implies a bidirectional process. This raises serious doubts as to the appropriateness to refer to these cells as stem cells. Perhaps a definition like "resistant cancer cells" would be more appropriate to portray the essential of these cells.

Last but not least, an important limitation in the development of antibodies and ADCs is represented by the animal models that are used for preclinical testing. As can be seen just going through the articles that are referenced here, it is rather quite common that the compounds tested in animal models lead to complete tumor regressions, yet the same compounds fail in the human setting (e.g., 31, i.e., the ADC rovalpituzumab tesirine). In other terms, the predictability of animal models is very limited and it seems appropriate to say that observing complete tumor regressions in animal models is, nowadays, a necessary but by far not sufficient condition. At this point, the obvious question arises as to why animal (essentially mouse) models are inadequate to predict efficacy of antibodies and ADCs? A detailed discussion of this aspect goes beyond the scope of this article. Nevertheless, we would like to briefly address two aspects that may contribute to this insufficiency. First, the percentage of injected dose of an antibody or antibody-like construct that is taken up by the tumor mass (expressed in grams) is much lower for humans than for mice (87). This is because an antibody dose diluted in a larger plasma volume gives a much lower percentage of injected dose/gram tumor tissue, three orders of magnitude lower for humans (∼3 l plasma volume) than mice (∼2 ml). Such a dose may be sufficient to eradicate a tumor in a mouse, but insufficient to eradicate an equivalent tumor in a human. Incidentally, it has been recently demonstrated that the intratumoral payload concentration correlates with the antitumor activity of ADCs (88), a result that underscores the importance of attaining a sufficient antibody or ADC concentration within a tumor tissue in order to be efficacious. The second aspect relates to the mouse tumor models that are commonly used to test antitumor drugs, including those that are considered mimicking most closely the human situation, PDXs. It seems very difficult that these models can fully mimic the heterogeneity encountered in the human setting, in particular as regards the possibility that stressors from the tumor microenvironment may induce a conversion of non-CSC to CSCs. Thus, in one of the articles that have been referenced here (41), the anti-5T4 ADC MEDI-0641 ablated CSCs in one PDX model of HNSCC, reduced the frequency of CSCs 5-fold in a second PDX model, and 2-fold in a third PDX model (M11). Yet, in spite of the relatively modest CSC reduction that was observed in the M11 model, MEDI-0641 was able to completely prevent tumor recurrence after surgical removal of the tumor in this model. This leads obviously to ask as to which is the predictability of ADC-induced reduction of CSC frequency on the *in vivo* efficacy of an ADC in PDX models.

In spite of all these limitations and even if we resort to a minimalist definition of CSCs as tumor cells that become drug-resistant in response to stressors generated in the tumor microenvironment, then we have to agree that it remains a desirable therapeutic goal to get rid of these cells together with the rest of the tumor cells, i.e., the proliferating, drug-sensitive tumor cells. The point is to how best attack these cells. Addressing this question, however, implies recapitulating all of the limitations that we have listed and discussed in the first part of this section. Now, how can we address this conundrum? There is no easy answer to this but, at present, we can envisage two possibilities that can to be implemented.

The first of these possibilities is to develop *in silico* models of human tumors that are better able to predict efficacy of antibodies or ADCs than do the present animal models. Efforts in this direction are ongoing (89). Thus, *in silico* models for tumor growth and tumor treatment have been described (90, 91). In perspective, it seems reasonable to predict that these models may incorporate a number of variables allowing them to match the situation(s) encountered in the human setting more closely than currently used animal models, including PDX models, are able to do.

Second, the identification of biomarkers that are predictive of clinical efficacy of anti-CSC ADCs, similarly to the overexpression of HER2 that is used to predict efficacy of trastuzumab in $HER2^{+}$ breast cancer patients. Biomarkers,

including circulating tumor cells (CTC), witnessing antitumor effects in general and anti-CSC effects in particular (e.g., CTCs with a mesenchymal phenotype or soluble markers from mesenchymal CSCs), may allow to predict in a more reliable manner the overall clinical efficacy of anti-CSC ADCs. Such an approach could be systemically applied to the anti-CSC ADCs that are currently in clinical development. Elucidation of the relationship/lack of relationship between anti-CSC activity and clinical efficacy could allow to identify the contribution of anti-CSC activity to the overall antitumor activity and predict the efficacy of novel ADCs.

Overall, the present scenario regarding the clinical efficacy of anti-CSC ADCs is not very encouraging, yet the biological functions of CSCs suggest that further efforts should be devoted to this goal. Here, we have proposed some activities that could help moving forward in this field.

AUTHOR CONTRIBUTIONS

FM, CC, ER, and CR contributed to the conception of the work, drafted or revisited it critically, approved the final version, and agreed to be accountable for all aspects of the work.

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Pre-clinical Models for Studying the Interaction Between Mesenchymal Stromal Cells and Cancer Cells and the Induction of Stemness

Sofia Avnet[1], *Silvia Lemma*[1], *Margherita Cortini*[1], *Gemma Di Pompo*[1], *Francesca Perut*[1] *and Nicola Baldini*[1,2]*

[1] Orthopaedic Pathophysiology and Regenerative Medicine Unit, IRCCS Istituto Ortopedico Rizzoli, Bologna, Italy,
[2] Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

Mesenchymal stromal cells (MSC) have essential functions in building and supporting the tumour microenvironment, providing metastatic niches, and maintaining cancer hallmarks, and it is increasingly evident that the study of the role of MSC in cancer is crucial for paving the way to clinical opportunities for novel anti-cancer therapies. To date, the vast majority of preclinical models that have been used for studying the effect of reactive MSC on cancer growth, metastasis, and response to therapy has been mainly based on *in vitro* flat biology, including the co-culturing with cell compartmentalization or with cell-to-cell contact, and on *in vivo* cancer models with different routes of MSC inoculation. More complex *in vitro* 3D models based on spheroid structures that are formed by intermingled MSC and tumour cells are also capturing the interest in cancer research. These are innovative culture systems tailored on the specific tumour type and that can be combined with a synthetic extracellular matrix, or included in *in silico* technologies, to more properly mimic the *in vivo* biological, spatial, biochemical, and biophysical features of tumour tissues. In this review, we summarized the most popular and currently available preclinical models for evaluating the role of MSC in cancer and their specific suitability, for example, in assaying the MSC-driven induction of epithelial-to-mesenchymal transition or of stem-like traits in cancer cells. Finally, we enlightened the need to carefully consider those parameters that might unintentionally strongly affect the secretome in MSC-cancer interplay and introduce confounding variables for the interpretation of results.

Keywords: mesenchymal stromal cells, tumour microenvironment, stemness, secretome, 3D models, metastasis

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National Autonomous University of Mexico, Mexico

***Correspondence:**
Nicola Baldini
fisiopatologia@ior.it

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INTRODUCTION

Cancer is a complex disease that thrives in a heterogeneous and adaptive tumour microenvironment (TME) admixed with reactive elements surrounding or infiltrating the tumour cells. Among these, endothelial, immune, and mesenchymal stromal cells (MSC) or cancer-associated fibroblasts (CAF) are frequently observed, playing an important role during carcinogenesis and cancer progression (1, 2). As a part of the tumour-supporting mesenchymal stroma, CAF have been suggested to originate from MSC, thereby sharing several features (3–5). For the distinguishing between MSC and CAF we recommend other previous review (5),

since in this review, we will focus on MSC. Regardless the tissue origins, MSC are inherently tumour-homing and are a considerable component of the general host response to tissue damage caused by cancer cells. Cancer has been associated with MSC at all stages of disease progression with contradicting conclusions. Indeed, MSC have been also shown to have anti-cancer activities (6). More often, MSC are considered a foe in cancer for the immunosuppressive ability that creates a protective milieu for tumour cells by recruiting immunosuppressive tumour-associated macrophage TAM (7), for the promotion of tumour angiogenesis, proliferation, and metastasis, but also tumour dormancy and drug resistance (5).

CSC AND MSC

Under physiological conditions, MSC have a major role in the maintenance of stem cell niches, like for the hematopoietic niche (8). Similarly, in cancer, MSC are relevant for the formation and maintenance of cancer stem cells (CSC). CSC are considered quiescent cells that have been isolated from a number of tumours (e.g., hematopoietic malignancies, breast carcinoma, glioblastoma, and sarcomas) by using different techniques (9–12).

Research on CSC has defined them as the driving force in tumour formation as they are characterized by self-renewal ability and can give rise to heterogeneous lineages that recapitulate the main tumour features (13). Yet, it has gradually become clear that CSC, like normal stem cells, do not necessarily have to be rare and/or quiescent; multiple examples now show that they can be abundant and can proliferate vigorously. Furthermore, it is emerging that stem cell hierarchies may be much more plastic than previously appreciated, a phenomenon that complicates the identification and eradication of CSC (14).

Despite recent data are therefore questioning the validity of the CSC model, the CSC-MSC interaction is well documented and is crucial for 3D growth and stemness of tumour cells (15), or for the generation of an hybrid MSC-CSC cell populations, occurring via entosis, i.e., a form of cell-to-cell internalization, or via fusion, as demonstrated in different tumours (16–20). Indeed, the selection processes of hybrid cells after MSC–tumour cell fusion contribute to CSC development (20, 21). CSC-MSC interaction occurs via soluble factors (22), exosomes, or direct interaction (23). Another form of contact has been described for glioblastoma, where the CSC state is regulated by a transient interaction between cancer cells and platelets. This contact induces an epithelial-to-mesenchymal (EMT) transition program leading to the expression of mesenchymal features which may coincide with a CSC-like state. In turn, once CSC have seeded a distant organ, they can orchestrate stromal cells to their needs (23) as CSC may promote, as an example, the release of TGF-β by MSC that further fosters EMT and increases the CSC stem-like state (24).

Given their mesenchymal origin, EMT cannot be proposed in sarcomas. However, after we demonstrated the existence of CSC (25), we showed that, by using a 2D co-culture system that also included CSC spheroids, MSC increased the CSC migratory capacity via TGF-β1 that, in turn, stimulated the secretion of the pro-inflammatory cytokine IL-6 that fostered osteosarcoma stemness and aggressiveness (26). Blocking the TGF- β1 signalling pathway in the same MSC-CSC co-cultures inhibited the CSC dedifferentiation, clonogenicity, and self-renewal capacity (27).

It follows that the study of the interaction between MSC and cancer cells, with or without stem-like properties, is crucial to bring out clinical opportunities for new cancer therapies. However, the set up of the appropriate models according to the specific study aim is crucial. Here, we summarized the currently available and most popular preclinical models and their specific features for modelling MSC-cancer cells interplays.

IN VITRO MODELS

The set up of pre-clinical models with MSC requires *in vitro* expansion thereby possibly causing meaningful changes in MSC behaviour, and affecting the interpretation of results. However, this is a bias that, to date, cannot be overcome to meet the demand for MSC-cancer cells preclinical modelling. The current *in vitro* preclinical models are mainly based on two-dimensional (2D) surfaces and include co-culture systems. These type of models have the enormous advantage to easily allow the control of the experimental conditions, and the analysis of expression of specific molecular signalling that can be distinguished between the two different cell populations. The first and simplest example is the treatment of cancer cells with conditioned medium of MSC cultures and is useful to study the effect of MSC-secreted soluble factors on cancer cell behaviour (28–30). A more complex system is based on the use of transwell with the two cell types seeded onto separate compartments and is suitable for keeping the reciprocal paracrine interactions for studying the effect of MSC secretome on tumour migratory, invasive, and stemness potentials (26, 31, 32). Finally, the co-seeding of the two cell populations on the same compartment of the culture support is also possible and has been used, for example, to evaluate the transfer of mitochondria from MSC to breast carcinoma cells (33), or to evaluate the metabolic symbiosis (34). Notably, the co-seeding is the most appropriate 2D approach to resemble the *in vivo* phenotype: it allows the cell-to-cell direct contact interactions. For this model, immunostaining is the easiest way of analysis, combined with the observation of either fixed or live cells through confocal or optical microscopes. However, for more complex molecular analysis, expensive techniques to sort single cells are needed, like immunomagnetic separation or, after the cell transfection with a fluorescent reporter, the cell retrieval by flow cytometry (34, 35). In conclusion, 2D *in vitro* culture systems are easily handled but they are falling short in predicting biological responses since they cannot thoroughly recapitulate both the complexity and the specificity of living tissues. Indeed, tumours are not merely clusters of proliferating cancer cells that grow on plastic 2D surfaces, but rather highly complex 3D structures with a dynamic extracellular matrix (ECM) and reactive stromal cells with a precise spatial relationship. Thus, 2D models should be not used for studying complex processes that

cannot be reproduced in this type of culture, like drug perfusion in the tissue, intravasion, or extravasion of tumour cells, invasion of tumour cells through an ECM with a 3D structure, cytotoxicity of anticancer-drugs that might be affected by solute or gas gradient or by different transcriptome or proteome signature that is affected by the tumour-stroma interaction in 3D structures. Based on that, significant effort has been put forward to develop more sophisticated 3D structures, like cells aggregates alone or combined with bioprinting or microfluidics techniques.

The key point to improve 3D co-culturing model is that functional unit of the tissue must be considered rather than single cells, including cell-cell contact and, depending on the cell types, a polarized morphology, a basement membrane and an ECM. For a list and brief description of the most commonly used 3D culturing systems see **Table 1**. The simplest model of 3D cultures is based on multicellular tumour spheroids without the addition of external ECM component. Despite cells can *per se* secrete ECM proteins, 3D spheroids are commonly considered ECM-free models. Spheroid cultures have been established from several cancers, also to study tumour-MSC interaction, including glioma, breast, colon, ovary, and prostate carcinoma (36). These multicellular structures mimic *in vivo* growth via the formation of a central necrotic core, a solute/ion gradient from the periphery to the centre, and a 3D cellular spatial organization. Forced-floating, hanging drop spheroids, spheroids obtained by using bioreactors are examples of this type of cell aggregates mixed with MSC and tumour cells, at different ratio (e.g., 3:1, respectively) (36, 37). Forced-floating cell aggregates are obtained by avoiding cell attachment to the well bottom. The hanging-drop method is the most widely used and is obtained by seeding a small aliquot of single-cell suspension in a volume that exceeds the well volume. By inverting the plate, the volume generates a drop in which cells are kept in place by surface tension and are then densely packed in spheroid-like structures with high reproducibility. Both forced-floating and hanging-drop spheroids are extensively used for drug screening (38, 39), thanks to the high number of spheroids/plate that can be obtained and the low cost. On the contrary, rotating cell culture bioreactor and spinner flasks force spheroid formation by continuous agitation (37, 40). However, the different size and the fact that spheroids formed in bioreactors must be related to be tested for drug screening, makes them unsuitable for this application. Nevertheless, bioreactors are the best options when long term culture and carefully monitoring of the environmental conditions (such as oxygen and nutrients) are required.

To recreate the interstitial space, it is essential to add the ECM component to the multicellular spheroids (41). For this aim, tumour cell and MSC co-cultures can be admixed to high biocompatible scaffolds of natural origin (i.g. collagen, hyaluronan, matrigel, elastin), or synthetic origin (polyethylenglycol, polyvinyl alcohol, ceramics, or biomaterials), or also ECM isolated from tumour biopsies to mimic microenvironmental conditions (42). Within the scaffold, cells can interact one with the other (essential in the case of MSC-tumour studies), migrate through the pores and re-create *in vivo*-like communication strategies that mimic physiology. More the used matrix resembles the real tumour matrix and more predictive is assumed to be the model. For the addition of ECM in 3D cancer models, 3D bioprinting has stolen the spotlight since it allows the formation of high-resolution 3D structures by dispensing cell-laden biomaterials in a precisely and spatially defined way (43). In this technique, a hydrogel-like pre-polymer solution with encapsulated cells is stored into the ink cartridge that is connected to a printer head. The printer heads are deformed by a thermal or piezoelectric actuators and squeezed to generate bioink droplets of controllable size. However, to date, with very few exceptions (44), the bioprinting has been barely explored to study the MSC and cancer cells interactions.

Finally, tailored innovative platforms that combine spheroid technologies with disease-specific *in silico* models by using microfluidics, the so called "organ-on-a-chip" technologies that reconstitute organ-level *in vivo* characteristics (45, 46), have emerged also in cancer research. Although more expensive and less practical, this cutting-edge approach facilitates the identification of molecular mechanisms behind the disease or the identification of novel biomarkers, and is also particularly useful for drug screening. Microfluidics allows the study of complex phenomena under the combination of multiple biochemical and biophysical parameters, coupled with high-resolution real-time imaging. This type of approach has been mainly developed to study the interaction of tumour cells with blood vessels that can be recreated in the microfluidic chips. Few examples have also been reported for co-culturing MSC with tumour cells, like for lung cancer (47), or to recapitulate the bone metastatic niche that also includes MSC, like for acute lymphoid myeloma, acute lymphoblastic leukaemia, or breast carcinoma (48–50). By using this approach, it is possible to evaluate on real-time the induction of tumour apoptosis, proliferation, migration and invasion, the activation of the reactive stroma, the secretion of cytokines by tumour cells, the activation of specific oncogenes, and stroma-mediated extravasion and intravasion.

IN VIVO MODELS

To study the role of MSC on cancer development and progression, several animal models has been developed, mainly xenograft and syngenic small rodents, with MSC co-injected with tumour cells (51–53). In these models, MSC participate to tumour pathophysiology, ultimately facilitating the metastatic spread of weakly metastatic cancer (52, 54–56). MSC/tumour cells ratio seems to be particularly relevant like for tumour dormancy/growth, as demonstrated in melanoma or osteosarcoma models (57, 58). However, the isolation of MSC with different methods and from different tissues (e.g., bone marrow and adipose tissues) have made it difficult to reach consistent conclusions.

Heterotopic injections are the most used and include the subcutaneous injections (52), the easiest and most reproducible model that rarely gives origin to metastases, and that is quite far from the human disease since the host tissue surrounding the tumor might be very different from the tumor-associated stroma of the normal tissue where the tumor develops.

Systemically infused MSC localize within injured, inflamed, and cancerous tissues. Thus, to study the tropism of MSC to

TABLE 1 | 3D preclinical models to study the interaction between MSC and cancer cells.

Model	General properties	Advantages	Disadvantages	Applications of the model
Forced-floating spheroids	Aggregation induced by preventing attachment	Low cost and high reproducibility	Variability in cell size and shape	High-throughput investigations for efficacy vs. toxicity of drugs
Hanging drop	Aggregation induced by agitation at the tip of a drop formed by surface tension	Relative low cost and high reproducibility. Suitable for drug screening and high-throughput testing	The spheroid forms a necrotic core	High-throughput drug screening
Rotating cell culture bioreactors	Forced spheroid formation by continuous agitation	Easy to produce spheroids on a large scale	Specialized equipment required. Variability in size and shape	Ideal for cells that require long-term cultures and controlled amounts of nutrients and oxygen
Scaffold-based	Cells are seeded within a gel-like scaffold of natural or synthetic origin	Provides a 3D support that mimics the physiological tissue for ECM composition	Higher costs. Difficult to retrieve cells from the biomaterial. Lack of reproducibility	3D structures where the cells are free to migrate or form *in vivo*-like cues
3D printing	Cells are printed within scaffold of natural or synthetic origin	Provides a 3D support that mimics the physiological tissue for ECM composition and the spatial organization	Specialized equipment required. Higher costs. Difficult to retrieve cells from the biomaterial. Lack of reproducibility	Allow formation of custom-specific ECMs or scaffolds
Microfluidics	Cells are seeded on microfluidic device that, by using multiple channel and gel-like scaffolds perfused by fluid medium	Provides a 3D support that reconstitute organ-level *in vivo* characteristics. Live observation.	Specialized equipment required. Higher costs. Difficult to retrieve cells from the biomaterial. Lack of reproducibility. N. of cells that can be used is limited	Identification of molecular cellular mechanisms or biomarkers. High-throughput drug screening

the tumour, MSC have been injected into circulation through the tail vein (59, 60). MSC have been also systemically administered via alternative routes, like via intratracheal (61), internal carotid artery (62), intraperitoneal (63), like for gliomas, breast, colon, ovarian, and lung carcinomas (52, 53, 61–66). Systemic injection of MSC may be useful also to enhance their viability along the experiment. Indeed, as it appeared from studies on MSC-based cell therapy, MSC survival is very low (67–70). Thus, periodic injections of MSC after the first injection might enhance their engraftment in the tumour and ensure the continuous secretion of MSC-derived protein factors.

Finally, the use of orthotropic injection site mimics the fate of MSC that have been already chemoattracted by tumour cells and have migrated at the primary tumour site, and it better recapitulates the human disease. However, this model has a great variability and requires a higher number of animals to obtain results that may be correctly interpreted.

It is clear that, due to the short-term survival of injected MSC, monitoring their fate *in vivo* is crucial to support the conclusions about their pro- or anti-cancer activities. As for tumour cells, tracking MSC fate has been obtained by different approaches (see **Table 2**). In principle, the most powerful technique should allow non-invasive live imaging by optical or not-optical methods to gain real-time information. After animal sacrifice, also histology, immunofluorescence, immunohistochemistry, or flow cytometry techniques on isolated live or fixed cells can be used. Among the live-imaging techniques, the most used are the pre-labelling of MSC with a lipophilic fluorescent dyes (e.g., DiL or Cell TrackerTM) (54), or the pre-tagging of MSC by specific gene transfection, like luciferase or green fluorescent protein for the detection of bioluminescence (60, 71, 84) or fluorescence (55), respectively. Notably, bioluminescent imaging of luciferase-expressing cells is also a quantitative technique for the direct assessment of tumour growth (51, 53), whereas non-optical methods, such as magnetic resonance imaging (MRI) (82), positron emission tomography (PET) (65) and single photon emission computed tomography (SPECT) (83) provide a high spatial resolution and three-dimensional whole-body imaging.

In conclusion, to develop a more clinical relevant *in vivo* model that accurately reflects the human tumour biology is an urgent need to better predict the response of the tumour to the treatments and for identifying those steps that are crucial for tumour progression to be targeted or prevented for an improved clinical outcome. The addition of MSC in the model is a step forward in this direction, although for the development of *in vivo* pre-clinical models of MSC-tumour cells interaction several parameters need to be carefully considered according to the specific aim, like MSC:tumour cells ratio, the via of co-injection, and the tracking of MSC to check their fate. Last but not least, variables affecting the secretome should be very carefully analysed.

THE SECRETOME

Studies on MSC-cancer interplay and analyses of cell conditioned media in the mentioned preclinical models have allowed the

TABLE 2 | Methods for imaging MSC-tumour interplay *in vivo*.

Method	Cellular modification	Contrast agent	Model of implantation	Tumour model	Reference for tracking MSC	Reference for tracking tumour cells
BLI-live imaging	Luciferase transduction	Bioluminescence from luciferase/luciferin reaction	Orthotopic and heterotopic	Osteosarcoma, breast, ovarian cancers	(53, 59, 60, 63, 71, 72)	(51, 73)
Fluorescent-live imaging	GFP or fluorescent dye labeling of membrane	Fluorescence from fluorescent proteins or fluorescent dyes	Orthotopic and heterotopic	Glioblastoma, gliomas, breast, colon carcinoma	(62, 64, 65, 74–77)	(52, 62, 64, 65)
PET	Genetic modification of cells (PET reporter gene) or uptake of radioisotope labels	Positron-emitting radionucleotides	Heterotopic	Colon cancer, clear cell sarcoma	(65, 72, 78–81)	
MRI	Magnetic nanoparticles added to cells or coupled to ligands	Superparamagnetic iron oxide contrast agent, internalized iron, metal chelates, etc.	Orthotopic and heterotopic	Melanoma, gliomas	(78, 82)	
SPECT	Uptake of radioisotope labels	Radionucleotides (gamma-emitting radioisotopes)	Heterotopic	Breast cancer	(81, 83)	

identification of soluble mediators of the indirect communication between MSC and tumour cells. To summarise, cancer cells frequently secrete IL-1 and TGF-β which switch MSC to a pro-inflammatory phenotype (85, 86), and the monocyte chemotactic protein-1 (MCP-1) which stimulates MSC migration (87). Conversely, MSC produce a plethora of cytokines which, in turn, modulates cancer cell behaviour: IL-6 and IL-8 that promote tumour cell proliferation, survival, migration, and invasion of different tumour cells (26, 88–90), CCL5 that support metastasis in several cancers (52, 91–93), the pro-angiogenic cytokine VEGF that enhances tumour growth and metastasis (94, 95), and TGF-β that fosters tumour invasion and metastasis via EMT (96). Cell communication within the tumour microenvironment is mediated also by exosomes, extracellular nanovesicles that deliver a functional cargo of proteins, lipids, and nucleic acids (97). Tumour-derived exosomes are able to co-opt and reprogram MSC by enhancing their pro-tumourigenic functions, including the pro-angiogenic activity and the production of the pro-inflammatory cytokines IL6 (51, 98). On the other side, exosomes derived from MSC are able to influence tumour development (99), and to increase the tumour stemness (100). Besides, several metabolites, like glutamine, lactate, and ketone bodies, that are released both by tumours cells and by MSC in the extracellular space might fuel the energetic metabolism of cells of the TME (101) or may act as signalling molecules, ultimately stimulating cancer motility, survival, or self-renewal (2, 102–106). Also in osteosarcoma, we recently demonstrated that tumour cells cause an oxidative stress in MSC that, in response, acquire a Warburg phenotype and produce a large amount of lactate.

In this context, it is worth to highlight that the *in vitro* conditions, both 2D and 3D, might induce secretory modifications *per se* (107), thereby affecting the interpretation of results, like by using experimental conditions that can unintentionally exert a stressing stimulus for the cells. Thus, we suggest to evaluate results by considering cells, secretome, and three-dimension as an integrated whole. This add complexity to the system, and careful attention has to be paid when setting up the experiment according to the specific aim and during the interpretation of data.

CONCLUSIONS

Several model systems are now available to characterize the MSC-tumour interplay in the TME. These offer early promise in establishing robust preclinical platforms for the identification of crucial molecular pathways and for the assessment of clinical efficacy of novel drugs to inhibit cancer development and progression. However, selection of the right model for a given study should be shaped on the purpose, and should also consider fixed biological, biochemical, and biophysical parameters according to the specific tumour type. Finally, in order to get reliable and useful results to be translated to the clinic, it should be always kept in mind the careful comparisons in the prediction of human outcomes by the developed model.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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NAMPT as a Dedifferentiation-Inducer Gene: NAD^+ as Core Axis for Glioma Cancer Stem-Like Cells Maintenance

Antonio Lucena-Cacace[1], *Masayuki Umeda*[1], *Lola E. Navas*[2,3] *and Amancio Carnero*[2,3]*

[1] Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan, [2] CIBERONC, ISCIII, Madrid, Spain, [3] Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío (HUVR), CSIC, Universidad de Sevilla, Sevilla, Spain

Glioma Cancer Stem-Like Cells (GSCs) are a small subset of $CD133^+$ cells with self-renewal properties and capable of initiating new tumors contributing to Glioma progression, maintenance, hierarchy, and complexity. GSCs are highly resistant to chemo and radiotherapy. These cells are believed to be responsible for tumor relapses and patients' fatal outcome after developing a recurrent Glioblastoma (GBM) or High Grade Glioma (HGG). GSCs are cells under replicative stress with high demands on NAD^+ supply to repair DNA, maintain self-renewal capacity and to induce tumor plasticity. NAD^+ feeds Poly-ADP polymerases (PARP) and NAD^+-dependent deacetylases (SIRTUINS) contributing to GSC phenotype. This energetic core axis is mainly controlled by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), an important oncogene contributing to tumor dedifferentiation. Targeting GSCs depicts a new frontier in Glioma therapy; hence NAMPT could represent a key regulator for GSCs maintenance. Its inhibition may attenuate GSCs properties by decreasing NAD^+ supply, consequently contributing to a better outcome together with current therapies for Glioma control.

Keywords: NAD, NAMPT, Glioma, GSCs, PARP, SIRT, TMZ, GBM

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Versha Banerji, University of Manitoba, Canada

***Correspondence:**
Amancio Carnero
acarnero-ibis@us.es

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INTRODUCTION

Gliomas are the most prevalent primary brain cancer in adults. Arising from glia cells, Gliomas involve a broad category of tumors including astrocytoma, oligodendroglioma, and glioblastoma (GBM). Regardless of tumor aggressiveness, malignancy and infiltration, these glia-derived tumors rarely exceed a median survival time of 12–14 months (1, 2). Driven by the infiltrative nature of these tumors, the clinical approach is difficult and relapses often occur with fatal consequences.

Therapeutic responses and patient survival rely on intratumoral heterogeneity ruled by genetic and epigenetic effectors. Nonetheless, there are many physiological barriers to the development of successful treatments. Blood-brain barrier (BBB) is a major limitation when it comes to deliver a chemotherapy-based treatment. Surgical resection is an ineffective long-term procedure since Gliomas infiltrate together with healthy brain tissue and their resection become virtually impossible. Invasive procedures compromise the patient's life quality and radiotherapy drives harmful side effects. As a final outcome, gliomas ultimately relapse in highly radio- and chemo-resistant forms.

These unsuccessful attempts to control glioma's fate have fostered research looking for more effective therapies.

Glioma Stem-like cells (GSCs) are Cancer initiating cells (CICs) maintaining self-renewal properties, sustaining cellular hierarchy, and partially explaining the tumor heterogeneity since they maintain a progeny responsible for tumor complexity (3, 4). GSCs express certain stem cell-like markers and were first characterized as $CD133^+$ cells able to initiate new tumors in mice (5). GSCs are chemo- and radio resistant, contributing to tumor relapse. Hence, targeting the molecular networks controlling GSCs maintenance is a promising new treatment frontier for glioma control.

According to the most recent World Health Organization for Glioma classification, GBM is labeled as a grade IV glioma (6). Although GBM often originates from 5 different cell types, neuronal stem cells, transit amplification cells, glial/neural progenitors, astrocytes, and oligodendrocytes (**Figure 1**), it may be classified into two types depending on its severity: primary and secondary GBM. A low grade diffuse astrocytoma (Grade II glioma) or an anaplastic astrocytoma (Grade III glioma) might evolve to a secondary GBM (**Figure 1**). Counting up to 95% of total cases, primary GBM tumors are the most frequent. Since primary and secondary GBMs display a markedly micro vascularization and high mitotic activity, they are mostly indistinguishable histologically. High mitotic activity turns Gliomas in very fast growing tumors. Since the growth rate is often greater than its angiogenesis capacity, the tumor's core is often hypoxic and necrotic (7). Nonetheless, secondary GBMs only count for 5% of the total cases.

Representing up to 16% of brain tumors, GBM risk and incidence are age-related. Normally it affects an average of 7.2 individuals per 100,000 adults aged 19 and above. Nonetheless, 14.6 individuals per 100,000 are aged between 74 and 84, representing the highest incidence peak (7).

Regardless of gender, GBM patient prognosis rarely exceeds 12 months after diagnosis. Nonetheless, GBM shows gender preference, being 40% more frequent in men (1, 2). Broadly, the standard therapeutic strategy for Gliomas consists of a primary resection surgery followed by radiotherapy and chemotherapy. However, since surgery is virtually unable to remove the neoplastic tissue, GSCs will ultimately reconstitute the whole tumor, leading to the consequence that 100% of patients end up with a relapse of the tumor in a peripheral area up to 2 cm from the focus of origin.

Following surgery, radiotherapy is often applied with Temozolomide (TMZ) as an adjuvant treatment. TMZ is a DNA alkylating agent able to permeate through the brain blood barrier (8). Since the early 2000s, it has been one of the few FDA approved drugs for GBM control, providing a survival advantage of 2.5 months. Recently, FDA approved Bevacizumab and Lomustine for recurrent GBM and Carmustine wafer for newly diagnosed high grade-malignant glioma (HGG) and recurrent GBM. To date, all these new treatments show inconclusive results regarding the benefits or survival advantages over TMZ, merely providing disease-related symptoms.

Even though treatments in other cancers have greatly evolved in recent years, the clinical approach to Glioma treatment remains challenging. Despite treatment, <5% of patients survive more than 5 years after the diagnosis.

An unmonitored cluster of gene expression data from 200 GBM samples from TCGA in 2011 established four subtypes of GBM according to their molecular profile: Proneural, Classical, Mesenchymal, and Neural (9, 10).

Proneural subtype is characterized by abnormalities in platelet-derived growth factor alpha receptor (PDGFRA) or in isocitrate dehydrogenase 1 (IDH1). The Classical subtype is mainly characterized by mutations in the epidermal growth factor receptor (EGFR). Mesenchymal subtype features mutations in neurofibromine 1 (NF1). Neural subtype was not completely defined but it is known to contain amplification and overexpression of EGFR. Nonetheless, molecular markers defining the neural subtype of GBM could be contaminated with normal neural tissue in the tumor margin, thus not representing a true subtype.

The mutational spectrum of GBM is varied. They are highly molecularly complex tumors and the number of coding mutations per tumor sample is assorted.

Ranging from 3 to 179 mutations per tumor, the average mutational burden per tumor is 53 (9). The most frequent mutations in GBMs are PTEN (29%), TP53 (29%), EGFR (20%), 21 NF1 (9%), RB1 (8%), phosphatidylinositol-4,5-bisphosphate alpha catalytic subunit - 3- kinase (PIK3CA; 7%), regulatory subunit 1 of 3-phosphoinositide (PIK3R1; 6%), and IDH1(5%) (11, 12).

All GBM subtypes and HGGs have in common a deep dependence on glucose consumption as the main source of metabolic energy. Gliomas are highly metabolic and rely on glycolytic pathways. This is why most gliomas may have a strong dependence on NAD^+ metabolism as the main intermediary in the reduction-oxidation reactions (13, 14). Additionally, GSCs are cells under replicative stress with high demands on NAD^+ supply to repair DNA, maintain self-renewal capacity and to induce tumor plasticity. NAD^+ feeds PARP and SIRTUINS contributing to GSC phenotype. This energetic core axis is mainly controlled by the rate-limiting enzyme NAMPT, an important oncogene contributing to tumor dedifferentiation.

In addition, NAMPT is particularly overexpressed in mesenchymal GSCs, where its expression is correlated to a hypomethylation state driven by depletion of methionine and *de novo* methyltransferases, sustaining mesenchymal GSCs rapid growth and NAM consumption to support NAD^+ utilization and sustain DNA hypomethylation (15), another marker of poor GBM prognosis.

In this review we will explore the importance of NAD^+ as core axis for Glioma Cancer Stem-Like Cells maintenance and how targeting NAMPT over GSCs could represent a promising new frontier therapy for Glioma control.

MALIGNANT CLONAL EVOLUTION ON GLIOMAS

Intertumoral diversity relies on a particular clonal evolution driven by a cell of origin acquiring cancer-initiating capacity

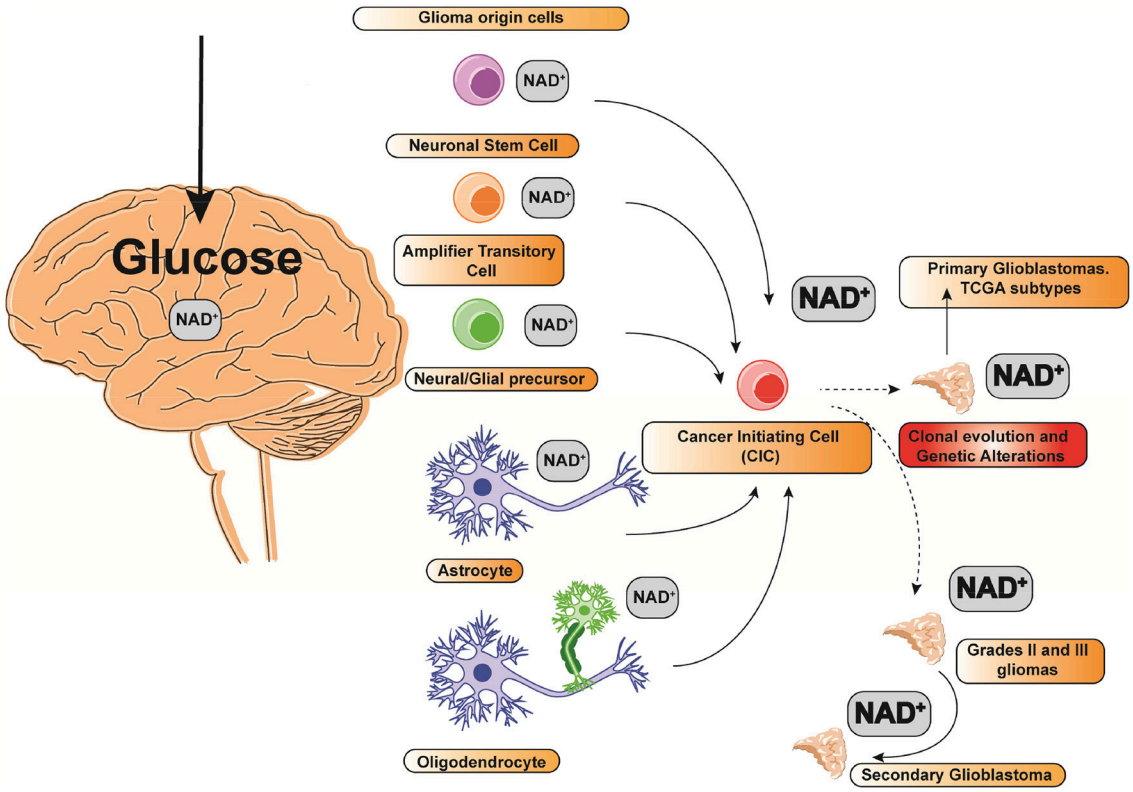

FIGURE 1 | Glioma cell(s) of origin and NAD^+ relative consumption. The size of the font indicates the relative NAD^+ consumption.

(**Figure 1**) (16). Whether specific glioma cells of origin are susceptible to certain cancer-initiating mutations driving to GSCs is unclear. Nonetheless, there are many studies suggesting that certain progenitors and stem cells are markedly susceptible to a variety of different mutations (**Figure 1**) (17). Some cells of origins might potentially display a preferential vulnerability to specific mutations. To note, TCGA molecular subtypes are augmented for lineage markers characteristic of distinct glia-derived cell types, suggesting that molecular and/or epigenetic profiles of the Glioma initiating cell are maintained during tumorigenesis (18). Clonal evolution on Gliomas can be an important determinant defining tumor phenotype and genotype in both GBM and HGGs. Nonetheless, given the diversity of driver mutations representing different subtypes of GBM, the contribution of the cancer initiating cell is unknown (**Figure 1**) (11). Indeed, recent studies in adult neural stem cells (NSCs) and oligodendrocyte precursor cells (OPCs) reveal that they might have different transformation capacity as a cellular origin candidacy since their self-renewal capacity in the adult human brain is again under re-evaluation (19). Any cell type out of the five candidates to drive into a primary GBM can evolve to a defined molecular GBM subtype, mainly depending on their driver mutation leading to the cancer-initiating phenotype (19) (**Figure 1**). Since single GBM cells are not mere genetic phenocopies, analyses from tumor cells taken from the same patient show notably heterogeneous tumors consisting of mutant cells carrying different genetic burden, expression of different cell markers and different levels of aneuploidy (20). The inherent heterogeneity and complexity of GBM, HGG, and GSCs show a variable expression of transcriptional programs embracing different cellular processes involving cell cycle, hypoxia, and immune signaling (10).

Alterations such as chromosomal aberrations, genomic rearrangements, and focal copy number aberrations can give rise to GSCs and eventually to a glioma.

GSCs are a subpopulation of cells that explain part of tumor heterogeneity. Circulating GSCs also display stem cell-like properties (21). They are cells with capacity for differentiation and self-renewal, responsible for the hierarchical clonal development and able to regenerate *de novo* tumor from a single cell. The metabolism of NAD^+ could play a relevant role in the mechanisms associated with chemoresistance in CICs and their maintenance mechanisms.

NICOTINAMIDE ADENINE DINUCLEOTIDE METABOLISM

Four major molecules are used as substrates for the synthesis of NAD^+. These molecules are dietary tryptophan (L-Trp), nicotinic acid (NA), nicotinamide (NAM), and nicotinamide riboside (NR) (22).

These four major metabolic molecules are involved in the synthesis of NAD^+ through two major pathways:

De Novo Pathway and Salvage Pathway. Some metabolic intermediates such as nicotinamide mononucleotide (NMN) might also stimulate the direct synthesis of NAD^+ (**Figure 2**) (23, 24).

De novo synthesis of NAD^+ takes place intracellularly in an eight-step reaction (25–29). This pathway takes the L-Trp acquired through daily diet as a conversion molecule when obtaining NAD^+. Tryptophan-derived quinolinic acid is produced and used by quinolinate phosphoribosyl transferase (NAPT) to form nicotinic acid mononucleotide (NAMN). NAMN is converted to nicotinic acid adenine mononucleotide (NAAD) in a NMN adenylyltransferase (NMNAT) -mediated reaction with ATP consumption (25). There are three isoforms of NMNAT (NMNAT 1-3) with different tissue and cellular locations depending on the metabolic requirements (30). NMNAT-1 is an ubiquitously expressed nuclear protein. NMNAT-2 is normally present in the golgi apparatus and the cytosol (31–35). NMNAT-3 may be present in both the cytosol and mitochondrial compartments. The efficiency of obtaining NAD^+ by dietary tryptophan is very low compared to that obtained by the Salvage pathway.

This NAD^+ synthesis pathway is also known as the Preiss-Handler pathway. In this pathway the NAD^+ is generated from Niacin (Vitamin B3) in a three-step reaction (36). NAM can also be a precursor of NAD^+ through its conversion to NMN by the limiting enzyme nicotinamide phosphoribosyl transferase (NAMPT). NMN acts as an intermediate by catalyzing the reversible addition of a ribose group from 5-phosphoribosyl-D-ribosyl-1-pyrophosphate to NAM. In the mitochondrial respiratory chain, NADH acts as the main donor of electrons, which end up in the generation of ATP by oxidative phosphorylation (37–39).

The liver is the major organ involving NAD^+ metabolic activity as it expresses all enzymes for either metabolism or recycling. Hepatic cells can actually convert all its precursors: NA, NAM, and their ribosides as well as L-Trp. For that, NAMPT and NAPRT mRNA levels are particularly high in the liver, so this organ is one of the major NAD^+ recycling and synthesis engine cores in humans (40, 41).

All tissues have the potential to at least convert NAM and NR into NAD^+. That is why the NAMPT enzyme and at least two NMNAT isoforms are ubiquitously expressed in all cells and tissues. Some others, such as NMNAT2, are brain specific (42). Other NMNAT isoforms are expressed in a greater proportion in the pancreas, thyroid gland and lymphocytes (31). In mammalian cells, the metabolism of NAD^+ is compartmentalized. The formation of NAD^+ from NMN takes place in the nucleus and in the mitochondria (37, 38, 43). These two organelles are particularly important as the most important NAD^+-dependent intracellular signaling pathways occur in them.

The most important cytosolic NAD^+ precursor for mitochondrial synthesis is NMN. Therefore, NAD^+-dependent cellular processes are intimately linked to the most important molecular events that could lead to cancer: genomic alterations, metabolic imbalances, and changes in the transcription patterns of candidate oncogenes or tumor suppressors.

Nicotinamide phosphoribosyl transferase (NAMPT) is the limiting enzyme of the NAD^+ Salvage Pathway, the major NAD^+ source in living cells, being the major contributor to NAD^+ maintenance, recycling, and homeostasis.

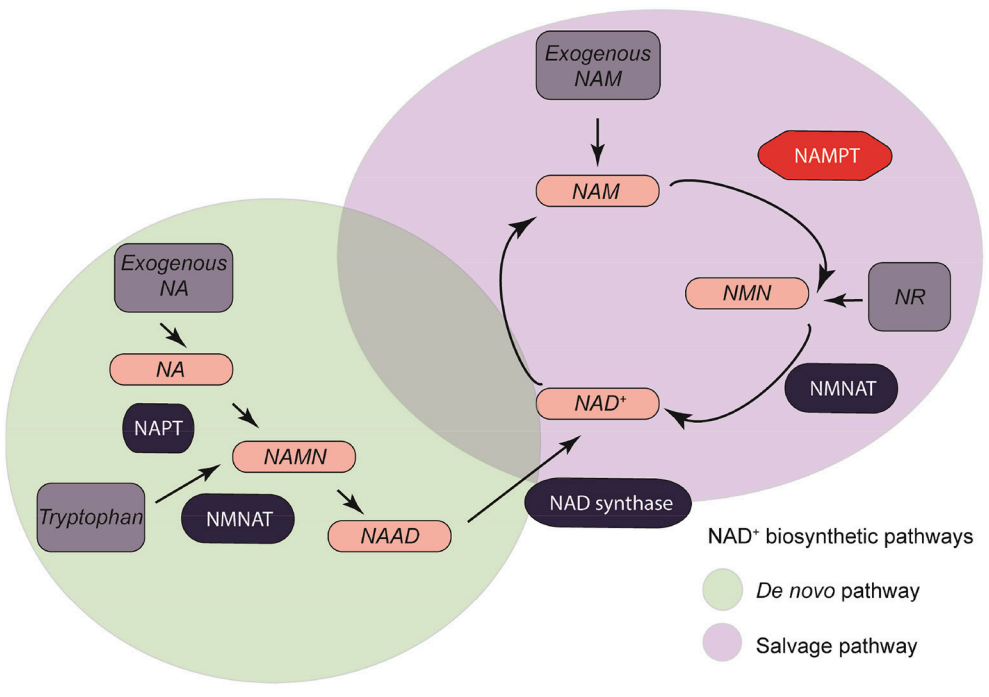

FIGURE 2 | NAMPT/Nicotinamide adenine dinucleotide metabolism. Four major synthesis precursors (Exogenous NA, exogenous NAM, NR, and Tryptophan, dark gray) are divided between two major pathways: *de novo* pathway (light green), and salvage pathway (light purple).

NAMPT is a highly conserved protein among mammals. It was cloned and isolated for the first time in the organism Haemophilus ducreyi and it has been extensively studied.

It was originally characterized as the human homologous protein pre-B cell colony-enhancer factor (PBEF). In its role as PBEF it acts as a cytokine, which stimulates early B cell formation in a synergistic effect with interleukin 7 (IL-7) and stem cell factor (SFC) (44, 45).

The gene encoding NAMPT is found on human chromosome 7, specifically at the 7q22.3 locus. The size of the gene within the DNA is 3.7 kilobases (kb) and contains 11 exons and 10 introns that encode a coding DNA (cDNA) of 2.357 kb. The protein has a weight of 52 kilodaltons (kDa) and contains 491 amino acids (aa) (46). The protein lacks a cellular export signal and also contains 6 cysteine residues, so it has been suggested a structure of the same as zinc finger.

In silico, up to 13 messengers of different NAMPT RNAs (mRNA) are predicted by alternative splicing mechanisms, of which only four have been found at the biological level. Of the four, only the first messenger translating into the 491 amino acid protein is able to make the conversion to its enzymatic product NMN.

NAMPT is expressed in all tissues, with higher levels in bone marrow, liver, and muscle fiber cells, where the energy intake is greater.

The extracellular form of NAMPT, PBEF, or Visfatin is known as extracellular NAMPT (eNAMPT, as opposed to intracellular NAMPT, iNAMPT), an adipocytokine that is expressed in visceral fat tissue and whose circulating levels correlate with obesity (43). The extracellular role of NAMPT is unknown and could have a function of activation or silencing in signaling pathways within the cell other than those related to its enzymatic function such as NAMPT.

As a limiting enzyme of the pathway which plays a key role in the maintenance of intracellular NAD+, NAMPT could be an oncogene contributing to the onset, progression and relapse of cancer (47).

NAMPT IN CANCER

NAMPT is overexpressed in a broad range of solid tumors including colorectal, ovarian, breast, gastric, prostate, well-differentiated thyroid cancers, melanoma, gliomas, and endometrial carcinomas (48–51). Clinically, higher NAMPT expression is associated with worse prognosis correlating with tumor growth, metastases and cellular dedifferentiation in melanoma (52, 53). High levels of NAMPT have been found in hematological malignancies such as diffuse large B-cell lymphoma, Hodgkin's lymphoma, follicular B-cell lymphoma, and peripheral T-cell lymphoma. In these tumors, it associates to a more aggressive malignant lymphoma phenotype (54).

Besides, NAMPT levels have been associated to increased chemoresistance to certain therapeutic agents such as doxorubicin, paclitaxel, etoposide, fluorouracil, and phenylethyl isothiocyanate (55, 56).

Many studies have shown that NAD^+ depletion by NAMPT inhibition causes cell death through apoptosis. Many pro-apoptotic proteins were found activated when NAMPT is inhibited in leukemias, multiple myeloma, breast cancer, and lymphoma cells (57–64). It has been found that NAMPT inhibition-mediated apoptosis requires functional apoptotic machinery because blocking apoptosis with several factors such as: L-type calcium channels with verapamil or nimodipine, capase 3 with Z-Asp-Glu Val-Asp-fluoromethylketone, capase 9 with Z-Leu-Glu-His-Asp-fluoromethylketone, or the mitochondrial permeability transition with bongkrekic acid blocks the effect of NAMPT inhibition-mediated apoptosis (62, 64).

Three NAMPT inhibitors (APO866/FK866, GMX1778, and GMX1777) entered clinical trials and completed phase I, however, further evaluation was discontinued primarily due to dose-limiting toxicities (ClinicalTrials.gov identifiers: NCT00457574, NCT00724841, NCT00432107, NCT00435084, NCT00431912).

More enzymes of the salvage pathway have been suggested to be potential therapeutic targets in cancer. Mitochondrial NMNAT3 knockdown had minimal effect over mitochondrial NAD+ levels (37, 65, 66). On the other hand, NMNAT2 cytosolic inhibition decreased mitochondrial NAD+ levels, suggesting that NAD+ in the mitochondrial is partially supported by NAD+ intake from the cytosol (65). However, shortly after it was found that NAMPT inhibition had no effect on mitochondrial NAD^+ pool, discarding the previous theory, and highlighting the role of NAMPT as the main potential target of the pathway in cancer by depleting NAD^+ pool (40). NAMPT inhibition seems to be particularly effective over cells harboring naturally high glycolysis (67).

CAUSAL ROLE OF NAD+ IN GLIOMA STEM-LIKE CELLS TRANSFORMATION

NAD^+ is an important cofactor for cells requiring high energetics demands and helps to maintain a proficient neural function. Indeed, the increase in NAD^+ levels shown as Nicotinamide nucleotide transhydrogenase (NNT) and Nicotinamide nucleotide adenylyltransferase 3 (NMNAT3) delays senescence in mesenchymal stem cells (68). In relation, it is known that NAMPT and NAD^+ biosynthesis decreases with age in the hippocampus (69). Also, NAMPT downregulation and NAD^+ depletion reduces pool and proliferation of Neural stem progenitor cells (NSPCs) *in vivo* (70). Increased NR decreases senescence in both neural and melanocyte stem cells by improving mitochondrial function relying on SIRT1 function (68). It has been reported that NAD^+ replenishment reduces the severity of Ataxia telangiectasia (A-T) neuropathology, normalizing neuro-muscular function, extending lifespan in animal models, and delaying memory loss (71). Moreover, intracellular NAD^+ levels stimulates neural DNA repair through PARP proteins and improves mitochondrial activity via mitophagy (71).

Cancer cells exhibit a dependency on metabolic pathways regulated by NAD^+. Nonetheless, the regulatory network interfacing with signal transduction remains poorly understood in GBM and HGG. NAMPT downregulation reduces *in vivo* tumorigenicity (72). Indeed, RNA-seq reveals the transcription factor E2F2 in the center of an NAD^+-dependent transcriptional

network, required to self-renewal maintenance in GSCs. Interestingly, downstream to E2F2 we can find members of the inhibitor of differentiation (ID) helix-loop-helix gene family (72).

NAMPT and NAD^+ levels also mediate GSCs radio-resistance. NAD^+ pool decreases with aging and their levels are critical in cell bioenergetics and adaptive stress responses (73).

Another important consequence driven by NAD^+ supply is the epigenetic reprogramming in tumors. Nicotinamide N-methyltransferase (NNMT) is a critical node in methyl donor metabolism and is markedly upregulated in GBM (15). NNMT is also overexpressed in mesenchymal stem cells. Increases in NNMT lead to a decrease in S-Adenosyl methionine (SAM), a methyl donor generated from methionine. GSCs show a decrease in methionine and SAM, thereby decreased levels of Nicotinamide (15). However, GSCs show notably increased NAD^+ levels and the dramatic hypomethylation state in GBM, leading tumors to shift toward mesenchymal phenotype and accelerated growth, where NAMPT is particularly overexpressed (15). Targeting NNMT decreases cellular proliferation and diminishes methyl donor availability, thus decreasing methionine levels. This fact also leads to the induction of decreasing unmethylated cytosine levels, increased DNA methyltransferases and induced expression of *de novo* methylases like DNMT1 and DNMT3A (15).

NAMPT is ultimately required for G1/S progression of the Neural Stem Progenitor Cells (NSPC) cell cycle (69). Indeed, NAMPT is crucial for oligodendrocytic lineage fate decisions through an overlapping mechanism mediated by Sirt1 and Sirt2. Reported studies on this topic proved that NAMPT downregulation *in vivo* leads to a decreased NSPC-mediated oligodendrogenesis (69).

ROLE OF SIRTUINS IN GLIOMA PROGRESSION

Sirtuins are NAD^+ dependent deacetylases that regulate numerous cellular processes including aging, cell cycle, metabolism, DNA repair, and survival under stress conditions. Nonetheless, the role of Sirtuins in glioma remains unclear, with some studies counteracting others. Whether they act as an oncogene or tumor suppressor in Glioma progression is unclear, but their contribution seems to be correlated with tumor heterogeneity.

On one hand, SIRT1 is characterized by some authors as a promoter factor in tumorigenesis of human glioma. The role of SIRT1 in glioma may be related with PTEN/PI3K/Akt axis promoting tumorigenicity (74).

On the other hand, some studies point that SIRT2 is required for GSCs proliferation arrest, highlighting a potential tumor suppressive effect over GSCs (75). Nonetheless, Funato et al. proposed a mechanism where SIRT2-mediated inactivation of p73 is required for GBM tumorigenicity (76).

Accordingly, Li et al. found that SIRT2 expression is markedly down-regulated in human glioma. Indeed, its expression decreases cell growth and colony formation via apoptosis. Mechanistically, they claim that mIR-21 is essential for the functions of SIRT2 in these tumors. SIRT2 specifically deacetylases p65 to decrease mIR-21 expression (77). They conclude in their study that SIRT2 suppresses glioma cell growth through targeting NF-kB-mIR-21 axis (77).

Other Sirtuins like SIRT6 suppresses cell proliferation, migration, and invasion via inhibition of oxidative stress through NOTCH3 (78) or inhibition of the activation of the JAK2/STAT3 pathway in glioma (79).

Mechanistically, miR-33a targets SIRT6 and promotes tumor development in human glioma by regulating its expression (80).

REPLICATION STRESS: ROLE OF PARP PROTEINS IN GLIOMA PROGRESSION

GSCs are Mismatch repair-proficient (MMR-proficient) cells. TMZ-resistant GSCs are either O^6-methylguanine-DNA methyltransferase (MGMT) active or displaying low proliferation rate. MMR-proficiency leads GSCs to select networks to effectively repair DNA driven by TMZ and radiotherapy (81). GSCs are also highly radioresistant. This radio resistance was originally overcome for the first time through CHK1 and CHK2 inhibition (82).

NAD^+ plays a key role enhancing Base excision repair (BER) pathway through Poly(ADP-ribose) polymerases (PARPs) (83). PARP1 expression is increased in GBM at both mRNA and protein level. Increased PARP1 levels show a positive correlation with increasing tumor grades in Gliomas. PARP1 is ultimately essential for DNA repair during TMZ-based treatment and radiation therapy (**Figure 3**) (83). PARP1 expression is associated with TP53 and ataxia telangiectasia—Rad3 related kinase (ATR) mutations (84). GSCs display elevated basal levels of activated ATR and CHK1 along with increased replication stress (RS) expression markers including foci marked with the single-stranded DNA binding protein, replication protein A, DNA damage markers γH2AX and 53BP1 (82). PARP1 is mainly overexpressed in Proneural and Classical GBM subtypes. PARP1 overexpression decreases OS in patients with classical type GBM (84). GSCs display a heightened DNA damage response (DDR) with a markedly enhanced replication stress. In order to overcome radiation resistance relying on G_2-M activation, combined inhibition of ATR and PARP inhibitors proved to be effective (85–87). Since chemical inhibition of PARP1 through Olaparib also impairs BER, it significantly enhances TMZ-induced damage (**Figure 3**), exerting synergistic anti-tumor effects in GSCs lines.

NAD^+ availability is substantially decreased in IDH1-mutants', thus, PARP1-associated DNA repair pathway is compromised. Targeting DNA repair pathway through PARP inhibition sensitizes IDH1-mutated glioma cells to TMZ in combinational therapy (83).

When radio- or chemo-driven DNA damage occurs, PARP1 and PARP2 enzymes are recruited as a systemic damage response to bind to ssDNA breaks and transduce signals within

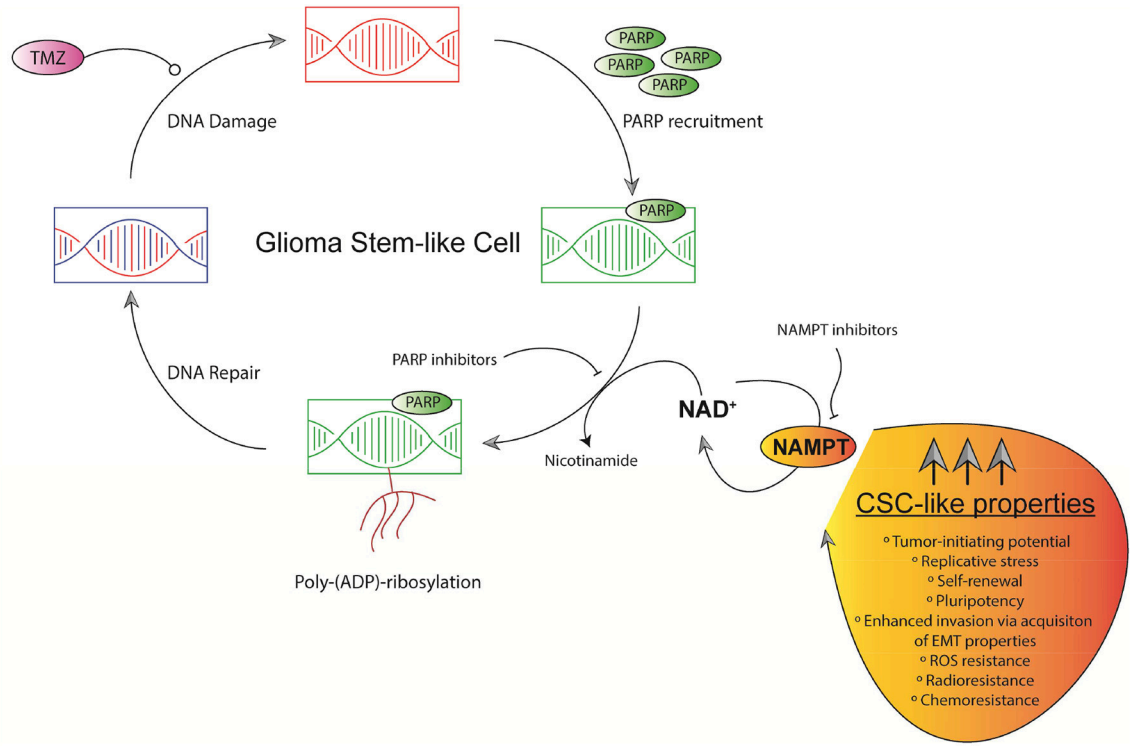

FIGURE 3 | Schematic representation of GSCs' replication stress and NAD^+-dependent DNA repair mechanisms. TMZ induces DNA damage in chromosomal DNA in GSCs. PARPs-based DNA repair defines chemosensitivity. PARPs efficacy relies on NAD^+ levels mainly controlled by NAMPT. NAMPT dually governs NAD^+ production and maintain CSC-like properties in GSCs.

the DDR pathways (82). Once the bond to ssDNA breaks, damaged cells activate PARP1/2 to post-translationally modify themselves together with proteins by synthesizing negatively charged poly(ADP-ribose) chains.

Poly-ADP ribosylation recruits proteins involved in ssDNA break repair, for example XRCC1 modifying chromatin structure. Regulating fork stabilization and restart, PARP proteins work on DNA replication, RS response ligation of lagging strand Okazaki fragments, elongation velocity, and homologous recombination repair of stalled DNA replication forks (88–92).

PARP "trapping" through PARP inhibitors leads to loss of auto-PARylation, consequently facilitating removal of PARP protein from DNA. ATR inhibitors decreases GSCs formation *in vitro*. PARP inhibitors plus ATR inhibitors dramatically decrease GSC formation and sensitize to radiotherapy. This phenotype is greater in GSCs-$CD133^+$ cells compared to bulk population (82, 93, 94).

To note, given the well-characterized immunosuppressive tumor microenvironment associated with GBM, ATR treatment may present two weapons against this disease: targeting the addiction to the DDR pathway and reinvigorating T cells to attack GBM cells following radiotherapy (87, 95).

One of the major limitations establishing PARP or ATR inhibitors as a standard therapy is the BBB, which means an ineffective drug penetration into the central nervous system.

NAMPT AS A DEDIFFERENTIATION-INDUCER GENE IN GLIOMA

NAMPT is markedly overexpressed in HGG and GBM tumors, correlating with tumor grade and able to predict patients' prognosis. Ectopic overexpression of NAMPT in Glioma cell lines increases tumorigenic properties controlling stem cell pathways and enriching the GSCs population (**Figure 3**) (96). Given the infiltrative nature of GBM portraying high plasticity, many studies have tried to focus on identifying key factors governing self-renewal properties driving to tumor relapses. Gujar et al. demonstrated that a NAD^+ transcriptional network governs self-renewal properties in GSCs and radiation resistance in GBM (72). NAMPT expression, indeed, correlates with high levels of NANOG as a final effector of pluripotency and stem cell-like properties, $CD133^+$, and GSCs in primary GBM tumors. NAMPT is also a key factor inducing cancer stem-like pathways in glioma cells. NAMPT also increases number of *de novo* GSCs formation (96). Moreover, NAMPT is also a key factor inducing cancer stem pathway effectors in colon cancer tumors. In colon cancer, this phenotype is mediated by PARP and SIRT1. NAMPT also increases the number of tumorspheres *in vitro* in colon cancer cells lines, correlating with important endpoints of CSC pathways activation (97).

CURRENT THERAPIES ON GLIOMA TREATMENT

Sixty gray (Gy) dose of radiotherapy following the maximum safe surgical resection provides the highest benefits regarding GBM median survival (98). TMZ addition after radiotherapy or in concurrence is the only regimen markedly improving the patients' overall survival (OS) (99, 100). The major relevant biomarker predicting response to TMZ treatment is MGMT (O^6- methylguanine-DNA methyltransferase), a gene involved in DNA-repair (101). Silencing MGMT expression through promoter methylation impairs the ability to repair TMZ-driven DNA damage, decreasing tumor cell survival (102).

Since GBM tumors are often overexpressing numerous angiogenic effectors, Bevacizumab, a humanized antibody binding to vascular endothelial growth factor A (VEGF-A) is effective in impairing tumor angiogenesis. Bevacizumab is actually the only FDA approved agent for recurrent GBM (103, 104). Irinotecan (topoisomerase I inhibitor) in combination with Bevacizumab proved an increase in OS from 4.1 to 9.2 months in a phase II clinical trial (104). 6 and 12 months' survival rates were 77 and 31%, respectively (105, 106). Bevacizumab has been also tested in phase III trials for newly diagnosed GBM. However, there was no effect on patients' OS. TMZ administration in concurrence with Bevacizumab on newly diagnosed GBM is being tested (NCT00943826 and NCT00884741) (106, 107).

Proven that most of the current attempts on therapies seem to be insufficient to achieve relevant outcomes regarding OS, targeting NAD^+ through NAMPT as the central core axis of GSCs energetics maintenance could represent a ground-breaking therapy approach.

TARGETING NAMPT ON GLIOMA CANCER STEM-LIKE CELLS FOR GLIOMA CONTROL

NAMPT is markedly overexpressed in HGG and GBM. NAMPT overexpression is correlated with patient survival (96). NAMPT downregulation triggers a markedly decrease in *in vivo* tumorigenicity and induces a decrease in GSCs self-renewal capacity (72). Indeed, first generations of tumorspheres *in vitro* are particularly sensitive to NAMPT inhibitors, particularly tumorspheres with high levels of NAMPT expression. Unlike glioma cells, NAMPT inhibitors will ultimately target cells in an active cell cycle under replicative stress, two main hallmarks of GSCs (96). This fact potentially ensures that anti-NAMPT therapies in either monotherapy or in combination with TMZ or PARP inhibitors will be ultimately effective. Targeting proliferating cells relying on NAD+ may have effects over GSCs in two main ways: first, by suppressing self-renewal properties based on NAD+ pool restoration and recycling and second, by taking down ADP-ribosylation required for DNA repair process through PARP recruitment.

CONCLUSION AND PERSPECTIVES

Gliomas are the most prevalent primary brain cancer in adults and include a broad category of tumors including astrocytoma, oligodendroglioma, and GBM. Regardless of tumor aggressiveness, malignancy, and infiltration, these glia-derived tumors rarely exceed a median survival time of 12–14 months. Driven by the infiltrative nature of these tumors, the clinical approach is difficult and relapses often occur with fatal consequences. These unsuccessful attempts to control glioma's fate have fostered research looking for more effective therapies.

(GSCs) are a small subset of $CD133^+$ cells with self-renewal properties and capable of initiating new tumors contributing to Glioma progression, maintenance, hierarchy and complexity. GSCs are highly resistant to chemo and radiotherapy. These cells are believed to be responsible for tumor relapses and patients' fatal outcome after developing a recurrent GBM or High Grade Glioma (HGG). GSCs are cells under replicative stress with high demands on NAD^+ supply to repair DNA, maintain self-renewal capacity and to induce tumor plasticity. NAD^+ feeds Poly-ADP polymerases (PARP) and NAD^+-dependent deacetylases (SIRTUINS) contributing to GSC phenotype. Ablation of NAMPT showed to impair Schwann cell differentiation program associated to reduced NAD^+ levels, counteracting the phenotype found in tumor cells but reinforcing the idea that an adequate NAD^+ level is required for a fine tuned balance on cell-fate definition toward differentiation programs (108). This energetic core axis is mainly controlled by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), an important oncogene contributing to tumor dedifferentiation. Supporting these conclusions, Jung et al. also highlight the importance of the epigenetic reprogramming on GSC, supported by a high expression of NNMT together with NAMPT counteracting with a hypomethylation state, preferentially occurring within mesenchymal GBM subtype, where NAMPT shows its maximum expression profile (96). They raise the idea that one causal role of methionine depletion, an important upstream methyl donor, is to ultimately drive GBMs to evolve to mesenchymal subtype, where NAMPT plays a key role promoting tumor growth and dedifferentiating tumors (15).

We have queried public datasets to dissect these proposed mechanisms at a single cell level. For that, we have analyzed a single-cell RNA-Seq dataset from Darmanis et al. (109) performing comparative transcriptomic analysis of neoplastic and stromal cells within and proximal to primary GBMs (109).

In this dataset, three clusters out of 11 are identified as tumor clusters (**Figure 4A**). We found that NAMPT is particularly expressed in vascular, myeloid, and tumor cells (**Figure 4C**). Within tumor cells, NAMPT is particularly overexpressed in cluster 9 and 10 but not in cluster 11 (**Figure 4B**), which is a cluster with a pro-neural profiling and therefore with low NAMPT levels, in accordance with the results showed by Lucena et al. and Jung et al. Cluster 10 is classified as GSCs subset, where NAMPT is highly overexpressed. According to the high contribution to data dimensionality of XY axis in the t-distributed stochastic neighbor embedding (tSNE), the GSC cluster shows a high heterogeneity, reinforcing the idea

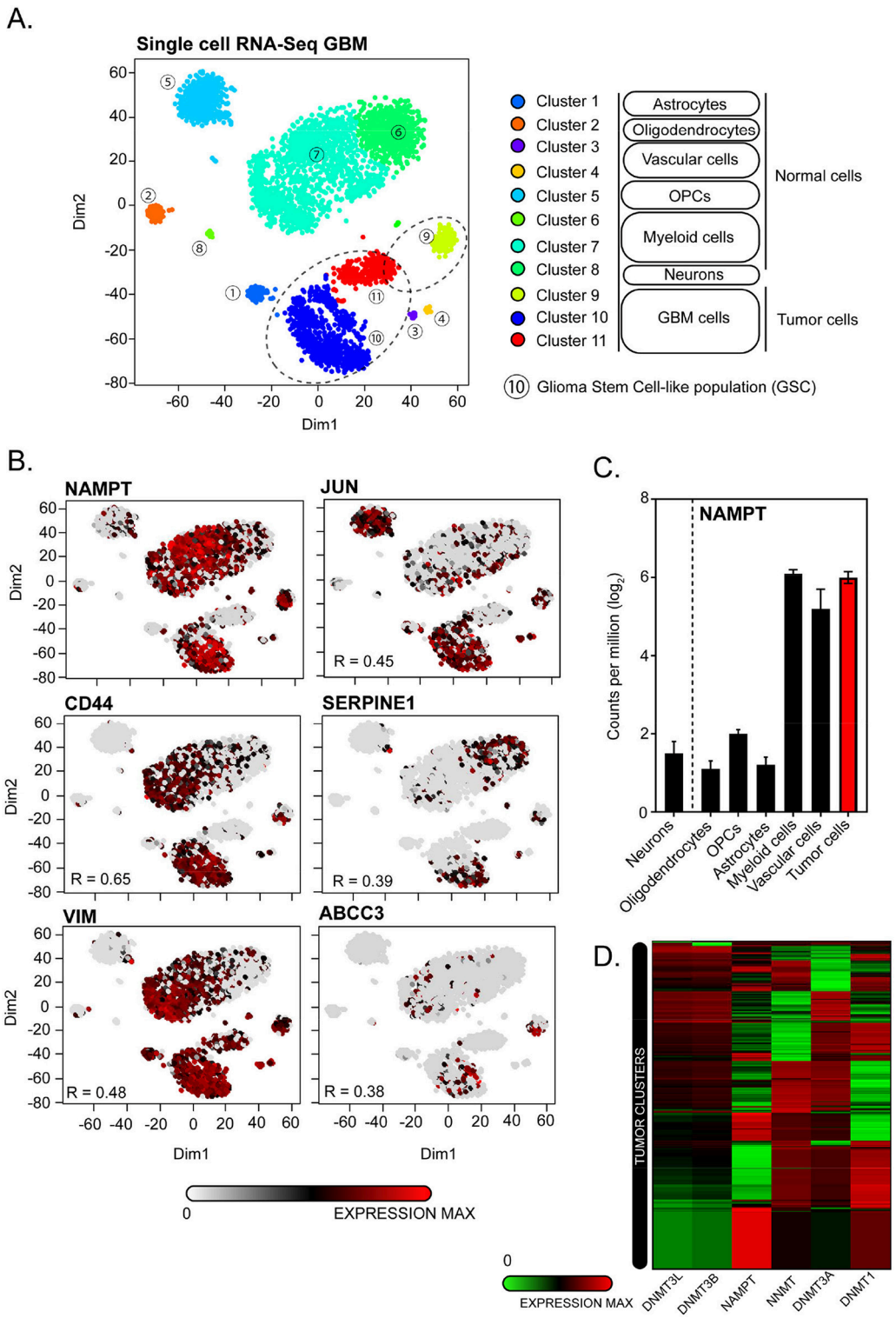

FIGURE 4 | Single-cell transcriptomic analysis of GSE84465 for *NAMPT* expression in GBM. **(A)** 3,589 cells in a cohort of 4 patients are categorized by cell identity (Normal vs. Tumoral) and clusters based on differences on transcriptomic profiles based on heterogenic distribution on dimensions 1 and 2 of t-distributed stochastic neighbor embedding (tSNE). Three tumors clusters are highlighted on dashed circles. Cluster number 10 is transcriptionally allotted to GSC-cells subset. **(B)** Single-cell transcriptomic distribution for GSC-related markers expression in correlation to *NAMPT* expression. **(C)** *NAMPT* expression analysis classified by different cell identities from normal and tumor tissue. **(D)** Supervised hierarchical clustering of *NAMPT*, *NNMT*, *DNMT1*, *DNMT3A*, *DNMT3B,* and *DNMT3L* mRNA expression in tumor cell clusters (9, 10, and 11).

that GSCs rely on a high diversity in terms of molecular-profiling, sustaining an ever-changing identity contributing to tumor plasticity. With the aim of confirming the expression of factors that induce cell stemness and pluripotency in correlation to NAMPT levels, we observed a clear transcriptional activation of JUN, CD44, SERPINE1, VIM, and ABCC3 putative markers of GSCs maintenance as previously described by Lucena et al. within the tumor clusters.

In addition, NAMPT expression negatively correlates with *de novo* methyltransferases DNMT1, DNMT3A, DNMT3B, and DNMT3L, supporting the hypothesis raised by Jung et al. where the poor outcome of GBMs is related to a hypomethylation state driven by overexpression of NAMPT controlling NAD^+ pool since mesenchymal GSCs rapidly consume NAM to support NAD^+ utilization and sustain DNA hypomethylation (**Figure 4D**) (15).

Altogether, targeting GSCs depicts a new frontier in Glioma therapy; hence NAMPT could represent a key regulator for GSCs maintenance. Its inhibition may attenuate GSCs properties by decreasing NAD^+ supply, consequently contributing a better outcome together with current therapies for Glioma control.

AUTHOR CONTRIBUTIONS

AL-C and AC conceived and designed the project. AL-C, MU, and LEN collected, processed, and analyzed experimental data. AL-C and AC wrote the manuscript. All authors discussed the results.

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REVIEW
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Transcriptional Reprogramming and Novel Therapeutic Approaches for Targeting Prostate Cancer Stem Cells

*Gianluca Civenni, Domenico Albino, Dheeraj Shinde, Ramiro Vázquez, Jessica Merulla, Aleksandra Kokanovic, Sarah N. Mapelli, Giuseppina M. Carbone and Carlo V. Catapano**

Institute of Oncology (IOR), Università della Svizzera Italiana, Bellinzona, Switzerland

Prostate cancer is the most common malignancy in men and the second cause of cancer-related deaths in western countries. Despite the progress in the treatment of localized prostate cancer, there is still lack of effective therapies for the advanced forms of the disease. Most patients with advanced prostate cancer become resistant to androgen deprivation therapy (ADT), which remains the main therapeutic option in this setting, and progress to lethal metastatic castration-resistant prostate cancer (mCRPC). Current therapies for prostate cancer preferentially target proliferating, partially differentiated, and AR-dependent cancer cells that constitute the bulk of the tumor mass. However, the subpopulation of tumor-initiating or tumor-propagating stem-like cancer cells is virtually resistant to the standard treatments causing tumor relapse at the primary or metastatic sites. Understanding the pathways controlling the establishment, expansion and maintenance of the cancer stem cell (CSC) subpopulation is an important step toward the development of more effective treatment for prostate cancer, which might enable ablation or exhaustion of CSCs and prevent treatment resistance and disease recurrence. In this review, we focus on the impact of transcriptional regulators on phenotypic reprogramming of prostate CSCs and provide examples supporting the possibility of inhibiting maintenance and expansion of the CSC pool in human prostate cancer along with the currently available methodological approaches. Transcription factors are key elements for instructing specific transcriptional programs and inducing CSC-associated phenotypic changes implicated in disease progression and treatment resistance. Recent studies have shown that interfering with these processes causes exhaustion of CSCs with loss of self-renewal and tumorigenic capability in prostate cancer models. Targeting key transcriptional regulators in prostate CSCs is a valid therapeutic strategy waiting to be tested in clinical trials.

Keywords: prostate cancer, cancer stem cells, transcription factors, ERG, ESE3/EHF, c-Myc, STAT3, NF-κB

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Cyril Corbet,
Catholic University of Louvain, Belgium

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Vincent Goffin,
INSERM U1151 Institut Necker Enfants Malades Centre de Médecine Moléculaire (INEM), France
Collene Jeter,
University of Texas MD Anderson Cancer Center, United States

****Correspondence:***
Carlo V. Catapano
carlo.catapano@ior.usi.ch

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INTRODUCTION

To date there is compelling evidence supporting the presence of tumor-initiating, tumor-propagating stem-like cells or cancer stem cells (CSCs) in human cancers (1, 2). At any given time, the CSCs likely constitute only a minority of tumor cells within the tumor mass (1, 2). However, CSCs contribute substantially to the biological and clinical heterogeneity of human cancers (3, 4). The CSC model proposes that tumor

cells maintain a lineage hierarchy similar to normal tissues (2). The small population of stem-like cancer cells that sustain this hierarchical organization is able both to self-renew by symmetric cell division and to produce, through asymmetric cell divisions, phenotypically distinct daughter cells with limited self-renewal but greater proliferative activity (**Figure 1A**). Similar to normal stem cells, the balance between self-renewal, differentiation, and senescence is essential to maintain the CSC subpopulation (2). Importantly, these processes lead to the expansion and maintenance or, alternatively, to progressive loss of proliferative potential and exhaustion of the CSC pool. According to the stem cell model, CSCs are key elements driving tumor heterogeneity and contributing to tumor progression and metastases (2, 4). Importantly, CSCs contribute substantially to treatment failure and disease recurrence by virtue of their intrinsic resistance to chemotherapy, radiotherapy, and even molecular-targeted drugs (2, 3, 5). Despite even massive reduction of bulk tumor cells after effective treatment, the CSC subpopulation can survive, expand and reconstitute, through a combination of symmetric and asymmetric cell divisions, the population of bulk tumor cells leading to tumor re-growth and relapse (**Figure 1B**). Indeed, the inability of current therapies to affect the CSC subpopulation contributes to their limited success and the almost inevitable progression to treatment-resistant disease. In this scenario, a significant increase in treatment efficacy, duration of clinical response, and patient survival may depend on the clinical implementation of new treatment strategies aimed at eliminating or reprogramming CSCs toward differentiation and senescence (**Figure 1C**). In this context, the knowledge of the pathways underlying the peculiar properties of CSCs can provide ideal targets for development of CSC-directed therapies (4–6).

In this review, we focus on prostate cancer and the role of transcriptional regulators on phenotypic reprogramming of prostate CSCs. We provide examples supporting the possibility of interfering with maintenance and expansion of the CSC subpopulation in human prostate cancer by targeting transcriptional regulators. Transcriptional and epigenetic regulatory factors are key elements for instructing specific transcriptional programs and phenotypic changes in CSCs (3, 6). Notably, recent work has established that interfering with these processes can induce loss of self-renewal capability and exhaustion of the tumorigenic potential of CSCs. Promising compounds are emerging from preclinical studies. Thus, targeting transcriptional regulators in prostate CSCs might be a valid therapeutic strategy to explore further in the preclinical and clinical setting.

PROSTATE CANCER AND THE CURRENT TREATMENT PERSPECTIVE

Prostate cancer is the most common malignancy in men and the second cause of cancer-related deaths in developed countries (7). Despite the progress in the treatment of localized prostate cancer, management of locally advanced and metastatic disease is still a critical unmet need (8, 9). Recent genomic studies have shown that multiple genetic and epigenetic events contribute to prostate cancer initiation and progression (10–12). Deregulated expression and activity of transcriptional and epigenetic regulators occur at early stages of disease and are particularly relevant during the progression from localized to metastatic disease and development of treatment-resistant prostate cancer (13, 14). Moreover, complex transcriptional and epigenetic reprogramming contribute to cancer cell plasticity or trans-differentiation leading to the acquisition of tumorigenic, stem-like, mesenchymal, or neuroendocrine features (15–17).

The prostate is an exocrine gland that is located around the urethra at the base of the bladder and produces the alkaline seminal fluid (18). Histologically, the human prostate is composed of a pseudostratified epithelium containing basal and luminal epithelial cells with rare neuroendocrine cells (19, 20). Luminal cells are differentiated secretory epithelial cells that line the lumen of the ducts and secretes the alkaline prostatic fluid (20). Luminal secretory cells express cytokeratin 8, cytokeratin 18 and the androgen receptor (AR). Basal cells lie on the basement membrane between luminal cells. Basal cells have low levels of AR and express cytokeratin 5, cytokeratin 14 and p63. Basal cells are considered the main niche for stem and progenitor cells within the normal prostate epithelium, although more recent lineage-tracing studies suggest that both basal and luminal cells contain lineage-restricted stem/progenitor cells in the mouse prostate (19, 20). Rare neuroendocrine cells, which express chromogranin A and synaptophysin, are scattered in the prostate gland. Neuroendocrine cells are AR negative and androgen-independent (19).

Most prostate tumors are adenocarcinomas arising from the peripheral zone of the prostate gland (18). The majority of human prostatic adenocarcinomas have a predominant luminal phenotype, with a limited number of primary tumors showing features of neuroendocrine, small cell or sarcomatoid carcinomas. Some 15% of patients diagnosed with a prostate cancer will ultimately develop metastatic lesions, with about 90% of these cases presenting with osteoblastic bone metastases (18). In about 85% of the metastatic patients, the bone is the sole site of metastasis. Notably, aggressive prostate adenocarcinomas with neuroendocrine features (NEPC) form preferentially osteoclastic bone metastases and metastasize more frequently to brain, liver, bladder, and adrenal gland than adenocarcinoma-type tumors (16).

The clinical evolution of prostate cancer is highly heterogeneous, ranging from indolent to very aggressive tumors that rapidly progress to metastatic and treatment refractory prostate cancer (9, 18). Surgery and radiotherapy are highly effective for treatment of low-risk localized prostate tumors (21). Because most prostate cancers are androgen-dependent at the time of diagnosis, patients with locally advanced or metastatic diseases are treated with androgen deprivation therapy (ADT), which limits disease progression (**Figure 2**) (8). Nevertheless, most tumors eventually become resistant to ADT and progress to lethal metastatic castration-resistant prostate cancer (mCRPC), for which there are limited treatment options (22, 23). Despite the reduced efficacy of ADT, many mCRPC continues to have active AR signaling through a variety of mechanisms including

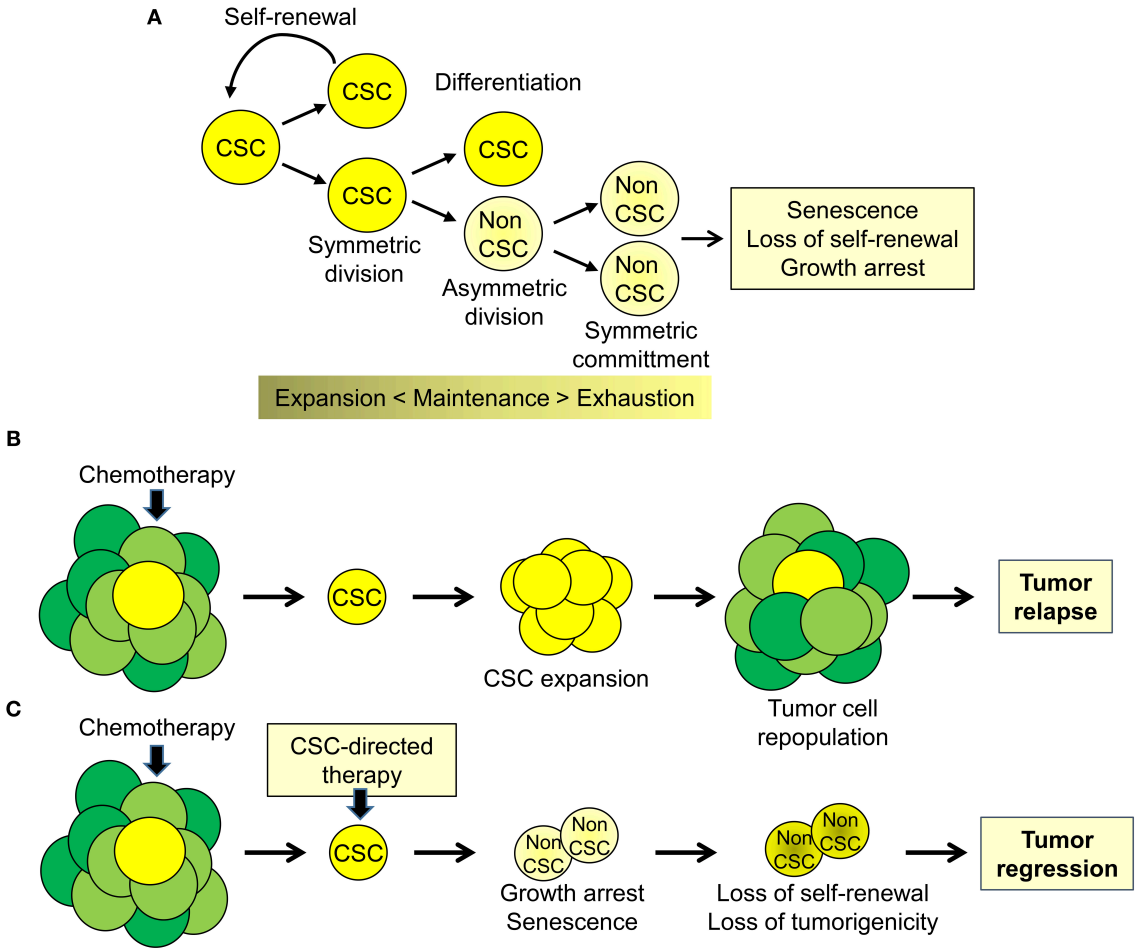

FIGURE 1 | Cancer stem cell biology and perspectives for cancer therapy. **(A)** Cancer stem cell (CSC) are a subpopulation of tumor cells capable of self-renewing through symmetric cell division and of generating, through asymmetric division, more differentiated proliferating daughter cells (non-CSC) that, through successive cell divisions (symmetric commitment) constitute the bulk of the tumor mass. **(B)** CSC are intrinsically resistant to chemotherapy and other therapeutic modalities and cause disease recurrence by reconstituting the original tumor cell population at the primary or metastatic sites. **(C)** Targeting CSC could impair tumor regrowth and decrease the likelihood of tumor progression and disease recurrence.

AR gene amplification, splice variants, point mutations, transcriptional upregulation, ligand-independent activation, and increased androgen and dihydrotestosterone (DHT) synthesis by the adrenal glands or the tumor (9, 24). The continued reliance on AR signaling makes a fraction of mCRPC still potentially responsive to new AR pathway inhibitors (ARPI), such as the anti-androgen receptor antagonist enzalutamide and the androgen-biosynthesis inhibitor abiraterone (**Figure 2**) (8). However, mCRPC can activate additional escape mechanisms and become resistant to the AR-targeted drugs (9).

An emerging modality of escape from ADT is phenotypic plasticity with the acquisition of neuroendocrine features and expression of characteristic markers such as synaptophysin and chromogranin (15, 25, 26). This process involves a complex interplay of multiple signaling pathways linked to transcriptional activators (e.g., STAT3, MYC family members, SOX2) and epigenetic effectors (e.g., EZH2) (16). In this context, expansion of AR-indifferent CSCs followed by differentiation toward a NE phenotype leads to a progeny of poorly differentiated tumor cells insensitive to androgen ablation or suppression (**Figure 2**). Thus, chronic ADT can induce dedifferentiation or transdifferentiation in mCRPCs with the NEPC variant considerably increasing among patients with metastatic castration-resistant disease. Neuroendocrine differentiation may represent an extreme form of evolution of prostate adenocarcinomas to an androgen-independent status.

mCRPCs non-responsive to ADT and AR-targeted therapeutics are treated with chemotherapy (27). Docetaxel is now the standard therapy for these patients, although the beneficial effect in this setting is rarely durable (28). Many patients do not respond or, after an initial response, become refractory to the treatment. Patients with docetaxel-refractory tumors generally receive cabazitaxel, a second-generation taxane, or platinum (Pt)-based compounds such as cisplatin

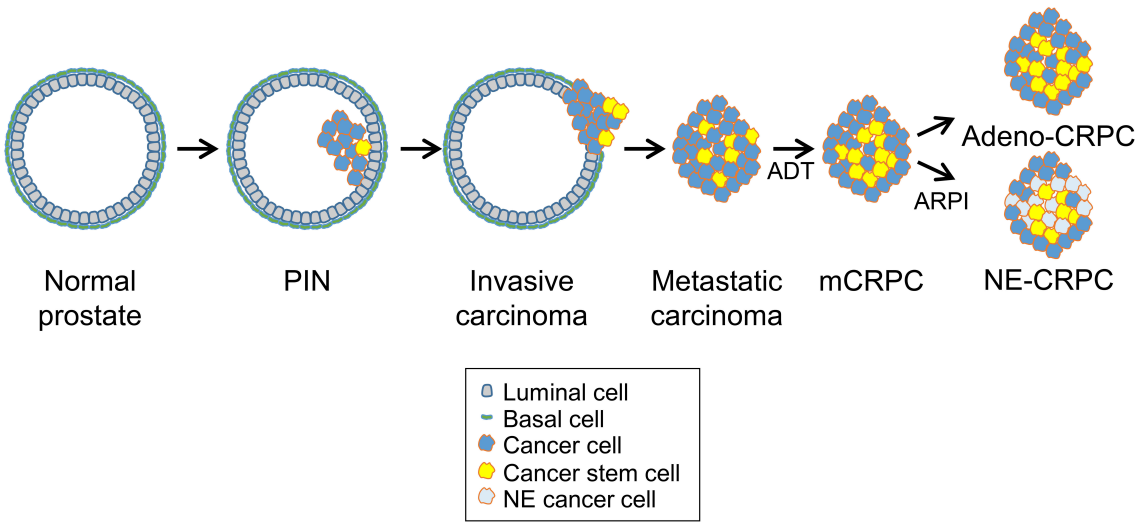

FIGURE 2 | Prostate cancer progression and cancer stem cells. Prostate cancers initiate as *in situ* carcinoma called prostatic intraepithelial neoplasia (PIN) and then evolve into invasive carcinomas and later, after androgen deprivation therapy (ADT), progress to metastatic castration-resistant prostate carcinomas (mCRPC). After continuous ADT or treatment with new AR-pathway inhibitors (ARPI), treatment-resistant tumors emerge that either retain adenocarcinoma features with enhanced AR signaling (Adeno-CRPC) or acquire neuroendocrine features with attenuated AR signaling (NE-CRPC). Progression through these stages and development of castration-resistance are driven likely by the expansion and specific behavior of prostate cancer stem cells.

and carboplatin (21, 29). Chemotherapy with carboplatin, docetaxel, or cabazitaxel is currently the preferred treatment for patients presenting with low PSA/tumor burden ratio and rapid metastatic progression or features of small cell carcinoma or NEPC (28). Inevitably, rapid development of resistance severely limits the duration of response and efficacy of any form of treatment in these patients.

CANCER STEM CELLS IN PROSTATE CANCER

Prostate cancer is highly heterogeneous in cell composition (19). The presence of stem-like tumor cells with tumor-propagating and metastasis-generating properties can greatly influence the biological heterogeneity, clinical progression and treatment response (19). CSCs within primary tumors are likely the main cause of metastatic spread and disease recurrence in prostate cancer patients (**Figure 2**). Moreover, expansion of CSCs, which are independent of AR signaling, can contribute to the development of castration-resistance as well as to reduced sensitivity to chemotherapy and radiotherapy (19, 20, 30, 31). Furthermore, CSCs that derive from basal or luminal-type progenitor/stem cells may exhibit different characteristics and contribute diversely to the biological and clinical heterogeneity of prostate tumors and their propensity to aggressive behavior and treatment resistance (19, 20, 31).

CSCs display three main characteristics: the ability to initiate tumor (tumorigenesis), to maintain their cellular properties in at least one daughter cell (self-renewal) and to reproduce the cellular composition of the original tumor (differentiation program) (32). Several studies provide evidence for the presence of self-renewing tumor-initiating stem-like cancer cells in prostate tumors (19). Putative CSCs can be purified using appropriate cell surface markers to define specific cell populations and their properties can be assessed using *in vitro* tumor-sphere and *in vivo* transplantation assays (33–36). Broad and heterogeneous sets of extracellular markers have been used to identify and isolate prostate CSCs (37, 38). However, the reproducibility and reliability in different settings and experimental models as well as the clinical relevance of most markers have not been demonstrated with any certainty (38). Increased expression of intracellular markers (e.g., ALDH), stem cell reprogramming factor, transcriptional and epigenetic regulators (e.g., Oct3/4, Sox2, Klf4, Nanog, Myc, BMI1) characterize prostate CSCs and provide additional tools for their identification (36, 37, 39–41).

In the experimental setting, *in vitro*, *ex vivo*, and *in vivo* functional assays are highly relevant to isolate CSCs and assess their content and properties (33–36, 42). Culturing prostate cancer cells in adherent monolayers in presence of serum-supplemented cell culture medium allows propagation of the heterogeneous bulk population of tumor cells (**Figure 3A**). Prostato-sphere or tumor-sphere cultures in serum-free liquid or semi-solid media and non-adherent conditions favors the expansion of single-cell derived colonies (spheroids), which are enriched of stem-like tumor cells able to survive and proliferate in this setting (34–36, 42). Organoid cultures derived from human or mouse tumors are an alternative method to preserve the heterogeneity of the cell composition of the original tumor and test drug efficacy in a three-dimensional, microenvironment-inclusive system (43, 44). However, unlike tumor-sphere culture systems, organoids do not enrich specifically for CSCs and do not allow a direct assessment of tumor cells with stem-like

properties. Xenografts of established human cancer cell lines or patient-derived tumor cells by subcutaneous or orthotopic implantation in immunodeficient mice can be a reliable and reproducible source of stem-like tumor-initiating cells and are used to assess *in vivo* tumorigenicity and self-renewal properties of the isolated CSCs by serial re-implantation in mice (**Figure 3B**). Long-term tumor regeneration in mice as well as reproducible tumor-sphere forming ability *in vitro* are paramount evidence of stem-like capability of the isolated tumor cells (35, 36, 45). Furthermore, an emerging area of research involves the isolation, characterization and propagation of CSCs derived from genetically engineered mouse (GEM) models of prostate cancer (**Figure 3C**). These GEM models reproduce prostate tumors that mimic human cancer with similar defined genetic alterations within the orthotopic prostatic microenvironment and in presence of an intact immune system and thus are becoming a valuable resource to study prostate CSC behavior and response to treatment (19, 46, 47).

When properly applied, collectively, these experimental systems represent reliable tools to monitor the effects of genetic and pharmacological interventions on CSCs. Furthermore, these *in vitro/in vivo* assays along with supplementary approaches (e.g., gene signatures, surface markers) need to be implemented rigorously in preclinical and clinical studies to demonstrate the efficacy of CSC-directed strategies and monitor the dynamic changes in tumor cell subpopulations upon treatment (5, 48). Such studies would provide a great deal of essential information for defining the best strategies to improve cancer treatment in a precision medicine approach.

CANONICAL SIGNALING PATHWAYS IN PROSTATE CANCER STEM CELLS

Current therapies for prostate cancer target preferentially partially differentiated, AR-dependent and proliferating tumor cells that constitute the bulk of the tumor mass in locally advanced and metastatic tumors (8, 21, 28, 29). However, the subpopulation of CSCs is virtually insensitive to these therapies and can repopulate the tumors at primary and metastatic sites (19, 49). Understanding the pathways controlling the establishment, expansion and maintenance of the CSC pool would be an important step toward the development of more effective therapies for prostate cancer enabling the ablation or exhaustion of CSC and preventing treatment resistance and disease recurrence. Much emphasis has been put on canonical pathways identified as drivers of stemness features in normal stem cells and proven to have similar functions in CSCs.

Canonical stem cell-associated pathways, such as Sonic-Hedgehog, Wnt and Notch, play important roles in CSC maintenance and represent promising targets to explore for the eradication of prostate CSCs (50, 51). In the canonical Wnt pathway, Wnt ligands bind to Frizzled receptor and co-receptor LRP 5/6 leading to stabilization and nuclear translocation of β-catenin that acts as transcriptional activator of the expression of pro-tumorigenic genes (50). Altered expression and localization of β-catenin is frequent in advanced prostate cancer and the Wnt signaling pathway can directly promote self-renewal of prostate CSCs (52–55). The Hedgehog pathway controls cell renewal and survival in normal stem cells during embryogenesis and adulthood (50). Hedgehog signaling is activated by binding of a specific set of ligands (Desert, Indian and Sonic) to the membrane receptors Patched (Ptch1 and 2) and Smoothened (SMO). In the presence of the ligands, SMO is relieved from the repression by PTCH and promotes the nuclear translocation of transcription factor Gli, which triggers the expression of specific target genes. Prostate tumors, like other cancers, frequently exhibit abnormally activated Hedgehog signaling (56, 57), which promotes the expansion of prostate CSCs (58, 59). A complex set of receptors (Notch1-4) and ligands (DLL 1, DLL 3, DLL 4, Jagged 1, and Jagged 2) controls Notch signaling (50). Upon ligand binding, the cytoplasmic domain of the receptor is cleaved by proteolytic enzymes (ADAMs and γ-secretase) leading to the release of the Notch intracellular domain (NICD), which moves in the nucleus and activates transcription of target genes. The Notch signaling pathway is activated improperly in human cancers, including prostate tumors, where it alters normal differentiation programs and contribute to CSC expansion (50, 60–62). In prostate cancer, combined upregulation of Notch and Hedgehog signaling promotes the stem-like phenotype and treatment resistance (63, 64).

Inhibitors of the Hedgehog, Wnt and Notch pathway have been developed and some have been tested in clinical trials in oncological patients (50). Targeting these stemness-related pathways with selective inhibitors has potent anti-CSC effect and influences positively the response to other cancer treatments in preclinical models (65, 66). Notch pathway inhibitors have shown efficacy enhancing the activity of both chemotherapy and ADT in prostate cancer preclinical models (67–71). Hedgehog inhibitors have anti-CSC effects in prostate cancer reducing expression of stemness-related genes and growth of tumor xenografts in mice (63, 72–74). Wnt pathway inhibitors also have been tested successfully in preclinical models of prostate cancer, although the evidence of a direct anti-CSC effect is not systematically provided (54, 65, 66, 75). Wnt inhibitors include promising compounds that have shown relevant activity in various experimental cancer models (53, 76–78).

Ongoing trials in multiple cancer types, including prostate cancer, are testing the efficacy of canonical stemness pathway inhibitors (48, 79, 80). Notably, vismodegib (GDC-0449), the first inhibitor approved for clinical use, and other Hedgehog inhibitors are in clinical trials for prostate cancer patients. Similarly, several Wnt and Notch pathway inhibitors are currently undergoing clinical evaluation for treatment of patients with various types of tumors including patients with prostate cancer (80–82). These early phase clinical studies are set to determine the efficacy and toxicity of these compounds and they will provide insightful information for further development as single agents or in combinatorial regimens (48). However, it would be important in the future to assess specifically whether the compounds affect the prostate CSC subpopulation taking advantage of some of the assays developed in preclinical studies. It might also be difficult to exclude effects of these

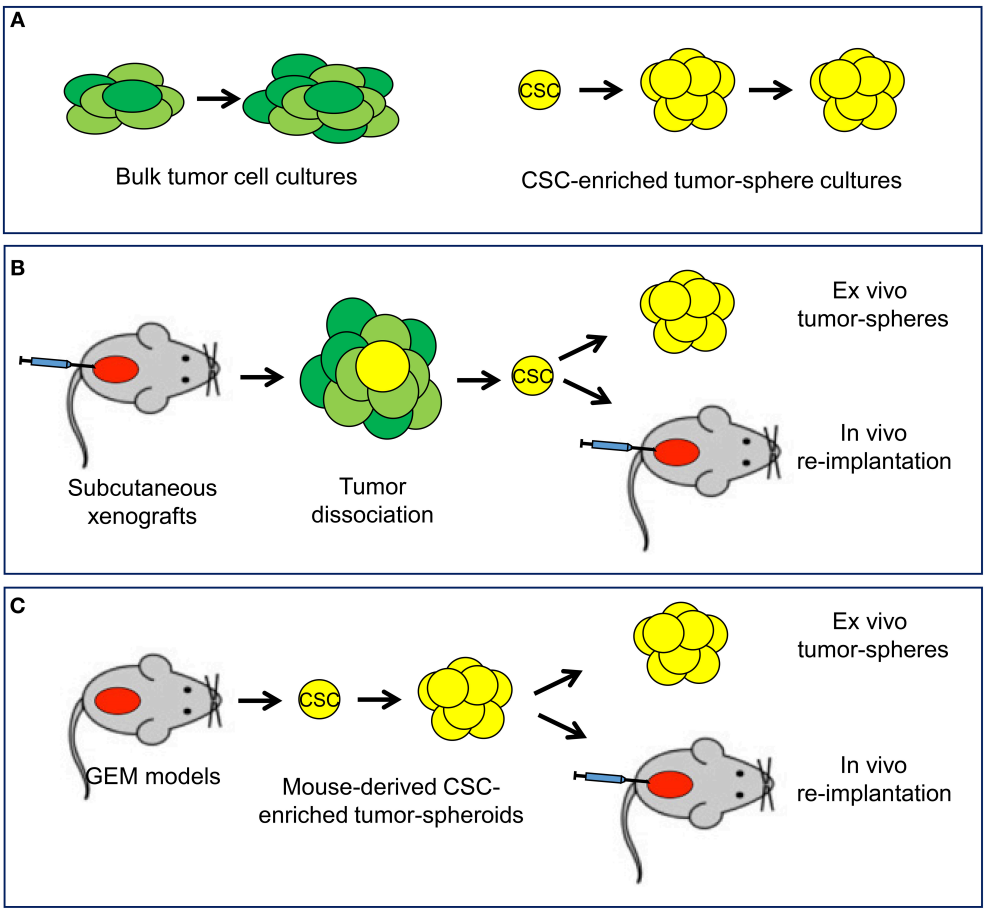

FIGURE 3 | *In vitro* and *in vivo* experimental models available to study cancer stem cells. **(A)** *In vitro* systems include standard cultures of adherent bulk tumor cells and tumor-sphere cultures of cancer stem cell (CSC)-enriched subpopulation capable of self-renewal. **(B)** Mouse xenograft models allow the isolation of CSC and monitoring self-renewal and tumorigenicity in *ex vivo* tumor-sphere assays and *in vivo* serial re-implantation assays. **(C)** Tumor-sphere assays and *in vivo* serial re-implantation can be performed with genetically engineered mouse (GEM) models through the isolation and propagation of CSC-enriched tumor-spheroids.

pharmacological pathway inhibitors on normal stem cells and prevent toxicity due to a limited therapeutic window (5, 48).

TRANSCRIPTIONAL REGULATORS IN PROSTATE CSCs

Prostate CSCs present over-expression of various transcriptional and epigenetic regulators (e.g., Nanog, SOX2, BMI1, and EZH2) that are directly involved in reprogramming the CSC transcriptome and sustaining the stem-like phenotype. Some of these factors have been effectively targeted to induce CSC depletion and counteract treatment resistance (41, 83–87). Small molecule inhibitors of EZH2 and BMI1, two epigenetic effectors, are available and have shown efficacy in prostate cancer preclinical models (25, 26, 85, 88–92). Furthermore, EZH2 inhibitors are undergoing clinical testing in patients with advanced tumors.

Additional transcriptional regulators are emerging as targetable elements in prostate CSCs opening new opportunities for anti-CSC therapeutic interventions. In the following sections, we describe the recent data and provide proof of principle examples of the effectiveness of such approaches for targeting prostate CSCs.

c-Myc

Additional pathways controlling the enhanced self-renewal capability and reduced differentiation potential of CSCs could provide ideal targets for development of CSC-specific treatment strategies. Several transcription factors aberrantly activated in advanced and mCRPCs can be directly responsible for expansion and tumorigenic potential of prostate CSCs. c-Myc (Myc) is a transcription factor involved in many biological processes, including transcription, replication, cell division, protein synthesis and metabolism (93). Amplification, chromosomal translocations, and deregulated expression of Myc are among the most common alterations occurring in human cancers (93). Myc is frequently upregulated in primary and metastatic prostate cancers and its overexpression has been associated with progression to CRPC (94). Many lines of evidence

suggest that Myc has an important role in ensuring tumor development and maintenance of CSCs in human cancers (31, 95–97). Myc, along with other stem cell genes like SOX2, BMI1 and OCT-4, is highly expressed in prostate cancer cells having the CD44+/CD24– phenotype, which is considered a hallmark of stem/progenitor cells (36, 98). However, similar to many other transcription factors, Myc is a difficult target to address directly with conventional small-molecule drugs (99). Various approaches have been attempted to target Myc by blocking Myc-protein interactions, Myc-DNA interactions and Myc transcription or translation using small molecules, peptides, oligonucleotides and small interfering RNAs (99–102). Few compounds inhibiting Myc have entered early phases of clinical investigation (100).

Following previous studies on Myc transcription and promoter regulation by oligonucleotide-based approaches (103, 104), we showed more recently that Myc transcription could be epigenetically silenced using a novel strategy based on promoter-targeting siRNAs (105). This approach relies on the presence of a cis-acting non-coding promoter-associated RNA (paRNA) overlapping the gene transcription start site and positively regulating Myc gene transcription (105). siRNA directed to this paRNA inhibited Myc transcription by interfering with the formation of the transcription pre-initiation complex at the gene promoter (105). This strategy resulted in prolonged repression of Myc transcription. Interestingly, a single transfection of prostate cancer cells with the promoter-targeting siRNA induced long-lasting effects on cell proliferation and colony formation in CRPC models such as the DU145 and PC3 cell lines, indicative of persistent loss of proliferative potential as consequence of Myc silencing (105). Notably, using this promoter-targeting strategy we were able to show that Myc silencing impaired maintenance and induced senescence in the prostate CSC subpopulation blocking their expansion and tumorigenic potential (36). We showed that tumor-sphere forming cells derived from these human cancer cell lines and grown in stem cell selective conditions retained high self-renewal capability and had high tumorigenic potential and ability to reconstitute the original tumor cell population. Myc silencing impaired propagation of tumor-spheres *in vitro*, growth of subcutaneous tumors and formation of metastasis in mice (36). Consistent with an impact on CSCs, tumors formed by Myc-depleted cells had reduced content of stem-like tumor cells capable of forming *ex vivo* tumor-spheres and generating secondary tumor xenografts in mice. Thus, these *ex vivo* assays provided direct confirmation of the anti-CSC effect of Myc silencing. Notably, the reduced CSC content and tumorigenic capability was associated with increased senescence in CSCs both *in vitro* and *in vivo*. Thus, Myc silencing led to depletion of CSCs and reduced their tumorigenic and metastatic potential through the activation of a latent senescence program in CSCs (36). This study, thus, provided direct evidence of the role of Myc in the maintenance of CSCs in human tumors and identified loss of self-renewal and induction of senescence as primary mechanisms of the depletion of tumor-initiating and metastatic prostate CSCs. These data also demonstrated that RNAi-based targeting of regulatory non-coding RNA could be an effective strategy to modulate gene expression for therapeutic applications.

Targeting upstream regulators or downstream effectors of Myc is also a valid approach (100, 102). Notably, bromodomain and extra-terminal domain (BET) proteins, such as BRD4, bind to acetylated histones and cooperate with multiple oncogenic transcription factors including Myc (106). Importantly, chemical inhibitors designed to disrupt BET protein-chromatin interactions interfere with expression and activity of Myc and other transcription factors (107–110). BET inhibitors are effective anticancer agents in preclinical models of multiple types of cancers (111). Currently, several BET protein inhibitors (e.g., ZEN003694, OTX015/MK-8628, ABBV-075, INCB057643, GSK525762/I-BET762, GS-5829) are in phase I/II clinical trials, with some studies specifically assessing their efficacy in prostate cancer patients alone or in combination with AR-targeted therapies (112). In prostate cancer, BET protein inhibitors modulate AR signaling and enhance the anti-androgenic effect of AR-targeted therapies in AR positive prostate cancer cells such as VCaP and LNCaP cells, making them suitable drugs for treatment of mCRPCs (113–116). Interestingly, BET protein inhibitors interfere with Myc functions in preclinical cancer models (106, 108, 110) and, therefore, have the potential to inhibit Myc-dependent processes also in prostate CSCs.

STAT3

Signal transducer and activator of transcription 3 (STAT3) is a key element in multiple signaling pathways and is activated aberrantly in many human cancers (117–120). Phosphorylation at tyrosine 705 (Tyr705), which is catalyzed by protein tyrosine kinases such as Janus kinases (JAK), regulates STAT3 transcriptional activity by inducing dimerization of STAT3 monomers, nuclear accumulation and DNA binding (117, 118). The IL-6/JAK pathway is the main responsible of Tyr705 phosphorylation and activation of this pathway contributes to tumor development in many experimental models (117, 118). STAT3 activation is associated with advanced disease, metastasis and clinical progression (118). Despite some recent controversial observations, the evidence of a role of this transcription factor in tumorigenesis in clinical and experimental systems and of its potential as therapeutic target is rather overwhelming [for an extensive discussion of these issues see (119–121)].

Increasing evidence indicates that STAT3 also localizes to mitochondria and is important in controlling mitochondria function (120, 122, 123). Mitochondrial STAT3 is phosphorylated at serine 727 (Ser727) by various serine protein kinases, whereas nuclear STAT3 is predominantly phosphorylated at Tyr705 by tyrosine protein kinases, like JAK family kinases (124, 125). Interestingly, constitutively Ser727-phosphorylated STAT3 is present in many human cancers and is sufficient to drive tumorigenesis independent of Tyr705 phosphorylation in various models (124, 126, 127). Moreover, mitochondrial STAT3 is critical for survival of tumor cells under microenvironment or treatment induced stress conditions, reflecting a tumor-specific dependency on STAT3 mitochondrial functions (124, 128).

A large set of evidence reveals a critical role of the STAT3 in prostate cancer. Over-activity of STAT3 in human

cancers, including prostate cancer, is frequently the result of deregulation of upstream pathways with activation of protein tyrosine kinases associated with cytokine and growth factor receptors, like JAK family kinases (123). Increased levels of IL-6, IL-6 receptor, JAK1, and pSTAT3 have been detected in patients with metastatic tumors and CRPCs (129, 130) and are associated with poor prognosis (131, 132). The IL-6/JAK/STAT3 pathway contributes to treatment resistance promoting tumor cell survival after targeted anticancer drugs or ADT (133, 134). The pathway is at the center of tumor-microenvironment crosstalks that promote treatment resistance and stemness (134, 135). Activation of STAT3 can promote also immune-tolerance and chemo-resistance in prostate cancer through the secretion of immunosuppressive cytokines in the tumor microenvironment (136). Conversely, inhibition of the IL-6/JAK/STAT3 pathway reduces tumor cell proliferation and restores sensitivity to AR-targeted drugs (137–139). Importantly, in recent years antibodies (e.g., siltuximab) targeting the IL-6/JAK/STAT3 pathway have been tested as monotherapy or in combination with cytotoxic drugs in various clinical trials for treatment of cancers, including prostate cancer (119, 140–142). However, despite the positive data in preclinical models, the clinical activity in advanced prostate cancer patients was modest or not significant (119, 140–142), suggesting that anti-IL-6 therapies may not be the most effective approach to block STAT3 signaling in this setting.

Increased STAT3 levels and higher Tyr705 and Ser727 phosphorylation are frequent in human prostate cancer both at early (androgen-dependent) and late (castration-resistant) stages of the disease (143). STAT3 activation is associated with poor clinical outcome in prostate cancer patients (144, 145). Importantly, activation of STAT3 has been associated with promotion and maintenance of CSC, tumorigenicity and metastatic capability in prostate cancer (133, 146, 147). Alternative activation pathways and non-transcriptional functions of STAT3 may also be important in CSC maintenance (122). In prostate cancer, induction of Ser727 phosphorylation can promote cell transformation and tumor development in the absence of Tyr705 phosphorylation (126). The oncogenic effect of STAT3 in this experimental system depended strictly on phosphorylation of Ser727 and both transcriptional dependent and independent functions of STAT3 (126). Interestingly, we found that in a subset of prostate cancer, characterized by reduced expression of the ETS factor ESE3/EHF, STAT3 upregulation and activation depended on the over-expression of a microRNA, miR-424, which prevented proteasomal degradation of STAT3 and led to increased levels of total STAT3 protein (148). Remarkably, miR-424 upregulation correlated with the acquisition of CSC features in cell lines and human tumors, confirming the relevance of this non-canonical STAT3 activation pathway for stemness and tumorigenicity of prostate CSC (148).

The anti-CSC effects of interfering with IL-6/JAK signaling using chemical inhibitors or soluble IL-6R support the relevance of STAT3 activation in the CSC compartment (133, 146, 149–152). Napabucasin (BBI608), a small molecule inhibitor proposed to interfere with STAT3 signaling, has been shown to inhibit stem-like tumor cells in *ad-hoc* designed preclinical models (153). The compound has been extensively studied in preclinical setting as single agent and in drug combinations to take advantage of the concomitant targeting of CSC and non-CSC populations of tumor cells and is currently tested in several clinical trials in combination with standard therapies for advanced cancers (152, 154). In addition to STAT3 pathway or indirect inhibitors, various direct STAT3 inhibitors have been developed and some have been tested in prostate cancer models (119). We recently showed that small molecule inhibitors of STAT3 OPB-31121 and OPB-51602, which directly bind to the SH2 domain and effectively block global downstream signaling through multiple STAT3-dependent pathways, were very active in prostate cancer cell models and specifically highly effective on the CSC compartment (128, 155). OPB-31121, OPB-51602 and a third structurally related compound, OPB-111077, have entered phase I/II clinical trials showing some limited efficacy as single agents in advanced patients with solid tumors (156–159). These inhibitors block both Tyr705 and Ser727 phosphorylation and impair functioning of both nuclear and mitochondrial STAT3 (128). Importantly, in DU145 tumor xenografts, a CRPC model, OPB-51602 profoundly inhibited tumor growth and blocked tumor cell re-population after treatment withdrawal (128). These effects correlated with significant depletion of the fraction of stem-like tumor cells in the tumor xenografts after OPB-51602 treatment as assessed by *ex vivo* flow cytometry and tumor-sphere assays (128).

In human cancers, STAT3 activation occurs often concomitantly with activation of the NF-κB transcription factor pathway (160). NF-κB is frequently activated in advanced prostate cancer and has been implicated in expansion of CSC (37). Notably, STAT3 and NF-κB induce highly overlapping sets of pro-tumorigenic genes that might have important functions in prostate CSC (160). Activation of NF-κB and crosstalk with the IL6/JAK/STAT3 signaling pathway were essential for the acquisition of the epithelial-to-mesenchymal transition (EMT) and CSC features in aggressive prostate tumors (161). Furthermore, multiple positive and negative feedback loops link the two pathways leading to reciprocal activation or inhibition, depending on the cell context and microenvironment stimuli (160). Interestingly, we found that the activity of both STAT3 and NF-κB was strikingly higher in prostate CSC compared to bulk tumor cells and took advantage of the availability of a novel chimeric multi-kinase inhibitor, EC-70124, generated by genetic engineering of biosynthetic pathway of natural compounds (151). The novel compound was particularly effective against IKKβ and JAK kinases, which catalyze the critical steps for activation of NF-κB and STAT3, respectively (162). Thus, we reasoned that the ability of EC-70124 to target concomitantly NF-κB and STAT3 could provide an innovative strategy to disrupt the pro-tumorigenic crosstalk between the two transcription factors and avoid the downsides of individual pathway targeting and activation of alternative survival pathways. EC-70124 blocked effectively both NF-κB and STAT3 activity in prostate cancer cells and particularly in tumor-sphere cells with constitutive activation of these pathways (151). Moreover, the drug reduced tumor-sphere formation *in vitro* and tumor growth *in vivo* (151). Notably, EC-70124 had

profound effect on the CSC subpopulation in tumor xenografts. This latter aspect was investigated by performing *ex vivo* assays with cells directly isolated from tumor xenografts at the end of the *in vivo* treatment and determining the fraction of tumor cells retaining CSC features and self-renewal capability (151). Thus, dual inhibition of STAT3 and NF-κB by EC-70124 impairs CSC maintenance and tumor development in mice and provides the basis for new therapeutic strategies for treatment of prostate cancer.

ERG

ETS transcription factors constitute a large family of transcriptional regulators with important roles in cell differentiation and carcinogenesis (163). The ETS family includes 27 members that share the highly conserved ETS domain (163). Individual ETS factors have different patterns of cell and tissue specific expression and induce distinct transcriptional and biological responses. This diversity among individual ETS factors are reflected in different roles in tumorigenesis (163). ETS factors are deregulated in many human cancers and can either promote or suppress tumorigenesis (163).

A significant percentage of prostate cancers exhibit a specific gene fusion of the ETS gene ERG and the 5′ region of TMPRSS2 gene (164). The TMPRSS2 gene encodes a serine protease highly expressed in the prostate epithelium. This genetic event results in overexpression of full length (or minimally truncated) ERG protein driven by the androgen-regulated TMPRSS2 promoter in prostate epithelial cells (164–166). Interestingly, recent studies indicate the new options for targeting pharmacologically ERG for prostate cancer treatment (167–169). Ectopic expression of ERG results in complex changes in the cell transcriptome and acquisition of tumorigenic properties. However, the biological impact of aberrantly expressed ERG in prostate cancer progression and the underlying mechanisms are still unclear (170, 171). In a relevant number of human prostate cancers, ERG gene fusion occurs concomitantly with PTEN loss (172). The coexistence of the two events is generally associated with a more aggressive disease (172). Importantly, the cooperation of ERG gain and PTEN loss was recapitulated in mouse models whereby ERG transgenic mice crossed with PTEN-deficient mice developed frank malignant lesions and progression to invasive adenocarcinomas (172–174).

We recently used these GEM models with prostate-specific expression of ERG (Pb-Cre4; Rosa26ERG/ERG) with and without PTEN deletion to examine the mechanisms underlying tumor progression in ERG-fusion positive prostate cancers. ERG transgenic mice fail to develop invasive adenocarcinomas while the combined ERG/PTEN (Pb-Cre4; Ptenflox/flox; Rosa26ERG/ER) mice develop large invasive tumors (172). Thus, these GEM models represent good systems to assess events associated with prostate cancer progression. Moreover, to examine the relation between tumor progression and CSC, we took advantage of the established protocols for isolation and analysis of tumor-propagating stem-like tumor cells from *in vivo* models. Importantly, we found that prostate tumors from ERG/PTEN mice were highly enriched of stem-like cancer cells that formed large tumor-spheroids when plated in prostate-sphere culture conditions (47). Tumor-spheroids were positive for cytokeratins confirming their epithelial origin and expressed typical stem cell markers. Moreover, the ERG/PTEN derived tumor-spheroids were endowed of high *in vitro* self-renewal potential and were capable of generating tumors with high efficiency when re-implanted in mice (47).

Using this system, we recently evaluated the activity of compounds that could interfere with ERG induced transcriptonal and phenotypic reprogramming. Based on the finding of a relevant fraction of ERG/Sp1 co-regulated genes among the ERG activated targets in ERG-fusion positive tumor, we tested the activity of a novel DNA binding and Sp1 interfering compound, demycarosyl-3D-β-D-digitoxosyl-mithramycin SK (EC-8042), in ERG positive models (47). Specifically, we found that EC-8042 was a potent inhibitor of tumor-sphere formation by ERG fusion positive VCaP cells, a measure of the drug's anti-CSC activity. Interestingly, this effect was associated with reduced expression of ERG/Sp1 target genes and impaired invasive and metastatic property *in vivo* in the Chick Chorioallantoic Membrane (CAM) system (47). The CAM assay provide a simplified system to assess tumor growth, invasion, migration, circulation in blood vessels and metastasis in live chicken embryos. To investigate further the impact of EC-8042 on tumor-propagating stem-like cells, we took advantage of the ERG/PTEN GEM model. Treatment with EC-8042 reduced formation of tumor-spheroids from ERG/PTEN mice *in vitro* and impaired the re-implantation of tumor-spheroid cells in mice (47). Systemic treatment with EC-8042 inhibited tumor progression reducing invasive and proliferative areas in prostate adenocarcinomas in ERG/PTEN mice. Moreover, EC-8042 had a significant impact of the CSC subpopulation in ERG/PTEN mice as indicated by reduced *ex vivo* tumor-sphere formation and CSC marker expression (47). These data established for the first time the efficacy of antagonizing ERG oncogenic activity to block maintenance and expansion of CSC in ERG positive prostate tumor models opening new possibilities for treatment of this disease.

ESE3/EHF

ESE3/EHF is an ETS family transcription factor of the epithelial-specific subfamily. ESE3/EHF is highly expressed in normal prostate epithelial cells and is essential for epithelial cell differentiation. Interestingly, we found that ESE3/EHF, along with ERG, was one of the most frequently deregulated ETS factors in human prostate cancer (175, 176). Importantly, down-regulation of ESE3/EHF in immortalized human prostate epithelial cells resulted in transformation, dedifferentiation, EMT, and acquisition of CSC properties (35). Furthermore, we identified a group of prostate tumors that exhibited marked reduction of ESE3/EHF expression in the absence of alterations of other ETS genes, including ERG. Enrichment of transcriptional features associated with EMT and CSC phenotype along with adverse clinical outcome characterized tumors with loss of ESE3/EHF expression (35). In follow up studies, we made further progress in understanding the tumor suppressor role of ESE3/EHF, particularly with respect to its function in cell differentiation and stemness. The link between ESE3/EHF and

CSC properties was investigated by *in vitro* tumor-sphere and *in vivo* xenograft re-implantation assays (35, 45). ESE3/EHF knockdown in immortalized normal prostate epithelial cells, such as RWPE1 and LHS, was a potent inducer of stem-like, tumorigenic and self-renewal capability in prostate epithelial cells (35). Furthermore, we established that ESE3/EHF controls key genes and microRNAs specifically involved in epithelial differentiation and CSC maintenance (35, 45, 148). Collectively, these findings suggested also various strategies to target tumors with loss of ESE3/EHF expression and reverse their aggressive phenotype.

We found that ESE3/EHF downregulation led to increased expression of stem cell factors Lin28A/B along with other stemness-related factors (45). Lin28 A/B are key elements in the processing of mature microRNA of the let-7 family, which are potent tumor suppressors and anti-CSC effectors (177). Accordingly, we evaluated the effect of knocking down Lin28 on tumorigenic and stem-like properties of transformed prostate epithelial cells with ESE3/EHF downregulation. Lin28 knockdown reduced the expression of CSC markers and the ability to sustain tumor formation in mice (45). Accordingly, *ex vivo* tumor-sphere assays showed a significant and persistent reduction of stem-like cells in Lin28-depleted tumor xenografts. Moreover, in serial re-implantation experiments Lin28 knockdown decreased profoundly the *in vivo* self-renewal and tumorigenic potential of prostate CSC (45). Thus, targeting Lin28 could re-activate a latent differentiation/senescence program in prostate CSC and lead to their ablation in ESE3/EHF^{low} prostate tumors (45). Based on these findings, we recently evaluated a first chemical inhibitor of Lin28A/B, ID1632, and demonstrated *in vitro* its significant activity in CSC culture systems (178), suggesting an alternative to the siRNA and short-oligonucleotide based approaches (45, 179). All these modalities to target Lin28A/B and counteract the effects of ESE3/EHF silencing are in early preclinical stages of investigation.

We observed that ESE3/EHF had also a relevant impact on the activation state of STAT3. By performing miRNA expression profiling in a cohort of primary prostate tumors and normal prostate for which we had matching gene expression data we found that many microRNAs were significantly deregulated in tumors compared to normal prostate. We identified miR-424 as one of the most upregulated miRNAs in ESE3^{low} tumors and cell lines (148). Functional assays demonstrated that ESE3/EHF repressed transcription of miR-424 in normal prostate epithelial cells and loss of ESE3/EHF triggered miR-424 upregulation in cancer cells (148). Among the potential targets of miR-424, we found that the E3 ubiquitin ligase COP1 had a key role in miR-424 induced phenotypes in ESE3/EHF under-expressing prostate tumor cells. Interestingly, follow up studies revealed that miR-424 mediated silencing of COP1 led to impaired proteasomal degradation of STAT3 leading to stabilization and constitutive activation of this oncogenic transcription factor (148). Importantly, miR-424 upregulation promoted EMT and tumor-sphere formation, features associated with the CSC phenotype. Moreover, a synthetic antagonist of miR-424 reduced tumor-sphere formation *in vitro* and impaired the ability to generate tumors in mice (148). Several miRNA-based therapeutics are currently in clinical trials and represent promising tools for targeting oncogenic and tumor suppressor pathways (180).

ESE3/EHF modulates STAT3 activity also by controlling IL-6 transcription (150). We observed that expression of ESE3/EHF and IL-6 were significantly anti-correlated in primary and metastatic prostate cancers. ESE3/EHF bound to the IL-6 promoter and repressed IL-6 transcription (150). Moreover, IL-6, phosphorylated STAT3 and STAT3 transcriptional activity were consistently upregulated in tumor-spheres from ESE3/EHF under-expressing tumor cells, in line with aberrant activation of IL-6/JAK/STAT3 pathway in prostate CSC. To test the effect of antagonizing IL-6/JAK/STAT3 pathway in ESE3/EHF under-expressing tumors, we used the JAK2 inhibitor NVP-BSK805 (150). NVP-BSK805 significantly reduced tumor-sphere formation in ESE3/EHF low expressing models. Moreover, treatment with NVP-BSK805 inhibited growth of tumor xenografts and self-renewal capability of tumor-sphere cells derived from ESE3/EHF knockdown models, indicating that the CSC compartment was compromised persistently by disrupting the IL-6/JAK/STAT3 axis in the context of ESE3/EHF^{low} tumors (150). Many JAK inhibitors are currently in clinical trials for oncological and non-oncological indications (181), making their use for counteracting CSC expansion in specific subtypes of prostate cancer a reasonably testable hypothesis. Collectively, these data indicate that ESE3/EHF activity is essential to maintain the balance between differentiation and self-renewal in the prostate epithelium and that loss of expression of this transcriptional regulator characterize aggressive tumors specifically susceptible to approaches aimed at restoring the tumor suppressor function of ESE3/EHF.

CONCLUSIONS

In recent years, a growing body of evidence has accumulated on the role of CSC in the genesis and progression of prostate cancer. Prostate CSC play a pivotal role in castration-resistance and phenotypic plasticity that underlie treatment failure and disease recurrence in advanced stage patients. Therapies targeting prostate CSCs can lead to effective treatment for these patients. Anti-CSC strategies should complement the current therapeutic approaches that aim at reducing AR-dependent and proliferating bulk tumor cells. The dissection of the molecular mechanisms controlling the dynamic phenotypic changes that characterize the CSC subpopulation is a mandatory prerequisite to design precise therapeutic interventions aimed at eradicating the CSC. Stem cell reprogramming factors, transcriptional regulators, and epigenetic effectors sustain the maintenance and expansion of prostate CSC and may represent valid therapeutic targets. We have shown that blocking expression and function of transcription factors that are aberrantly upregulated in prostate CSC derived from human cell lines, xenografts and GEM models results in substantial depletion of the CSC subpopulation and severe impairment of the self-renewal and tumorigenic capability. These approaches based on the use of small-molecule chemical inhibitors or synthetic siRNA provide innovative

strategies to disrupt the pro-tumorigenic signaling sustaining the prostate CSC phenotype. Nevertheless, despite the enormous progress seen in the last decades, many questions on the heterogeneity and plasticity of prostate CSCs and their evolution during tumor progression and treatment remain open and the results will influence the successful implementation of anti-CSC therapies (1, 3, 5, 48). The application of emerging technologies such as single-cell genomics and spatial transcriptomics (182–186) will allow addressing the important questions of stem cell niche composition, anatomical location, biological and genomic heterogeneity of prostate CSCs in longitudinal studies in mouse models and human samples. Genomic and proteomic approaches may lead to the development of specific CSC signatures to apply to preclinical models and human samples and probe the CSC population and characterize their heterogeneity and evolution during the course of the disease and in response to treatments (31, 95, 187). Likely, combinations of standard therapies targeting bulk tumor cells with more selective anti-CSC therapies would be the most reliable treatment approach for most patients. Combined targeting of multiple CSC pathways might also be required to achieve effective control of the CSC subpopulation within highly heterogeneous tumors and avoid CSC escape. Properly designed preclinical studies and clinical trials should investigate the feasibility and efficacy of the diverse strategies matching the genotypic and epigenetic features of the tumors.

AUTHOR CONTRIBUTIONS

GC, DA, DS, RV, JM, AK, SM, GMC, and CC reviewed the literature, analyzed the data, and contribute to writing the manuscript.

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REVIEW
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Stem Cell Plasticity and Dormancy in the Development of Cancer Therapy Resistance

Maria Laura De Angelis[1], *Federica Francescangeli*[1], *Filippo La Torre*[2] *and Ann Zeuner*[1]*

[1] Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy, [2] Department of Surgical Sciences Policlinico Umberto I, Sapienza University of Rome, Rome, Italy

Cancer treatment with either standard chemotherapy or targeted agents often results in the emergence of drug-refractory cell populations, ultimately leading to therapy failure. The biological features of drug resistant cells are largely overlapping with those of cancer stem cells and include heterogeneity, plasticity, self-renewal ability, and tumor-initiating capacity. Moreover, drug resistance is usually characterized by a suppression of proliferation that can manifest as quiescence, dormancy, senescence, or proliferative slowdown. Alterations in key cellular pathways such as autophagy, unfolded protein response or redox signaling, as well as metabolic adaptations also contribute to the establishment of drug resistance, thus representing attractive therapeutic targets. Moreover, a complex interplay of drug resistant cells with the micro/macroenvironment and with the immune system plays a key role in dictating and maintaining the resistant phenotype. Recent studies have challenged traditional views of cancer drug resistance providing innovative perspectives, establishing new connections between drug resistant cells and their environment and indicating unexpected therapeutic strategies. In this review we discuss recent advancements in understanding the mechanisms underlying drug resistance and we report novel targeting agents able to overcome the drug resistant status, with particular focus on strategies directed against dormant cells. Research on drug resistant cancer cells will take us one step forward toward the development of novel treatment approaches and the improvement of relapse-free survival in solid and hematological cancer patients.

Keywords: cancer stem cells, chemoresistance, dormancy, quiescence, plasticity, drug resistance, target therapies

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University of Turin, Italy
Benjamin Le Calvé,
University of Namur, Belgium

***Correspondence:**
Ann Zeuner
ann.zeuner@iss.it

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INTRODUCTION

Resistance to chemotherapy and molecularly targeted therapies is a major problem that limits the effectiveness of cancer treatments. While some tumors are intrinsically insensitive to therapies due to pre-existing resistance factors (primary or intrinsic resistance), others become resistant during drug treatment (1). The development of resistance after an initial period of response (acquired resistance) is due to the molecular heterogeneity of tumor cells which, together with their ability to evolve at the genetic, epigenetic, and phenotypic level, is able to overcome the action of cancer therapies. The emergence of resistant cells has been observed upon treatment with

chemotherapy, radiotherapy, and targeted therapies, including EGFR tyrosine kinase inhibitors in lung cancer, anti-HER2 therapies in breast cancer, and BRAF inhibitors in melanoma. Even cancer immunotherapies, which exploit a dynamic interaction between the host immune system and tumor cells thus achieving lasting antitumor responses, are linked to the development of resistance and consequent cancer progression (2). Treatment with chemotherapeutic or targeted drugs is increasingly recognized to promote the emergence of resistant cells with features of cancer stem cells (CSCs) (3). This process clearly involves a Darwinian selection of cell populations with novel genetic mutations conferring drug resistance (4–6). However, non-genetic events involving both chromatin remodeling and the activation of stress-related pathways are responsible for the establishment of drug tolerance, a process more rapid, and massive than genetic mutation (7–9). Drug tolerance is habitually associated to a transient state of slow proliferation, thus identifying a population of Drug Tolerant Persisters (DTPs) that are largely quiescent and maintain viability in conditions where other cancer cells are killed (9). Drug tolerance is a temporary condition, which can revert after the cessation of cytotoxic stimuli. Differently, in the presence of continuous drug stimulation or other cellular stresses such as hypoxia, drug tolerance stabilizes into an enduring drug resistant state (9, 10). Besides quiescence, senescence has also been proposed as a process adopted by tumor cells to escape from therapy (11), suggesting that drug resistance is a composite picture of heterogeneous cell states. This picture is further complicated by a plethora of cell-intrinsic and extrinsic factors that contribute to the establishment of drug resistance including hypoxia, cytokines (among which IL-6, IL-8, and TGF-β play a prominent role), cellular composition and stiffness of the extracellular matrix. Drug resistant cells are found not only within bulk tumor populations but are also scattered in distant organs as disseminated tumor cells (DTCs), which have been recognized as the seeds of metastasis. DTCs are in a state of dormancy, which is induced and maintained by interactions with the target organ niche (12). The neutralization of DTCs is a primary goal in patients with cancers subject to late relapses such as breast and prostate cancer: in fact, recent insights on the mechanisms by which DTCs persist and reawaken are paving the way for new therapeutic avenues (13). This review will draw a picture of drug resistant cells in different contexts such as primary tumors or pre-metastatic niches and discuss a surge of recent findings that shed new light on their strengths and weaknesses, making drug resistance one of the most fertile fields of cancer research.

Abbreviations: SA-β-Gal, senescence- associated-β-galactosidase; H3K9me3, trimethylated lysine 9 at histone H3; CML, chronic myeloid leukemia; p38 MAPK, p38 mitogen-activated protein kinase; ERK 1/2, extracellular signal-regulated kinase ½; RARβ, retinoic acid receptor beta; RORγ, nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma; SOX9 (sex-determining region Y [SRY]–containing box 9); GRP78, glucose regulatory protein 78; EZH2, enhancer of Zeste homolog 2; OXPHOS, oxidative phosphorylation; NSAIDs, non-steroidal antinflammatory drugs; IGF1, insulin growth factor 1; EGFR, epidermal growth factor receptor; NGFR, nerve growth factor receptor; NSCLC, non-small cell lung cancer.

DRUG RESISTANT CANCER STEM CELLS: A CONCENTRATE OF ROBUSTNESS AND PLASTICITY

The concept of CSCs originated as a hierarchical model where, in parallel to normal tissues, a small number of undifferentiated elements give rise to intermediate progenitors and finally to a differentiated progeny. While the hierarchical model remains fundamentally valid for normal tissues (with the exception of rare dedifferentiation events occurring during tissue regeneration or artificial reprogramming), it is becoming clear that boundaries between stem and non-stem cells are much weaker in cancer. In fact, in tumors state transitions seem to be very frequent and chaotic, thus generating high levels of heterogeneity that constitute the foundation of drug resistance (14–16). Not surprisingly, state transitions also affect the expression of molecules expressed on the cell membrane, such as surface markers used for CSCs isolation. Expression of surface or intracellular markers can in some cases identify a population of cells with enhanced self-renewal and/or metastatic capacity in several tumors (**Supplementary Table 1**). However, it should be kept in mind that such expression is transient, dynamic, and variable both among individual tumors (17, 18). In fact, few past studies on the expression of CSCs markers analyzed the expression of such markers over time (particularly upon flow cytometry isolation of positive and negative populations) or the variation of CSCs markers upon microenvironmental stimuli. Consequently, the phenotypic plasticity and dynamic properties of CSCs populations were often overlooked. Functional features such as tumor-repopulating ability in limiting dilution/serial transplantation assays are more suitable to identify CSCs populations. Such assays may nonetheless select for particularly robust cells able to thrive in harsh experimental conditions. Genetic barcoding makes use of lentiviral infection systems to tag human cells and has been employed to analyze and track stem cell hierarchies, particularly in colorectal cancer. In this context, molecular tracking studies revealed a stable functional heterogeneity of the colorectal CSCs population during serial xenografting despite profound changes in genomic subclone contribution (19), thus highlighting the functional robustness of cancer cell hierarchies. In addition to cell-intrinsic features, interactions with the tumor microenvironment are increasingly recognized as crucial determinants of stemness. The fact that soluble molecules released by the tumor microenvironment have the potential to initiate CSC-like programs was first demonstrated in brain tumors, where the self-renewal and proliferation of stem-like cells were shown to crucially depend from their interaction with endothelial cells (20). The tumor endothelium has also been shown to produce nitric oxide, which diffuses to neighboring glioma cells and activates the Notch pathway to induce stem-like characteristics (21). Later studies in colorectal cancer showed that cancer-associated fibroblasts secrete hepatocyte growth factor, osteopontin, and stromal-derived factor 1α, which activate the WNT pathway to promote cancer cell stemness (22, 23). Tumor-associated macrophages play also a role in supporting breast CSCs and brain CSCs, further supporting the importance of the niche in dictating

a cancer stem cell phenotype (24, 25). Moreover, exosomes and microvesicles produced by niche cells are increasingly recognized to influence CSCs and drug resistance. For example, microvesicles produced by breast cancer-associated fibroblasts transfer miR-221 to cancer cells thus increasing the drug resistant $CD133^{hi}$ stem cell population (26). In addition to soluble factors, other microenvironmental features such as clone location have been recently shown to determine the self-renewal capacity of colorectal cancer cells (27). In light of these evidences, stemness in cancer can be defined as a transient state of enhanced plasticity and robustness crucially influenced by microenvironmental signals, including interactions with niche elements, tumor, and non-tumoral cells, soluble factors, and anticancer therapies. The link between stemness and drug resistance derives mainly from three observations: (1) CSCs populations are more resistant to therapy (28), (2) cancers with a stemness-related gene expression have a worse prognosis (29–33), and (3) cells with combined features of stemness, drug resistance, and dormancy have been identified in several tumors including pancreatic carcinoma (34, 35), ovarian cancer (36), melanoma (37), lung cancer (38), and CML (39). More recently, dormant/slow cycling CSCs have been identified in acute leukemia (40), glioblastoma (8, 41, 42), breast (43), and colorectal cancer (44, 45). An interesting association between stemness and dormancy, together with enhanced migratory features, has also been reported in early metastatic cells which are largely responsible for tumor dissemination (46–49). While increasing evidences point to the presence of drug resistant CSCs in multiple cancers, the effect of conventional, and targeted therapies is not usually evaluated specifically on the CSCs compartment, rendering difficult to estimate CSCs permanence after therapy. Likewise, current diagnostic and therapeutic approaches include few tools for the identification, quantification, and elimination of drug resistant cells and DTCs. The elucidation of mechanisms of drug resistance and the identification of biomarkers of resistant cells are therefore essential to improve the clinical management of cancer patients.

HETEROGENEITY OF THERAPY RESISTANT CANCER STEM CELLS

Previously thought to be a quite homogeneous condition, drug resistance is emerging as a surprisingly heterogeneous state that includes quiescent, drug-tolerant, and persister cells (50). This scenario is further complicated by the recent addition of post-senescent cells to drug-resistant cells responsible for disease recurrence (11). In this section we propose a functional distinction of drug resistant cells based on their origin, location, and cellular state. **Figure 1** illustrates schematically three main populations associated with drug resistance such as (1) "spontaneous" drug-resistant cells in untreated tumors, (2) stress-induced drug resistant cells (including drug-tolerant persisters, post-senescent cells, and cells resident in hypoxic tumor areas), and (3) DTCs. As mentioned previously, a small population of drug-resistant cells is already present in tumors before any kind of treatment (32, 34, 37–40, 42, 44, 45). Recent studies showed that populations of endogenous drug resistant cells transiently arise as the result of stochastic state transitions that induce a high expression of resistance factors (9, 51). Such factors have been identified in melanoma cells and include EGFR, NGFR, WNT5A, AXL, PDGFRB, and JUN, with cells expressing more than one factor displaying a higher level of resistance (51). The emergence of drug tolerant cells has been detected and quantified in multiple tumors upon treatment with chemotherapeutics or targeted agents including cisplatin (in NSCLC), erlotinib, and gefitinib (NSCLC), lapatinib (breast cancer), the RAF kinase inhibitor AZ628 (melanoma and colorectal cancer), and the MET inhibitor PF-2341066 (MET-amplified gastric cancer) (9). DTPs represent a variable percentage of the parental cell population ranging approximately from 0.2 to 5% and have been identified as largely quiescent cells, although a minor part of them can resume proliferation even in the presence of the drug (9). A particularly interesting mechanism that leads to the emergence of DTPs has been recently elucidated in melanoma cells that develop resistance to BRAF inhibitors (BRAFi). A subset of melanoma cells constitutively activates the Aryl hydrocarbon Receptor (AhR), a basic helix-loop-helix transcription factor responsible for the de-differentiation of melanoma cells and the expression of BRAFi- resistance genes. Treatment with BRAFi results in the enrichment of a small subpopulation of AhR-activated BRAFi-persister cells, responsible for melanoma relapse (52). While spontaneous resistant cells and DTPs can be appropriately defined as "quiescent" as their cell cycle interruption is transient and programmed to last until the subsequent change of gene expression or drug concentration. However, such quiescent state can be stabilized by a protracted environmental stresses like hypoxia, thus shifting the balance from a short-term quiescent state to medium- or long-term dormancy. Interestingly, cells deriving from a hypoxic tumor microenvironment have been shown to activate a dormancy program giving rise to chemoresistant DTCs, further strengthening the link between dormancy and drug resistance (10). Recently, senescence has emerged as another key cellular response to drug resistance, further contributing to the heterogeneous picture of drug resistant cells. Chemotherapy and targeted therapies can induce senescence in tumor cells, intended as a stable form of growth arrest (11, 53, 54). Senescent cells are characterized by peculiar morphological features, by the expression of senescent markers (mainly SA-β-Gal and H3K9me3) and by the so-called Senescence-Associated Secretory Phenotype (11, 55). Moreover, senescent cells are arrested in the G1 or G2/M phase of the cell cycle, differently from quiescent cells that are in G0 or G0/G1 transition. Importantly, an estimated $1/10^6$ cells can escape from senescence and re-enter the cell cycle, gaining increased aggressiveness and tumor-initiating potential (56). Cells able to escape from senescence express nuclear β-catenin and stem cell markers, indicating they underwent a process of cellular reprogramming that rendered them a fully functional cancer stem cell population (56). Finally, DTCs represent a category of drug resistant stem cells located in lymph nodes or distant organs that can persist for decades after removal of the primary tumor. DTCs are crucially dependent from niche interactions and can be

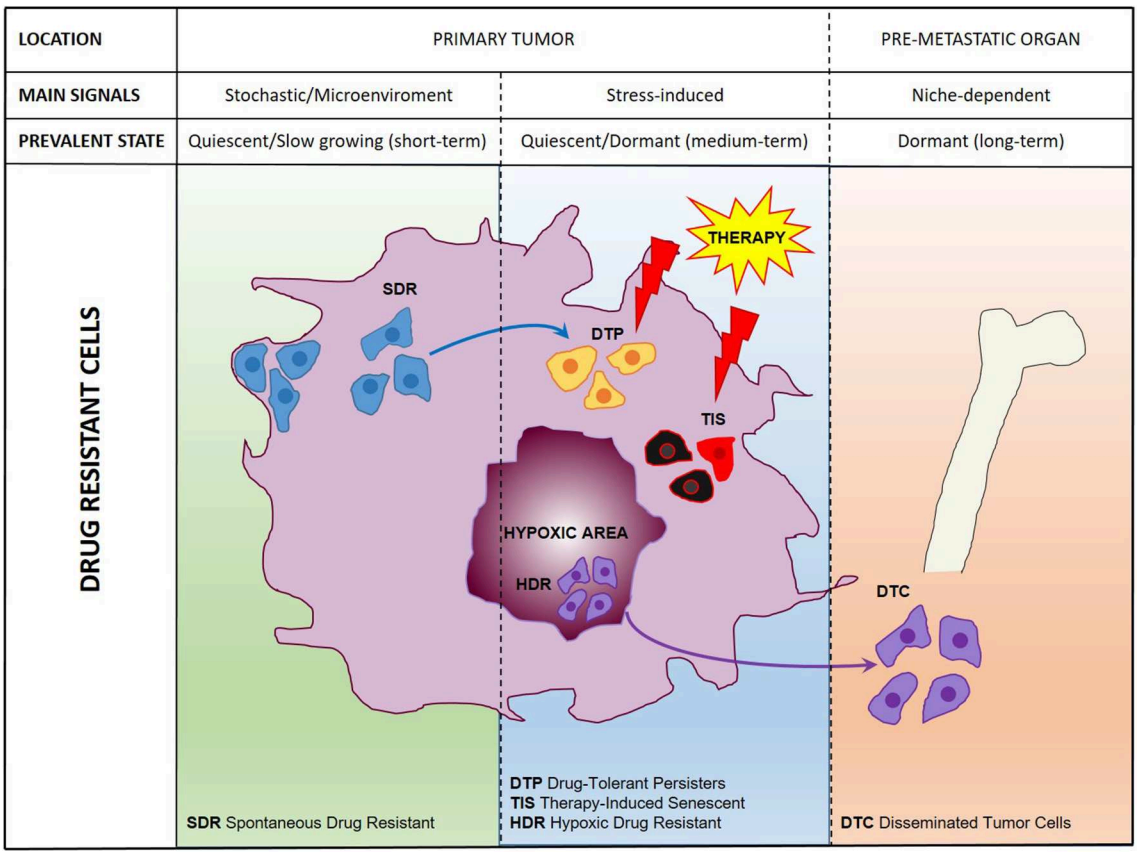

FIGURE 1 | A comprehensive scheme of drug resistant cancer stem cells. Representation of main cell populations responsible for drug resistance present in the untreated tumor (SDR, Spontaneous Drug Resistant) induced by therapeutic treatment (DTPs, Drug-Tolerant Persisters; TIS, Therapy-Induced Senescent; HDR, Hypoxic Drug Resistant) or disseminated in pre-metastatic organs (DTCs, Disseminated Tumor Cells).

resistant to chemotherapy, targeted therapies, and hormonal therapy (57–59). Thus, understanding the biology of DTCs is crucial for devising alternative strategies aimed at eradicating DTCs while dormant or preventing their awakening (12, 60). Many efforts are currently dedicated to answer key unresolved questions regarding DTCs, such as which pathways are involved in maintaining DTCs dormancy, how DTCs evade immune surveillance and what triggers their awakening (12, 60, 61). However, it is also of note that physiologic models for cancer cell dissemination are represented by orthotopic/metastatic tumors in mice, which are relatively straightforward in the case of breast cancer but less feasible in other tumors. An increased use of orthotopic/metastatic models is therefore, warranted to improve the knowledge on tumor cell dissemination, dormancy, and reawakening. A provocative contribution to the field of DTCs came from recent studies showing that disseminated breast cancer cells are protected from chemotherapy through integrin-mediated interactions irrespectively from their cell cycle status (57). Disrupting the interactions between DTCs and the perivascular niche with integrin inhibitors results in DTCs chemosensitization and may represent a clinical strategy to eradicate minimal residual disease (57). In summary, it appears that both cell-intrinsic factors and cell-extrinsic signals (either local or systemic) crucially contribute to drug resistance (**Figure 2**). Therefore, integrated approaches able to interfere with the establishment of drug resistance at multiple levels are urgently needed to increase the life expectancy of cancer patients.

NON-GENETIC PATHWAYS INVOLVED IN DRUG RESISTANCE

Pioneer studies on dormant cells and their microenvironment have led to a deeper understanding of drug resistance (62), thus paving the way for a flurry of recent studies in this field. Here, we will discuss the main categories of factors crucially involved in the determination of a dormant/drug resistant status with particular focus on recent discoveries. It is important to acknowledge that factors responsible for dormancy/drug resistance are not mutually exclusive, but many of them are likely active at the same time and crosstalk to reinforce each other.

Stress-Induced Pathways Part 1: The p38 Hub

Early studies pointed to a key role for p38 stress-activated protein kinase activation in dormant cells indicating that the

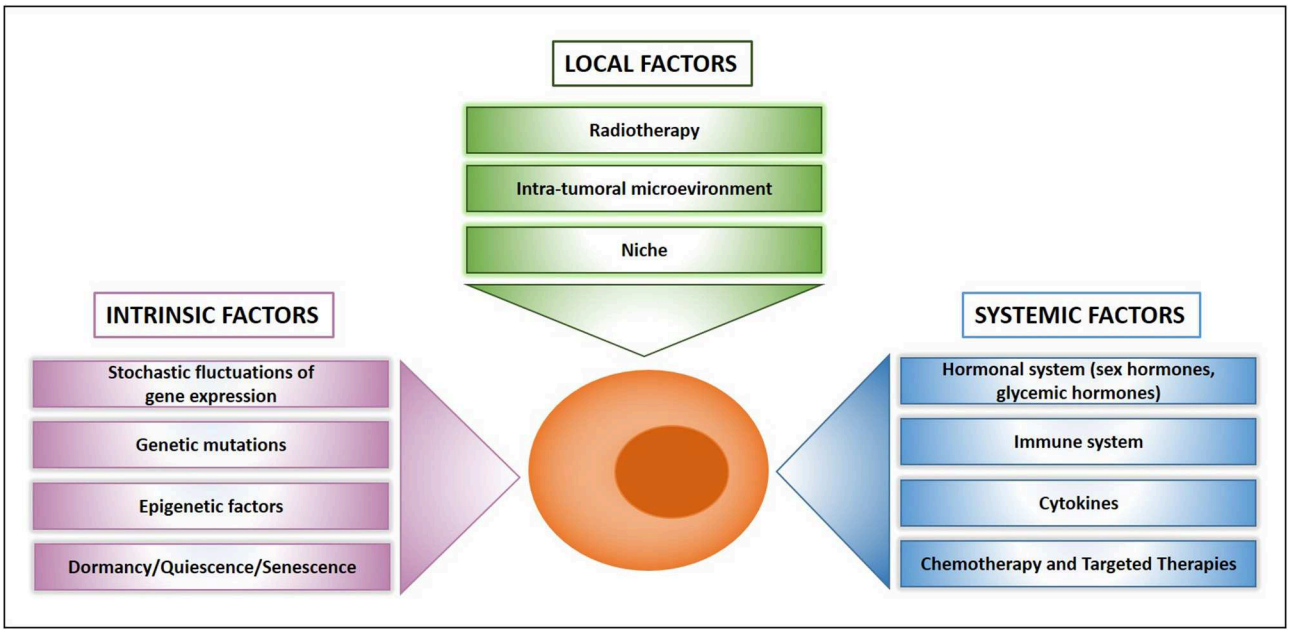

FIGURE 2 | What makes a drug resistant cell. Schematic presentation of main factors that influence drug resistance at the cellular, local, and systemic level.

balance between proliferation and dormancy is determined by the ratio between the activity of p38 and ERK1/2 (63, 64). Since then, p38 has emerged as a hub in the control of multiple pathways involved in both drug resistance and cellular stress and has itself reported to induce chemoresistance in a variety of tumors. Further insights into p38-activated pathways led to the key discovery that the orphan nuclear receptor NR2F1 upon activation by p38 induces dormancy through SOX9, RARβ, CDK inhibitors, and global chromatin repression in head and neck squamous cell carcinoma (65). Recently, NR2F1 has emerged as a clinical marker of dormancy, its expression being able to discriminate breast cancer patients with short term systemic relapse from those with long disease-free intervals (66). Most interestingly, factors involved in the p38/NR2F1/retinoic acid receptors pathway are possibly emerging as part of a general program of dormancy/drug resistance active across several types of cancer. In line with this hypothesis another retinoic acid-binding nuclear receptor, RORγ, emerged during a recent mapping of molecular traits related to stemness and drug resistance in pancreatic cancer (67). RORγ, which is also known for its role in immune modulation ad inflammation, was correlated with the aggressiveness of pancreatic cancer and its inhibition led to a striking defect in tumor growth (67). A current clinical trial based on the use of 5-azacytidine/all-trans retinoic acid aimed at inhibiting DTCs reawakening in prostate cancer patients (NCT03572387) will provide clinical evidence on the efficacy of epigenetic therapies that induce histone demethylation and NR2F1 activation. SOX9 is another factor downstream of p38 that has recently been involved in drug resistance of CSCs in breast and esophageal cancer (68, 69) and in chemoresistance of cholangiocarcinoma (70). Interestingly, SOX9 expression is regulated by the SCF^{FBW7} (Skp1/Cul1/F-box), a component of the ubiquitin ligase complex (71, 72), which has been recently shown to regulate dormancy/drug resistance in breast cancer. In fact, Fbxw7 ablation sensitizes disseminated breast tumor cells to chemotherapy, arguing for a key role of the ubiquitination pathway in dictating drug resistance by selective substrate degradation (72). SOX2 and SOX9 transcription factors are also typically expressed by dormant CSCs in breast and lung cancer. Interestingly, these cells maintain dormancy in an autocrine fashion by inhibiting Wnt signals through expression of the Wnt inhibitor DKK1 (73). Other tumors have been recently reported to modulate Wnt signaling in order to maintain dormancy: prostate cancer cells receive Wnt5a from the osteoblastic niche and activate a non-canonical signaling that represses canonical Wnt3a/β-catenin signaling (74). Notably, osteoblast-produced Wnt5a acts by inducing the Siah E3 Ubiquitin Protein Ligase expression, further supporting the role of E3 ligases in dormancy/drug resistance (75). Wnt signals have also been implicated in the survival of dormant tumor cells in colorectal cancer, which have been identified as a population of partially differentiated cells characterized by high clonogenic capacity and chemoresistance (45).

Stress-Induced Pathways Part 2: Hypoxia, Endoplasmic Reticulum Stress, and Autophagy

Global stress responses such as the hypoxia, unfolded protein response (UPR), endoplasmic reticulum stress and autophagy have all been implicated in drug resistance and pre-metastatic dormancy. Hypoxia has been habitually linked to tumor aggressiveness and poor survival but the underlying

mechanisms are still under elucidation. Cells in low oxygen microenvironments activate hypoxia-inducible factors and increase the expression of key dormancy genes such as N2RF1, p27, and MIG6, inducing a combined state of dormancy and drug resistance (10, 76). Hypoxic responses can be triggered by hypoxic microenvironments in primary tumors but can also be induced by chemotherapy, which promotes a signaling cascade involving calcium release from the endoplasmic reticulum and expression of pluripotency genes, leading to an enrichment of stem cells in breast cancer (77). The endoplasmic reticulum and the related UPR, which is responsible for re-establishing endoplasmic reticulum homeostasis following cellular stress, are implicated in several steps of the drug resistance process (78). The endoplasmic reticulum stress sensor GRP78, previously shown to be downstream of activated p38 (79), seems to play a central role in the induction of drug resistance and has been particularly investigated in pancreatic cancer, where it has been involved in both chemoresistance and maintenance of the stem cell population (80, 81). Besides chemoresistance, endoplasmic reticulum stress has been demonstrated to be involved also in resistance to tyrosine kinase inhibitors in lung cancer, where drug persister cells activate the recently described ufmylation pathway and downstream UPR to upregulate key survival signals such as Bcl-xL (82). An interesting crosstalk occurs between endoplasmic reticulum stress/UPR and autophagy, which occur simultaneously and are both implicated in tumorigenesis and chemoresistance. In fact, GRP78, PERK, and ATF6 lie at the crossroads between UPR and autophagy, being able to modulate both pathways (83). Autophagy was recognized a decade ago as being implicated in the regulation of tumor cell survival and dormancy in ovarian and gastrointestinal tumors (84, 85). In fact, autophagy is required during quiescence for recycling of aminoacids and nucleotides (86), but new evidence adds to a specific role of autophagy in dictating chemoresistance in colorectal cancer (87), liver cancer (88), brain tumors (89), and melanoma (90). Recently, autophagy has been shown to be essential for the survival of disseminated dormant breast cancer cells and its inhibition with antimalarial hydroxychloroquine eliminates DTCs while dormant (91). In KRAS-dependent tumors such as pancreatic adenocarcinomas (PDAC), KRAS inhibition has demonstrated to increase autophagic signaling resulting in autophagy dependance. Removing this protective mechanism through the combined use of MEK/ERK inhibitors and autophagy inhibitors may be therapeutically beneficial in patients with PDAC, NRAS-driven melanoma, and BRAF-mutant colorectal cancer (92). By contrast, the activation of autophagic/lysosomal pathways can occur as the consequence of anticancer therapies, as has been demonstrated in melanoma treated with anti-BRAF targeted agents (93). In this case, autophagy blockade has detrimental effects, resulting in enhanced tumor progression, metastatic dissemination, and chemoresistance. Thus, autophagy may play different roles in multiple contexts and further studies are needed to clarify the potential utility of autophagy modulators in cancer therapy.

METABOLIC REPROGRAMMING OF DRUG RESISTANT CELLS

Metabolic deregulation is recognized as a hallmark of cancer, and increasing evidences suggest that it can be exploited by neoplastic cells in order to acquire a drug resistant phenotype. Intuitively, since chemotherapy kills highly proliferative cells that rely on aerobic glycolysis, it also induces a selective pressure toward the emergence of slow growing cells switched to OXPHOS metabolism (94). However, this apparently straightforward hypothesis is contradicted by very different metabolic patterns found in resistant tumor cells (95). On one hand, several studies indicate that chemotherapy-resistant cells become OXPHOS-dependent (96–99). However, other reports showed that chemoresistant cells rely on high ATP levels (100) and express glycolytic markers (101–104). A possible explanation for such divergences can be found in the timing of data collection relatively to drug treatment: during therapy, resistant cells may activate a survival program based on proliferative slowdown and switch to OXPHOS, while some time after therapy cessation cells may recover a high proliferative rate associated to aerobic glycolysis. However, the main explanation for the different metabolic patterns found in drug resistant cells probably resides in the high plasticity of the metabolic response to cytotoxic challenges. A recent study confirmed this hypothesis by showing that cancer cells are able to switch between OXPHOS and glycolysis to circumvent the inhibition of either process (105). A therapeutic strategy targeting metabolic plasticity based on intermittent fasting (to reduce glucose availability) plus the OXPHOS inhibitor metformin effectively restrained tumor growth by activating PP2A, GSK3β, and the pro-apoptotic protein Mcl-1 (105). These results also suggest that an optimization of metformin administration schedules may potentiate its ability on tumor metabolism and increase its therapeutic efficacy. Metabolic stress is a condition often encountered by tumor cells, particularly by the quiescent population resident in poorly vascularized/hypoxic areas. The combination of hypoxia and reduced nutrient availability limits the metabolic plasticity of tumor cells, which become more sensitive to drugs that target mitochondrial respiration. In fact, drugs targeting mitochondrial bioenergetics have been proposed to eliminate metabolically stressed quiescent cells, alone or in combination with autophagy inhibitors (106). Interesting insights into the mechanisms of drug-induced metabolic reprogramming have come from the study of estrogen receptor (ER)-positive breast cancers. In these cancers, hormonal therapy has been shown to result in the emergence of dormant $CD133^{high}/ER^{low}$ cells responsible for metastatic progression and to induce an OXPHOS metabolic editing of breast cancer cells through IL6/Notch3 signaling (107). Hormonal therapy resistance and the generation of breast CSCs have been correlated to microvesicle-mediated horizontal transfer of microRNAs from host stromal cells (26). Recently, extracellular microRNAs have been further implicated in the metabolic crosstalk between tumor cells and their microenvironment by showing that cancer

cell-secreted miR-105 instructs cancer-associated fibroblasts to display different metabolic features, thus helping the tumor to face changes in the metabolic environment (108). Finally, metabolic reprogramming related to the development of drug resistance has been also shown to occur during antiangiogenic therapies (109). Interestingly, overexpression of the glucose transporter 3 (GLUT3) recapitulates all the metabolic features of bevacizumab-resistant cells indicating GLUT3 as a potential metabolic target in glioblastoma (110). In summary, it appears increasingly clear that metabolic heterogeneity can be driven by both intrinsic (either genetic or epigenetic) mechanisms or as an adaptation to environmental changes and plays a key role in the development of drug resistance, representing a potential avenue for targeted therapies (111).

EPIGENETIC PLASTICITY IN THE REGULATION OF DRUG RESISTANCE

Epigenetic deregulation is a feature of virtually all human cancers (112). Tumors exhibit a continuously changing epigenetic landscape that includes altered modifications of DNA promoter regions, deregulated acetylation, or methylation of histone proteins or inappropriate expression of repetitive regions, contributing to tumors biological properties (113). The involvement of epigenetic (rather than genetic) mechanisms in drug resistance is particularly evident when drug resistant states are transient, rapidly emerging, and functionally heterogeneous. A number of past studies have demonstrated a contribution of epigenetic modifiers such as histone deacetylases (HDACs) to oncogenesis with different mechanisms strongly depending on the cellular context (114). Recent observations point to a crucial role of histone demethylases, and in particular KDM2, KDM3, KDM5, KDM6, and KDM7, in generating a drug resistant state and often a concomitant slow-dividing and stem cell-like state (8, 9, 115–121). Additional interesting insights into the epigenetic mechanisms of drug resistance came from the observation that drug treatment induces a rapid reprogramming of spontaneous resistant cells in primary tumors, converting the transient quiescent state into a stably resistant state and generating DTPs, the cells that actually survive drug-induced toxicity (**Figure 1**) (51). In breast cancer, multiple epigenetic enzymes including KDM5B, bromodomain, and extraterminal (BET) proteins and the histone methyltransferase EZH2 have been shown to be responsible for the generation of persister cells through a dynamic remodeling of the chromatin architecture, and such state transitions can be counteracted with inhibitors of chromatin-modifying enzymes (117). Recently, BET inhibitors were found to revert drug resistance and to block the pro-tumorigenic activity exerted by YAP/TAZ binding to the epigenetic coactivator bromodomain-containing protein 4 (BRD4) (122). However, cancer cells can develop also resistance to epigenetic inhibitors. In neuroblastoma, PI3K pathway activation and transcriptional reprogramming can confer resistance to BET inhibitors, indicating that sequential or combination therapies will likely be required to achieve durable antitumor effects (123). In this regard, the combined inhibition of BET proteins and HDACs is increasingly regarded as a strategy to improve the effectiveness of these drugs in cancer (124). A novel link between epigenetic regulation and chemoresistance has come from colorectal cancer, where the epigenetic dioxygenase TET2 has been shown to control a population of slow cycling cells responsible for chemoresistance and tumor recurrence (44). Slow cycling cell populations generated by epigenetic factors in multiple tumor settings likely represent a reservoir for the subsequent emergence of heterogeneous proliferating drug resistant cells. In fact, further epigenetic rearrangements and even genetic mutations can occur in quiescent cells giving rise to a variety of survival strategies (125). In line with this hypothesis, a single lung cancer persister cell was shown to generate a variety of colonies with different mechanisms of erlotinib resistance (126). Finally, recent discoveries suggest that repetitive transposable elements may be involved in the epigenetic determination of drug resistance. Repetitive elements constitute nearly half of the human genome and in normal cells they are tightly regulated to avoid dangerous inappropriate activation events. In cancer cells repetitive elements are often aberrantly activated, in part due to decreases in DNA methylation (127). Chemotherapeutic and targeted drugs can also induce a strong activation of repeated elements in cancer cells that results in cell death (116, 128). By contrast, DTPs within the heterogeneous cancer cell population are able to maintain the epigenetic repression of repetitive elements through increased histone H3K9 and H3K27 methylation even during drug treatment, exploiting this strategy to survive drug exposure (116). In line with these observations, a new generation of drugs targeting epigenetic modulators are finding their way to the clinic, in the attempt to exploit cancer-associated epigenetic traits for therapeutic intervention (129). Clinically induced derepression of genomic repeat elements also harbors the potential to enhance the immunogenicity of cancer cells and enhance the response to immunotherapeutic approaches (127), fostering further investigations on the mechanisms that deregulate repeat element expression in tumor cells.

INTERACTIONS BETWEEN THE IMMUNE SYSTEM AND THERAPY RESISTANT CELLS

The importance of the immune system in controlling tumor growth, metastatization, and relapse is undisputed, as witnessed by the expanding role of immunotherapies in cancer treatment. New challenges concerning the use of immunotherapeutic drugs are often related to properties of CSCs, which have been reported to have a low immunogenic profile and peculiar interactions with immune cells (130). A striking example of interactions between CSCs and immune cells resulting in immunotherapy resistance has been recently highlighted in squamous cell carcinoma. Here, a population of tumor-initiating cells responsive to TGF-β acquires the expression of CD80 (a molecule previously identified on cells of the immune system) and hinders cytotoxic T cell activity leading to tumor relapse (131). The relationships between drug resistant cancer cells and the immune system are the

object of particularly intense investigations in the pre-metastatic context. In fact, while tumor cells in the primary tumor are often surrounded by an immune-suppressive microenvironment (132), DTCs are theoretically more vulnerable to immune attack, providing a window for therapeutic interventions aimed at preventing metastasis formation. Recent reviews have addressed in detail the role of the immune system in cancer (133) and the mechanisms of DTCs immune escape (60, 134). Here, we will focus our discussion to a limited number of recent breakthroughs in the relationships between drug resistant cells and the immune system. New insights on how DTCs evade immune recognition came from the observation that dormant cells activate the UPR, which in turn causes the downregulation of major histocompatibility complex class I (MHC I) molecules required for antigen presentation to $CD8^+$ T cells. This mechanism rendered DTCs undetectable by $CD8^+$ T cells, while targeting the UPR led to MHC I re-expression and reversal of the immune-evasive phenotype (135). The most important soluble factors in cancer-immune system interactions are interferons, and particularly IFNγ produced by T cells and NK cells (136). IFNγ has been shown to induce cancer cell dormancy through multiple pathways and, interestingly, to exert different effects in indolent ($Ki67^{low}$) cells, and in dormant ($Ki67^-$) cells (137). Besides its established role in contributing to anti-tumor immunity, IFNγ is also implicated in mediating resistance to various cancer therapies, including anti-PD1 therapies via downregulation of MHC I molecules (138, 139). An interesting link between IFNγ and CSCs metabolism came from the observation that IFNγ triggers cancer dormancy through indolamine 2,3 dioxygenase (IDO1), an enzyme that catalyzes tryptophan metabolism. Blocking IDO1 metabolic circuitry abrogates dormancy and induces apoptosis of tumor-repopulating cells (140). The same metabolic pathway was found to be involved in IFNβ-induced dormancy in melanoma (141). The Stimulator of Interferon Genes (STING) is a central component of the intracellular DNA sensing pathway and has been initially characterized for its capacity to mediate type I interferon inflammatory responses in immune cells during infections. Recent breakthroughs indicate that the STING pathway has much broader functions, being implicated also in fundamental cancer-related processes such as cellular transformation (142, 143), metastasis (144), and response to radio- and chemotherapy (145, 146). STING has been recently identified as an activator of autophagy downstream of the ancestral cyclic GMP-AMP synthase (cGAS) pathway (147) and may also be implicated in chemoresistance-related autophagy. Additional evidences indicate a direct link between STING and LKB1, which is a crucial regulator of stem cell quiescence, metabolism, and anti-tumor immunity (148). Specifically, loss of LKB1 leads to the suppression of STING and insensitivity to cytoplasmic double-strand DNA detection, resulting in resistance of lung cancer to immunotherapy (149). Therefore, therapies that reactivate LKB1 or the STING pathway may boost anticancer immune response in cancers with resistance to immune-checkpoint blockade (150). Finally, immune cells have been recently implicated in DTC reawakening from dormancy in a study showing a key role for Neutrophil Extracellular Traps (NETs) produced by neutrophils in the lung parenchyma upon inflammation. NETs trigger integrin-mediated activation of focal-adesion kinase in DTCs and subsequent exit from dormancy, while integrin-blocking antibodies prevent DTC reactivation in NET-enriched lungs (151). The latter study confirms the involvement of integrins in chemo- and radiotherapy resistance of multiple cancers, raising hopes for the future development of effective therapeutic agents blocking integrin signaling (152).

CONSIDERATIONS ON TARGETING THERAPY RESISTANT CANCER STEM CELLS

Therapies directed against drug resistant cells resident in either primary tumors, pre-metastatic niches, or metastatic cancers have to face an array of genetic and epigenetic survival strategies exploited by cancer cells. Conventional therapies such as radio- and chemotherapy, although representing the mainstay of cancer therapy, are intrinsically limited in their capacity to face drug resistance and may themselves promote the emergence of more aggressive cells. In recent years the mechanisms underlying cancer cell plasticity, heterogeneity, stress responses, and dormancy have begun to be elucidated, indicating new routes of therapeutic intervention. At the same time, new therapeutic approaches should undergo careful pre-clinical evaluation for their effects on the CSCs compartment, which would provide indications on the development of resistant cell populations. New therapeutic strategies directed against dormant/drug resistant cells in primary tumors are progressively focusing on epigenetic modulators such as inhibitors of KDMs, HDACs, or BET proteins. By contrast, therapeutic strategies directed against pre-metastatic DTCs are divided in three main workstreams: the so-called "sleeping strategies" include drugs that suppress proliferative signals such as anti-estrogen therapies (153), inhibitors of CDK4/6 (154), and inhibitors of ERK or Src (155). Prolonging dormant states can also be obtained with drugs that increase the expression of dormancy factors such as p38, DYRK1A, and N2RF1 (63, 65, 156, 157). Sleeping strategies such as anti-estrogen therapies for breast and prostate cancer had a profound impact in the clinical setting. However, sleeping strategies must be long-lasting (or even lifetime long) and therefore must deal with unwanted side effects that can limit their long-term usage and reduce patient compliance. In this regard, the use of retinoic acid or fenretinide derivatives to induce cancer cell quiescence appear as a feasible and relatively non-toxic therapeutic approach (12, 158). An additional problem related to sleeping strategies is that not all tumor cells are responsive, as ER-positive breast tumors can give rise to metastases even during hormonal therapy. Thus, the combination or sequential administration of "sleeping" therapeutics should be explored, with the caution of avoiding toxic side effects. A second type of strategies directed against dormant cells is represented by cell cycle reactivation, classically with G-CSF or IFNα (159). However, treatment with reactivating agents may not be effective on all the tumor cells, leaving behind some dormant persisters. Also, therapeutic reactivation

may render tumor cells more aggressive and potentially resistant to subsequent chemotherapy. The third strategy consists in eliminating dormant/drug resistant cells while dormant, a difficult but not impossible challenge. Elimination of dormant cells has been achieved in experimental brain tumors with mithramycin (160), HDACs or KDMs inhibition (8, 9, 116, 119), while in pancreatic tumors resistant cells were eliminated by IGF1 inhibition (161). Activators of ferroptosis, a form of cell death characterized by the accumulation of lipid peroxidation products and lethal reactive oxygen species derived from iron metabolism, were shown to kill drug tolerant cells in multiple tumors (162, 163). Mitochondrial respiration is also considered a promising target (106, 164–167), although metabolic plasticity could result in the unresponsiveness of some resistant cells. Besides the inhibition of specific cellular proteins/pathways, other strategies with a broader mechanism of action may be useful to cope with drug resistance. First, immunotherapy alone or in combination with targeted agents is proving effective in several cancers even in the metastatic setting (168, 169). Adoptive transfer of tumor-reactive lymphocytes has led to striking anti-tumor immune responses in breast cancer and other tumors (170–174), indicating that totally drug resistant cells such as those in advanced metastatic tumors can still be eliminated by the immune system. Also, emerging evidences suggest that micro- and macro-environmental signals can profoundly impact on the biology of drug resistant cells. While inflammation has been shown to facilitate metastatic outgrowth (151, 175, 176), anti-inflammatory agents such as NSAIDs seem to dramatically decrease the risk of metastatic relapse, possibly by preventing the reawakening of dormant cells caused by niche alterations that occur during inflammation (177, 178). Finally, therapeutic strategies that include lifestyle-related factors such as exercise and nutrition are emerging as an important tool not only in cancer prevention but also in managing established cancers (179). Diet and lifestyle likely act through reinforcing the immune system, modulating hormone levels, shaping gut microbiota, preventing inflammatory conditions, and influencing the pre-metastatic niche to become less favorable to DTCs awakening (179, 180). A link between diet-related factors and therapy has recently emerged by studies showing that a hypoglycemic diet improves the effectiveness of PI3K inhibitors (181). Likewise, fasting or fasting-mimicking diets are increasingly considered as valid supports in cancer therapy due to their ability to induce wide alterations in growth factors and metabolite levels that generate unfavorable environments for cancer cells and improve the effects of cancer therapies (182). Finally, physical exercise is being explored for its ability to promote and restore antitumor immunity. In fact, the infiltration and antitumor activity of immune cells are limited by a scarcely oxygenated and acidic tumor microenvironment. Exercise has been reported to modulate oxygen concentration and pH in the tumor bed and to directly stimulate tumor cell killing by immune cells, thus appearing as a potential tool to improve the effectiveness of immunotherapy (183). In summary, drug resistance increasingly appears as a multifactorial process (**Figure 2**) where each class of factors can be considered as a target for novel therapeutic strategies.

CONCLUSIONS

Understanding the mechanisms of drug resistance is mandatory in order to improve the effectiveness of cancer therapies. While novel and unexpected mechanisms of drug resistance continue to emerge, translational research is moving toward new therapeutic approaches involving not only cancer cells and peritumoral cells but also other components of the body such as the immune and the hormonal system. As a result of the discoveries made in the last decade, drug resistant cancer cells in their different contexts are starting to appear as a treatable target. However, increasing efforts are required to explore the mechanisms that regulate drug resistance of cancer cells either in primary tumors, pre-metastatic niches, and overt metastases in order to find new therapeutic avenues.

AUTHOR CONTRIBUTIONS

AZ, MD, and FF wrote the manuscript. FL provided essential expertise.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00626/full#supplementary-material

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REVIEW
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Targeting the Interplay Between Cancer Fibroblasts, Mesenchymal Stem Cells, and Cancer Stem Cells in Desmoplastic Cancers

Tze-Sian Chan[1,2,3,4], *Yuval Shaked*[5,6] *and Kelvin K. Tsai*[1,2,3,7,8]*

[1] *Laboratory of Advanced Molecular Therapeutics, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan,* [2] *Division of Gastroenterology, Department of Internal Medicine, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan,* [3] *Integrative Therapy Center for Gastroenterologic Cancers, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan,* [4] *School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan,* [5] *Department of Cell Biology and Cancer Science, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel,* [6] *Technion Integrated Cancer Center, Technion – Israel Institute of Technology, Haifa, Israel,* [7] *Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan,* [8] *National Institute of Cancer Research, National Health Research Institutes, Taipei, Taiwan*

Malignant tumors are highly heterogeneous and likely contain a subset of cancer cells termed cancer stem cells (CSCs). CSCs exist in a dynamic equilibrium with their microenvironments and the CSC phenotype is tightly regulated by both cell-intrinsic and cell-extrinsic factors including those derived from their surrounding cells or stroma. Many human solid tumors like breast, lung, colorectal and pancreatic cancers are characterized by a pronounced stromal reaction termed "the desmoplastic response." Carcinoma-associated fibroblasts (CAFs) derived either from resident fibroblasts or tumor-infiltrating mesenchymal stem cells (MSCs) are a major component of the stroma in desmoplastic cancers. Recent studies identified subpopulations of CAFs proficient in secreting a plethora of factors to foster CSCs, tumor growth, and invasion. In addition, cytotoxic therapy can lead to the enrichment of functionally perturbed CAFs, which are endowed with additional capabilities to enhance cancer stemness, leading to treatment resistance and tumor aggressiveness. When recruited into the tumor stroma, bone-marrow-derived MSCs can promote cancer stemness by secreting a specific set of paracrine factors or converting into pro-stemness CAFs. Thus, blockade of the crosstalk of pro-stemness CAFs and MSCs with CSCs may provide a new avenue to improving the therapeutic outcome of desmoplastic tumors. This up-to-date, in-depth and balanced review describes the recent progress in understanding the pro-stemness roles of CAFs and tumor-infiltrating MSCs and the associated paracrine signaling processes. We emphasize the effects of systemic chemotherapy on the CAF/MSC–CSC interplay. We summarize various promising and novel approaches in mitigating the stimulatory effect of CAFs or MSCs on CSCs that have shown efficacies in preclinical models of desmoplastic tumors and highlight the unique advantages of CAF- or MSC-targeted therapies. We also discuss potential challenges in the clinical development of CSC- or MSC-targeted therapies and propose CAF-related biomarkers that can guide the next-generation clinical studies.

Keywords: cancer-associated fibroblasts, mesenchymal stem cells, cancer stem cells, paracrine signaling, desmoplasia

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Alexandre Prieur,
ECS-Screening SA, Switzerland

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Aamir Ahmad,
Mitchell Cancer Institute,
United States
Nate Brennen,
Johns Hopkins Medicine,
United States

***Correspondence:**
Kelvin K. Tsai
tsaik@tmu.edu.tw

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INTRODUCTION

Cancer Stem Cells (CSCs) as the Driving Force of Tumor Progression

An emerging concept of cancer biology emphasizes the critical role of the hierarchical organization in tumors in the maintenance as well as the progression of the malignant phenotypes. In support of this paradigm, mounting data over recent years, including large-scale genomic analysis and single-cell RNA sequencing analysis, have consistently indicated the existence of a subset of cancer cells termed the tumor-initiating cells (TICs) or CSCs, which are stem-like and have the capability of self-renewing and sustaining tumorigenesis and thereby serve as the driving force of cancer growth, metastasis, and treatment resistance (1–3). CSCs have been found to exist in leukemia and multiple solid tumors, such as glioma, breast cancer, pancreatic ductal adenocarcinoma (PDAC), head and neck squamous cell carcinoma, hepatocellular carcinoma, non-small cell lung cancer (NSCLC), and colorectal cancer (CRC) (4–8).

The recent insights into the complex nature of cancer stemness reveal that CSCs exist in a dynamic equilibrium with their microenvironments and the CSC phenotype is regulated by both cell-intrinsic and cell-extrinsic factors derived by their surrounding cells or stroma cells. The notable examples of the "pro-stemness" or "pro-CSC" factors identified from these studies are inflammatory cytokines, such as interleukin-6 (IL-6), IL-8, and C-C motif chemokine ligand 5 (CCL-5), which play an essential role in CSC regulation as well as invasion and metastasis of tumors (9–11).

CAFs and MSCs Foster Cancer Stemness

Many types of human solid tumors, especially those derived from glandular epithelium, such as breast cancer, NSCLC, PDAC, the scirrhous subtype of gastric adenocarcinoma, and the "stem/serrated/mesenchymal (SSM)" molecular subtype of CRC, are characterized by a pronounced stromal reaction termed "the desmoplastic response" (12–17). CAFs and their collagen matrix products are a major component of the stroma in desmoplastic cancers, comprising a substantial proportion of the tumor mass (18, 19). Instead of being functional inert, there is circumstantial evidence that CAFs are pro-inflammatory due to activation of nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT)-1 and−3, and transforming growth factor (TGF)-β/SMAD signaling and are engaged in active cross-talk with cancer cells (19, 20). Therefore, CAFs can foster tumor cell growth, angiogenesis and invasion (21) by secreting paracrine factors, such as pro-inflammatory cytokines (19), chemokines (14, 19), prostaglandins (PGE) (22), growth factors (23), and proteases (24), and by remodeling the extracellular matrix (ECM) (25–28). CAFs also help foster an immunosuppressive microenvironment in tumors by promoting regulatory T cells (29). Recent studies demonstrated that exosomes derived from CAFs promote cancer progression and treatment resistance (30, 31). Intriguingly, CAFs can even travel with malignant cells to distant sites, where they significantly promote metastasis (32). One of the major mechanisms by which CAFs promote oncogenesis is mediated through their pro-stemness abilities. Recent studies have identified specific subpopulations of CAFs that are proficient in secreting pro-stemness paracrine factors (9–11, 23, 33–35), thereby promoting the conversion of cancer cells into CSCs or supporting the self-renewal and the stemness properties of existing CSCs in tumors. Upon stimulation by cytotoxic stress such as chemotherapy, CAFs can be further induced to secrete pro-stemness cytokines or acquire a senescence-like secretory phenotype and produce large amounts of pro-stemness chemokines to further enhance tumor stemness and aggressiveness following therapy (36, 37).

Although the majority of CAFs in the tumor stroma may be derived from resident stromal fibroblasts, there are now multiple lines of evidence suggesting that a significant proportion of CAFs in tumors are derived from bone marrow-derived mesenchymal stem cells (MSCs). MSCs are pluripotent stem cells that contribute to bone, adipose, cartilage, and muscle tissues and are involved in tissue remodeling, chronic inflammation, immune response, and cancer progression (38). Bone marrow-derived MSCs can be recruited to sites of tissue damages or inflammation by endocrinal signals to exert their tissue repairing functions (39), whereas the tissue-regenerative function of MSCs may go awry in malignant tumors. For instance, in mouse models of breast cancer, PDAC or gastric cancer, bone marrow-derived MSCs are recruited to the tumor microenvironment where they differentiate into CAFs (40–42). Indeed, in a gastric cancer model, approximately 20% of CAFs were found to originate from bone marrow-derived MSCs, which were recruited into the tumors in a transforming growth factor (TGF)-β and C-X-C motif chemokine ligand (CXCL)-12-dependent manner (43). Similarly, MSCs introduced into the tibia trafficked to sites of breast tumor xenografts (44). In an orthotopic murine PDAC model, MSCs were actively recruited into the growing pancreatic tumors (45). Like CAFs, MSCs can significantly influence tumor behaviors and contribute to tumor progression. Most importantly, MSCs promote CSCs by secreting a plethora of pro-stemness cytokines and growth factors or indirectly by differentiating into pro-stemness CAFs (44, 46, 47).

Cancer Therapy Can Alter Tumor Stroma and Promote Tumor Stemness

In clinical scenarios, most cancers are treated with certain types of cytotoxic therapies, such as chemotherapy and radiation therapy, which may have profound impacts on the characteristics of tumors including the epithelial and the stromal compartments. Indeed, chemotherapy has been shown to enrich tumor cells for those with mesenchymal and/or CSC features in different types of cancers. CSCs are intrinsically more resistant to therapy and consequently increase disproportionately following systemic chemotherapy and are thought to contribute to tumor relapse and treatment resistance (1, 48, 49). For instance, breast cancers after neoadjuvant chemotherapy are enriched from $CD44^{+}CD24^{-}$ CSCs that also express mesenchymal markers (48, 49). Chronic oxaliplatin or paclitaxel treatment induces an epithelial-mesenchymal transition (EMT) and the enrichment of CSCs in CRC and ovarian cancer (50, 51). Chemotherapy has also been shown to expand CSCs that are dependent on

the interleukin (IL)-8–CXCR-1 signaling axis (52). Importantly, CAFs are enriched in chemotherapy-treated human tumor tissues wherein they promote cancer growth, treatment resistance and the self-renewal of CSCs by secreting paracrine factors (36, 53). Moreover, chemotherapy-modulated CAFs secrete a panel of CXCL chemokines to expand CSCs in the treated tumor, leading to paradoxical tumor aggression and treatment failure (37). Thus, adjuvant strategies that target CAFs to temper the chemotherapy-induced enrichment of CSCs may further improve the therapeutic outcome of patients with desmoplastic cancers.

The past two decades of investigations into CSCs and their biology have led to the identification of a number of potentially druggable targets, based on which many CSC-directed therapies have been developed with some of them entering clinical trials (54). Unfortunately, the idea of therapeutic targeting of CSCs has suffered from a series of notable clinical trial failures over recent years, including the focal adhesion kinase (FAK) inhibitor defactinib, the STAT-3 inhibitor napabucasion, the anti-NOTCH-2/3 antibody tarextumab, the anti-delta like canonical notch ligand (DLL)-4 antibody demcizumab, and most recently the multibillion-dollar anti-DLL-3 antibody-drug conjugate rovalpituzumab tesirine (Rova-T). Apparently, there is an urgent need for new and more viable strategies of successfully and safely targeting CSCs. As opposed to the direct targeting of the rare, dynamic and plastic CSC populations, targeting the more abundant, favorably spaced and stable CAFs and MSCs, especially their pro-stemness subsets, presents an attractive strategy to indirectly target cancer stemness to enhance the efficacy of current anti-cancer therapies.

In this review, we describe how CAFs and MSCs initiate crosstalk with CSCs and augment cancer stemness in human solid tumors. We emphasize the effects of systemic chemotherapy on CAFs and how these effects can modulate their pro-stemness functions in the treated tumor. We discuss the advantages of targeting CAFs or MSCs over directly targeting CSCs, as well as various promising approaches that aim at disengaging the CAF/MSC–CSC link in preclinical models. This review finally lists potential challenges in the clinical development of pro-stemness-CAF- or MSC-targeted therapies and explores potential biomarkers of pro-stemness CAFs to guide the development of therapeutic strategies to disengage the dangerous interplay between CAFs, MSCs, and CSCs that can be quickly deployed in clinical trials in the treatment of human desmoplastic cancers.

CAFs AND CSCs IN DESMOPLASTIC CANCERS: THE MESENCHYMAL–EPITHELIAL CROSSTALK GOES AWRY

As described above, CAFs are proficient in paracrine signaling and are capable of secreting a plethora of paracrine factors that have been implicated in the maintenance and/or the expansion of CSCs (**Figure 1** and **Table 1**). Among the most extensively studied pro-stemness cytokines secreted by CAFs are IL-6 and IL-8, which have been shown to play an essential role in the regulation of CSCs as well as cancer invasion and metastasis (9–11). Several mechanistic studies have demonstrated that IL-6 participates in the regulation and maintenance of the CSC phenotype mainly through the STAT-3–NF-κB signaling pathway (10, 11, 57). Constitutive IL-6 expression in breast cancer cells maintains their EMT phenotype, which has been implicated in the generation of a CSC phenotype (58, 59). As opposed to the role of the IL-6 inflammatory loop in inducing CSCs with mesenchymal features in breast cancer, IL-8 mainly regulates a subpopulation of epithelial-like CSCs that express high aldehyde dehydrogenase (ALDH) activity and are highly proliferative (52). Consistently, IL-8 was found to profoundly enhance the stemness property of breast cancer and PDAC cells (60–62). A recent proteomic screening identified leukemia inhibitory factor (LIF)-induced STAT-3 activation as the major signaling event in PDAC cells induced by PSCs, leading to activation of stemness programs, including Hippo, Wnt, and STAT-3 (35). Notably, LIF expression is significantly up-regulated in PDAC tissues while the expression of IL-6 does not, underscoring the importance of LIF over IL-6 in PDAC. Aside from interleukins, a multitude of other secretory factors has also been implicated in mediating the pro-stemness capability of CAFs. For instance, in CRC models established using primary carcinoma cells, CAF-derived osteopontin (OPN) has been shown to support the clonogenic capacity of CSCs, which predominantly reside at the tumor edge in close proximity to CAFs (64). Another study also showed that the CAFs freshly isolated from human CRC tumors produced significantly higher levels of CXCL-12, OPN, TGF-β, and hepatocyte growth factor (HGF), which coordinately activated Wnt–β-catenin signaling to induce the expression of the novel CSC marker CD44 variant 6, resulting in an EMT in cancer cells and tumor invasion and metastasis (56). In another CRC model established using freshly isolated carcinoma cells and the paired CAFs, CAFs up-regulated the expression of TGF-β2 and IL-6, which activated the expression of GLI family zinc finger (GLI)-2 in the sonic hedgehog (SHH) pathway, resulting in the transdifferentiation of cancer cells into CSCs and chemotherapy resistance (65). Pancreatic stellate cells (PSCs), a specialized type of CAFs present in the stroma of PDAC, secrete the TGF-β family protein Nodal, which binds to its receptor Activin-like (Alk)-4 and−7 on CSCs to promote their stemness properties (34, 63). In an NSCLC model, insulin-like growth factor (IGF)-II and allied autocrine/paracrine factors secreted by CAFs synergistically activated IGF-1R signaling to induce the expression of the stemness-related gene Nanog, thereby converting cancer cells into CSCs (23). CAFs isolated from human breast cancer secrete abundant levels of PGE-2, which enhances the secretion of IL-6 to expand CSCs (22). Moreover, when co-cultivated with cancer cells, CAFs produced a higher level of CCL-2, which stimulate CSCs by inducing Notch-1 expression and thereby activating the Notch signaling pathway (33).

Mounting data accumulated over recent years have suggested that CAFs in desmoplastic cancers are phenotypically, functionally and genetically heterogeneous and are likely dynamically controlled by their environments and origins (29, 71–76). Indeed, CAFs have been classified into various functional subtypes according to a panel of surface markers,

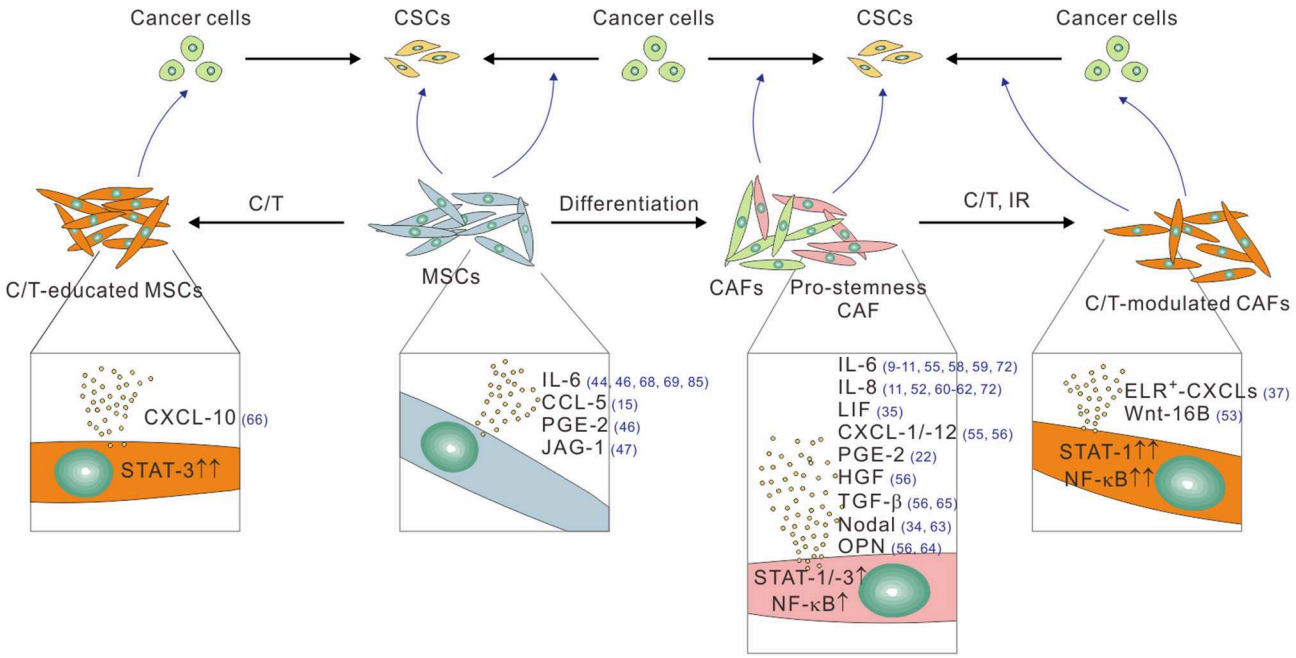

FIGURE 1 | The pro-stemness functions of CAFs and tumor infiltrating MSCs linked to their different functional and treatment status. CAFs, especially their pro-stemness subset, secrete assorted cytokine and chemokines, including IL-6, IL-8, LIF, PGE-2, CXCL-1, CXCL-12, HGF, and TGF-β through heightened STAT, and NF-κB signaling activity to promote the reprogramming of cancer cells into CSCs and/or directly expand the CSC population. CAFs also secret Nodal and osteopontin (OPN) to promote CSCs. Cytotoxic therapies such as chemotherapy (C/T) and ionizing radiation (IR) further potentiate the pro-stemness functions of CAFs by further activating STAT-1 and NF-κB signaling, thereby inducing the secretion of a different panel of pro-stemness factors, including ELR^+-CXCL chemokines, and Wnt-16B. Bone marrow-derived and tumor-infiltrating MSCs can promote CSCs by converting into CAFs or by secreting several pro-stemness chemokines such as IL-6, CCL-5, PGE-2, and JAG-1. C/T-educated MSCs further secrete the pro-stemness chemokine CXCL-10 by activating STAT-1 signaling. The reference numbers are shown in blue.

including such as α-SMA, fibroblast activation protein (FAP), fibroblast specific protein (FSP)-1, and platelet-derived growth factor receptor (PDGFR)-α/β (19, 77, 78). At the functional level, FAP^+ CAFs are enriched in low stiffness and fibronectin-rich ECMs, whereas α-SMA^+ CAFs are found in stiffer ECM contexts (76). In a transgenic model of PDAC, depletion of α-SMA^+ CAFs did not affect the number of FAP^+ CAFs, indicating that they represent different CAF subpopulations (79). Interestingly, FAP^+ CAFs are predominantly involved in the synthesis and the turnover of ECM while α-SMA^+ CAFs mediate contraction. Importantly, the recently identified CAF heterogeneity relates to the pro-stemness and pro-oncogenic capabilities of CAFs. For instance, in oral squamous cell carcinoma, a subgroup of CAFs termed "CAF-D" has been shown to induce EMT of malignant keratinocytes through secreting TGF-β (74). Since the EMT program in cancer cells imparts them with CSC features (59, 80, 81), it is likely that this specific subpopulation of CAFs might have induced the phenotypic conversion of keratinocytes into CSCs. In PDAC, two distinct subgroups of PSCs have been identified in mouse and human PDAC tissues (71). Remarkably, only those PSCs located away from tumor cells, denoted as "inflammatory CAFs (iCAFs)," were proficient in secreting pro-stemness factors, including IL-6, CXCL-1, and CXCL-2, through activation of IL-1α-Janus kina (JAK)–STAT signaling (55). By contrast, PSCs located adjacent to tumor cells have the propensity of differentiating into collagen-producing and α-smooth muscle actin (α-SMA)-positive myofibroblasts. In analogous to this emerging paradigm of the functional heterogeneity of CAFs, in human breast cancer and NSCLC tissues, a distinct subpopulation of CAFs was found to express CD10 as well as the complement 5 a receptor G-protein coupled receptor 77 (GPR-77) and are proficient in promoting CSCs and their stemness properties and inducing chemoresistance of tumor cells through persistent NF-κB activation along with the resultant IL-6 and IL-8 secretion (72). Of note, these pro-stemness subset of CAFs were either defined by surface markers (e.g., CD10 and GPR-77), their transcriptome and secretome (e.g., CAF-D), or a specific set of secretory factors (e.g., IL-6 and LIF in iCAFs). It remains to be established if the pro-stemness subset of CAFs indeed vary among different types of cancers or can be molecularly defined in a more precise manner.

In chemotherapy-treated desmoplastic cancers, CAFs are endowed with additional pro-stemness and pro-oncogenic capabilities as a result of the stress-induced chronic phenotypic and functional alterations. For instance, in prostate cancer, the genotoxic agent mitoxantrone stimulated Wnt-16B secretion by stromal fibroblasts, which promoted the proliferation and invasion of carcinoma cells, which likely contained the enriched CSCs (53). In human CRC, chemotherapy led to the enrichment of IL-17A-producing CAFs within the tumor stroma, which in turn promoted the self-renewal of CSCs and tumor growth (36). In addition, following systemic chemotherapy, breast and

TABLE 1 | Pro-stemness factors secreted by CAFs and MSCs.

Factors	Cancer types	Functions	Stemness pathway involved	References
CAFs				
CCL-2	BC	Stimulates CSCs by inducing Notch-1 expression	Notch-1	(33)
CXCL-1	PDAC	Promotes cancer stemness	IL-1α/JAK/STAT	(55)
CXCL-12	CRC	Induces the expression of CSC markers	Wnt/CD44v6, PI3K	(56)
CXCL-2	PDAC	Promotes cancer stemness	IL-1α/JAK/STAT	(55)
ELR^+ CXCLs	BC, PDAC	Secreted by chemotherapy-altered CAFs and promote CSC expansion	STAT-1, NF-κB	(37)
HGF	CRC	Induces the expression of CSC markers	PI3K	(56)
IGF-II	NSCLC	Induces conversion of cancer cells into CSCs	IGF1R, EMT, PI3K, TGF-β, Wnt, and Hedgehog	(23)
IL-17A	CRC	Promotes the self-renewal of CSCs and tumor growth		(36)
IL-6	BC	Promotes and maintains CSCs	STAT-3 and NF-κB	(10, 11, 57)
	BC	Maintains EMT phenotype and stem cell properties	EMT	(58, 59)
	PDAC	Pro-stemness factor	IL-1α/JAK/STAT	(55)
IL-8	BC	Promotes epithelial-like $ALDH^+$ CSCs	FAK/AKT/FOXO-3A	(52)
	BC, PDAC	Enhances stemness property		(60–62)
LIF	PDAC	Activates stemness program, including Hippo, Wnt and STAT-3	STAT-3	(35)
Nodal	PDAC	Binds to Alk-4/-7 to promote stemness in cancer cells	Nodal/activin	(34, 63)
OPN	CRC	Supports the clonogenic capacity of CSCs Induces expression of CSC marker	Wnt/CD44v6, PI3K	(56, 64)
PGE-2	BC	Promotes secretion of IL-6 and expansion of CSCs	NF-κB	(22)
TGF-β	CRC	Induces the expression of CSC marker	Wnt/CD44v6, PI3K	(56)
TGF-β2	CRC	Induces trans-differentiation of cancer cells into CSCs and confers chemo-resistance	Hedgehog/GLI-2	(65)
WNT16B	PC	Enriches CSCs and promotes proliferation and invasion of cancer cells	Wnt, EMT	(53)
MSCs				
CCL-5	BC	Promotes cancer stemness and tumor metastasis		(15)
CXCL-10	PDAC	Promotes cancer stemness and expand the number of MSCs.	CXCR-3	(66)
CXCL-3	PDAC	Promote CSCs following gemcitabine therapy	STAT-3/CXCR-3	(67)
CXCL-7	BC	Promotes CSCs and tumor growth	IL-6	(68)
IL-6	BC	Regulates CSCs and promotes tumor growth	β-catenin	(46)
	CRC	Promotes drug resistance following paclitaxel therapy	JAK-2/STAT-3	(69)
JAG-1	PDAC	Maintains CSCs	Notch-1	(47)
PGE-2	CRC	Induces the formation of CSCs by inducing the expressions of IL-6, IL-8, and CXCL-1.	Wnt	(46)
PUFA	BC, CRC, LC	Promotes the regrowth of tumors following chemotherapy	Cyclooxygenase-1/thromboxane synthase	(70)

Alk-4, activin-like 4; BC, breast cancer; CCL, chemokine C-C motif ligand; CRC, colorectal cancer; CXCL, C-X-C motif chemokine ligand; ELR^+ CXCLs, ELR motif–positive chemokines; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; FOXO-3A, Forkhead box O3; GLI-2, GLI family zinc finger 2; HER-2, human epidermal growth factor receptor 2; HGF, hepatocyte growth factor; IGF, insulin growth factor; IL, interleukin; JAG, Jagged; JAK, Janus kinase; LC, lung carcinoma; LIF, leukemia inhibitory factor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; OPN, osteopontin; PC, prostate cancer; PDAC, pancreatic ductal adenocarcinoma; PGE, prostaglandin; PI3K, phosphatidylinositol 3-kinase; PUFA, polyunsaturated fatty acids; STAT, signal transducer and activator of transcription; TGF, transforming growth factor.

pancreatic CAFs secreted large amounts of the "ELR-motif-positive" (ELR^+) CXCL chemokines through chronic activation of the STAT-1 and NF-κB transcriptional activities, which stimulated CXCR-2 signaling in cancer cells to elicit their transdifferentiation into CSCs and thereby promoted post-treatment tumor aggression and treatment failure (37).

Despite the multiple lines of evidence supporting the pro-stemness functions of CAFs and therapy-modulated CAFs, it is worth noting that, as highlighted above, CAFs are capable of promoting tumor progression and treatment resistance through a multitude of mechanisms. Therefore, the tumor-promoting effects of CAFs summarized herein may likely be mediated by the concerted actions of a plurality of mechanisms and should not be attributed only to the pro-stemness functions of CAFs. Moreover, since most of these studies were conducted in immuno-compromised or immune-deficient animal models,

caution should be exercised while interpreting the results related to the CAF-derived pro-stemness cytokines and chemokines that are actively involved in inflammation and immune regulation. Whether or not CAFs exert similar positive regulatory effects on CSCs in immunocompetent backgrounds and how these effects work in concert with other tumor-promoting mechanisms of CAFs await further in-depth investigation.

TUMOR-INFILTRATING MSCs AND THEIR INTERPLAY WITH CSCs

MSCs were initially considered to be cells promoting the regenerative properties of wounds and damaged tissues. A growing body of evidence indicated that the regenerative function of MSCs are hijacked by malignant tumors such that a significant number of bone marrow-derived MSCs are recruited to the tumor microenvironment, where a considerable proportion of them differentiate into CAFs (40, 41). Like CAFs, MSCs can secrete a plethora of cytokines and growth factors, which make them proficient in paracrine and heterotypic signaling processes. For instance, a recent comprehensive cytokine secretion profile of human MSCs identified IL-6, IL-8, TIMP metallopeptidase inhibitor 2 (TIMP-2), CCL-2 (MCP-1), and vascular endothelial growth factor (VEGF) as the most abundantly secreted factors (82). Other studies reported that MSCs promote cancer metastasis by secreting CCL-5 (15), CXCL-12, and IGF-1 (83). MSCs also contribute to tumor angiogenesis by secreting vascular endothelial growth factor (VEGF), and β-fibroblast growth factor (FGF) (67). Furthermore, MSCs promote immunomodulation by upregulating cytokines such as IL-6, IL-8, and TGF-β (68, 69, 84). In breast cancer, the cancer cells stimulate the secretion of CCL-5 from MSCs, which acts in a paracrine fashion on the cancer cells to enhance their motility, invasion, and metastasis (15). Importantly, several studies have directly implicated MSCs in the regulation of CSCs (**Figure 1**). MSCs in breast cancer regulate CSCs through cytokine loops involving IL-6 and CXCL-7, thereby accelerating tumor growth (44). In CRC, MSCs secrete prostaglandin E2 (PGE-2) in response to IL-1 released by carcinoma cells, which act in an autocrine fashion to induce the expression of IL-6, IL-8 and CXCL-1, which together induce the formation of CSCs (46). Once differentiated into CAFs, MSCs can maintain CSCs through secreting the Notch ligand Jagged-1 (47). In analogous to the effect of chemotherapy on the number and the pro-stemness property of CAFs, the number of bone marrow-derived MSCs significantly increased following gemcitabine treatment in the tumor stroma in a mouse xenograft model of PDAC (66). Importantly, these gemcitabine-educated MSCs were found to have a positive regulatory effect on CSCs through the STAT-3–CXCL-10–CXCR-3 paracrine signaling axis. Similarly, following paclitaxel treatment or hyperthermia therapy, MSCs secreted IL-6, IL-7, IL-8, EGF, and IGF, which supported drug resistance (85, 86). In another study, cisplatin-activated MSCs produced specific polyunsaturated fatty acids which in turn promoted the regrowth of tumors following therapy (70).

Collectively, the ample evidence underscore the important role of CAFs and tumor-infiltrating MSCs in the maintenance and the expansion of CSCs and suggest that targeting this component of the tumor stroma may provide a new avenue to improving the therapeutic outcome of human desmoplastic cancers.

TARGETING THE CROSSTALK BETWEEN CAFs AND CSCs

Given that CAFs positively regulate CSCs through the secretion of pro-stemness paracrine factors, a number of preclinical studies have exploited the therapeutic potential of the functional blockade of the CAF-to-CSC paracrine signaling process to improve the treatment of desmoplastic cancers (**Figure 2**). For instance, loss of PTEN in a HER2-overexpression genetic background or the trastuzumab resistance in breast cancer cells has been linked to activation of the IL-6/STAT-3/NF-κB inflammatory loop, which induced an EMT phenotype and expansion of the CSC population. Therefore, blocking this loop by a function-blocking anti-IL-6 receptor antibody could effectively revert these phenotypes (10). In another study, functional inhibition of the subgroup of IL-6- and IL-8-secreting $CD10^{+}GPR\text{-}77^{+}$ CAFs with anti-IL-6 and anti-IL-8 antibodies, together with docetaxel chemotherapy, has led to a near complete remission of tumors in a patient-derived xenograft (PDX) model of breast cancer (72). Interestingly, this study also demonstrated that an anti-GPR-77 antibody in combination with docetaxel therapy exerted anti-tumor efficacy comparable to that induced by the combination anti-IL-6/anti-IL-8 therapy, which significantly reduced the number of $CD10^{+}GPR\text{-}77^{+}$ CAFs and the proportion of CSCs in the treated tumors (72). In keeping with the critical role of IL-8 in cancer stemness, a function-blocking antibody against its receptor, CXCR-1, or a small-molecule inhibitor of CXCR-1 and CXCR-2, repertaxin, could deplete CSCs and inhibit tumor aggressiveness in human breast cancer xenografts (5). Of particular clinical relevance was the recent finding that repertaxin in combination with paclitaxel demonstrated a 30% response rate in a phase 1b study of metastatic breast cancer (87). Other small-molecule inhibitors of CXCR-2, including AZ13381758 and SB225002, have also shown preclinical efficacy in transgenic or PDX models of PDAC (37, 88). Of note, since that CXCR-2 is also expressed by myeloid-derived immunosuppressive cells in PDAC (88), its inhibitor may exert anti-tumor efficacy through multiple mechanisms of action. Aside from the molecular targeting of IL-6, IL-8 and their receptors, targeting their downstream signaling components in cancer cells and/or CSCs offer other viable opportunities for disabling the CAF–CSC crosstalk. For instance, a small-molecule inhibitor of STAT-3, BBI608, has been reported to significantly inhibit cancer stemness in a variety of cancer types (89), whereas the results from some of the recent clinical trials were discouraging. Furthermore, several novel therapeutics targeting the CAF-to-CSC IL-6–STAT-3 signaling axis are under development. These include a high-affinity anti-IL-6 antibody, MEDI5117, which has been shown to enhance the anti-tumor

efficacy of chemotherapy or gefitinib in several types of tumors that are known to be driven by the IL-6–STAT-3 signaling and especially target the $CD44^+CD24^-$ CSCs in trastuzumab-resistant and HER-2^+ breast cancer cells (90). Another example is a cyclic oligonucleotide decoy that corresponds to the STAT-3 response element of STAT-3-targeted genes, which showed promising anti-tumor efficacy in NSCLC models (91). Recently, another interleukin family protein, LIF, was found to be the major PSC-derived factors that promote CSCs in PDAC cells and tissues (35). Accordingly, systemic administration of a LIF-neutralizing antibody in combination with chemotherapy reduced the percentage of CSCs and mesenchymal-transited cancer cells and extended the survival of tumor-bearing mice in a transgenic model of PDAC.

Aside from inhibiting interleukin paracrine signaling, a number of studies have explored the therapeutic potential of inhibiting other CAF-derived pro-stemness factors. For instance, the CCL-2 neutralizing antibody has been shown to significantly suppress tumorigenesis and inhibit pro-stemness Notch signaling in an orthotopic breast cancer model involving the co-implantation of cancer cells and CAFs (33). The TGF-β inhibitor SD208 has been shown to reduce the CAF-induced expression of stemness markers and simultaneously induced the expression of differentiation markers in CAF-cocultivated CRC cells (65). Consequently, SD208 in combination with the small molecule inhibitor of GLI-2, a transcriptional factor in the SHH pathway, restored the sensitivity of the tumors to chemotherapy in mouse PDX models of CRC. SB431542, an inhibitor of the Nodal receptor Alk-4/7 expressed on pancreatic CSCs, could block the stemness and invasive capacities of CSCs and thereby inhibited PDAC progression especially when used in conjunction with a Smoothened (a SHH pathway receptor) inhibitor that depleted the tumor stroma (34). Nevertheless, it is worthy of note that these promising results should be interpreted with caution since most of the studies were conducted in immunodeficient mice without the potential influence from the immune system, which is especially relevant as most of the pro-stemness mediators studied also have pro-inflammatory and immune-related functions.

Given the profound impacts of CAFs on the tumor stemness and aggressiveness in desmoplastic cancers, depleting CAFs in the tumor stroma provided another viable option in attenuating the CAF–CSC interplay (**Figure 2**). Along this line, an oral DNA vaccine targeting FAP, a CAF-specific marker, has been demonstrated to suppress tumor growth and metastasis and confer a survival benefit in murine models of CRC and breast cancer (92). Moreover, adoptive transfer of FAP-targeted chimeric antigen receptor (CAR) T cells could specifically kill FAP^+ CAFs and induce multiple beneficial stroma alterations, leading to delayed tumor growth and survival extension in mouse models of NSCLC and PDAC (93, 94). Interestingly, a combined targeting of FAP^+ CAFs and EPH receptor A2 $(EphA2)^+$ cancer cells led to a nearly complete remission of the tumors (93), suggesting that CAF-targeted approaches have the potential to supplement and synergize with conventional cancer-cell-targeted therapies. Notwithstanding these promising results, caution must be exercised with the application of CAF-depleting strategy as the genetic depletion of CAFs using the conditional knockout of SHH signaling or the ganciclovir-induced depletion of CAFs in PDAC unexpectedly led to invasive and undifferentiated tumors along with unfavorable immunosuppression (79, 95). Furthermore, depletion of FAP^+ stromal cells in a transgenic mouse model has been associated with muscle wasting and impaired erythropoiesis (96), implicating the potential adverse effects of CAF-depleting therapies. In this regard, the functional inhibition of CAFs instead of their depletion may be a safer and more desirable therapeutic approach than the direct depletion of CAFs. Several recent studies toward this direction have shown promises. For instance, vitamin D receptor (VDR) signaling has been shown to antagonize TGF-β/SMAD signaling-induced activation of PSCs in PDAC tissues, which was mediated by the pro-stemness factors IL-6, CCL-2, and CXCL-1 (20). As such, calcipotriol, a potent vitamin D analog that controls VDR induction, inhibited inflammatory signaling in CAFs and reduced the expressions of IL-6, CCL-2, and CXCL-1. When combined with gemcitabine treatment, calcipotriol synergized with chemotherapy to control tumor growth and extend survival in transgenic mouse models of PDAC. Another approach involved transducing CAFs with a nanocarrier-formulated plasmid encoding a secretable form of the death ligand TNF-related apoptosis-inducing ligand (TRAIL) termed sTRAIL. Since CAFs are intrinsically resistant the killing effect of TRAIL, once transduced with the sTRAIL-expressing plasmid, they were converted into sTRAIL-producing cells and thereby triggered apoptosis of neighboring cancer cells (97). Surprisingly, the expression of sTRAIL also reprogrammed CAFs into a quiescent state. This approach demonstrated strong anti-tumor efficacy in a PDAC model.

Recently, low-dose metronomic (LDM) chemotherapy has emerged as a highly clinical applicable strategy to enhance the tumoral treatment response by tempering the therapy-induced stromal alterations in desmoplastic cancers (37, 98, 99). Compelling evidence from laboratory-based and clinical correlative studies have demonstrated that conventional chemotherapy administered at a maximum tolerated dose (MTD) induces myriad alterations in stromal cells, including endothelial cells and their progenitor cells, immune cells, and CAFs (53, 100). In keeping with this paradigm, our group recently demonstrated that systemic MTD chemotherapy of assorted agents, including paclitaxel, gemcitabine, doxorubicin, and cyclophosphamide, had profound impacts on CAFs in human breast cancer and PDAC tissues, which acquired the ability to secret large amounts of pro-stemness ELR^+ CXCL chemokines through the chronic activation of STAT-1 and NF-κB signaling (37). The CSC niche microenvironment generated by therapy-modulated CAFs could be attenuated by pretreating the tumors with a CXCR-2 inhibitor or by switching the dosing schedule to LDM regimens, which had a much less stimulatory effect on CAFs. We envisage that this approach has multiple benefits. First, it obviates the lengthy and costly process of developing new CAF- and/or CSC-targeted agents, which has an especially high attrition rate according to past experiences. Second, an increasing number of oral chemotherapeutic agents are available for clinical use, making the concept of

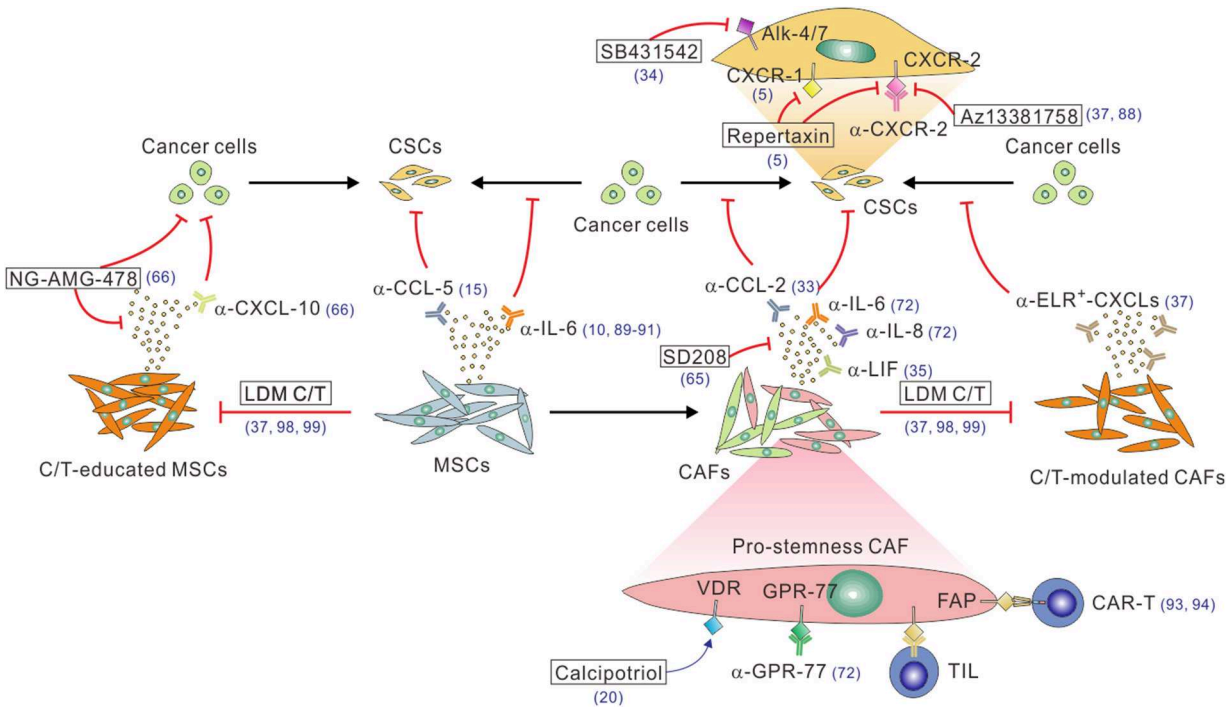

FIGURE 2 | A multitude of approaches to block the CAF/MSC–CSC crosstalk. Function-blocking antibodies, including α-IL-6, α-IL-8, α-LIF, α-CCL-2, and α-CCL5, or small molecular inhibitors, such as the TGF-β inhibitor SD208, can be used to block the stimulatory effect of these pro-stemness factors secreted by CAFs or C/T-modulated CAFs. On the other hand, the CAF-CSC paracrine signaling can be blocked by function-blocking antibodies (e.g., α-CXCR-1, α-CXCR-2) or small molecule inhibitors (e.g., repertaxin, AZ13381758, SB431542) of the receptors on CSCs and/or cancer cells. Likewise, the pro-stemness functions of tumor-infiltrating MSCs can be antagonized by function-blocking antibodies against IL-6 or CCL-5. The enhanced pro-stemness functions of C/T-modulated CAFs or MSCs can be potentially blocked by function-blocking anti-ELR$^+$-CXCL-chemokine antibodies, anti-CXCL-10 antibody, the CXCL-10 inhibitor AMG-478 (encapsulated by MSC-derived nano-ghost, NG), or adopting low-dose metronomic (LDM) C/T regimens. Function-blocking α-GRP-77 antibodies can be used to reduce the tumor infiltration of CD10$^+$GPR-77$^+$ pro-stemness CAFs. Calcipotriol can activate VDR signaling to inhibit IL-6, CCL-2, and CXCL-1 production by CAFs. Finally, FAP$^+$ or GPR-77$^+$ CAFs can be depleted by using DNA vaccines to induce CAF-specific tumor-infiltrating T cells (TILs) or administrating CAF-specific CAR-T cell or other types of engineered immune cells. The reference numbers are shown in blue.

LDM chemotherapy immediately clinical applicable. Third, as mentioned earlier, LDM chemotherapy not only may prevent the CAF–CSC interplay but may also exert multiple favorable effects on other cells in the tumor stroma, including tumor-associated macrophages, myeloid-derived suppressor cells, and blood vessel cells (98, 99, 101, 102). We thus foresee that LDM chemotherapy will become the treatment of choice in many types of desmoplastic cancers.

TARGETING THE CROSSTALK BETWEEN MSCs AND CSCs

Due to their pro-tumorigenic activities, a number of studies had been carried out to try and target MSCs as a therapeutic approach in cancer (103). Specifically, given that tumor-infiltrating MSCs can directly support CSCs through multiple paracrine signaling pathways, including IL-6, IL-7, CXCL-1, PGE-2, Jagged-1, and CXCL-10 (44, 46, 47, 66), blockade of the molecular crosstalk between MSCs and CSCs may be potentially useful in inhibiting cancer stemness in desmoplastic cancers. Indeed, a recently study exemplified the potential utility of this approach (66). In a mouse model of PDAC, MSCs were found in close proximity to CSCs following gemcitabine chemotherapy, implicating MSCs as the CSC niche. Mechanistically, gemcitabine-exposed MSCs secrete high levels of CXCL-10 that activate its receptor CXCR-3 on CSCs, activating STAT-3 signaling and promoting the survival of CSCs. Consistently, systemic administration of the CXCL-10 inhibitor AMG487 formulated with MSC-derived membrane-based nanoparticles termed "nano-ghost (NG)" led to its intratumoral accumulation in close proximity to CSCs, thereby reducing the percentage of CSCs and augmenting the therapeutic efficacy of gemcitabine. In analogous to the directly depletion of CAFs, the direct depletion of MSCs might provide an alternative approach to nullify their crosstalk with CSCs. However, whether MSC-deprived host for a limited time may cause toxicity on its own remains an open question. Alternatively, given that MSCs secrete CXCL-10 in response to gemcitabine treatments, and that gemcitabine given at an LDM regimen could attenuate therapy-induced production of pro-stemness chemokines from CAFs in PDAC (37), it is highly likely that LDM chemotherapy may also prevent or at least attenuate chemotherapy-induced activation of MSCs and their secretion of pro-stemness chemokines. This possibility merits further investigations. On the other hand, since MSCs secrete the

pro-stemness cytokine IL-6 (46), the various anti-IL-6 antibodies and/or STAT-3 inhibitors developed to inhibit the CAF–CSC crosstalk can also be adopted to block the interaction between MSCs and CSCs (10, 89–91). This raises the possibility that MSC- and CAF-targeted therapeutics may synergize with each other. We thus envisage that the CAFs and MSCs dual targeting approach may provide an opportunity to more thoroughly block the stroma-derived pro-stemness signals to maximize the anti-tumor efficacy in the treatment of desmoplastic cancers.

THE UNIQUE ADVANTAGES OF TARGETING PRO-STEMNESS CAFs AND MSCs

As opposed to the direct targeting of CSCs, which poses significant challenges, targeting CAFs or MSCs along with the pro-stemness niches they generate may have several advantages in the treatment of desmoplastic cancers (**Table 2**). First and foremost, a growing body of evidence now suggests that CSCs are highly heterogeneous and plastic and the conversion between different CSC populations plays an important role in tumor progression and treatment response (104). For instance, breast cancer CSCs exist in alternative mesenchymal-like and epithelial-like states which can transition between each other (105–107). CSCs can also be derived from differentiated cancer cells through cellular reprogramming or transdifferentiation (11), which can be particularly facilitated by cytotoxic stresses such as chemotherapy and ionizing radiation (37, 108). The highly dynamic nature of CSCs makes them moving targets in cancer therapy, which presents a daunting challenge to therapeutic efforts aiming at completely eradicating them. Echoing this notion, two recent studies in the organoid models of CRC highlighted the difficulty of eradicating CSCs. Specifically, ablation of LGR-5$^+$ CSCs halted tumor growth, whereas the tumors resumed growth following the removal of the cell death inducers due to the re-emergence of CSCs from differentiated tumor cells (109, 110). As a comparison, CAFs are both genetically and phenotypically stable; therefore, CAF-directed therapies may lead to a more stable and sustainable anti-CSC effect compared with that results from the direct targeting of CSCs. Second, the recent discoveries of specific subpopulations of pro-stemness CAFs have rendered CAF-directed therapy more feasible as they not only provide novel therapeutic targets, such as GPR-77 (72), but also rendered the related therapies more specific and safer than the non-specific targeting of CAFs (79). Another unique advantage of targeting pro-stemness CAFs relates to their spatial distributions within desmoplastic cancers. Specifically, CAFs and tumor-infiltrating MSCs exist in large numbers in the tumor stroma, which contrasts sharply with CSCs that comprise only a small or even a rare subpopulation of cancer cells and exist within cancer cell nests or as individually dispersed cells or small cell clusters at the tumor periphery or the invasive front (111, 112). In desmoplastic cancers such as PDAC, there are abundant CAFs in the stroma, which can account for more than 90% of the total tumor volume (113, 114). Thus, there are a far larger number of CAFs or MSCs that can be exposed to the therapeutics administrated at a given tissue concentration than that of CSCs. Accordingly, CAF- or MSC-targeted therapeutics may have better pharmacodynamic effects than CSC-targeted agents in the treatment of desmoplastic cancers. Moreover, CAFs are often localized to the periphery of the tumor cell nests or glands and close to blood vessels, rendering them directly accessible to the therapeutics diffused from the blood circulation (115). By contrast, carcinoma cells, including the small population of CSCs, are frequently distantly spaced from blood vessels in desmoplastic tumors. In fact, CAFs *per se* constitute a significant barrier for the therapeutic delivery of drugs and even nanoparticles to cancer cells (97, 116). Echoing the importance of the spatial distribution of cells in the treatment of poorly perfused desmoplastic tumors, clinical data has confirmed that the majority of therapeutics, such as gemcitabine, can only reach the stroma of human PDAC tissues (117). Collectively, these factors make targeting the link between CAFs or MSCs with CSCs more justified, feasible and clinically promising than the direct targeting of CSCs in the treatment of desmoplastic cancers.

TABLE 2 | The potential advantages of targeting pro-stemness CAFs and MSCs.

Characteristics	CAFs or MSCs	CSCs	Advantages of CAF targeting
Genotype	Relatively stable	Heterogeneous	More constant effects and less treatment failure
Phenotype	Relatively stable	Highly dynamic and plastic	
Density in tumor	High (especially in desmoplastic cancer)	Rare to low	Favorable pharmacodynamic effects
Localization in tumor	Tumor periphery or surrounding blood vessels	Within tumor cell nests or at the invasive front	More accessible to therapeutics

BIOMARKERS OF PRO-STEMNESS CAFs

Tumor cells are highly heterogeneous in terms of their phenotypes, genotypes, and functions. As aforementioned, it is increasingly recognized that the intra-tumoral heterogeneity not only exists in the epithelial compartment but also the stromal compartment of the tumors, including CAFs (29, 71, 72). As such, human desmoplastic cancers may vary considerably with respect to the number as well as the composition of CAFs, including those with pro-stemness properties. Clinical trials investigating therapies targeting the CAF-to-CSC crosstalk should be ideally conducted in a patient- and tumor-tailored manner based on surrogate markers of CAF activation and/or their pro-stemness functions. We list a number of CAF-related biomarkers that may potentially fulfill this purpose (**Table 3**). First, a high density of α-SMA$^+$ CAFs in tumors has been linked to the resistance to neoadjuvant chemotherapy in breast cancer (72). Therefore, the density of CAFs may serve as a simple and immediately clinically applicable biomarker based on which CAF-targeted therapies can be implemented. Likewise, the density of CAFs also significantly increased following systemic chemotherapy in

TABLE 3 | Biomarkers linked to pro-stemness CAFs that can guide clinical studies.

Biomarker	Significance	Clinical setting	Cancer type	References
CAF density[a]	A high density of CAFs is associated with resistance to chemotherapy	Adjuvant or combination therapy	BC, CRC	(31, 62)
Phosphorylated STAT-1$^+$ fibroblasts	Positive staining indicates pro-CSC CAFs following chemotherapy	Adjuvant or combination therapy	BC, PDAC	(32)
SMA$^-$PDGF-Rα^+IL-6$^+$ fibroblasts	Reflects the number of pro-CSC CAFs in breast cancer or NSCLC	Neoadjuvant or combination therapy	PDAC	(61)
CD10$^+$GPR-77$^+$ fibroblasts	Reflects the number of pro-CSC CAFs in PDAC	Neoadjuvant or combination therapy	BC, NSCLC	(62)
ALDH$^+$, CD133$^+$, CD44$^+$, CD24$^+$, CD90$^+$ and/or EpCAM$^+$ cancer cells[b]	Reflects the density of CSCs	Neoadjuvant or combination therapy	When applicable	(108, 109)

BC, breast cancer; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; NSCLC, non-small cell lung cancer.
[a] *Identified using reported CAF markers, including FAP, α-SMA, FSP-1, PDGFR-α/β, etc, or their combinations.*
[b] *Used in combination with CAF-related markers.*

human CRC tissues (36). A plausible corollary is that the density of CAFs positively correlates with the likelihood of treatment resistance in most desmoplastic cancer and thus can serve as a universal biomarker to guide CAF-targeted therapies. Notably, since different CAF markers, including such as α-SMA, FAP, and FSP-1, may identify functionally distinct CAF populations that vary among different cancer types of subtypes (76, 77, 79), it remains to be established which CAF marker or any of their combinations can serve as a clinically informed biomarker. Beyond simply measuring the density of CAFs, the staining intensity of phosphorylated STAT-1 in CAFs, which reflects their ability to produce pro-stemness chemokines following chemotherapy (37), may also aid the clinical decision-making regarding when CAF-directed therapies should be implemented. On the other hand, in untreated tumors, the density of pro-stemness CAFs, such as α-SMA$^-$PDGF-Rα^+IL-6$^+$ iCAFs in PDAC and CD10$^+$GPR-77$^+$ CAFs in breast cancer and NSCLC (71, 72), can serve as a companion diagnostic to guide the selection of patients for anti-CAF/CSC therapies, especially those targeting the IL-6 and/or the IL-8 paracrine signaling pathways. In theory, these CAF-related biomarkers can be further combined with widely used surrogate markers of CSCs, such as ALDH, CD133, CD44, CD24, CD90, and EpCAM (118, 119), to increase their predictive power and clinical utility. We predict that the application of these CAF-related stemness markers may increase the success rate of the related clinical trials and pave the road for the next-generation patient-tailored anti-cancer therapies.

IMPORTANT CONSIDERATIONS AND POTENTIAL CHALLENGES IN THE CLINICAL DEVELOPMENT OF PRO-STEMNESS-CAF- OR MSC-TARGETED THERAPIES

Whilst targeting pro-stemness CAFs and MSCs have multiple theoretical advantages over the direct targeting of CSCs, several potential challenges remain and require careful considerations at the various developmental stages of the therapies. First, since CAFs or MSCs maintain their crosstalk with CSCs mainly through pro-stemness cytokines and chemokines, the majority of CAF- or MSC-targeted therapeutics are function-blocking antibodies (**Figure 2**). It is widely accepted that large-molecule therapeutics like antibodies have very limited penetration into desmoplastic tissues and may only be able to reach CAFs or MSCs spaced at the outer rim of tumors or those located surrounding or near blood vessels. If so, their anti-CSC and anti-tumor efficacy will be severely compromised (120). One potential solution for this problem is pre-treating desmoplastic tumors with agents that can reduce the number of CAFs and/or the desmoplastic reaction they produce, which can be exemplified by the stroma-reducing effect of *nab*-paclitaxel and SHH inhibitors in human PDAC (34, 117). Another solution is by using small-molecule inhibitors or nanoparticles designed to block pro-stemness factors or their receptors, such as repertaxin, SD208, BBI608, calcipotriol, and NG-AMG487, which have the ability to diffuse deeply into the desmoplastic stroma and reach their intended target cells compared with antibodies. Second, CSC-directed therapies, which target only a small subpopulation of cancer cells, would not be expected to produce measurable changes in tumor burden according to conventional Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Therefore, more pertinent, "stemness-informed" surrogate markers of response that are applicable to anti-CSC agents should be developed to guide the conduction of clinical trials, especially at the phase II stage (121). This concern should be also taken into consideration when conducting clinical trial testing CAF- or MSC-targeted therapies designed to specifically target CSCs. We propose that this problem can be at least partially tackled by introducing stemness-informed CAF- or MSC-related biomarkers as described in **Table 2**. Third, as described above, CAFs or MSCs are spaced in the tumor stroma, whereas CSCs exist mainly within tumor nests or as individually dispersed cells or small cell clusters at the tumor periphery or the invasive front. Therefore, a plausible corollary is that the CAF/MSC–CSC crosstalk through paracrine signaling will predominantly take place at the tumor periphery. If so, pathological biomarkers and criteria that reflect the distance between CAFs or MSCs and CSCs should be developed to select those tumors that most likely respond to therapies directed at disrupting the CAF/MSC–CSC interplay. Finally, the

timing of implementing pro-stemness-CAF- or MSC-targeted therapies will be another important consideration in the design of the related clinical trials. For the agents designed to target treatment-naïve CAFs or MSCs, it is critical to dose patients in early phases of cancer treatment before or concurrently with neoadjuvant chemotherapy as CSCs are less frequent and may be more susceptible to CSC-directed agents (54). By contrast, for the therapeutics targeting chemotherapy-modulated CAFs or MSCs, they should be administered following the initiation or during the course of chemotherapy, depending on when and the extent to which the pro-stemness functions of CAFs or MSCs are activated. Another timing of CAF/MSC-CSC-directed therapy is at the adjuvant setting following the removal of primary tumors, at which the therapy is designed to target micro-metastatic and circulating tumor cells that are known to contain enriched CSC populations (122–124). In this scenario, the blockade of CAF- or MSC-derived pro-stemness factors is expected to prevent the formation of CSC niches in primary or distant sites to reduce tumor recurrence and/or metastasis following surgery. Again, appropriate stemness- and/or stroma-informed biomarkers will be required to guide patient selection as well as the prediction of response in this type of trial.

CONCLUSIONS AND FUTURE DIRECTIONS

The first generation of therapeutic strategies aiming at blocking the CAF-derived pro-stemness factors has remained largely in preclinical stages or been tested in early-phase clinical trials. Further optimization and improvements in the potency of antibody and small-molecule therapeutics or the introduction of novel therapeutic entities, such as the STAT-3-targeted oligonucleotide (91), may hold promises to overcoming current developmental hurdles. Alternatively, functional targeting or the specific depletion of the pro-stemness subpopulation of CAFs using such as FAP- or GPR-77-targeted antibodies, DNA vaccine, and immune cell therapeutics, provides promising next-generation approaches to preventing the cross-talk between CAFs and CSCs. Suppressing the pro-stemness factors secreted by MSCs or the direct depletion of MSCs also represents an interesting and promising opportunity of antagonizing their pro-oncogenic effects. The pro-stemness-CAF- or MSC-targeted therapies offer a novel opportunity of enhancing the treatment response of cytotoxic therapies such as chemotherapy and IR to prevent treatment-triggered expansion and activation of CSCs. Moreover, pro-stemness-CAF- and MSC-targeted therapies may synergize with CSC-targeted agents to reduce cancer stemness and aggressiveness, ultimately improving the therapeutic outcome of patients with desmoplastic cancers. Pro-stemness-CAF-related biomarkers are expected to aid the design of clinical trials and guide patient selection in CAF-/MSC-targeted therapies. Whilst these novel stroma-targeted approaches may potentially renew the interest in CSC-directed therapies in solid tumors, whether or not they can indeed fulfill their promise remains to be validated by more meticulously designed clinical trials.

AUTHOR CONTRIBUTIONS

T-SC: conceptualization, drafting of the manuscript, and critical revision of the manuscript. YS: drafting of the manuscript. KT: conceptualization, drafting of the manuscript, critical revision of the manuscript, obtained funding, final supervision, review, and editing.

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REVIEW
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Therapeutic Targeting of Cancer Stem Cells via Modulation of the Renin-Angiotensin System

*Imogen M. Roth[1], Agadha C. Wickremesekera[1,2], Susrutha K. Wickremesekera[1,3], Paul F. Davis[1] and Swee T. Tan[1,4]**

[1] Gillies McIndoe Research Institute, Wellington, New Zealand, [2] Department of Neurosurgery, Wellington Regional Hospital, Wellington, New Zealand, [3] Upper Gastrointestinal, Hepatobiliary and Pancreatic Section, Department of General Surgery, Wellington Regional Hospital, Wellington, New Zealand, [4] Wellington Regional Plastic, Maxillofacial and Burns Unit, Hutt Hospital, Wellington, New Zealand

Cancer stem cells (CSCs) are proposed to be the cells that initiate tumorigenesis and maintain tumor development due to their self-renewal and multipotency properties. CSCs have been identified in many cancer types and are thought to be responsible for treatment resistance, metastasis, and recurrence. As such, targeting CSCs specifically should result in durable cancer treatment. One potential option for targeting CSCs is by manipulation of the renin-angiotensin system (RAS) and pathways that converge on the RAS with numerous inexpensive medications currently in common clinical use. In addition to its crucial role in cardiovascular and body fluid homeostasis, the RAS is vital for stem cell maintenance and differentiation and plays a role in tumorigenesis and cancer prevention, suggesting that these roles may converge and result in modulation of CSC function by the RAS. In support of this, components of the RAS have been shown to be expressed in many cancer types and have been more recently localized to the CSCs in some tumors. Given these roles of the RAS in tumor development, clinical trials using RAS inhibitors either singly or in combination with other therapies are underway in different cancer types. This review outlines the roles of the RAS, with respect to CSCs, and suggests that the presence of components of the RAS in CSCs could offer an avenue for therapeutic targeting using RAS modulators. Due to the nature of the RAS and its crosstalk with numerous other signaling pathways, a systems approach using traditional RAS inhibitors in combination with inhibitors of bypass loops of the RAS and other signaling pathways that converge on the RAS may offer a novel therapeutic approach to cancer treatment.

Keywords: cancer stem cells, renin-angiotensin system, stem cell differentiation, tumorigenesis, bypass loops

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***Correspondence:**
Swee T. Tan
swee.tan@gmri.org.nz

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CANCER STEM CELLS

As in normal tissue, tumors consist of diverse cell populations. The cellular heterogeneity observed in tumors has led to the suggestion that cancer may be sustained by cancer stem cells (CSCs), which, like normal embryonic stem cells (ESCs), are able to self-renew and undergo differentiation into multiple cell types. This is supported by several observations in cancer biology, including that only some tumor cells can recapitulate a tumor when xenografted into immunodeficient mice, and that tumors grown from tumorigenic cells consist of a mixed population of both tumorigenic and non-tumorigenic

cancer cells (1). CSCs are thought to arise from either resident adult stem cells which have acquired oncogenic mutations or from progenitor cells which have an unlimited ability to replicate. CSCs share the properties of differentiation, self-renewal and homeostatic control with normal stem cells (1), express stem cell markers (2), and have subverted self-renewal pathways of normal stem cells (3).

The CSC concept proposes that cancer develops from a small subset of cells which can generate all the heterogeneous cell types seen within the tumor, including generating more CSCs as well as differentiated cancer cells. It has been shown in numerous tumor types that expression of certain markers can define populations of cancer cells which are able to generate a tumor, as well as their ability to respond to or resist cancer therapies, suggesting CSCs are present within these tumors (4). Studies transplanting mouse tumors into compatible wild-type mice have also shown that the cancer cells differ in their tumorigenic capacity, as only a small population of cancer cells are able to form tumors (4).

Given that adult human stem cells themselves are a diverse pool of cells expressing different markers, it is unsurprising that CSCs are also mixed populations of cells and are phenotypically and functionally diverse, and that the same tumor can contain multiple pools of CSCs (5). CSC diversity has also resulted in the emergence of a hierarchy, with a slow-cycling pool of cells giving rise to both a rapidly cycling population and non-proliferative cells, suggesting that targeting the cells with the potential to produce multiple types of tumor cells would be a beneficial approach to cancer treatment (6, 7). Heterogeneity within CSCs extends beyond tumorigenic potential and encompasses genetic and epigenetic changes as well as local environmental determinants and temporal and spatial differences (8). These differences have implications for effective therapies, as some cancer cells have been shown to resist chemotherapy and radiotherapy, and it has been suggested that they could be specifically targeted for differentiation as a therapeutic approach (8). Importantly, there is a level of plasticity within this system, as differentiated non-tumorigenic cancer cells can revert to CSCs (9, 10). This could be advanced by changes in the local environment driven by cues including hypoxia and inflammatory mediators to induce epithelial-mesenchymal transition and de-differentiation to increase the "stemness" of the tumor (5). This heterogeneity conferred by plasticity can result in treatment resistance (11).

CSCs have been shown to be capable of surviving radiotherapy and chemotherapy, which have no effect on the ability of the CSCs to regrow tumors (12). This resistance to radiotherapy is thought to occur by several mechanisms, including activation of DNA repair mechanisms, through activation of Wnt/β-catenin signaling, reactive oxygen species generation, and activation of other pro-survival signaling pathways (12). Resistance to chemotherapeutic agents is thought to occur via the use of drug efflux pumps and the expression of metabolic mediators (12). In addition, the quiescent, slow-cycling nature of CSCs is also likely to confer resistance to conventional treatments such as chemotherapy and radiotherapy which target rapidly dividing cells. The ability of CSCs to resist conventional cancer treatments has been well documented in breast cancer. Irradiation of mouse mammary primary epithelial cells enriches for progenitor cells (13), and breast cancer cells from patients following neoadjuvant chemotherapy are enriched for self-renewing cells (14). Furthermore, the number of CSCs and their ability to form mammospheres in culture is increased following chemotherapy of breast cancer patients (15) and Trastuzumab treatment of a breast cancer cell line (16).

Given their ability to generate a diverse cell population within a tumor and their ability to resist conventional cancer treatments, CSCs are proposed to be the cause of loco-regional recurrence and distant metastasis, and consequently treatment failure. This has implications for cancer therapy and suggests that the CSCs should be targeted for effective and durable cancer treatment. Consequently, several treatments targeting CSCs are currently in use in the clinic, with the main strategies being inhibiting key signaling pathways or directly targeting CSCs (17). These therapies include targeting CSC markers, such as CD44 and CD133, which have shown promise in a pre-clinical setting and therapies targeting these markers are in current clinical trials for acute myeloid leukemia and recurrent solid tumors, including liver, brain, pancreatic, breast, and colorectal cancers (18). In addition, a vaccination-based strategy against CSCs is in clinical trials for glioblastoma and other brain tumors (18), demonstrating the diverse approaches taken to target these cells. Given that CSCs express a unique set of markers, another approach toward identifying and eliminating these cells is to characterize other common features of CSCs and exploit these features for therapeutic targeting using drugs in common use, such as via modulation of signaling pathways such as the renin-angiotensin system (RAS).

THE RENIN-ANGIOTENSIN SYSTEM

Physiological Control of Blood Pressure and Fluid Balance

The RAS is an endocrine system crucial for the maintenance of homeostasis, as it regulates blood pressure and fluid balance via a signaling network (**Figure 1**). Physiologically, the RAS is activated in response to either reduced blood volume or blood pressure, and acts to restore homeostasis through the release of renin from the kidneys. Pro-renin is converted to active renin by binding to the pro-renin receptor (PRR). Renin then cleaves angiotensinogen, which is normally synthesized and released by the liver, giving rise to angiotensin I (ATI). ATI is then converted to angiotensin II (ATII) by angiotensin converting enzyme (ACE). Aminopeptidase A converts ATII to angiotensin III, and together they act on ATII receptors 1 and 2 (ATIIR1 and ATIIR2). These receptors have divergent actions, with ATIIR1 driving vasoconstriction and inhibiting renin to restore blood pressure, and ATIIR2 acting to promote vasodilation. Angiotensin 1-7 (Ang1-7) is the cleavage product of ATII and affects cardiovascular functions by binding to the G-protein coupled receptor MAS. However, there is considerable redundancy in the pathway with bypass loops involving proteases such as cathepsins B, D, and G, and the convergence of other signaling pathways on the RAS itself, including inflammatory pathways and Wnt/β-catenin signaling (**Figure 1**). Given the importance of

the RAS for maintaining blood pressure, numerous modulators that inhibit the RAS at different points in the pathway have been developed (**Figure 2**). These groups of RAS inhibitors are commonly used in the clinic for the treatment of hypertension and include β-blockers, ACE inhibitors (ACEI), and ATIIR1 blockers (ARBs) as well as newer agents targeting other points in the pathway (e.g., renin inhibitors, chymase inhibitors, ATIIR2 inhibitors), inhibitors targeting bypass loops in the RAS pathway (e.g., cathepsin inhibitors), and inhibitors used in other canonical signaling pathways that converge on the RAS (e.g., Wnt/β-catenin inhibitors, metformin, and non-steroidal anti-inflammatory drugs) (**Figure 2**).

Stem Cell Differentiation

Alongside its crucial role in fluid volume regulation, the RAS is also important for stem cell maintenance and differentiation in several cell types. ATII expression drives the differentiation of mesenchymal stem cells into adipocytes (19), while other components of the RAS drive differentiation into insulin producing cells (20). ACE is required for hemangioblast expansion, and modulation of ATIIR1 or ATIIR2 signaling can direct the fate of the blasts toward either an endothelial or hematopoietic lineage (21). The RAS also plays a role in hematopoiesis (22, 23), vasculogenesis (24), erythropoiesis (25, 26), and myeloid differentiation (27).

Importantly, the RAS not only acts to promote stem cell differentiation in diverse cell populations, but also appears to act in a feedback loop with Wnt/β-catenin signaling, where pro-renin receptor (PRR) can induce Wnt/β-catenin (28), and components of the RAS themselves are targets of Wnt/β-catenin (29). Wnt/β-catenin signaling is crucial for embryonic development and induces differentiation of pluripotent stem cells into progenitor cells (30). Given that Wnt signaling is also involved in cancer development (31), and downstream Wnt targets include the CSC markers CD44 and c-Myc (32), it may be that CSCs require activation of Wnt signaling (33). This suggests that RAS modulators could be employed in these cells to indirectly inhibit Wnt signaling and its effects.

Having identified these roles in normal stem cell maintenance and differentiation and feedback loops with a canonical developmental signaling pathway, it may be that the expression of the RAS also plays a role in the regulation or function of CSCs.

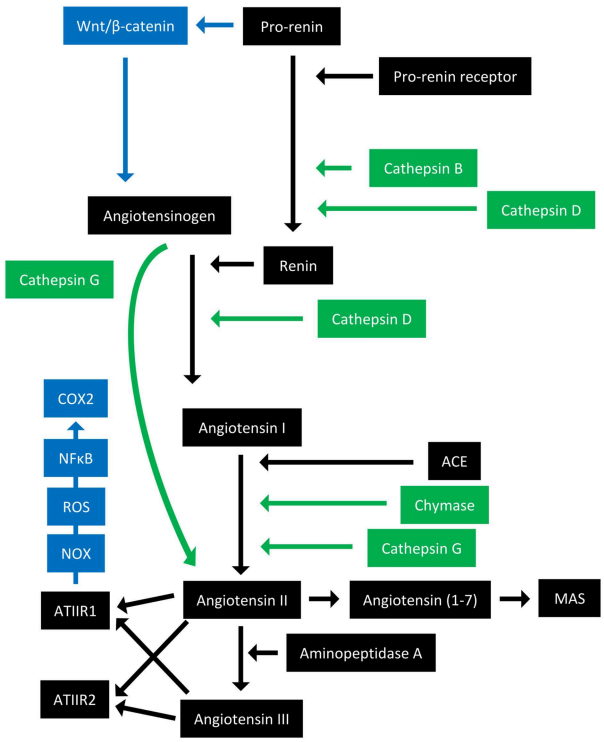

FIGURE 1 | Overview of the renin-angiotensin system with its bypass loops and convergent signaling pathways. The renin-angiotensin system (black) regulates blood pressure, stem cell differentiation, and tumor development. Bypass loops of the RAS involving enzymes such as chymase and cathepsins B, D, and G (green) provide redundancy, while convergent inflammatory and developmental signaling pathways (blue) have multiple roles and effects. Angiotensinogen (AGN) is physiologically synthesized and released by the liver and is cleaved by renin to form angiotensin I (ATI). Renin is formed following binding of pro-renin to the pro-renin receptor. ATI is converted to angiotensin II (ATII) by angiotensin converting enzyme (ACE). ATII interacts with the G-protein coupled receptors ATII receptor 1 (ATIIR1) and ATII receptor 2 (ATIIR2) to restore homeostasis, via vasoconstriction and vasodilation, respectively. ATII can also give rise to angiotensin III via the action of aminopeptidase A, and Angiotensin 1–7 which binds and activates the G-protein coupled receptor MAS. Cathepsins B and D are also renin-activating enzymes that convert pro-renin to renin. Cathepsin D converts AGN to ATI, and cathepsin G converts ATI to ATII or AGN directly to ATII. Chymase converts ATI to ATII. Pro-renin also induces Wnt/β-catenin signaling in a feedback loop. ATIIR1 can also result in inflammatory signaling via the NOX-ROS-NFκB-COX2 signaling axis. ROS, reactive oxygen species.

Retrospective Studies and Clinical Trials Indicate Potential Benefit of RAS Modulators in Reducing Cancer Risk

The widespread use of RAS modulators as anti-hypertensives and their potential effect on cancer risk have been extensively documented. A seminal study has shown that the use of ACEI and ARBs is associated with a reduced risk of developing some cancer types, particularly cancers affecting women (34). Many other retrospective population studies have reported differing effects on cancer risk depending on the cancer type, cohort characteristics, and the RAS inhibitor used. As a result, several meta-analyses have been undertaken (35), again with differing results, which could be due to the nature of the original studies included and inherent publication bias. Aside from the reported effects on cancer risk, many retrospective population studies have also assessed the effect of RAS inhibitors on cancer death. Again, these results have been mixed, though meta-analyses have indicated that β-blocker use is not associated with survival in breast cancer patients (36), and a meta-analysis looking at ACEI use in all cancers showed no effect on cancer survival (37). A more recent meta-analysis looking at the use of different RAS inhibitors in all cancers showed that RAS inhibitor use extended overall, progression-free and disease-free survival (38). This is mainly due to ARBs and not ACEI use, with some site-specific effects. These studies need

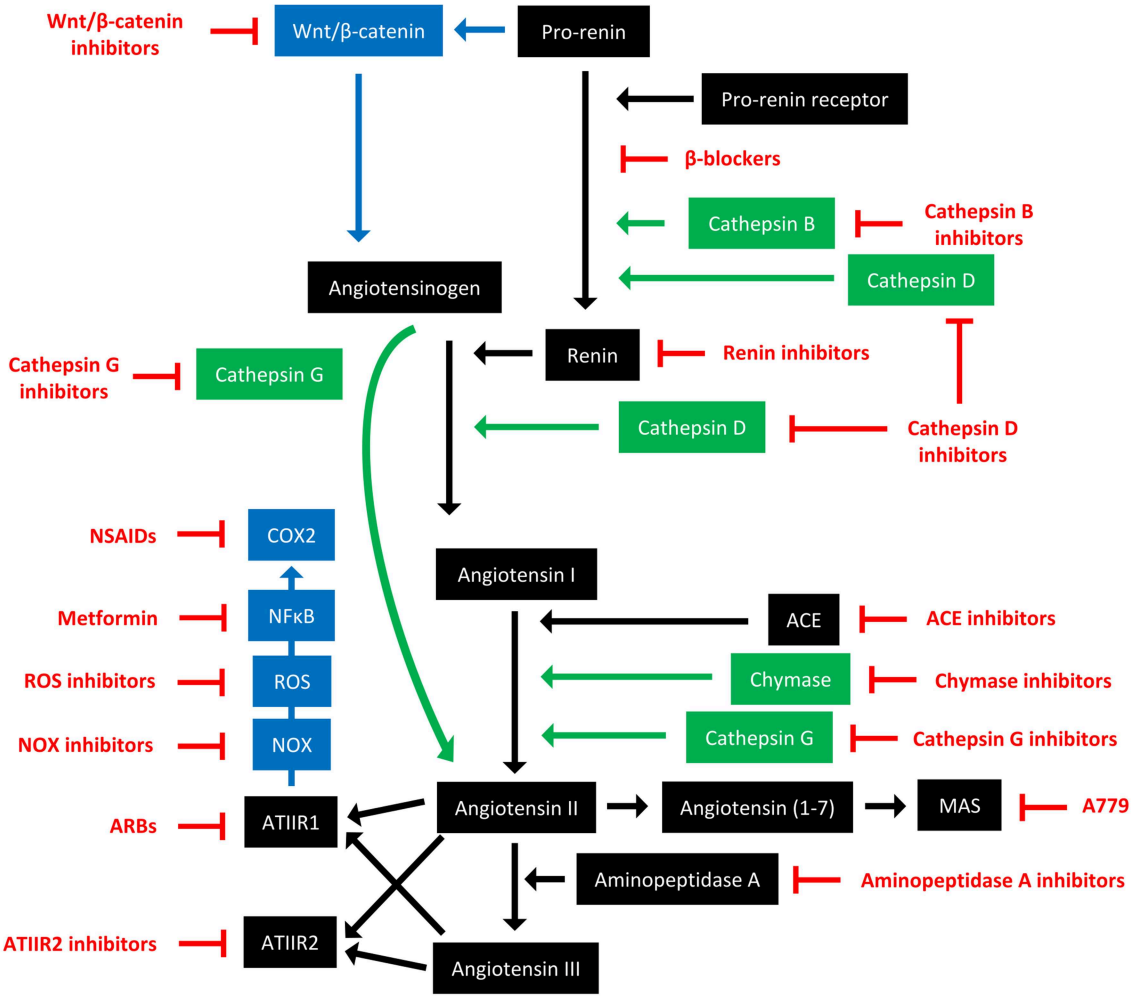

FIGURE 2 | The renin-angiotensin system and its bypass loops and converging signaling pathways can be targeted at different points. The renin-angiotensin system (black) regulates blood pressure, stem cell differentiation, and tumor development. Bypass loops in the system involving cathepsins and chymase (green) provide redundancy, while convergent inflammatory and development signaling pathways (blue) have multiple roles and effects. Multiple points of the pathway can be targeted by specific inhibitors (red). ACE, angiotensin converting enzyme; ARBs, ATIIR1 blockers; ROS, reactive oxygen species; NSAIDS, non-steroidal anti-inflammatory drugs.

to be interpreted critically and with caution as they do not prove causality and the effect on cancer risk and mortality could be due to other factors. It may also be that a defined patient group will derive benefit from these treatments and that a more holistic approach of targeting the RAS in cancer is required to achieve a sustained treatment for patients. Due to the nature of the RAS, with its inherent bypass loops conferring redundancies, and the presence of many other pathways that converge on the RAS, it is likely that a multi-faceted approach to target the RAS will be required for effective cancer treatment.

Despite these disparate observations in retrospective population studies, the data around the involvement of the RAS in tumor models is clear, leading to many clinical trials using RAS inhibitors and the development of new targeted agents (39–41). Several of these studies have trialed ARBs in cancer patients, with Losartan being shown to enhance the efficacy of chemotherapy and improve overall survival in ovarian cancer patients (42). Another ARB, Candesartan, has been shown to decrease prostate specific antigen levels in hormone-refractory prostate cancer patients (43), and is tolerated in advanced pancreatic cancer (44, 45). The ACEI Captopril is tolerated in patients with advanced cancer (46), and has been shown to reduce biochemical recurrence in prostate cancer patients (47), while Perindopril reduced the risk of recurrence of hepatocellular carcinoma as a combination therapy with other non-traditional treatments (48, 49). Several trials have targeted the Ang1-7/MAS axis in breast cancer before or after chemotherapy (50), and in metastatic sarcoma, where it is well tolerated (51), and a number of advanced solid tumors where it provides benefit for some patients (52).

TABLE 1 | Components of the RAS are expressed in tumors.

RAS component	Expression in tissue	Tumor types and references
Pro-renin receptor	Increased expression	Endometrial cancer (58)
Angiotensinogen	Increased expression	Lung cancer (59)
ACE	Increased expression	Prostate cancer (60), gastric cancer (61), endometrial cancer (58)
	Polymorphism correlated with metastases	Gastric cancer (62)
ATIIR1	Deficiency reduces tumor growth and angiogenesis	Melanoma (63), sarcoma (64), lung cancer (65), fibrosarcoma (66)
	Increased expression	Pancreatic cancer (67), ovarian cancer (68), prostate cancer (60), astrocytoma (69), breast cancer (70), renal clear cell carcinoma (71)
	Expression associated with disease progression	Ovarian cancer (68)
	Expression associated with poor survival	Intestinal type gastric cancer (72), astrocytoma (69)
ATIIR2	Deficiency increases tumor growth	Pancreatic cancer (73)
	Increased expression	Gastric cancer (61), endometrial cancer (58)
	Reduced expression	Lung cancer (59)
	Expression associated with poor survival	Astrocytoma (69), renal clear cell carcinoma (71)
Cathepsin B	Expression associated with poor survival	Gastric cancer (74)
Cathepsin D	Increased expression	Hepatocarcinoma (75), melanoma (76), colorectal cancer (77), prostate cancer (78)
	Expression increases metastasis	Liver metastases (79, 80)
	Expression associated with poor survival	Breast cancer (81–84)

TABLE 2 | β-blockers inhibit tumorigenesis in cell and animal models.

Drug name	Effect in tumor models or cell lines	Tumor types and references
Propranolol	Inhibition of growth and proliferation	Pancreatic ductal adenocarcinoma (89), breast cancer (90, 91), neuroblastoma (92), angiosarcoma (55, 93), melanoma (94–97), pancreatic cancer cells (98), gastric cancer cells (99, 100), neuroblastoma cells (92), hemangioendothelioma cells (93), angiosarcoma cells (55, 93), colorectal cancer cells (101), melanoma cells (94, 96), breast cancer cells (102), liver cancer cells (103), prostate cancer cells (104)
	Inhibition of migration	Colon carcinoma cells (105), breast cancer cells (106)
	Inhibition of invasion	Ovarian cancer cells (107), pancreatic cancer cells (108)
	Inhibition of metastasis	Prostate cancer (109), melanoma (95)
	Prolonged survival of tumor-bearing animals	Neuroblastoma (92)
Carvedilol	Inhibition of growth and proliferation	Neuroblastoma and neuroblastoma cells (92)
Nebivolol	Inhibition of growth and proliferation	Neuroblastoma and neuroblastoma cells (92)

β-blockers work by blocking β-adrenergic receptors to prevent neurotransmitter binding. This prevents renin secretion and its actions and subsequently results in lowered blood pressure (**Figure 2**). The non-selective β-blocker Propranolol has been shown in several case reports to be efficacious in treating angiosarcoma (53), and in combination with chemotherapy treatment induced responses in seven patients with advanced angiosarcoma (54). Another study showed that addition of Propranolol or another non-selective β-blocker Carvedilol to treatment regimens for metastatic angiosarcoma improved progression-free and overall survival (55). Propranolol has also been used in a proof of concept study in multiple myeloma patients receiving hematopoietic cell transplantations (56), and in a prospective cohort study in melanoma patients where its use was associated with reduced recurrence (57).

While these trials have demonstrated promise for targeting the RAS in cancer treatment, the mechanisms by which this is achieved are yet to be elucidated. Current clinical trials and the development of new RAS targets should help to further define which patient groups may benefit from these treatments.

In vitro and *in vivo* Cancer Models Rationalize the RAS as a Therapeutic Target

Given the potential effects on reducing cancer risk observed in retrospective population studies, expression of components of the RAS have been assessed in many different tumor types to clarify the potential role of the RAS in tumorigenesis (**Table 1**).

These studies have helped define the role of the RAS in tumorigenesis, and collectively show that components of the RAS are expressed in many different cancer types (39, 85). The effects on tumor growth, angiogenesis, metastasis and survival indicate that the RAS plays a role in the hallmarks of cancer (39, 86, 87). It is also thought to contribute to an immunosuppressive microenvironment in tumors and reduce infiltration of tumor-associated macrophages (88). The increased

TABLE 3 | ACE inhibitors inhibit tumorigenesis in cell and animal models.

Drug name	Effect in tumor models or cell lines	Tumor types and references
Captopril	Reduced growth	Renal cancer (110), lung cancer (111), colorectal cancer liver metastases (112, 113), lung cancer cells (111), esophageal squamous cell carcinoma cells (114)
	Increased growth	Fibrosarcoma (115)
	Reduced metastases	Lung cancer (111)
	Decreased survival of tumor-bearing animals	Renal cancer (115)
Enalapril	Inhibition of growth	Pancreatic cancer (116, 117), neuroendocrine cancer cells (117)
	Inhibition of invasion	Pancreatic cancer (116), gastric cancer cells (61)
Perindopril	Reduced growth and angiogenesis	Hepatocellular carcinoma (118–120)

TABLE 4 | ARBs inhibit tumorigenesis in cell and animal models.

Drug name	Effect in tumor models or cell lines	Tumor types and references
Candesartan	Inhibition of growth and proliferation	Gastric cancer cells (121), lung cancer cells (122)
	Reduced angiogenesis	Renal cancer (123), ovarian cancer (68), breast cancer (124)
	Reduced metastases	Renal cancer (123)
	Prolonged survival of tumor-bearing animals	Peritoneal carcinomatosis (121)
Irbesartan	Reduced growth	Colorectal cancer liver metastases (112), esophageal squamous cell carcinoma cells (114)
Losartan	Reduced growth	Breast cancer (70), esophageal squamous cell carcinoma cells (114)
	Increased proliferation	Melanoma cells (125)
	Reduced invasion	Breast cancer (70)
	Reduced angiogenesis	Pancreatic cancer (126)
Olmesartan	Reduced invasion	Gastric cancer cells (61)
Telmisartan	Inhibition of growth and proliferation	Prostate cancer cells (127), uterine leiomyoma cells (128), lung cancer cells (129)

expression of components of the RAS in different cancer types may contribute to tumorigenesis and the poor clinical outcome seen in some cancer types. This suggests that regulation of the RAS may be a general mechanism for cancer prevention and warrants further investigation to understand the precise underlying mechanisms.

Given that the RAS is over-expressed in many cancer types and the use of RAS modulators may affect cancer risk and cancer survival, numerous studies have assessed the effect of RAS inhibitors *in vitro* and on tumor models *in vivo*. These have focused on β-blockers (**Table 2**), ACEI (**Table 3**), and ARBs (**Table 4**) to assess the role of the RAS in tumor development.

Studies investigating β-blockers in cancer (**Table 2**) have largely used the β-blocker Propranolol and have shown that across a wide range of cancer types, Propranolol inhibits the growth of tumors and tumor cells. This suggests that Propranolol could be repurposed for cancer treatment (130, 131), as has been the case for the benign vascular tumor infantile hemangioma for which it is an effective treatment (132–134).

Given the effects of β-blockers on cancer and cancer cell growth, other studies have investigated the impact of other classes of drugs that modulate the RAS on neoplastic processes. One of these classes is ACEIs, which block the action of ACE and hence downstream production of ATII (**Figure 2**). Studies looking at ACEIs (**Table 3**) are extensive and demonstrate that this class of drugs (including Captopril, Enalapril, and Perindopril) appear to prevent tumor growth and invasion in many different tumor types and models.

Another broad class of drugs that modulate the RAS are ARBs, which block ATIIR1 (**Figure 2**). Studies using ARBs to assess cancer development in cell and animal models (**Table 4**) have also shown that different drugs within this class (Candesartan, Irbesartan, Losartan, Olmesartan, and Telmisartan) inhibit tumor development across several tumor types.

These studies underscore the complex nature of the RAS and suggests that different RAS modulators may have different effects in different tumor types. Taken together, they suggest that anti-hypertensive drugs which target the RAS have shown promise for repurposing in the cancer setting. Across several classes of drugs (β-blockers, ACEIs, and ARBs) in both *in vitro* and *in vivo* models, they have been shown to reduce tumor cell growth, migration, invasion, and metastasis in numerous cancer types. These processes comprise many of the characteristics of CSCs and of the hallmarks of cancer (135), and are consistent with the expression of some components of the RAS in high-grade disease and the associated poor survival (**Table 1**). This suggests that there is merit in repurposing RAS inhibitors for cancer treatment. Many clinical trials using this approach are currently underway, despite limited functional work and mechanistic understanding about how this approach might work in cancer patients. With the development of new agents targeting specific parts of the pathway, including the bypass loops, and the refinement of existing drugs, new

opportunities are emerging for modulating the RAS pathway, either in combination with current therapies or by targeting the entire RAS and its bypass loops, and pathways converging on the RAS.

CANCER STEM CELLS EXPRESS COMPONENTS OF THE RAS

Given the well characterized role of the RAS in both stem cell maintenance and tumorigenesis, it is possible that these functions are directed by RAS signaling in CSCs. In order to demonstrate this, it is important to first establish that CSCs express both CSC markers and components of the RAS. This has been shown to be the case in numerous cancer types, including glioblastoma (136–138), metastases to the liver from colon adenocarcinoma (139, 140), head and neck cutaneous squamous cell carcinoma (141), and oral cavity squamous cell carcinoma affecting the buccal mucosa (142, 143), oral tongue (144–146), and lip (147, 148). In addition, components of the RAS have also been demonstrated on the tumor stem cells of benign tumors such as meningioma (149, 150), infantile hemangioma (151, 152), and pyogenic granuloma (153). Importantly, the expression of cathepsins B, D, and G in some of these cancer types (74, 138, 140, 146, 150) suggests the presence of bypass loops of the RAS which could circumvent the action of traditional RAS inhibitors and offer a potential explanation for the differing findings of cancer risk and cancer survival with long-term use of traditional RAS inhibitors. Given the presence of components of the RAS in CSCs in these cancers, it is possible that the expression of these components is controlling the differentiation and function of the CSCs within these tumors.

Despite the indirect evidence from retrospective population studies and more substantial direct evidence from *in vitro* studies and *in vivo* tumor models, very little is known about the mechanism by which RAS modulators influence tumor development. Although expression of components of the RAS has been demonstrated in CSCs, their function and how they might respond to RAS modulators has yet to be characterized. However, the fact that many clinical trials involving targeting of the RAS in cancer have taken place and are currently underway underscores the role of RAS in tumorigenesis and the need for further investigations into this system. Importantly, the findings in tumor model systems are seen consistently across a broad range of tumor types, suggesting its common role in cancer biology which may be affected through CSCs and their functions.

The expression of both components of the RAS and CSC markers in several cancer types may indicate that the CSCs may be a novel therapeutic target through modulation of the RAS. It is possible that a multi-faceted strategy simultaneously targeting multiple critical points of the RAS and related signaling pathways may result in durable cancer treatments by altering CSC function. Indeed, Phase II trials in metastatic renal cell carcinoma using either Perindopril or Candesartan in combination with other agents, including a cyclooxygenase-2 inhibitor have shown potential for stabilizing the disease and reducing recurrence (154). Propranolol treatment in combination with a cyclooxygenase-2 inhibitor is well tolerated in breast cancer patients and transcriptional profiling showed the combination reduced markers of invasion and inflammation (155). Furthermore, targeting other pathways which converge on the RAS may also prove worthwhile, as Metformin selectively kills CSCs in mouse breast cancer models (156), and targeting Wnt signaling is known strategy for CSC elimination (157, 158).

CONCLUSION

The involvement of the RAS in both tumor development and stem cell maintenance suggests that these roles may converge on CSC maintenance and function. Given the ability of CSCs to promote cell migration, invasion and metastasis (17), and the reduction of these processes by RAS inhibitors *in vitro* and *in vivo*, it may be that the success of RAS inhibitors in reducing cancer risk and improving cancer survival is due to their effects on CSCs. In support of this, components of the RAS and enzymes that constitute bypass loops of the RAS have been shown to be expressed in CSCs of several different cancer types. This offers an avenue for targeted therapies using RAS inhibitors, modulators of the bypass loops, and agents targeting other signaling pathways that converge on the RAS. Importantly, RAS inhibitors are commonly available, well tolerated and inexpensive and have been shown to be effective in controlling tumor growth in several settings. However, many of these studies have relied on immortalized cancer cell lines and xenograft tumor models, and in order to better understand the mechanisms of these drugs and the discrepancies observed in their effects clinically, models closer to the patient need to be employed. In addition, the nature of the RAS and its crosstalk with other pathways means a system-wide approach simultaneously targeting multiple key steps of the RAS is needed to achieve effective cancer control.

AUTHOR CONTRIBUTIONS

IR drafted the manuscript. AW, SW, PD, and ST commented on the manuscript. All authors approved the manuscript.

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Therapeutic Strategies Targeting Cancer Stem Cells and Their Microenvironment

Hao-Ran Sun[1], Shun Wang[1], Shi-Can Yan[1], Yu Zhang[1], Peter J. Nelson[2], Hu-Liang Jia[1], Lun-Xiu Qin[1] and Qiong-Zhu Dong[1]**

[1] Department of General Surgery, Cancer Metastasis Institute, Institutes of Biomedical Sciences, Huashan Hospital, Fudan University, Shanghai, China, [2] Medizinische Klinik und Poliklinik IV, Ludwig-Maximilian-University (LMU), Munich, Germany

Cancer stem cells (CSCs) have been demonstrated in a variety of tumors and are thought to act as a clonogenic core for the genesis of new tumor growth. This small subpopulation of cancer cells has been proposed to help drive tumorigenesis, metastasis, recurrence and conventional therapy resistance. CSCs show self-renewal and flexible clonogenic properties and help define specific tumor microenvironments (TME). The interaction between CSCs and TME is thought to function as a dynamic support system that fosters the generation and maintenance of CSCs. Investigation of the interaction between CSCs and the TME is shedding light on the biologic mechanisms underlying the process of tumor malignancy, metastasis, and therapy resistance. We summarize recent advances in CSC biology and their environment, and discuss the challenges and future strategies for targeting this biology as a new therapeutic approach.

Keywords: cancer stem cells, tumor microenvironments, therapeutic resistance, mechanism, strategy

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***Correspondence:**
Lun-Xiu Qin
qinlx@fudan.edu.cn
Qiong-Zhu Dong
qzhdong@fudan.edu.cn

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INTRODUCTION

Cancer remains one of the leading causes of death worldwide (1). Tumor malignancy is linked to tumor heterogeneity, which has been proposed to be driven by a minor subpopulation of cancer cells referred to as cancer stem cells (CSCs) (2, 3). This subpopulation of tumor cells have the capacity to sustain tumorigenesis and drive tumor heterogeneity, processes that underlie tumor progression, metastasis, and resistance to anti-cancer therapies (4). To date, CSCs identification has been largely based on surface markers as well as their ability to self-renew and propagate. However, CSC surface markers alone are not a reliable means of identifying these populations which has led to some confusion and controversy in the field. It is unlikely that these methods can afford a universal specific marker for the identification of these cells. However, some functional markers including the ATP-binding cassette (ABC) transporter and aldehyde dehydrogenase (ALDH) activity (5), the activation of some key signaling pathways (6), live-cell RNA, and single-cell DNA detection (7) have been found to improve CSCs identification in some instances.

The self-renewal potential and extensive clonogenic properties of CSCs are dependent on the tumor microenvironment (TME) (8, 9). The interaction between CSCs and their tumor niche is strongly linked to the characterization of CSCs (10). Through this interaction, CSCs are able to preserve the tumor heterogeneity that underlies the important malignant behaviors of invasion, metastasis, and therapy resistance (11). The influence of TME on CSCs physiology has been shown to act through intrinsic and extrinsic actions. The intrinsic mechanisms include DNA methylation or demethylation, and gene mutation, while the extrinsic

actions involve the production of diverse growth factors and cytokines by the TME leading to the activation of specific signaling pathways (12). In addition, many studies have shown that CSCs may be responsible for tumor resistance to conventional cancer therapy (4, 13). The resistance that is enhanced through the cross-talk between CSCs and the TME include activation of the DNA repair system (8, 14), increased resistance to hypoxic environments (15), and the phenomenon of epithelial to mesenchymal transition (EMT) (16). These features may help explain the therapeutic failures that are often encountered in different tumor settings.

Despite the enormous challenges seen, a series of promising new therapeutic approaches based on this biology are currently under development. Notably, targeted therapeutic approaches have emerged as important tools in treatment strategies. To this end, both the CSCs and the TME represent important therapeutic targets. Emerging research has shown that CSC-targeted approaches have proven to be effective in prolonging survival time (17). In this review, recent advances in CSC biology are summarized, and the potential challenges and future strategies for targeted therapy and combination therapy to eliminate both cancer and CSC populations are discussed.

IDENTIFICATION OF CSC

CSCs are a small subpopulation of cells with characteristics that include the capacity to cycle slowly, self-renew, and initiate a novel tumor (18–20). Leukemic stem cells (LSCs) were first described in acute myeloid leukemia (AML), where it was demonstrated that $CD34^{+}CD38^{-}$ AML include a subpopulation of LSCs with a capacity to differentiate and self-renew (20). The early study first demonstrated the existence of a unique tumor subpopulation with the ability to drive tumor progression and recurrence. CSC-like subpopulations have been subsequently isolated from a variety of solid tumors (21). Because some CSCs have been identified via specific surface markers, a door has been opened for potential targeted therapy approaches directed against these cells (22). However, because of the wide diversity underlying this general biology across tumor types, general markers for the global identification of these cells are not available.

The functional relevance of surface markers for CSC identification is still disputed (23, 24). It is suggested that CSCs may arise from normal stem cells, progenitor cells, or even more differentiated cells. In tumor patients, the expression of CSC surface markers in normal organs implies potential metastasis and poor prognosis (25). CD24, CD26, CD44, CD133, CD166, and EpCAM (epithelial cell adhesion molecule, CD326) are surface markers commonly used in CSC characterization (26). CD133 is a special marker that has been widely used for identifying CSCs in different tumor settings (27), especially in solid tumors, such as prostate (28), pancreas (29), brain (30), liver (31), colorectal (32), ovarian (33), osteosarcoma (34), and lung cancer (35). In colorectal cancer, a subpopulation of cells expressing CD133, which comprise 1% of the tumor cells, was shown to efficiently induce xenografts *in vivo* (36). However, CD133 expression appears to represent only one subset of CSCs and the surface marker can also be found to be ubiquitously expressed on many differentiated cells (37). EpCAM is expessed by most adenocarcinomas and is thought to participatein tumor progression (38). In liver and pancreatic cancer (29, 39), a high expression of EpCAM is associated with the dedifferentiation of tumor cells that have regained stem cell-like features. CD24 is highly expressed in embryonic stem cells (40) and has been widely detected in different tumor settings. The combined surface markers C44/CD24 have been used to identify CSCs in breast tumors (41, 42). CD26 (dipeptidyl peptidase-4, DPP4) is expressed on various cell types, which includes cells with stem traits and is thought to influence progenitor cell migration (43). CD26 is widely detected in leukemic and colorectal cancer (44). Aldehyde dehydrogenase 1A1 (ALDH1A1) has also been identified as a potential CSC marker. ALDH expression is associated with the oxidation of aldehydes to carboxylic acid. ALDH activity has proven useful for the prediction of poor tumor outcome in prostate, breast and lung cancer (45, 46). The ABC transporters are able to pump chemotherapy agents out of the cells that express these proteins. These transporters are widely expressed by CSCs and are thus thought to represent an important component for the failure of cancer chemotherapy. The expression of ABC transporters has been used to identify or isolate CSCs from solid tumors (47). Importantly, CSCs have also been functionally identified in what would represent CSC negative populations based on surface markers (48). Thus, it is generally important to make use of multiple markers to more reliably identify CSCs. To this end, the activation of CSC-related signaling pathways such as the canonical Wnt pathway, has been shown to provide an addition level of information to better identify CSCs from colon and ovarian cancer (49).

Some surface markers used to characterize CSCs are also expressed by normal stem cells. CD29 (integrin β1) is widely expressed on CSCs and also on some normal cells, and is regarded as a marker for breast cancer CSCs. CD29 is important for breast cancer cell adhesion to extracellular matrix, and is thought to promote self-renewal and chemoresistance (50). CD9 (MRP-1) is widely expressed in normal tissues. However, it can also act as an effective marker to diagnose B-acute lymphoblastic leukemia (B-ALL) and is linked to drug resistance. CD44s is frequently used as a CSC marker (51). CD44 is composed of different subtypes (CD44V1-V10) (52, 53) and is expressed by both CSCs and normal cells. CD44 expression is associated with cancer progression and metastasis (51). For example, the CD44V9 is a predictive marker in solid tumors, including head and neck squamous carcinoma and gastric cancer. CD44V3 and V6 have been shown to be linked to invasion, metastasis, and resistance to apoptosis in colorectal cancer (54). The CD44V3-7 varients are highly expressed in non-small cell lung carcinoma (NSCLC) (55, 56). In addition, CD44V6 is associated with lymph node metastasis (6). In examples of breast cancer, high expression of CD44V3, V5, and V6 have been detected and shown to be related to the invasive properties of the tumor (57, 58). ABCB5 (ATP-binding cassette transporter) is a member of the ATP-binding cassette transporter family. ABCB5 expressed by normal cells and contributes to cell proliferation

and differentiation (59). However, the expression of ABCB5 has also been demonstrated in several malignant stem cells, including ocular surface squamous neoplasm (OSSN) (60) and melanoma (61, 62). The ABCB5 subpopulation was shown to have an unlimited self-renewal potential, and is thought to foster tumor progression, metastasis, and therapy resistance (63, 64).

CSCs with unlimited self-renewal potential express potential specific markers that can help dinstinguish them from other cells. By making use of markers in CSCs, it may be possible selectively eradicate CSCs in various tumors (22, 65). While there is a growing list of markers that have been used for identification and isolation of CSCs, very few reliable specific surface markers have been found that clearly identify CSCs because CSCs, for the most part, are heterogeneous. The identification of more universal CSC markers across diverse cancer types would clearly redine the field. Finally, what is emerging is that the application of multiple markers used in combination represents the most reliable means of characterizing these cells absence the functional criteria used to define CSCs.

CSC MICROENVIRONMENT

Accumulating evidence suggests that cancer cells acquire a "stemness" feature in part through environment input. Because of this, even differentiated cancer cells can revert to a more dedifferentiated state which has been linked to the ability to form tumors (66). CSCs co-injected into mice with stromal cells extracted from a tumor environment form more aggressive tumors that do CSCs alone suggesting an important role for the stromal matrix surrounding CSCs, also known as the "CSC niche" (67, 68). Cancer cells in a such a niche are capable of maintaining their stemness state (12, 69). The niche can contain various cell types and growth factors providing a tumor promoting microenvironment. This can involve endothelial cells, immune cells, cancer associated fibroblasts (CAFs), various growth factors, and cytokines. In addition to these components, environment changes, such as hypoxia, and pH have been proposed to contribute to the CSC niche (70–72). The perivascular niche, which is best studied in brain tumors, is recognized as a hallmark of glioblastoma (GBM). The perivascular niche enhances GBM stemness and ability for self-renewal and invasion (73).

Low levels of oxygen, referred to as hypoxia, is an important feature of TME. Hypoxia appears to help drive the maintenance of stemness and thus malignancy of CSCs, which promotes tumor survival and metastasis (74). The hypoxia-inducible factors (HIFs) are transcription factors that are increased in response to t hypoxia, and high expression of HIFs (HIF-1α, HIF-2α) is correlated with tumor malignancy (75). The octamer-binding transcription factor 4 (Oct4) is activated by HIF-2α and is linked to control of CSCs self-renewal and an increase in the malignant potential of embryonic stem cell-derived tumors (76). Another transcription factor-Sox2 is also linked to stemness through modulation of Oct4 levels in CSCs (77). A reduction in miR-145 was shown to significantly reduce expression Oct4 and Sox2, and thus lead to a decrease in the CSCs population and chemosensitivity in colon cancer (78). In pancreatic ductal adenocarcinoma (PDAC), YAP/HIF-1α signaling is activated by HGF stimulation through its receptor cMET (79). Dysregulation of YAP is related to tumor proliferation, epithelial mesenchymal transition (EMT) and therapy resistance. In the context of a low oxygen environment, CSCs can obtain energy by both OXPHOS and glycolysis activity. During hypoxia, glycolytic enzymes and glucose transporters become induced by HIF-1. Pyruvate dehydrogenase kinase 1(PKD1) plays a role in converting pyruvate to acetyl-coenzyme A (80). As an essential glycolytic enzyme, PDK1 is associated with tumor proliferation, metastasis and poor prognosis (81). In breast cancer, PDK1 stimulates glycolytic activity to stimulate cancer cells to take on stemness traits (82). The use of PDK1 inhibitors can help block glycolysis activity and also limit maintenance of breast cancer stem cells (82). In many instances, CSCs have been shown to be primarily glycolytic, or to preferentially shift from OXPHOS to glycolysis in a tumor type-dependent manner. In lung cancer (83), glioblastoma (84), and PDCA (85), CSCs were also shown to utilize OXPHOS as the preferred energy production process, however, the mechanism is still unclear. To target this metabolic biology it is thought that a combined therapy targeting both aerobic glycolysis and OXPHOS dependent cells may be the most effective therapy to block CSCs.

CAFs, as a part of TME, are believed to drive tumor progression and dedifferentiation by their secretion of key growth factors and their interplay with other stromal cells. HGF secreted by CAFs was found to activate the canonical Wnt pathway and promote cancer cells to dedifferentiate to the CSCs state (26). Cytokines secreted by CAFs, such as CCL2, IGF-1, and TGF-β affect the expansion and self-renewal of CSCs in breast, lung and gastric cancer (86, 87). In a hypoxic environment, CD44 is highly expressed by CAFs that in turns helps mediate cancer cell migration and stemness sustainability (88). The high-mobility group box 1 (HMGB1) released from CAFs was demonstrated to stimulate CSCs through the TLR4 receptor in breast cancer (89). CAFs induced expression of Notch3 is responsible for the activation of lysine demethylase 1 (LSD1) in CSCs, driving self-renewal in HCC (90, 91). In addition, CAFs facilitate tumor cells migration and metastasis indirectly through EMT. In prostate cancer, CAFs secret CXCL12 and promote EMT by inducing the expression of CXCR4 (one of the EMT phenotypes), which enhances metastasis (92). Recent reports also show that CSCs can differentiate into CAF-like cells through TGF-β secretion that promotes self-renewal and proliferation (93). CSCs also secret the Hedgehog ligand SHH that is known to increase the proliferation of CAF in the mammary tumor, and the CAFs secret factors to improve the ability of CSCs malignancy (94).

The biological cross-talk between CSCs and TME is quite complicated, and changes between different tumors and environments. By better understanding these processes, we can develop novel strategies to better target CSCs. **Figures 1**, **2** gives insight into illustration of hypoxia and CAF interactions on CSC.

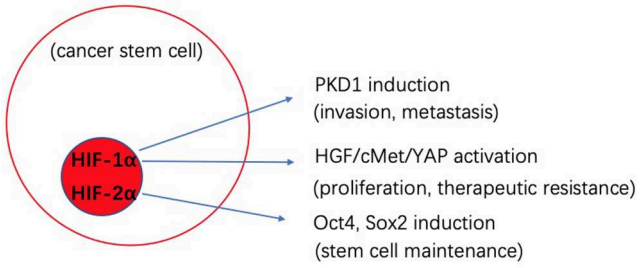

FIGURE 1 | Schematic illustration of the potential effects of hypoxia interaction on cancer stem cells (CSCs).

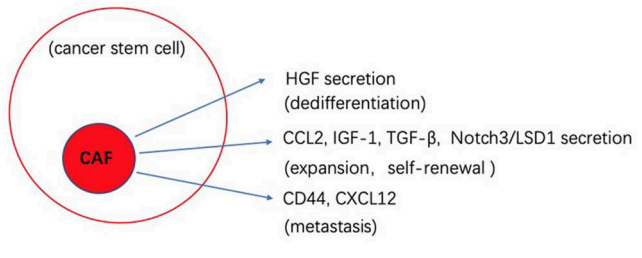

FIGURE 2 | Schematic illustration of the potential effects of CAF interaction on cancer stem cells (CSCs).

THERAPEUTIC RESISTANCE DRIVEN BY CSC AND THEIR MICROENVIRONMENT

Tumors recurrence often means poor prognosis and increased resistance to therapy (95, 96). Increasing evidence suggests that the interplay between CSCs and their TME is important in tumor development. The tumor is a complex tissue composed of different subpopulations of tumor cells and tumor-associated stromal cells. Through interaction with the microenvironment, CSCs can avoid target exposure and this may be a key to therapy resistance (97–99).

The general therapeutic resistance of CSCs allows them to escape from elimination and re-establish tumor. When the treatment cycle comes to an end, CSCs are revived from their quiescence and promote tumorgenesis (100, 101). The mechanisms by which CSCs achieve therapeutic resistance involves heightened DNA damage repair capacity, high expression of multiple drug resistance (MDR) transporters and high expression of anti-apoptosis proteins (102–105).

The DNA damaging repair (DDR) system is important in tumor progression. When under chemotherapy or radiotherapy, damaged DNA triggers the DDR, which enables CSCs to survive and thereby remain resistent to treatment. Several pathways can be activated in cancer cells includiong the double-strand breaks (DSBs) repair (homologous and non-homologous end joining), base excision repair (BER), transcription coupled nucleotide excision repair (NER), and mismatch repair (MMR) systems (106). Previous study have showen that the high expression of apurinic/apirimidinic endonuclease/redox effector factor (Ape1/Ref-1), corresponding to an activation of the BER pathway, has been implicated in the development of CSCs (107). Overexpressed Ape1/Ref-1 was also shown to maintain a low level of reactive oxygen radicals (ROS) that prevented DNA damage and cell death in CSCs (108–110). The Mre11-Rad50-Nbs1 (MRN) complex has the capacity to repair DNA and modulate cells apoptosis, and gene stability and is an important part in the DSBs pathway (111, 112). In nasopharyngeal and gastric cancer, MRN-ATM meditated DNA repair induced resistance to common chemotherapy agents, such as cisplatin and 5-FU (113, 114). The MRN complex also acts as one of the DSB pathway key elements to produce radio-resistance in various cancer types (115). Transcription factors such as forkhead box protein m1 (FOXM1), P53, glioma-associated oncogene (GLI1), and c-MYC, were also shown to be important for the DNA repair response (116, 117). Treating colon cancer with doxorubicin (a chemotherapy agent) was shown to lead to the activation of SMAD, which binds to P53 to produce chemoresistance (118).

The expression of multi-drug resistance (MDR) transporters in CSCs results in drug efflux and decreased intracellular drug concentration (119, 120). The ATP-binding cassette (ABC) transporters encompass 49 members in humans and are organized into seven subfamilies (ABCA-G) (121, 122). Three well-studied members of the family are ABCB1, ABCG2, and ABCC1 (123, 124). Overexpression of ABCG2 is associated with resistance to a large number of chemotherapy agents, such as mitoxantrone, camptothecins and flavopiridol (125). The human breast cancer resistance protein (BCRP/ABCG2), which was derived from the breast cancer cell line Mcf-7, was shown to induce resistance to mitoxantrone (126, 127). However, this resistance could be reverted by the MiR-487a target for the expression of BRCP (126). In ovarian cancer, c-MET/PI3K/AKT pathway activation was shown to induce the expression of BRCP/ABCG2, which is important in doxorubicin resistance (125, 128). ABCB1 is also called P-glycoprotein (P-gp) or multidrug resistance gene 1 (MDR1). In AML, P-gp acts as an adverse prognostic factor for drug resistance (129). It was also found that the absence of miR-298 is related to an over expression of P-gp. The upregulation of miR-298 could reduce the expression P-gp, leading to increased concentration and cytotoxicity of doxorubicin in doxorubicin-resistant breast cells (130). In addition, oncogene kinases such as MEK1/2, ERK1/2, c-Raf, EGF, and FGF, can increase the expression of P-gp and effect drug resistance and therefore may also represent potential targets (131). The ACBC1 transporter is encoded by the MRP1gene. This subfamily plays a role in affecting MDR in lung, bladder, and breast cancer (124). In neuroblastoma, the high expression of MRP1 is associated with a poor outcome and sensitivity to chemotherapy should be regained by targeting MRP1 (132).

Hypoxia influences cancer progression, and therapy resistance, and it leads to poor outcomes. The ROS level is affected by oxygen density. In tumors, hypoxia leads to a low ROS level that in turn can be protective for CSCs and lead to therapy failure (133). HIFs are considered as negative factors for effective tumor therapy. It has been suggested that HIFs influence the pathways that contribute to the quiescence of CSCs, such as cell cycle control via cyclin dependent kinase, metabolic control

via pyruvate dependent kinase, anti-apoptosis via BCL-XL, and self-renewal via OCT-4 (134–136). In some reports, HIFs are believed to be related to MDR, such as ABCG2, and to affect drug efficacy. VEGF has also been proven to be induced by hypoxia, leading to chemo/radiotherapy resistance (137, 138). In colon cancer, dual specificity phosphatase-2 (DUSP-2) was suppressed by hypoxic culture, which led to the upregulation of COX-2 expression. DUSP-2 has a negative function in cancer malignancy and COX-2 is closely related to cancer stemness, tumor growth and drug resistance (139).

Anti-apoptosis protein expression in CSCs is another component of therapy resistance. BCL2 and BCL-XL are highly expressed in breast and AML CSCs (136, 140). P53 is a tumor suppressor that is frequently mutated in most human cancers. Due to programs such as inactivation of caspase-9 and protease activating factor1 (Apaf-1), and the activation of gain-of-function, the mutant P53 shows acquisition of dedifferentiation and stemness that leads to drug resistance (141, 142). An altered apoptosis pathway has also been demonstrated to be involved in the formation of drug resistance. The high expression of Bcl-2 due to Notch and Hh signaling pathways translates into docetaxel resistance in prostate CSCs (143). Additionally, Hh signaling pathway activation in AML, especially in $CD34^{+}$ leukemic cell lymphoma, induces the function of anti-apoptosis that lead to chemotherapy (144).

EMT activities in the CSC environment include wound healing, tissue fibrosis, and carcinoma progression. Non-small lung cancer (NSCLC) treated with Erlotinib targeting EGFR mutation shows more drug resistance due to mesenchymal-like expression. With the reversion of EMT, an elevated epigenetic like the expression of E-cadherin, is associated with sensitivity to the EGFR kinase inhibitor (145). It is generally accepted that the Notch pathway is associated with gemcitabine resistance in PDAC and is regulated by EMT (146). The expression of mesenchymal-like regulators such as Zeb2/SIP1 can protect cells from DNA damage-induced apoptosis in bladder cancer, leading to poor prognosis (147). In non-small lung cancer (NSCLC), EMT is thought to act in the process of quiescence. Overexpressed CSC surface markers, such as ALDH1 and CD133 in NSCLC stem cells are proposed to develop resistance to conventional therapy agents 5-FU (148). CSC-mediated therapeutic resistance relies on different mechanisms. **Figure 3** gives insight into illustration of therapeutic resistance driven by CSC and microenvironment.

THERAPEUTIC STRATEGIES TARGETING CSC AND THEIR MICROENVIREMENTS

CSC Targeting Strategies

The selective targeting CSCs is a promising therapeutic strategy to eliminate the development of human cancer and reduce the risk of recurrence (149). Therapeutic strategies discussed include disrupting the central regulating signaling pathways important for the cell type, targeting specific markers, inhibition of the ABC transporters, manipulating miRNA expression, or inducing the differentiation and apoptosis of CSCs.

Signaling pathways that underlie CSC biology and have been identified as potential targets. Key pathways identified include Sonic hedgehog (Shh)/Patched (Ptch)/Smoothened (Smo), Notch/Delta-like ligand (DLL), CXC chemokine receptor 1-2/CXCL8/FAK, and Wnt pathways. Downstream transcription factors such as β-catenin, STAT3, and Nanog have also been identified as potential clinical targets (150). However, the fact that CSCs and normal stem cells share the expression of many genes and signaling pathways, as well as the redundancy of the regulatory pathways, may effectively limit the efficacy and clinical impact of the therapeutic approaches.

Drugs targeting CSC markers may play an adjunctive role together with surgery, chemotherapy, and radiotherapy (151). CD44, the transmembrane protein that is the receptor for matrix components such as hyaluronic acid selectin, collagen, and osteopontin, has been proven to help treat acute myeloid leukemia (AML) by inhibiting tumor proliferation, and increasing apoptosis (152, 153). CD133, the glycoprotein also known as prominin-1, is another well-known marker on the CSCs surface and it has been reported to be a useful target in cancers with a large CD133 subpopulation (154, 155). In addition, other drugs approved by FDA are also used for targeting CSC markers such as rituximab (anti-CD20) (156), cetuximab (anti-EGFR) (157–159).

The aberrant expression of ABC transporters, which are drug efflux pumps, is a major mechanism of chemoresistance in CSCs cells (160). Three generations of inhibitor drugs have been developed and the fourth generation is underway (49, 160), which should be more selective and less toxic. New technology has been applied to improve therapy efficacy, such as the application of miRNAs targeting specific RNAs or the use of nanomedicines for bypassing efflux pumps. However, currently no inhibitors have been approved for clinical practice. Accumulating studies have reported that different tyrosine kinase inhibitors (TKIs) including erlotinib, lapatinib, imatinib, and nilotinib can reverse drug resistance mediated by ABC transporters (161). The ability to inhibit the multiple regulatory targets of ABC transporters synergisticly, combined with other therapy strategies to overcome chemoresistance in CSCs may represent a promising approach.

CSCs have non-coding RNA profiles that are different from those seen in other cancer cells. Non-coding RNAs act as regulators in maintaining and modifying CSCs properties and functions (162). As such, they represent not only potential drugs but also therapeutic targets for the treatment of CSCs. Accumulating evidence suggests that non-coding RNAs, including microRNA (miRNA) and long non-coding RNA (LncRNA), regulate the stemness of CSCs by acting as suppressors or promoters of pathways that modulate the CSC carcinogenesis, differentiation, and EMT. In breast CSC and CD133 positive pancreatic cancer cells, miR-30 was found to be decreased and inhibited anoikis resistance (163). Three novel LncRNAs including Lnc TCF7, Lnc-b-Catm, and Lnc BRM were reported to sustain the self-renewal of CSCs by regulating the classic signaling pathways related to development and progression of liver CSC (164). Non-coding RNAs are regarded as very useful targets for potential therapeutic strategies due to their limited and selective expression in tumor tissues. MiRNA-based therapeutics are also emerging as tumor treatment options

and are currently entering clinical trials (165). For example, a phase 1 clinical trial that targomiRs, minicells loaded with miR-16-based mimic miRNA and targeted to EGFR, is being evaluated in patients with malignant pleural mesothelioma and non-small cell lung cancer (166).

The impairment of apoptosis contributes to cancer development and progression, and the reactivation of apoptosis programs might be useful in the treatment of cancer. It has been reported that targeting TRAIL could cause caspase-8 reactivation, ultimately initiating mitochondrial outer membrane permeabilization and triggering the apoptosis (167). In PACA, JNK inhibition attenuated resistance of TRAIL-induced apoptosis and reduced the self-renewal capacity of CSCs (168). Finally, inhibition of NF-κB by the molecule MG-132, has also been reported to induce cell death (169). Another approach that has been evaluated focused on various means to induce CSCs differentiation. Promising agents are under research currently include the use of retinoic acid and its analogs (ATRA) for the treatment of promyelocytic leukemia (170–172). These may also show utilityto induce differentiation of glioma and breast CSCs. Other molecules such as histone deacetylase inhibitors (HDACI), tyrosine kinase have also been proposed in many CSCs studies (151). Most recent antibody targets in CSC were summarized in **Table 1**.

Targeting the CSC Environment

The tumor environment is comprised of various components including CAFs, immune cells, multipotent stromal cells (MSCs), endothelial and perivascular cells, and their secreted factors including growth factors and cytokines. In addition, this environment is made up of extracellular matrix (ECM) components, and extracellular vesicles, within a prevailing hypoxic region (12). The tumor stroma is thought ot help foster the generation and maintenance of CSCs, protect the tumor from the immune system (173), and contribute to the induction of EMT, leading to enhanced tumor progression, invasion, and recolonization as secondary tumors. Furthermore, CSCs can acquire drug resistance by interacting with niche components in TME. Thus, targeting the TME may represent an effective indirect therapeutic strategy for the treatment of CSCs and for the prevention of drug resistance.

Stromal Cells in the CSC Niche

It is recognized that tumor stromal cells can not only provide physical shelter for CSC by limiting drug access, but also

TABLE 1 | The antibody target in CSC through different mechanisms in different tumors.

Antigen	Targeting mechanism	Inhibitor	Cancer tested	References
CD44	marker	H90	AML	(142, 143)
CD133	marker	Oxyteracycline FIBPi	Liver Colon	(144, 145)
CD20	Marker	Rituximab	Lymphoma	(146)
EGFR	Marker	Ectuximab	Head and neck Squamous cell Breast Esophagus	(147–149)
ABC transporters		Erlotinib Lapatinib Imatibib Nilotinib	Under test	(151)
targomiRs	EGFR	Clincal phase 1	Malignant pleural mesothelioma Lung	(156)
TRAIL	Apoptosis	JNKi	pancreas	(158)
NF-κB	Apoptosis	MG132	leukemic	(159)

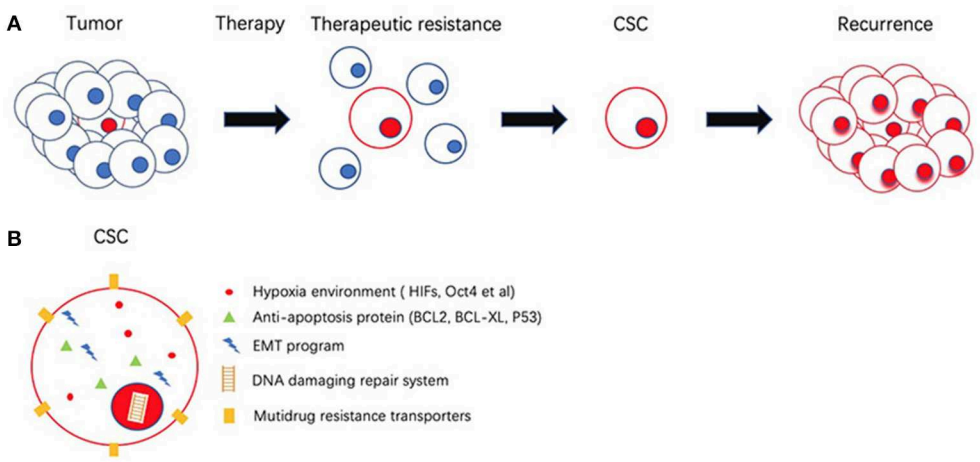

FIGURE 3 | Schematic representation of cancer stem cells (CSCs) and its role in therapeutic resistance. **(A)** Most of the cancer cells are eliminated by therapeutic resistance. CSC could escape from chemotherapy and re-establish tumor. **(B)** CSC possess several mechanisms to achieve therapeutic resistance involves Hypoxia environment, high expression of anti-apoptosis proteins, epithelial mesenchymal transition (EMT), DNA damaging repair system (DDR), multiple drug resistance transporters (MDR).

promote CSC growth, migration, and metastasis by producing important growth factors, cytokines and chemokines (174). Since the cross-talk between CSCs and stromal cells can stimulate tumor aggressiveness, directly targeting the stromal cells may serve as an alternative therapeutic strategy to treat CSCs (175).

Vascular endothelial cells (ECs), a type of stromal cell in CSC niche, which are required for angiogenesis, can also secrete growth factors and cytokines that enhance the proliferation of cancer cells, and promote the maintenance of CSCs properties in head and neck squamous cell carcinoma (176, 177). Interfering with tumor EC growth and survival could in theory inhibit not only angiogenesis but also the self-replication of CSCs (178). VEGF is a strong proangiogenic factor secreted by cancer cells, that is a well-recognized therapeutic target. Various angiogenic inhibitors have been developed that can also inhibit the self-renewal of CSCs leading to reduced tumor growth. In GBM, the VEGF tyrosine kinase inhibitor—bevacizumab has proven successful in expanding survival time by targeting the perivascular niche (179). Also in GBM, the VEGF-VEGF2-NRP1 axis is seen as an attractive target in order to decrease $CD133^{+}$GBM CSCs (180). Bevacizumab combined with anti-hepatoma-derived growth factor (HDGF) antibody has been shown to suppress CSC populations in NSCLC (181). However, in breast cancer, inhibition of VEGFR may increase CSCs population by inducing hypoxia (182). To address this it may be that use of a VEGFR inhibitor in combination witt HIF inhibition in combination therapy may provide a more effective treatment strategy (183).

CAFs represent the major component of tumor stroma andalso play an important role in cancer therapeutic resistance and radiotherapy resistance. Thus, the direct targeting of CAFs may enhance clinical outcomes. Surface markers of CAFs such as FAP, S1004A, and TEM8 have been directly targeted through administration of sibrotuzumab, 5C3, and ADC, respectively, in various tumor types (184–186). In breast cancer, CAF activation was blocked by inhibition of Hedgehog (Hh) signaling, which also increased the sensitivity of resident CSCs to chemotherapy agents (187). CAFs secret TGF-β, and the inhibition of TGF-β signaling by using LY364947 administered via nanoparticle therapy showed a potent therapeutic effect by disrupting CSC biology (188). PTK7 as a special marker of HNSCC stem cells and is demonstrated to have a close relationship with tumor persistence, metastasis, and recurrence. The use of a PTK7 inhibitor was found to increase the sensitivity of HNSCC to erlotinib (189). In addition to a direct activity on CSCs, the inhibition of important signaling pathways may represent a prospective strategy in tumor treatment. Notch signaling is over-activated in HCC, and is thought to help maintain stemness in liver CSCs by regulating LSD1 deacetylation in CAFs (190). CD10+GPR77+ CAFs were demonstrated to promote cancer stemness and chemoresistance. An antibody against GPR77 was demonstrated to reverse chemoresistance by targeting the CD10+GPR77+ CAF subset in solid tumors, such as in breast, lung cancer (191).

Tumor-associated macrophages (TAMs), feature M2-like characteristics and are important components of TME. TAMs have been demonstrated to promote CSC immunosuppressive traits leading to immune escape (192). It has been suggested that TAMs may represent potential targets for immunotherapy. In inflammatory breast cancer (IBC), tumor cells interact with immune suppressing M2-TAM leading to the production of high levels of IL-8 and CCL18 chemokines that in turn activate STAT3, which induces a CSCs-like phenotype in IBC cells and drives EMT during IBC progression (193). Targeting CXCL8/GRP/STAT3 may represent a therapeutic choice in the treatment of IBC (194). In lung cancer, the Src kinase is associated with metastasis and stemness (195). For example, it has been demonstrated that overexpressing Src in M2-TAMs induces cisplatin resistance in lung cancer. Inhibition of Src using the small molecular agent dasatinib, was found to re-sensitize lung cancer cells to cisplatin (196). CAF derived CXCL12 can help attract monocytes, which display M2 TAM characteristics. Blocking the CXCL12/CXCR4 axis significantly reduced the effect of M2 TME leading to reduced cell proliferation, migration and resistance to vineristine in OSCC chemotherapy (197). A member of the immunoglobulin family, CD47 is found to be overexpressed on the surface of many cancer types. It binds to the signal regulatory protein alpha (SIRPα) to prevent cancer cells from undergoing phagocytosis in the tumor environment (198, 199). Targeting CD47, or interfering with the CD47-SIRPα axis leads to enhanced tumor phagocytosis by macrophages and represents a promising therapeutic strategy to treat CSCs (200–202). It has been shown in HCC that the miRNA 125 delivered via TAM exosomes may significantly suppress the CSC phenotype and limit drug resistance (203). The same function of miRNA125 or TAM exosomes have also been seen in cervical cancer (204), nasopharyngeal cancer (205), and AML (206).

Immunotherapy

Immunotherapy is an emerging field and the exact mechanism by which these therapies may abrogate the ability of CSCs to reinitiate tumors is still under investigation. Over the past decade, therapeutic approaches have utilized the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (207) or programmed death 1 (PD-1)/programmed death receptor ligand-1 (PD-L1) (208) blocking antibodies, which have yielded notable response rates and have shown a remarkable clinical response in patients with advanced cancer. Despite the recent successes, the utilization of single antibodies is often limited and leads to poor treatment outcome. To achieve improved immune responses, the use of combination strategies for checkpoint inhibitors with other therapeutics may offer a stronger response against cancer as well as higher recovery rates (209, 210). PD-1 blockade was shown to enhance a specific antitumor efficacy of streptavidin-granulocyte-macrophage-CSF surface-modified bladder cancer stem cells vaccine (211). A recent study showed that targeting CSCs using the CSC-dendritic cell vaccine with CTLA-4 and PD-L1 blockades simultaneously enhanced the eradication of melanoma stem cells in the mouse model (212). In addition, CAR-T cells have produced remarkable antitumor activities in different types of tumors (213, 214). In prostate cancer and NSCLC tumor models, CAR T-cells targeted against EpCAM and EGFR antigens could successfully eradicate CSCs and cancer cells

TABLE 2 | Target factors and chemokines in different tumors.

Antigen	Derived	Inhibitor	Cancer tested	References
VEGF	EC	Bevacizumab	GBM Lung	(169, 170) (171)
FAP	CAF	Sibrotuzumab	Colon	(174)
S1004A	CAF/marcophage	5C3	Breast	(175)
TEM8	CAF	ADC	Pancreas Colon Breast	(176)
TGF-β	CAF	LY364947	Under test	(178)
PTK7	CAF		HNSCC	(179)
GPR77	CAF	Anti-GPR77	Lung Breast	(181)
IL8/GRP/STAT3	TAM	anti IL8/GRP/STAT3 axle	IBC	(183, 184)
Src	TAM	Anti Src/dasatinib	Lung	(186)
CXCL12/CXCR4	TAM	anti CXCL12/CXCR4	OSCC	(187)
CD47/CD47-SIRPα	TAM	Hu5f9-G4	NHL AML ALL	(190–192)
Immune-therapy		PD-1 PD-L1	Balder Melanoma	(201) (202)

(215, 216). Most recent target factors and chemokines in CSC were summarized in **Table 2**.

CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the central impact of CSCs on tumor progression with accompanying poor patient outcome, CSCs-targeted therapy approaches have emerged as an important new strategy for future tumor treatments. However, the identification of CSCs remains a challenge. Markers expressed on CSCs may also be displayed by normal stem cells, which may limit the accuracy of CSCs identification and compromise the targeted treatment. In addition, CSCs appear to represent by heterogeneous populations within tumor settings. Therefore, CSC surface markers alone are not broadly reliable for their identification. The best results, absence functional criteria, results from the use of multiple markers which provide a better means of identifying CSC in specific tumor types and may provide information regarding potential drug responsiveness and tumor recurrence.

CSCs have been demonstrated to influence tumor metastasis, immune escape, and drug resistance. Targeting CSCs via their unique signaling pathways, by metabolism reprogramming, hitting the ABC transporters, and even the use of non-coding RNA, represent promising strategies to control tumor progression through CSC-based targeting. However, due to the inherent heterogeneity of CSCs the targeting a single molecule or pathway may not be an effective strategy. Combination therapy may represent the most efficient means for the treatment of CSCs.

In cross-talk with CSCs, the tissue environment plays an important role in the development of tumor metastasis and recurrence. In the TME, CSCs are thought to reside in a special "CSC niche," which helps maintain their self–renewal and stemness. Immunotherapy represents an important emerging field in tumor therapy. Recent impressive results have been seen in immune targeting of CSCs through the use of PD-1/PDL-1 inhibitors. However, some studies have reported that CSCs are less immunogenic than non-CSCs, and thereby limiting antitumor response to CSCs. CAR-T cells also hold promise in overcoming cancer resistance in different types of tumors. Thus, targeting both CSCs and TME may represent the best option in the anti-cancer approach. Although the interconnected networks between CSCs and TME are complex, and most mechanisms are still obscure, various CSC targeting agents have been developed and successfully tested in several tumor types. In contrast with the single focus of CSCs-targeted therapy, because TME components include different types of stroma cells, cytokines, and growth factors, many of them have proved to be targeted in the eradication of CSCs. However, although there is growing literature in this promising area, the therapeutics of targeting CSCs and their environment are still in its infancy. Research on CSCs and their related environments will provide new targets for the development of anti-tumor strategies. In addition, clarifying the interconnectiveness of CSCs and TME will be important for the design of effective therapeutic approaches. The focus of future trials may include combination therapies that target multiple mechanisms in the tumor. However, this field is still undeveloped, and considerable research will be required to produce viable products. Many important challenges remain, including how to achieve drug selectivity and efficacy, reduce toxicity to normal cells, reduce adverse side effects, and explore new approaches to deliver and keep an effective drug concentration in place. In conclusion, the combined regimen of CSC-targeted therapy together with conventional treatment methods shows great potential and deserves further research.

AUTHOR CONTRIBUTIONS

Q-ZD and L-XQ designed and supervised the entire project. H-RS, SW, and S-CY wrote the manuscript. Q-ZD and PN made significant revisions to the manuscript. All authors read and approved the final manuscript.

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CPT1A/2-Mediated FAO Enhancement—A Metabolic Target in Radioresistant Breast Cancer

Shujun Han[1,2†], Ryan Wei[1,3†], Xiaodi Zhang[1‡], Nian Jiang[1‡], Ming Fan[1], Jie Hunter Huang[1], Bowen Xie[1‡], Lu Zhang[1‡], Weili Miao[4], Ashley Chen-Ping Butler[1], Matthew A. Coleman[1,5], Andrew T. Vaughan[1,5], Yinsheng Wang[4], Hong-Wu Chen[5,6], Jiankang Liu[2] and Jian Jian Li[1,5]**

[1] Department of Radiation Oncology, School of Medicine, University of California, Davis, Sacramento, CA, United States, [2] Center for Mitochondrial Biology and Medicine, Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology and Frontier Institute of Science and Technology, Xi'an Jiaotong University, Xi'an, Shaanxi, China, [3] Lewis Katz School of Medicine/St. Luke's University Regional Campus, Temple University, Philadelphia, PA, United States, [4] Department of Chemistry, University of California, Riverside, Riverside, CA, United States, [5] NCI-Designated Compressive Cancer Center, School of Medicine, University of California, Davis, Sacramento, CA, United States, [6] Department of Biochemistry and Molecular Medicine, University of California, Davis, Sacramento, CA, United States

Tumor cells, including cancer stem cells (CSCs) resistant to radio- and chemotherapy, must enhance metabolism to meet the extra energy demands to repair and survive such genotoxic conditions. However, such stress-induced adaptive metabolic alterations, especially in cancer cells that survive radiotherapy, remain unresolved. In this study, we found that CPT1 (Carnitine palmitoyl transferase I) and CPT2 (Carnitine palmitoyl transferase II), a pair of rate-limiting enzymes for mitochondrial fatty acid transportation, play a critical role in increasing fatty acid oxidation (FAO) required for the cellular fuel demands in radioresistant breast cancer cells (RBCs) and radiation-derived breast cancer stem cells (RD-BCSCs). Enhanced CPT1A/CPT2 expression was detected in the recurrent human breast cancers and associated with a worse prognosis in breast cancer patients. Blocking FAO via a FAO inhibitor or by CRISPR-mediated CPT1A/CPT2 gene deficiency inhibited radiation-induced ERK activation and aggressive growth and radioresistance of RBCs and RD-BCSCs. These results revealed that switching to FAO contributes to radiation-induced mitochondrial energy metabolism, and CPT1A/CPT2 is a potential metabolic target in cancer radiotherapy.

Keywords: breast cancer stem cells, CPT1A/CPT2, FAO, metabolism, radioresistance, breast cancer

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Patricia Sancho, Aragon Institute for Health Research (IIS Aragon), Spain

***Correspondence:**
Jiankang Liu
j.liu@mail.xjtu.edu.cn
Jian Jian Li
jijli@ucdavis.edu

[†] These authors have contributed equally to this work

[‡] Present address:
Xiaodi Zhang, Department of Toxicology, Forth Military Medical University, Xian, China
Nian Jiang, Bowen Xie and Lu Zhang, Xiangya Hospital, Southcentral University, Changsha, China

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INTRODUCTION

Radiation therapy (RT) is the major modality in treatment of solid cancer, including breast cancer (BC), with reported clinical benefits (1, 2). A meta-analysis of 10,801 women with or without RT after breast-conserving surgery in randomized trials demonstrated that RT reduced the 10-year risk of any first recurrence (locoregional or distant tumors) from 35.0 to 19.3% and reduced the 15-year risk of BC mortality rate from 25.2 to 21.4% (1). Cancer stem cells (CSCs; also termed as tumor-initiating cells, TICs) are suggested to be the carcinogenic cell source and responsible for tumor aggressive phenotype and failure of anti-tumor therapy (3, 4). To further improve the BC long-term efficacy by RT, the mechanisms linked with the adaptive radioresistance and recurrent risk in CSCs are to be investigated (5–8).

Elucidation of the metabolic dynamics of resistant breast cancer cells (RBCs) will help to identify metabolic targets to synergize the efficacy of RT. The theory of aerobic glycolysis in malignant cells (Warburg Effect) (9–11) is being updated, with emerging new evidence indicating an adaptive energy reprogramming in tumor cells with mitochondrial reactivation and oxidative phosphorylation (12, 13). We have reported that mitochondrial MnSOD activity is required for radioresistance in BC cells (14, 15) and radiation can enhance mitochondrial OXPHOS in tumor cells (16). Enhanced mitochondrial bioenergetics are found to be the major cellular fuel supplement for cell cycle progression (17–19), tumor aggressive phenotype and metastasis (20). Accumulating new evidence indicates that reprogramming in mitochondrial metabolism is actively involved in tumor proliferation and metastasis (12, 21–23). Mitochondrial energy output is also required for nuclear DNA repair after IR (24), and mitochondrial FAO is linked with BC metastasis (25, 26). With such a flexible adaptive energy metabolism detected in the malignant cells (13, 27), it is reasonable to look into the deep mechanistic insights of reprogramming mitochondrial bioenergetics in aggressive tumors, especially the RBCs.

Under genotoxic stress conditions, such as chemotherapy or radiotherapy, tumor cells must acquire additional cellular fuel resource to meet the increased demands of energy consumption for damage repair and survive (24). In addition to glucose, fatty acids with high ATP yield are a relevant energy-rich source in cancer cells under such genotoxic crisis. The FAO-mediated mitochondrial bioenergetics has been assumed to play a critical role in cell proliferation, cancer stemness and chemoresistance (28–30). Inhibition of mitochondrial FAO by etomoxir impairs NADPH production and increases reactive oxygen species (ROS), resulting in ATP depletion and cell death in human glioblastoma cells (31). In mitochondrial FAO pathways, targeting CPT1A generates clinic benefits in RT for nasopharyngeal carcinoma patients (32). However, the precise network of FAO enhancement in reprogramming mitochondrial energy metabolism, especially in resistant breast cancer cells, remains to be elucidated.

To mimic clinical radioresistance, in this study, RBCs were generated from wild type breast cancer cells via continuous ironizing radiation (IR), and the radioresistant BCSCs cells were sorted from the RBCs (8), termed as radiation-derived BCSCs (RD-BCSCs). Using proteomics of RD-BCSCs and CRISPR-mediated FAO gene editing, here we revealed a novel mitochondrial lipid metabolic reprogramming in RBCs and RD-BCSCs. The mitochondrial fatty acid oxidation (FAO) was enhanced in both RBCs and RD-BCSCs and linked with recurrent BC and a worse prognosis in BC patients. Blocking FAO by CRISPR-mediated CPT1A/CPT2 KO inhibited aggressive phenotype of the radioresistant BC with down-regulation of the ERK pathway, indicating a potential metabolic target in breast cancer radiotherapy.

MATERIALS AND METHODS

RBCs, RD-BCSCs, and Other Reagents

MCF7 and MDA-MB-231 human breast cancer cells were purchased from ATCC (Manassas, VA, USA). MCF7/C6 radioresistant clone is a single clone surviving from MCF7/WT cells after fractionated ionizing radiation treatment (2 Gy × 15) (33, 34). MDA-MB-231/C4 are radioresistant breast cancer cells surviving from MDA-MB-231/WT cells after fractionated radiation (2 Gy × 30) (35). MCF7, MCF7/C6, MDA-MB-231, and MDA-MB-231/C4 cells were cultured in DMEM medium with 10% fetal bovine serum (FBS, Life Technologies, NY, USA) and 1% penicillin/streptomycin (Life Technologies). RD-BCSCs were sorted as previously described (8, 36). Cell pellets of MCF7/C6 were rinsed with cold PBS with 2% FBS and then suspended with PBS containing 0.5% FBS and 0.5 mg/mL PI (Sigma, St. Louis, MO, USA). Then, the cell suspension was sorted using Cytopeia influx Cell Sorter (BD Biosciences, San Jose, CA, USA). The antibody against HER2/neu was conjugated to allophycocyanin (APC, BD Biosciences, San Jose, CA), anti-human CD44 was conjugated to FITC, and human CD24 was conjugated to phycoerythrin (PE; Invitrogen, Carlsbad, CA). Cell viability was assessed by 7-AA staining during cell sorting and then determined by trypan blue exclusion after sorting (**Figure S1A**). The isolated RD-BCSCs ($CD44^+/CD24^{-/low}/HER2^+$) were maintained in CSC medium containing free serum and supplemented with B27 (Life Technology, Carlsbad, CA, USA), 20 ng/ml EGF (Biovision, Mountain View, CA, USA), 20 ng/ml basic-FGF, and 4 μg/ml heparin (VWR, Philadelphia, PA, USA). Cells were cultured in ultralow-attachment Petri dishes with 5% CO_2 at 37°C. All cell lines were tested mycoplasma free before experiments. Etomoxir was purchased from Sigma-Aldrich. Oil Red O was obtained from Millipore Sigma (St. Louis, MO, USA).

Western Blot

Western blot was performed as previously described (37). Briefly, the cell lysates were separated on SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% milk for 1 h, and then incubated with primary antibodies with shaking at 4°C overnight. In the next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein blots were developed using an enhanced chemiluminescence western blot detection system (BioRad, Hercules, CA, USA). Antibodies against HER2/Neu (C-18, #SC284), CPT2 (G-5, #SC-377294), HADHA (E-8, #SC374497), HADHB (E-1, #SC-271495), ERK1/2 (C-9, #SC514302), and c-Fos (E-8, SC#166940) were all diluted at 1:500 and purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CPT1A (clone D3B3, #12252), Phospho-p44/42 MAPK (Thr202/Tyr204, clone 197G2, #4377), Phospho-GSK3 β (Ser9, #9336), GSK3 β (clone 27C10, #9315), Phospho-STAT3 (Ser727, clone D8C2Z, #94994), and Phospho-JNK (Thr183/Tyr185, #9251) were from Cell Signaling Technology (Beverly, MA, USA). Antibody against ACAD9 (#3170340688) was purchased from Novus Biologicals (Littleton, CO, USA). Anti-β-actin at 1:2000 (#A2066) was from Sigma-Aldrich.

Fatty Acid Oxidation (FAO) Assay

FAO measurement was following the manufacturer's instruction of Fatty Acid Oxidation Assay Kits from Abcam (ab217602). Briefly, 3×10^4 cells were seeded per well in 96-well plates and cultured overnight. Then the cells were rinsed twice with 100

μl prewarmed FA-Free medium followed by adding 90 μL pre-warmed FA Measurement Medium. The wells without cells were used as signal control. A total of 85 μL of FA-Free Measurement medium was added to the wells, and 5 μL of BSA control were included as the FA-free control. All wells except the blank control had 10 μL Extracellular O_2 Consumption Reagent added. The FAO activator FCCP (0.625 μM) and inhibitor Etomoxir (40 μM) were added as the positive and negative controls. Then the wells were sealed with 100 μL pre-warmed mineral oil, and the FAO was measured using the condition as Extracellular Oxygen Consumption. The results were normalized by the protein concentration with the cells in each sample under the BCA assay.

Lipid Accumulation Assay

Breast cancer cells were plated and cultured in 96-well plates. In the next day, cells were treated with 250 μM free fatty acid (oleic acid: palmitate acid = 2:1) for 48 h. After being washed with PBS twice, cells were fixed with 4% paraformaldehyde under room temperature (RT) for 30 min. Cells were washed with sterilized water once and added into 50 μL Oil Red working solution; they were then incubated for 15 min at RT. Then 50 μL 60% isopropanol was added to the cells for 20 s at RT. Finally, the cells were washed with water twice, and the images were obtained using a Nikon microscope (Eclipse, E1000M, Japan). The red oil dye was eluted with 50 μL DMSO and incubated for 10 min with gentle shaking. The lipid accumulation results were determined by the fluorescence microplate spectrophotometer (Molecular Devices) at 510 nm.

Oxygen Consumption

Extracellular Oxygen Consumption detection was performed following the instruction of kit from Abcam (ab197243,) with 3×10^4 cells seeded per well in 96-well plates and cultured at 37°C overnight. The cell medium was replaced with 150 μl fresh culture media followed by adding 10 μl of extracellular oxygen consumption reagent. The wells without oxygen consumption reagent were used as blank control. Wells with Etomoxir (40 μM) added were included as the negative control. Wells had 100 μl of pre-warmed mineral oil added to avoid the air bubbles. The plates were read immediately in a fluorescence microplate spectrophotometer (Molecular Devices) at 37°C. The signals were collected at 1.5 min intervals for 90–120 min at Ex/Em = 380/650 nm. The results were normalized by protein concentration of the cells in each sample under the BCA assay.

MTT Assay

Breast cancer cells were seeded in 96-well plates for 24 h. After they proliferated to about 90% confluence, the cells were added into 50∼100 μL MTT solution (M-2128, Sigma) and cultured at 37°C for 2 h. The results were measured in a microplate spectrophotometer (Molecular Devices) at 540 nm.

Cell Apoptosis Assay

Breast cancer cells were rinsed by cold PBS twice, collected and stained using the Annexin-V/PI kit (Biosource, Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Stained cells were analyzed by flow cytometry (Becton Dickinson canto II, BD, NJ, USA). Data were analyzed using Flowjo software (Three Star, Inc., Ashland, OR, USA).

Colony Formation Assay

For measuring cellular clonogenicity, 1×10^3 cells were seeded into 6-well plates and treated with or without 5 Gy radiation in the next day. Cells were then cultured for 14 days at 37°C. The colonies were fixed and stained with Coomassie blue, and then colony formation rate was determined by counting colonies in each group. Finally, the colony images were observed and recorded by a Nikon microscope (Eclipse, E1000M, Japan) (8).

Immunohistochemical Staining (IHC)

The slides of primary biopsy tissues and recurrent tumors from patients with breast cancer were tested by immunohistochemistry (IHC) using Vectastain ABC kit and DAB Peroxidase Substrate kit SK-4100 (Vector Laboratories, Burlingame, CA, USA). The prepared tumor slides were firstly deparaffinized, hydrated, and then covered with blocking buffer for 1 h after heat-induced epitope retrieval. The slides were incubated with anti-CPT1A (Cell Signaling Technology, #12252) and anti-CPT2 (Santa Cruz, sc-20671) at 1:200 at 4°C overnight, followed by washing with PBST three times, and then incubated with the secondary antibody for 30 min at RT. The slides were then covered with ABC solution for 30 min on the shaker at RT. The slides were incubated with DAB solution about 2 min and then transferred to hematoxylin, HCl solution and Li_2CO_3 solution quickly several times. Finally, the slides were dehydrated and sealed. The slides were observed and recorded by Nikon microscope (Eclipse, E1000M).

CRISPR-Mediated Gene Editing

The single guide RNA (sgRNA) was designed according the CRISPR Design in Zhang Lab https://zlab.bio/guide-design-resources. We created oligonucleotide to target genes CPT1A and CPT2. The sgRNA sequences are designed as follows: human CPT1A: CTCCGGACGGGATTGACCTG; human CPT2: CGGGGCCCCGCGGTTGGTCC. The LentiCRISPRv2 backbone was used, which contains the hSpCas9 and sgRNA expression cassettes. Plasmids were purchased from the Addgene plasmid repository (Addgene #52961) (https://www.addgene.org/). Backbone LentiCRISPRv2 was annealed to oligonucleotides following the Zhang Lab GeCKO protocol and packaged into lentiviruses. The Lentiviral particles were produced in HEK293T cells following the protocol from Addgene (38), and breast cancer cells were infected with lentiviruses and selected with 0.3 μg/ml puromycin. Western blot analysis was performed to identify cell colonies with gene deficiency.

Proteomics of RD-BCSCs and MCF7 Cells

The protein mixture from total cell lysates of RD-BCSCs and MCF7 was first treated with dithiothreitol and iodoacetamide for cysteine reduction and alkylation, respectively. The protein samples were then digested using modified sequencing-grade trypsin (Roche, Basel, Switzerland) at an enzyme/substrate ratio of 1:100 in 50 mM NH_4HCO_3 (pH 8.5) at 37°C overnight.

The peptide mixture was subsequently dried in a Speed-vacuum and desalted by employing OMIX C_{18} pipet tips (Agilent Technologies, Santa Clara, CA, USA), reconstituted in water and subjected to LC-MS and MS/MS analyses on a Q Exactive Plus mass spectrometer equipped with a nanoelectrospray ionization source. Samples were automatically loaded from a 48-well microplate autosampler using an EASY-nLC 1200 system (Thermo Fisher Scientific, Rockford, IL, USA) at 3 μL/min onto a biphasic precolumn (150 μm i.d.) comprised of a 3.5-cm column packed with 5 μm C_{18} 120 Å reversed-phase material (ReproSil-Pur 120 C_{18}-AQ, Dr. Maisch). The biphasic trapping column was connected to a 20-cm fused-silica analytical column (PicoTip Emitter, New Objective, 75 μm i.d.) with 3 μm C_{18} beads (ReproSil-Pur 120 C_{18}-AQ, Dr. Maisch). The peptides were then separated using a 180-min linear gradient of 2–45% acetonitrile in 0.1% formic acid and at a flow rate on 250 nL/min. The mass spectrometer was operated in a data-dependent scan mode. Full-scan mass spectra were acquired in the range of *m/z* 350–1,500 using the Orbitrap analyzer with a resolution of 70,000. Up to 25 of most abundant ions found in MS with a charge state of 2 or above were sequentially isolated and collisionally activated in the HCD cell with collision energy of 27 to yield MS/MS.

Bioinformatics Analysis

Maxquant (Version 1.5.2.8) was used to analyze the LC-MS and MS/MS data for the identification and quantification of proteins in the LFQ mode (39). The maximum number of mis-cleavages for trypsin was two per peptide. Cysteine carbamidomethylation was set as a fixed modification. Methionine oxidation and phosphorylation on serine, threonine, and tyrosine were set as variable modifications. The tolerances in mass accuracy for MS and MS/MS were both 20 ppm. Maximum false discovery rates (FDRs) were set to 0.01 at both peptide and protein levels, and minimum required peptide length was six amino acids. The LC-MS and MS/MS protein data were also analyzed with functional clustering. Of all proteins in our total protein array, only proteins that showed levels of detection were submitted to DAVID Bioinformatics Resources v6.7 (https://david.ncifcrf.gov/). Parameters were established for our functions of interest with a cutoff of $p < 0.05$.

Category Selection of Proteomics

DAVID Bioinformatics Resources provide a wealth of information within the Gene Ontology Tool for Biological Function (40, 41). Different broad categories were generated to profile the cluster of proteins related to varied cellular functions including mitochondrial bioenergetics and lipid metabolism as well as FAO. We used the Uniprotein tagging system, UPKeyword due to the high number of hits in the protein list.

Tumorsphere Formation

Tumorsphere assay was performed as described (42). Cells were sieved with 40 μm cell strainers (Fisher, Failawn, NJ, USA) and single-cell suspensions were seeded into 60 mm Petri dishes. The cells were grown in serum-free mammary epithelial basal medium (MEBM, Lonza, Walkersville, MD, USA), supplemented with B27, 20 ng/ml EGF, 20 ng/ml basic-FGF, and 4 μg/ml heparin. Cells were then cultured for 10 days, and tumor spheres were counted under light microscopy.

Three-Dimensional (3D) Morphogenesis Assay

MCF7 and RD-BCSC cells in 40 μL plug of Matrigel (growth factor reduced and phenol red free, Becton Dickinson, Plymouth, UK) were plated to the well of an 8-well LabTek Chambered coverglass (Nunc, Rochester, USA) at 37°C for 30 min. On ice, cells were prepared at a concentration of 5,000 cells/ml in KSFM supplemented with 5 ng/ml EGF, 2% (v/v) FCS, 4% (v/v) Matrigel, and 0.2 mL of this cell solution was plated onto the Matrigel plug and incubated for 30 min at 37°C, after which 0.2 mL of growth medium was added (KSFM supplemented with 5 ng/mL EGF, 2% (v/v) FCS). Culture medium was changed every 2–3 days. At day 10, morphology was assessed by phase microscopy and cells were fixed and processed for immunofluorescence microscopy analysis.

Statistical Analysis

Statistical significance of differences was evaluated using two-tailed student *t*-test for two groups' comparison or one-way ANOVA test where multiple groups were involved. $p < 0.05$ was considered statistically significant.

RESULTS

Mitochondrial FAO Is Enhanced in Radioresistant Breast Cancer Cells

Lipid metabolism has been linked with cancer therapy response (28–30). Here we address the question of whether reprogramming mitochondrial FAO plays a key role in breast cancer radioresistance. Two radioresistant BC (RBC) cell lines (MCF7/C6 and MDA-MB-231/C4) isolated from surviving MCF7 and MDA-MB-231 residues with HER2 induction and aggressive phenotype after chronical radiation (**Figure S1A**) (8, 35) showed enhanced expressions of mitochondrial FAO genes CPT1A, CPT2, HADHB, and ACAD9 (**Figure 1A**). We then measured the mitochondrial FAO activity by ^{18}C-labeled unsaturated fatty acid oleate as the substrates with CPT1A specific inhibitor Etomoxir (ETX) and the FAO activator FCCP as negative and positive controls, respectively, demonstrating a significant elevation of FAO activity in MCF7/C6 vs. wild type MCF7 cells (**Figure 1B**). Furthermore, enhanced lipid turnover rate was detected in MCF7/C6 cells loaded with free fatty acid (FFA), which was contrasted with the markedly accumulation of FFA in the wild type MCF7 cells (**Figure 1C**), indicating enhanced FAO metabolism in RBC cells.

We next determined whether the FAO is enhanced in the human recurrent breast tumors compared to the primary tumors. CPT1 and CPT2 are the rate-limiting transporters and play a key role in mitochondrial long-chain FAO and lipid metabolism. Remarkably, the enhanced co-expression of CPT1A (an isoform of CPT1) and CPT2 was mostly detected in the pathological sections of recurrent BC tumors compared to the paired original biopsy specimens in a group of 12 BC

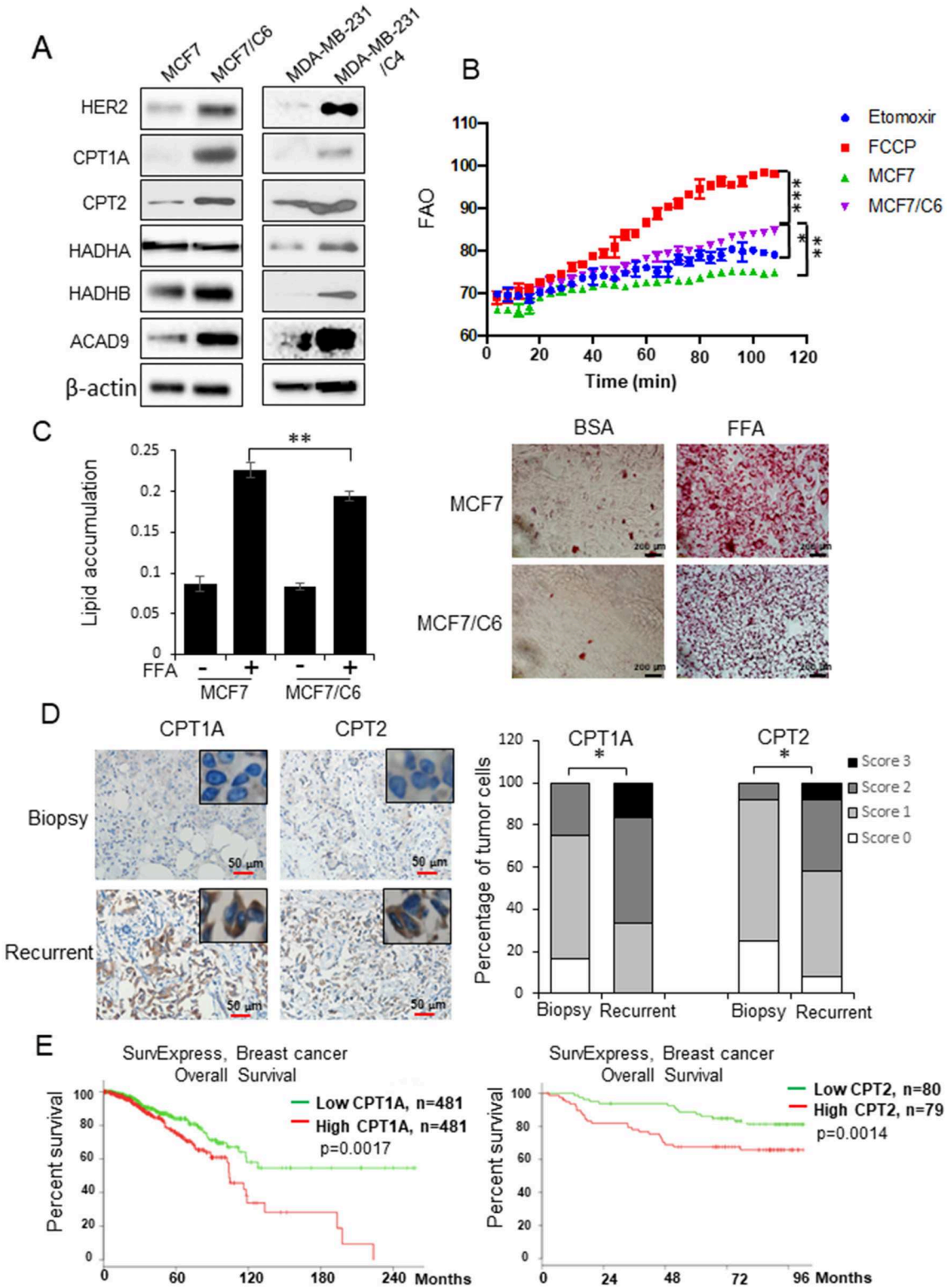

FIGURE 1 | FAO is enhanced in radioresistant BC cells and recurrent BC and linked with a poor prognosis in BC patients. **(A)** Western blot of a cluster of FAO enzymes and HER2 in wild type MCF7, MDA-MB-231, and their counterpart radioresistant MCF7/C6 and MDA-MB-231/C4 cells. **(B)** FAO activity assay of MCF7 and MCF7/C6 cells with MCF7/C6 treated with FAO inhibitor Etomoxir (40 μM) as a negative control and FAO enhancer FCCP (1 μM) as a positive control. **(C)** Fatty acid turnover rate in MCF7 and MCF7/C6 cells treated with or without 250 μM Free Fatty Acid (FFA; oleic acid: palmitic acid = 2:1) for 24 h before Oil Red staining

(Continued)

FIGURE 1 | (left: quantitation of lipid accumulation; right: representative images of FFA accumulation). **(D)** Representative IHC of CPT1A and CPT2 in biopsy and recurrent BC (left). Quantitation of IHC was achieved by scoring staining intensity and positive cells (right). **(E)** Elevated CPT1A/CPT2 expression correlates a worse overall survival of BC patients in the TCGA database http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp. Error bars in **(B–D)** represent the mean ± standard deviation. Significance was determined by one-way ANOVA test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

patients **(Figure 1D)**. In agreement, the TCGA database revealed a poor prognosis in BC patients with increased expression of CPT1A or CPT2 **(Figure 1E)** (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp). Together, these results indicate that reprograming mitochondrial FAO contributes to BC radioresistance and worse prognosis.

CPT1A/CPT2 Mediated FAO Is Required for Radioresistant Breast Cancer Stem Cells

Our previous results have indicated that mitochondrial energy enhancement is involved in BC aggressiveness due to HER2 expression (15, 43) that is confirmed in **Figure 1A**. Following the standard biomarkers of breast cancer stem cells (BCSCs) (4) and our established BCSCs from MCF7/C6 with HER2 induction as radiation-derived BCSCs (RD-BCSCs; $CD44^{+}/CD24^{-/low}/HER2^{+}$ with enhanced ALDH activity (8), we compared the tumorspheres from RD-BCSCs and MCF7 shown in **Figure 2A**. The tumorsphere of RD-BCSCs showed severely disorganized structure with altered distribution of cellular polarity protein (DLG, red) and enhanced HER2 expression (green), indicating an aggressive phenotype of RD-BCSCs.

Consistent with the results of RBC in **Figure 1A**, the key FAO enzymes, CPT1A, CPT2, and HADHB, were also enhanced in RD-BCSCs **(Figure 2B)**, and both basal and irradiated FAO levels were elevated in RD-BCSCs whereas no significant FAO elevation was detected in irradiated MCF7 compared to basal level, although the proteomics data showed enhanced mitochondrial proteins in both irradiated both MCF7 and RD-BCSCs (**Figure 2C**, **Figures S2A, S3**), suggesting that reprogramming FAO is an unique feature of RD-BCSCs. This is further evidenced by the specifically enhanced cluster of factors in lipid metabolism rather than other cellular proteins in irradiated RD-BCSCs (47 to 81 in RD-BCSCs vs. 55 to 76 in MCF7); and the protein intensity was also more increased in irradiated RD-BCSCs (5.24-fold) contrasted to 2.31-fold in MCF7 **(Figures S1B,C)**. The FAO inhibitor Etomoxir (ETX) significantly enhanced basal and radiation-induced apoptosis with inhibited tumorsphere formation and ERK activity in RD-BCSCs (**Figures 2D–F**, **Figure S2B**). In agreement, blocking CPT1A/CPT2 by CRISPR-mediated gene deficiency **(Figure 2G)**, enhanced apoptosis level and reduced the tumorsphere formation in RD-BCSCs upon IR (**Figures 2H,I**, **Figure S2C**). Additional proteomics evidence suggested that mitochondrial proteins were more enriched in the RD-BCSCs compared to MCF7 cells after radiation. The total mitochondrial protein counts increased from the same basal 117 in MCF7 and RD-BCSCs to 152 and 163, 29.9, and 39.3% respectively, whereas protein intensity had a 2.64-fold increase (4.50E+10 to 1.06E+11) in RD-BCSCs compared to a 1.83-fold increase in MCF7 cells **(Figures S3A,B)**. Additionally, we further analyzed the mitochondrial proteins and found that FAO metabolism was also demonstrated with an increased protein number, from 11 to 17, in irradiated RD-BCSCs including CPT1A and CPT2 (**Figures S4A,B**, **Table S1**), indicating that enhanced mitochondrial FAO contributes to radioresistance of RD-BCSCs.

ERK Activation Is Linked With Mitochondrial FAO Enhancement

To explore the key factors responsible for FAO-mediated radioresistance, we tested an array of cell proliferating factors in the two RBC cell lines by CRISPR-mediated knockout of CPT1A and CPT2 (**Figure 3A**). Strikingly, the activated form of ERK1/2 was absent in the CPT1A and CPT2 KO cells, although other cell growth factors including phosphor-GSK3β, phospho-STAT3, and phospho-JNK were also reduced to a certain degree (**Figure 3B**). Alternatively, a dose-dependent inhibition of phospho-ERK and its downstream effectors was also determined in MCF7/C6 cells with increasing concentrations of ETX (**Figure 3C**). It turned out that 200 μm ETX could dramatically block the phosphorylation of ERK1/2. The dependence of ERK1/2 kinase in the FAO-mediated resistance was again evaluated by a rescue experiment, in which the FAO activity was inhibited by ETX and then activated by L-carnitine. As expected, the induction of phospho-ERK1/2 upon radiation was significantly inhibited by etomoxir while enhanced by L-carnitine. Of note, the combination of etomoxir and L-carnitine treatment ablated the phospho-ERK1/2 induction by L-carnitine treatment upon radiation (**Figures 3D,E**). Together, our inhibition and rescue experiments consistently demonstrated that FAO mediated radioresistance is linked with ERK1/2 activation for the aggressive phenotype of radioresistant breast cancer cells.

Deficiency of CPT1A or CPT2 Reduces OCR, Cell Viability, and Slowdown Fatty Acid Turnover Rate in RBC Cells

By comparing the metabolic features of BC cells and RBCs, we found that both the oxygen consumption rate and cell viability were enhanced in MCF7/C6 cells compared to MCF7 cells, but remarkedly reduced by ETX or in CPT1A/CPT2 KO (**Figures 4A,B**). Additionally, ETX treatment and CPT1A/CPT2 KO increased FFA accumulation, indicating a slower fatty acid turnover (**Figure 4C**). The colony formation assay was used to evaluate the cell survival rates when given radiation therapy with FAO inhibition in RBCs. The colonies in the RBC MCF7/C6 and MDA-MB-231/C4 cells were more abundant than the basal clonogenic capacity of the parental MCF7 and MDA-MB-231 cells, indicating an enhanced aggressive phenotype of RBCs. However, the etomoxir treatment and

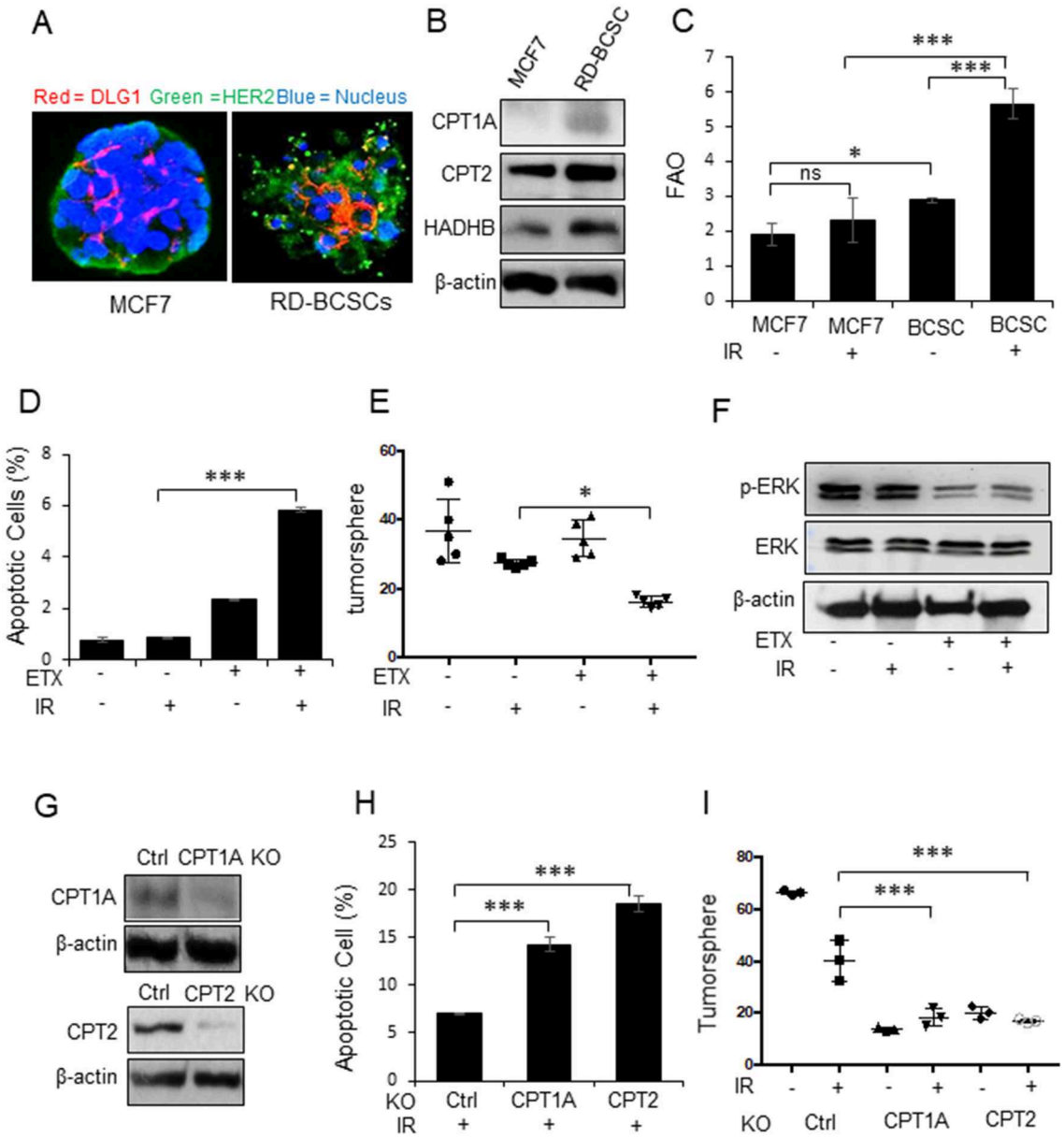

FIGURE 2 | CPT1A/CPT2 mediated FAO is required for radioresistant breast cancer stem cells. **(A)** Representative mammospheres formed from MCF7 and RD-BCSCs assessed by immunofluorescence microscopy (cell polarity protein DLG1, HER2, and nucleus are labeled by red, green, and blue respectively). **(B)** Western blot of FAO enzymes CPT1A, CPT2, and HADHB in MCF7 and RD-BCSCs cells. **(C)** FAO activity in MCF7 and RD-BCSCs with or without IR treatment for 24 h. **(D)** Radiation-induced apoptosis measured in RD-BCSCs with or without FAO inhibition (ETX, Etomoxir 200 μM for 48 h). **(E)** Tumorsphere formation assay of **(D)**. **(F)** Western blot of phosphorylated ERK in RD-BCSCs treated with ETX, IR or combined. **(G)** Western blot of CPT1A and CPT2 in RD-BCSCs with CRISPR-mediated CPT1A and CPT2 KO. **(H)** Radiation induced apoptosis in RD-BCSCs (Control), RD-BCSCs CPT1A KO, and RD-BCSCs CPT2 KO cells. **(I)** Tumorsphere formation of **(H)**. In **(C,D,E,H,I)** $n = 3$; mean ± SD; one-way ANOVA test, $^{*}p < 0.05$, $^{***}p < 0.001$.

CPT1A/CPT2 KO markedly reduced the survival colony rate in MCF7/C6 and MDA-MB-231/C4 cells (**Figures 5A–D**). IR induced cellular apoptosis was also evaluated in the RBCs. The FAO inhibition increased radiation-induced apoptosis from 10 to 34% (etomoxir treatment), 31% (CPT1A KO), and 36% (CPT2 KO) in MCF7/C6 cells and from 3.8 to 5% (etomoxir treatment), 9.2% (CPT1A KO), and 7.7% (CPT2 KO) in MDA-MB-231/C4 cells (**Figures 6A,B**). FAO inhibition also reduced tumorsphere formation from 58 to 34.5 (etomoxir treatment), 26.5 (CPT1A KO), and 24.5 (CPT2 KO) with IR in MDA-MB-231/C4 cells (**Figure 6C**). Together, these results indicate that CPT1A/CPT2 mediated FAO enhancement is required for the energy demands and cell survival in RBCs. In summary (**Figure 7**), our results reveal that

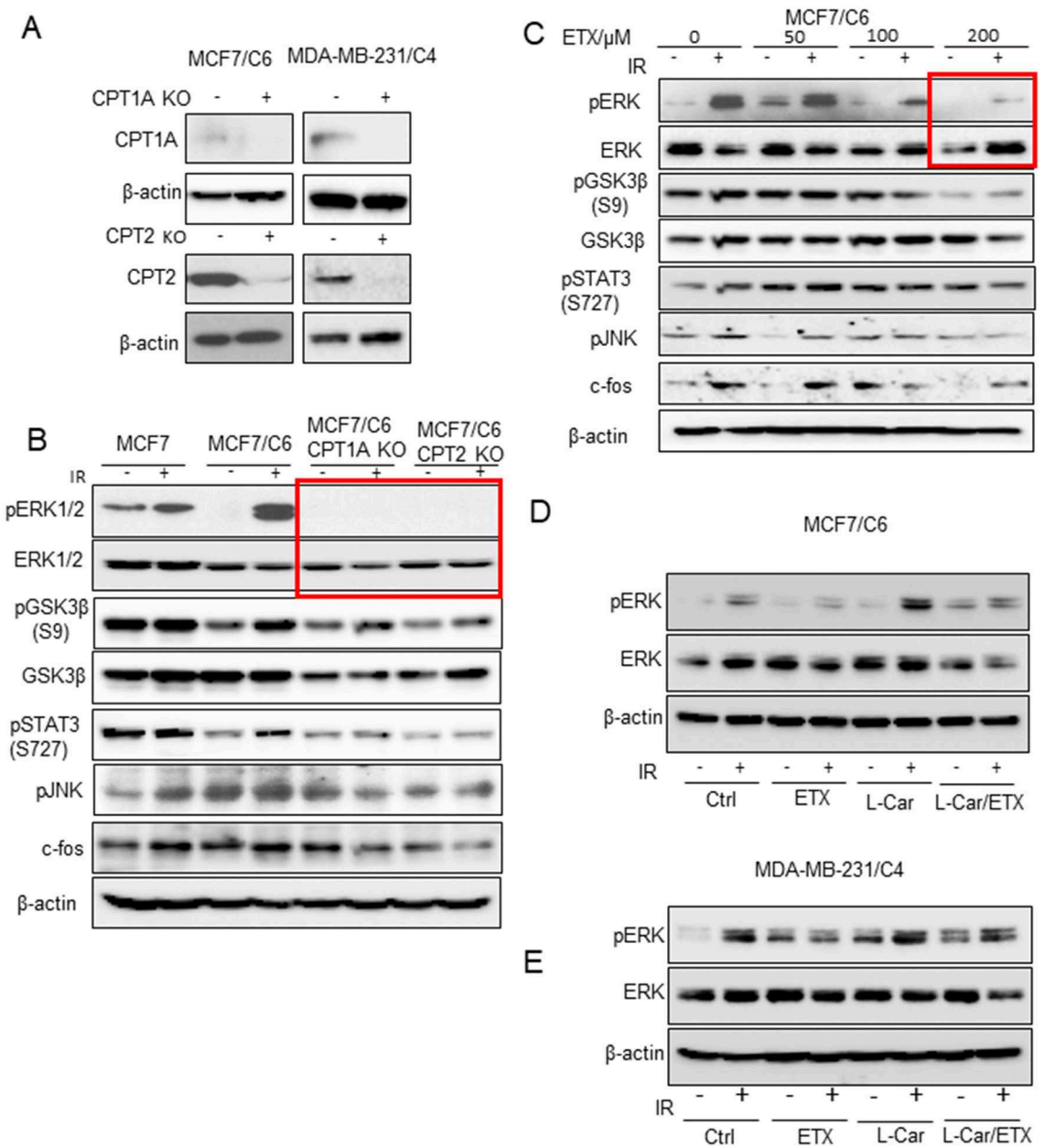

FIGURE 3 | ERK proliferative pathway is linked with FAO enhancement. **(A)** Western blot of CPT1A and CPT2 in control and MCF7/C6 and MDA-MB-231/C4 cells with CRISPR KO CPT1A and CPT2. **(B)** Western blot of proliferative genes in irradiated MCF7, MCF7/C6, MCF7/C6 CPT1A KO, and MCF7/C6 CPT2 KO cells. **(C)** Western blot of MCF7/C6 cells treated with increased doses of ETX for 24 h followed by 5 Gy IR. **(D)** Western blot of pERK in MCF7/C6 cells treated with ETX, L-carnitine (an FAO enhancer) or combined followed by 5 Gy IR. **(E)** Western blot of pERK in MDA-MB-231 cells were treated ETX, L-carnitine (an FAO enhancer) or combined followed by 5 Gy IR.

reprogramming mitochondrial FAO is the major cellular energy supplement in radioresistant breast cancer cells. Such adaptive mitochondrial energy metabolism is linked with the clinical outcome of BC patients treated with radiotherapy. We also reveal that the ERK-mediated prosurvival pathway is a potential downstream target in FAO-mediated aggressive proliferation in BC with enhanced activation of HER2 leading to promoted cell proliferation.

DISCUSSION

It is highly clinically relevant to reveal the major cellular energy driving the growth of therapy-resistant cancer cells. The concept of aerobic glycolysis in cancer metabolism (Warburg Effect), believing the deficient mitochondrial function in cancer cells, has been updated with accumulating results of active tumor metabolic to oncogenic (9) and genotoxic stress including

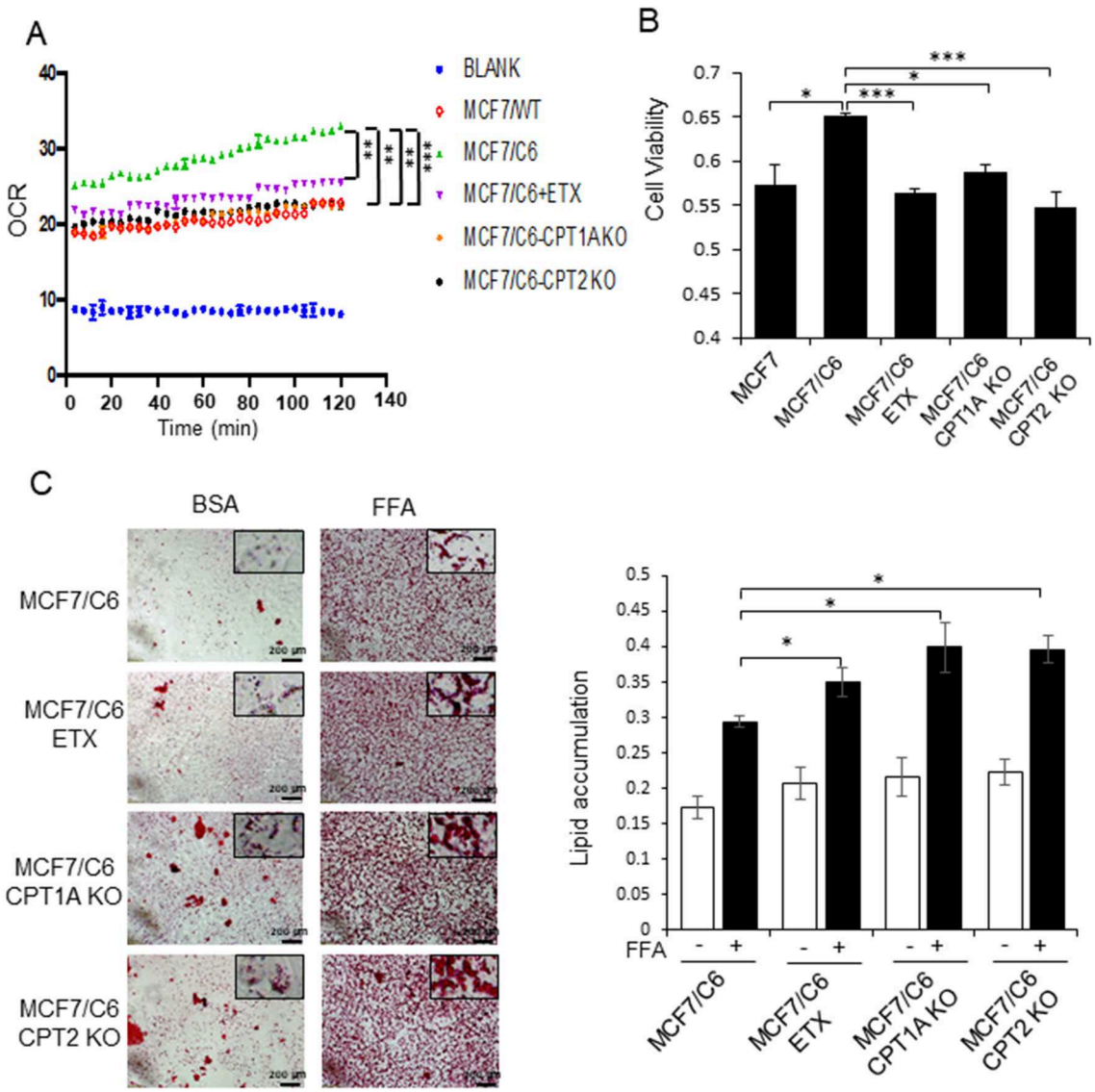

FIGURE 4 | Blocking CPT1A/CPT2 inhibits FAO and fatty acid turnover in RBC cells. **(A)** Basal oxygen consumption rate (OCR) measured in MCF7, MCF7/C6, and MCF7/C6 CPT1A KO and MCF7/C6 CPT2 KO cells with MCF7/C6 cells treated with 40 μM Etomoxir for 48 h as the FAO blockade control. **(B)** Cell viability measured by MTT of **(A)**. **(C)** Fatty acid turnover rate in MCF7/C6, MCF7/C6 CPT1A KO, and MCF7/C6 CPT2 KO cells with MCF7/C6 treated with 40 μM ETX for 48 h as FAO inhibition control; cells were incubated with 250 μM FFA (oleic acid: palmitic acid = 2:1) for an additional 24 h and then Oil Red staining; representative FA turnover rates were shown on the right. In **(A–C)**, $n = 3$; mean ± SD; significance was determined by one-way ANOVA test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ionizing radiation (16, 44, 45). A dynamic metabolic feature is linked to the adaptive reprogramming in energy supply, which is required to meet the increased cellular fuel demands for cancer cells to repair the damage and survive anti-cancer therapy. Identification of the metabolic targets required for tumor cell survival under genotoxic stress conditions such as chemo-radiotherapy are necessary for improving the therapeutic efficacy. Among other hallmarks of cancer cell progression, cancer cells can adjust energy metabolism to meet the demands of cellular fuel consumption for proliferation and survival to therapeutic stress conditions (11). In this study, we revealed a unique metabolic feature in RBCs and radioresistant breast cancer stem cells (RD-BCSCs). It showed that FAO enzyme expression and mitochondrial FAO activity were enhanced in response to radiation compared to wild type breast cancer cells. Elevated mitochondrial FAO is required for cell growth and survival in response of radiation therapy. Of note, CPT1A/CPT2 expression was also elevated in human recurrent breast cancer tissues compared to biopsy tumors. CRISPR/Cas9 mediated deficiency of CPT1A/CPT2 efficiently enhanced sensitivity of RBCs and RD-BCSCs to radiation treatment. Finally, we provided evidence that mitochondrial FAO likely functions through the ERK

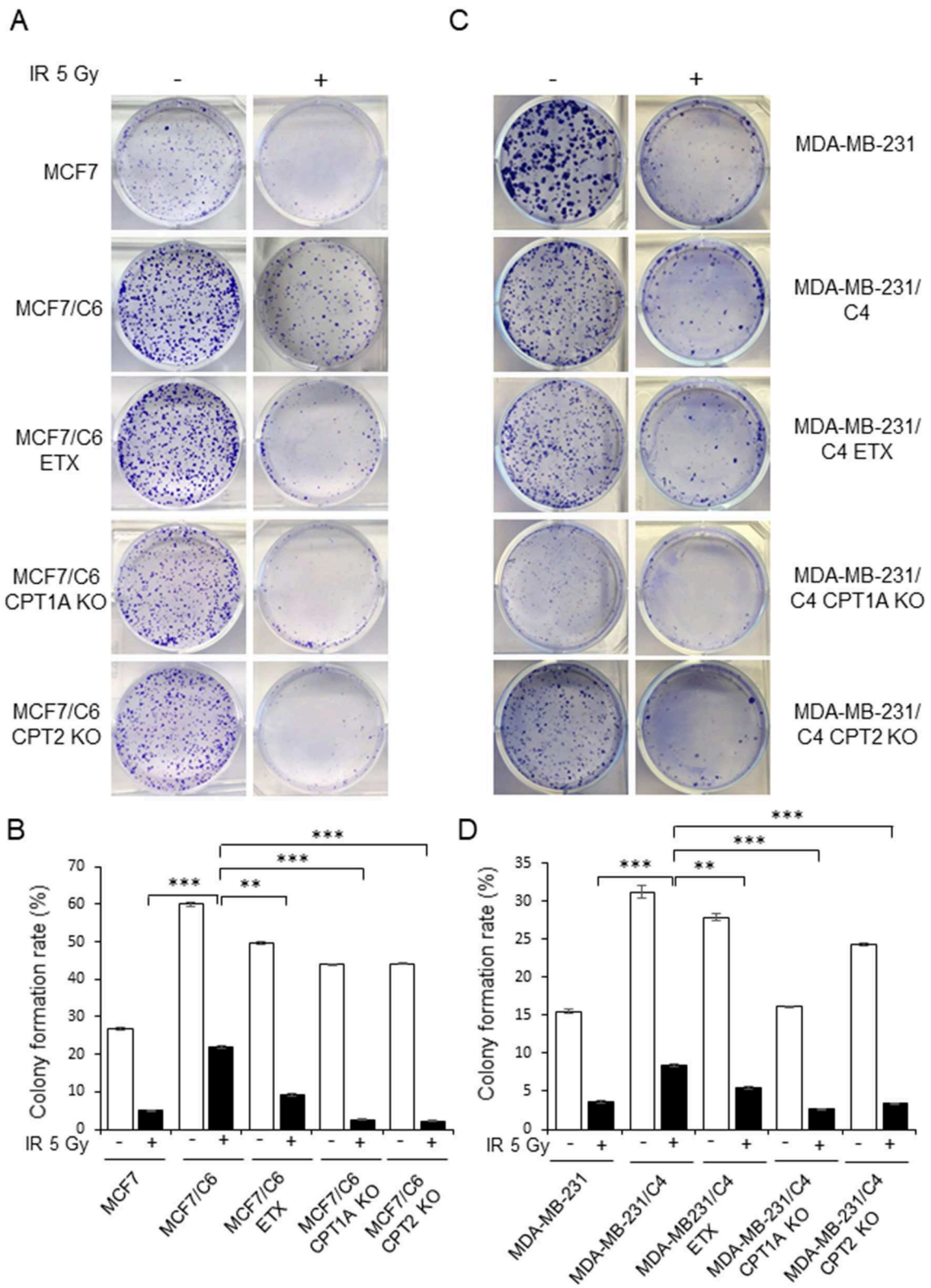

FIGURE 5 | Blocking CPT1A/CPT2 synergizes radiation in eliminating clonogenic RBCs cells. Representative images **(A)** of colonies generated with 1×10^3 MCF7, MCF7/C6, MCF7/C6 CPT1A KO, and MCF7/C6 CPT2 KO cells treated with or without 5 Gy IR, 40 μM ETX, or combined. All colonies were fixed at day 14 and quantitated **(B)**. **(C,D)** Same as **(A,B)** except that MDA231, MDA231/C4 CPT1A KO, MDA231/C4 CPT2 KO, and MDA231/C4 cells were tested. In all experiments, $n = 3$; Error bars in **(B,D)** represent mean ± SD; significance was determined by one-way ANOVA test, $^{**}p < 0.01$, $^{***}p < 0.001$.

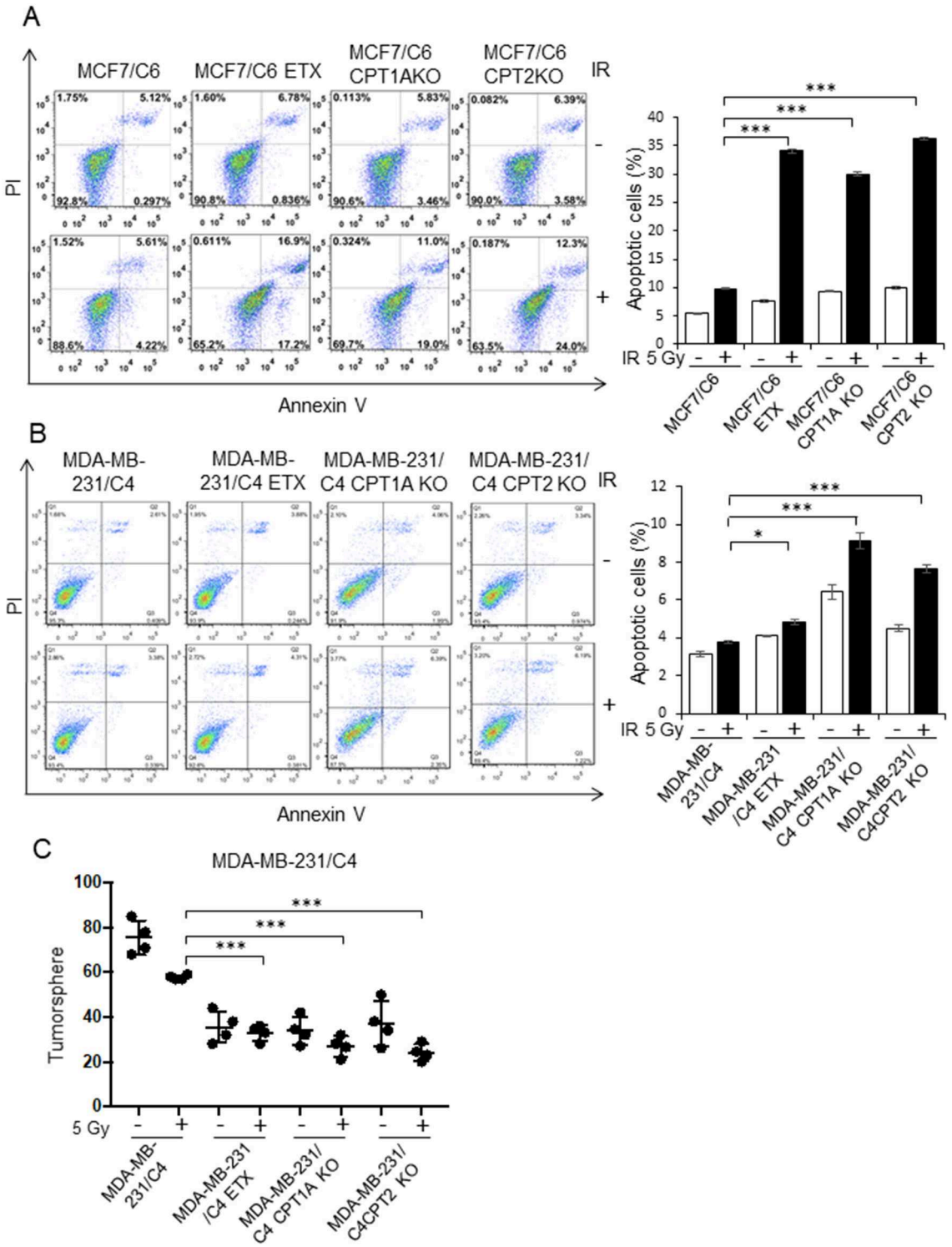

FIGURE 6 | Blocking CPT1A/CPT2 enhances apoptotic cell death and inhibits tumorsphere. **(A)** Representative flow cytometry of MCF7/C6, MCF7/C6 CPT1A KO, MCF7/C6 CPT2 KO, and MCF7/C6 cells treated with 5 Gy IR, ETX (40 μM, 48 h), or combined (left); quantitation of apoptotic cells is shown on the right. **(B)** Repeated experiments with RBC MDA-MB-231/C4 and CRISPR-KO counterparts. **(C)** Tumorsphere assay of **(B)**. In **(A–C)**, $n = 3$; mean ± SD; significance was determined by one-way ANOVA test, $^{*}p < 0.05$, $^{***}p < 0.001$.

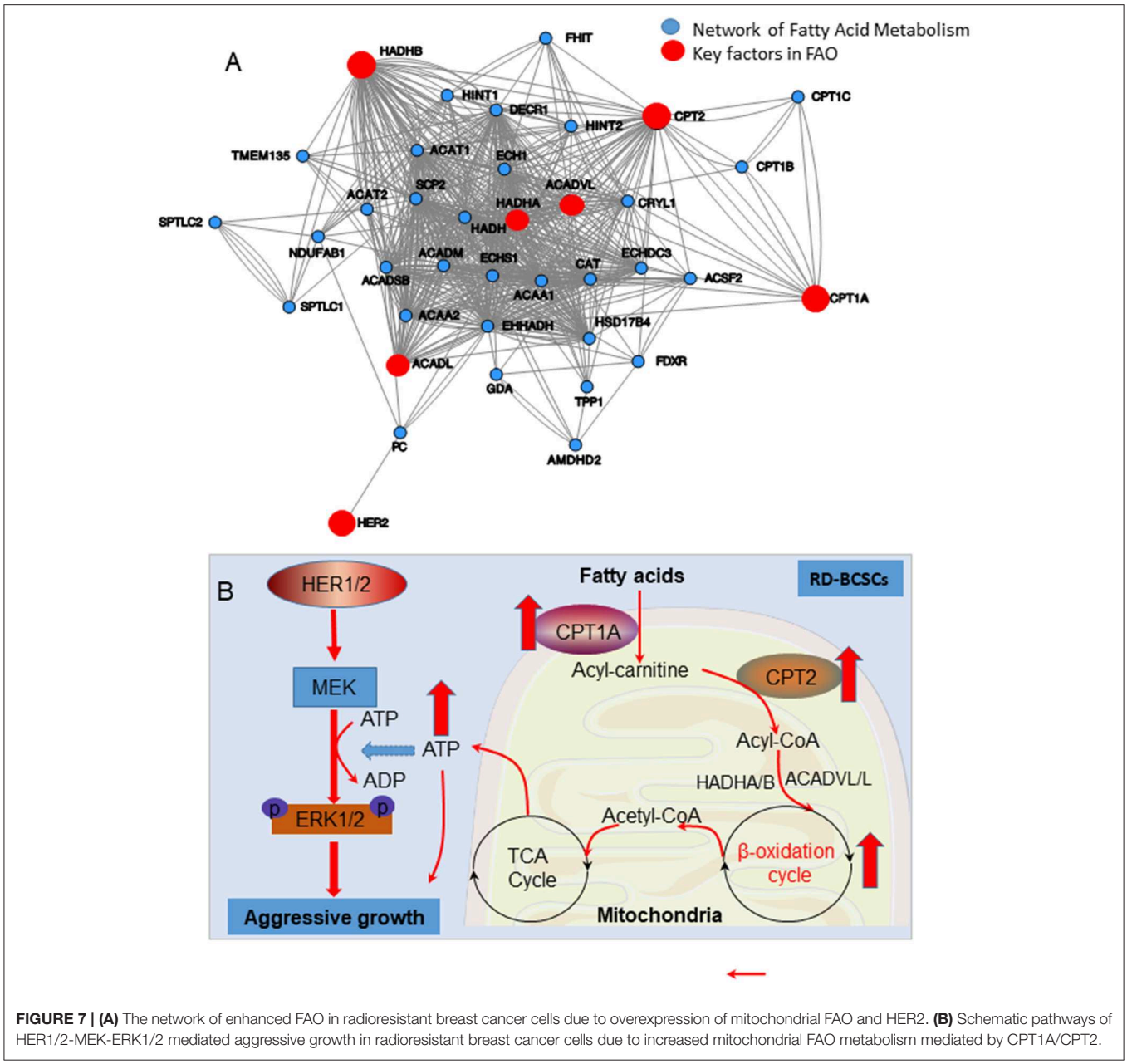

FIGURE 7 | (A) The network of enhanced FAO in radioresistant breast cancer cells due to overexpression of mitochondrial FAO and HER2. **(B)** Schematic pathways of HER1/2-MEK-ERK1/2 mediated aggressive growth in radioresistant breast cancer cells due to increased mitochondrial FAO metabolism mediated by CPT1A/CPT2.

signaling pathway to confer resistance to radiation therapy in RBCs.

Our proteomics data demonstrate a different adaptive scale of RD-BCSCs vs. wild type MCF7 cells with a higher mitochondrial protein number and density in RD-BCSCs than MCF7 cells. Such differential stress response may apply to the varied response to therapeutic radiation in primary and recurrent/metastatic breast cancers. The plasticity of human cancer cells and the genetic-independent acquisition of therapeutic resistance may be tightly associated with metabolic reprogramming. CSCs are capable of adjusting their unique metabolic plasticity in order to respond in a timely manner and adapt to hostile environments (46). The current study revealed that enhanced FAO could be a critical step for therapy-resistant cancer cells, especially cancer stem cells, to have a survival advantage, thus they could be used for identifying and developing effective metabolic targets. Altered metabolism is served as one of the hallmarks of cancer and has also been observed in CSCs (47, 48). CSCs have been identified in many types of solid tumors and often result in recurrence and both chemo- and radioresistant in tumors because of their self-renewal and tumorigenic properties (49–51). It has been shown that blocking thioredoxin- and glutathione-dependent metabolism can enhance radiation response of BCSCs (52), indicating that in addition to FAO enhancement, other metabolites could be critical for the survival advantage of CSCs. Accordingly, metabolic adjustment in the CSC populations under different therapeutic modalities should be further investigated.

The cellular energy shift may require a different signaling network to drive cancer cell proliferation and radioresistance. Our current data also reveal a potential crosstalk between CPT1A/CPT2-mediated lipid metabolism and ERK1/2-controlled cell proliferation (**Figure 7**), implicating a cooperative network of mitochondrial FAO in response to radiation in resistant cancer cells. Although currently there is no direct evidence supporting mitochondrial FAO-mediated ERK1/2 activation, the enhanced mitochondrial products including ATP from TCA cycle may increase the MEK-ERK1/2 cascade. High ATP concentration is indicated in the tumor microenvironment that can activate P2Y2 receptors to enhance BC cell migration through the activation of a MEK-ERK1/2 pathway (53). In addition, mitochondrial OXPHOS can be enhanced by Cyclin B1/CDK1 that can be relocated into mitochondria by radiation leading to phosphorylation of SIRT3, a key keeper for mitochondria homeostasis at the Thr150/Ser159 (54). Furthermore, we have recently observed that mitochondrial homeostasis is enhanced by SIRT3-regulated CTP2 activity in normal mouse liver cells via FAO (unpublished data). Thus, the CPT1A/CPT2-mediated FAO activity may be differently regulated in normal and cancer cells, which warrants further studies.

In summary, this study reveals a previously unknown feature of reprogramming mitochondrial FAO in RBCs due to enhanced CPT1A/CPT2. Thus, targeting CPT1A/CPT2 as well as other mitochondrial FAO elements may serve as a metabolic target to enhance the efficacy of breast cancer radiotherapy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JL, JJL, and SH designed the study, wrote the manuscript, and were involved in revising the manuscript. SH, RW, and MF performed experiments with the help of NJ, BX. LZ, XZ, AB, MC, JH, and AV helped to analyze the data, prepare and revise the manuscript. H-WC provided the instruction for CRISPR technology. WM and YW generated and analyzed the proteomics data. All authors have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01201/full#supplementary-material

Figure S1 | (A) Schematic representation of the process for generating RBCs and RD-BCSCs. **(B)** Functional clustering of proteomics of MCF7 and RD-BCSCs via DAVID bioinformatics indicating the protein numbers in categories inducing lipid metabolism, oxidation reduction, DNA repair, stress response, glycolysis, and ATP synthesis in MCF7 cells with or without IR. The intensities of proteins in lipid metabolism were shown in right. **(C)** Repeated proteomics analysis with RD-BCSCs cells, note a group of key FAO enzymes including CPT1A and CPT2 were enhanced by radiation (marked in red in the right).

Figure S2 | (A) FAO measured in MCF7 and RD-BCSCs cells with or without 5 Gy IR treatment for 24 h. RD-BCSCs treated with 40 μM Etomoxir (ETX) were used as FAO inhibition control, and RD-BCSCs cells treated with 1 μM FCCP as an FAO enhancement control. **(B)** Radiation-induced apoptotic cell death measured by Annexin-V/PI flow cytometry in RD-BCSCs cells, RD-BCSCs treated with 5 Gy IR, ETX (200 μM, 48 h) or combined. **(C)** Repeated experiment in **(B)** using RD-BCSCs CPT1A KO and RD-BCSCs CPT2 KO cells.

Figure S3 | (A) Mitochondrial fractions were prepared from MCF7 and RD-BCSCs cells treated with or without 5 Gy IR treatment and analyzed by LC-MS and MS/MS on a Q Exactive Plus mass spectrometer. Numbers of proteins detected comparing MCF7 and RD-BCSCs before and after IR are shown on the left. Percentage of enhanced quantitation of the increased protein numbers by radiation are shown in the pie on the right. **(B)** The intensities of mitochondrial protein expression of MCF7 and RD-BCSCs treated with –/+ IR are shown.

Figure S4 | (A) Functional clustering of mitochondrial proteins via DAVID bioinformatics show the relatively high enhancement of protein numbers in lipid metabolism, oxidation reduction and ATP synthesis in the mitochondria of irradiated RD-BCSCs. **(B)** The two key enzymes in mitochondrial FAO metabolism, CPT1A, and CPT2 (in red), were enhanced by IR in RD-BCSCs.

Table S1 | The cluster of proteins involved in fatty acid metabolism enhanced by radiation in RD-BCSCs. The mitochondrial proteomics data were generated with mitochondrial proteins isolated from RD-BCSCs treated with or without IR, followed by digestion with trypsin and analyses by LC-MS and MS/MS on a Q Exactive Plus mass spectrometer. The 17 listed proteins were detected in the proteomic analysis of mitochondrial proteins of RD-BCSCs and classified by UniProtein under the category Lipid Metabolism. The gene symbols, descriptions, and comparison with or without 5 Gy IR are shown in the table, and the CPT1A/CPT2 are marked with yellow.

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