

Review Article

Cancer stem cells (CSCs): metabolic strategies for their identification and eradication

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Phenotypic and functional heterogeneity is one of the most relevant features of cancer cells within different tumor types and is responsible for treatment failure. Cancer stem cells (CSCs) are a population of cells with stem cell-like properties that are considered to be the root cause of tumor heterogeneity, because of their ability to generate the full repertoire of cancer cell types. Moreover, CSCs have been invoked as the main drivers of metastatic dissemination and therapeutic resistance. As such, targeting CSCs may be a useful strategy to improve the effectiveness of classical anticancer therapies. Recently, metabolism has been considered as a relevant player in CSC biology, and indeed, oncogenic alterations trigger the metabolite-driven dissemination of CSCs. More interestingly, the action of metabolic pathways in CSC maintenance might not be merely a consequence of genomic alterations. Indeed, certain metabolotypic phenotypes may play a causative role in maintaining the stem traits, acting as an orchestrator of stemness. Here, we review the current studies on the metabolic features of CSCs, focusing on the biochemical energy pathways involved in CSC maintenance and propagation. We provide a detailed overview of the plastic metabolic behavior of CSCs in response to microenvironment changes, genetic aberrations, and pharmacological stressors. In addition, we describe the potential of comprehensive metabolic approaches to identify and selectively eradicate CSCs, together with the possibility to ‘force’ CSCs within certain metabolic dependences, in order to effectively target such metabolic biochemical inflexibilities. Finally, we focus on targeting mitochondria to halt CSC dissemination and effectively eradicate cancer.

Tumor heterogeneity and cell plasticity: a metabolic matter

Phenotypic and functional heterogeneity in cancer cells within many tumor types has highlighted the need to further dissect inter- and intratumor complexity, to more effectively eradicate neoplastic disease. Indeed, differences in size, morphology, antigen expression, membrane composition, and biochemical behavior may account for a large range of variability in crucial biological responses like cell proliferation, metastatic dissemination, and sensitivity to chemotherapy [1]. Rather than acting as randomly disorganized compartments, tumor cells employ a hierarchical structure that allows them to integrate and simplify the complexity of isolated subpopulations, in order to guarantee cancer cell survival and the malignant evolution of the disease [1]. Looking at the root of such complex organization, two main models have been postulated: the ‘clonal evolution model’ and the ‘cancer stem cell hypothesis’ (Figure 1). According to the first model, proposed by Peter Nowell in 1976, cancer may be regarded as an evolutionary process, initiated by multiple stepwise mutations occurring in somatic cells, and accompanied by natural selection of those clones with the most favorable phenotype [1,2].

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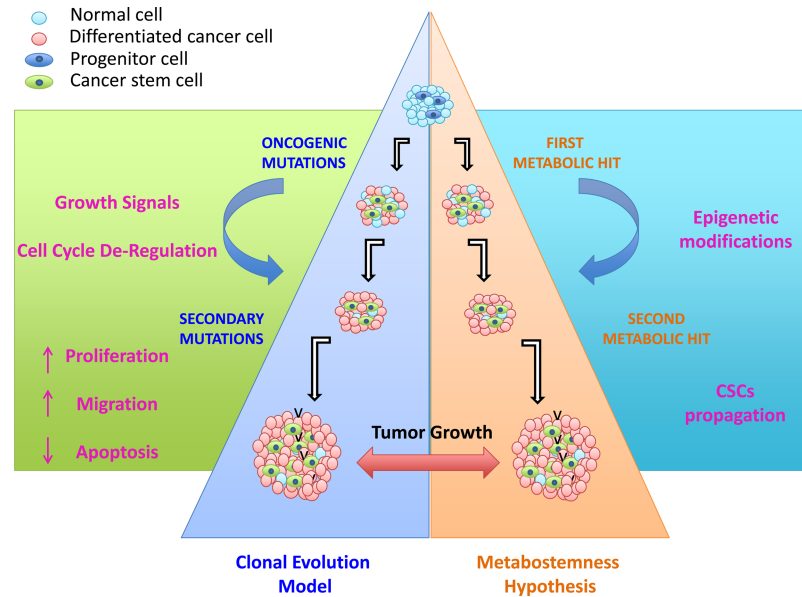


Figure 1. Metabolism drives stemness features and tumor heterogeneity.

Advanced genome sequencing techniques have clarified that cancer is heterogeneous within a single patient, with multiple subclones arising from primitive mutations. To explain this genetic and functional heterogeneity, two models have been proposed: the clonal evolution model and the cancer stem cell hypothesis. According to the first model, cancer is the result of oncogenic mutations that promote cell dedifferentiation and phenotypic regression with loss of function, uncontrolled proliferation, and inability to activate cell death pathways. The CSC hypothesis recognizes a rare subpopulation of self-renewing and tumorigenic cells as responsible for the generation of all the cells within the tumor bulk and their hierarchical organization. Mutations in the progenitor cells may account for the generation of CSCs. However, the origin of CSCs is still a topic under debate. Interestingly, a combined analysis of biological, biochemical, pharmacological, and genetic studies has recently revealed that CSCs may rise from metabolic events occurring in non-CSCs. Certain early and late metabolic hits are thought to affect chromatin organization and activate epigenetic program involved in the metabolic-driven reprogramming of CSCs. According to this new paradigm, the identification of key metabolic processes involved in CSC reprogramming might be useful to identify and target CSCs.

The accumulation of additional mutations and the parallel evolutionary pressure exerted by dominant clones support the idea of a clonal architecture model whose dynamic alterations favor the progression of tumor toward aggressive phenotype. Adding to this Darwinian model, a better understanding of the cross-communications between cancer cells and the surrounding ecosystem habitat has paved the way for further considering these reciprocal interactions as remarkable therapeutic targets to halt cancer cell proliferation, invasion, tumor angiogenesis, pharmacological resistance, and disease recurrence [2,3]. Based on this idea, significant progress has now been made in understanding cancer biology, on dissecting multilevel cell dynamics and disclosing adaptive microenvironmental responses, which can be translated into clinical practice with the result of achieving the better overall management of cancer patients. However, effective control of malignant disease still represents an unmet clinical need that highlights the limits of the clonal evolution model, suggesting that other approaches are still needed to understand the complexity of cancer.

According to the cancer stem cell (CSC) hypothesis, the complete eradication of malignancy can only be achieved by targeting the small cell population driving the origin of cancer, which is also responsible for progression and its resurgence after therapy [4–6]. CSCs or tumor-initiating cells (TICs) are a rare population of cells with stem cell-like properties that exhibit self-renewal, tumorigenicity, and multilineage differentiation capacity [4–6]. CSCs are thought to trigger tumor initiation and growth by generating the full repertoire of cancer cell types; in addition, these cells have been invoked as the main drivers of metastatic dissemination and therapeutic resistance [4–6]. On the basis of these observations, integrating the clonal evolution model with the CSC hypothesis may represent a promising strategy to fully eradicate cancer [7].

Evolving from the above concepts, the dynamic changes occurring in the bioenergetics machinery of cancer cells strongly contribute to tumor heterogeneity and add a further level of complexity to the overall scenario [8,9] (Figure 1). Indeed, the ability of a tumor mass to cope with the increased bioenergetic demand mainly relies on the highly plastic features of both cancer cells and the surrounding tumor stroma, which can adjust its metabolism in order to guarantee the elevated proliferation rate required for cancer outgrowth and expansion [10].

There is now a general consensus on the variable metabolic behavior of cancer cells, which allows them to adapt to transient bioenergetics crises caused by hypoxia or a lack of nutrients. Supporting these observations, metabolic heterogeneity has been detected not only between different tumor types, but also within the same tumor, thus confirming that local factors like oxygenation, pH, glucose, and metabolites levels can all contribute to create specialized metabolic compartments [10,11]. Bioenergetic dysregulation, metabolic flexibility, and symbiotic relationships between cancer and stromal cells define oncometabolism as one of the emerging hallmarks of cancer, to be regarded not an insuperable barrier, but as a challenging opportunity to selectively target aberrant features of transformed cells, on our way to defeating cancer [12].

As cancer cells may obtain energy from different sources, including glucose, lactate, pyruvate, hydroxybutyrate, acetate, glutamine, and fatty acids, it is not surprising that the utilization of such a large range of fuels may occur at the crossroads of different cellular sub-compartments and may involve different cell types [10]. Moreover, in tumors different metabolic activities may coexist within the same cell type, thus contributing to metabolic heterogeneity [10]. A nice example of intratumor metabolic heterogeneity was recently provided by Hensley et al. who demonstrated that non-small cell lung cancers (NSCLCs) get energy from different substrates depending on their proximity to blood vessels. In particular, the authors showed that poorly perfused areas mainly get energy from glucose, while well-vascularized areas utilize other fuels such as fatty acids, amino acids, ketones, and lactate to obtain ATP from oxidative phosphorylation [9]. Furthermore, the peculiar oncometabolic features of NSCLCs may also rely on aberrant genetic background, corroborating that diverse oncogenes can deeply affect tumor metabolism. For instance, Kerr et al. demonstrated that the acquisition of an additional mutant KRAS^{G12D} allele is associated with a glycolytic switch and a more aggressive NSCLC phenotype in mice. The authors also highlight that the relative mutant allelic content may generate unique metabolic features to be exploited in a therapeutic setting. These observations suggest that both tissue oxygenation and genetic background favor more plasticity and heterogeneity [13].

A further player in the metabolic plasticity of tumor cells is the surrounding catabolic microenvironment. The remarkable contribution of the stromal compartment to the anabolic requirements of malignant cells has been extensively demonstrated and recapitulated in the Reverse Warburg effect [14–16]. According to this model, cancer cells get energy to sustain their highly proliferation rate also from metabolic intermediates released by surrounding catabolic cells. In particular, lactate, free fatty acids, and ketones generated from the activation of glycolytic and autophagic programs in the stromal cells have been shown to fuel mitochondrial metabolism and ATP production in anabolic cancer cells [14–17]. Parallel autophagic responses activated in distal and poorly oxygenated tumor areas provide catabolic intermediate to sustain anabolic demands and support cancer outgrowth [17]. Overall, this scenario indicates that in cancer cells, as well as in tumor microenvironment, plastic bioenergetics dynamics fulfill different biochemical demands in synergistically operating tissue compartments. Likewise, such metabolic heterogeneity and plasticity have been emerging as one of the key feature allowing cancer cells to proliferate and hence, targeting cell metabolism may represent a useful tool to halt cancer progression [12].

In an effort to gain therapeutic advantage from the metabolic complexity at the basis of cancer, one of the possible strategies would be identifying the biochemical energetic reactions that occur within the CSCs, which, as mentioned above, represent the basic cell units giving rise to the developmental hierarchical pyramid. In particular, metabolism has currently been shown to deeply influence the maintenance and dissemination of CSCs, although the metabolic features of CSCs and their impact on CSC properties have not been comprehensively explored yet [18,19]. If, on the one hand, glucose seems to be an essential nutrient for CSCs [20], on the other hand it has been recently proposed that CSCs may use mitochondrial oxidative metabolism as favorite source of energy [21,22]. Being a relatively unexplored and emergent field in the study of CSCs, metabolism has been directly investigated in few studies, which possibly explains the existence of some of the discrepant outcomes in these reports. Several authors proposed that CSCs possess unique metabolic features when compared with the differentiated bulk of tumor cells, as well as with normal stem cells [11]. Hence, metabolism might be regarded as a useful tool and an innovative strategy to identify CSCs, together with the classic CSC markers. In addition,

hitting the biochemical reactions that allow CSC maintenance and dissemination might represent an effective approach to ablate the cells at the origin of cancer [23]. The possibility that the metabolic features of CSCs might be exploited in a therapeutic setting paves the way for further investigating the biochemical status of these peculiar tumor components, unveiling unexpected opportunities of selective clinical intervention.

CSCs at the origin of cancer: what, when, where, and why?

What are CSCs?

CSCs are a rare population of tumor cells with stem cell properties, which are thought to generate the tumor bulk [6]. They are considered the main drivers of malignant growth, treatment resistance, minimal residual disease, and metastases [6,24–28]. CSCs, also known as TICs, exhibit self-renewal properties, tumorigenicity, and multilineage differentiation capacity [24–28]. A lot of progress in understanding CSC biology has been made from the nineteenth century, when, for the first time, Virchow and Conheim hypothesized that cancer arises from the activation, in adult tissues, of dormant cells that recalls embryonic cells but are present in adult tissues. Later evidence, dating back at ~30 years ago, corroborated the hypothesis of the existence of specific types of cancer cells responsible for tumor progression and pharmacological resistance [29–34]. Indeed, in 1997, Bonnet and Dick [34] identified a subpopulation of CD34⁺/CD38⁻ cells from acute myeloid leukemia (AML) patients that exhibited the ability to form hematopoietic malignancy in NOD/SCID mice, together with the ability of self-renew, differentiate, and proliferate.

Lying at the apex of a figurative hierarchical pyramid, undifferentiated CSCs typically represent the minority of the tumor cell population, but undoubtedly one of the most important for their unique capability to generate all the differentiated progeny of tumor cells [31–33]. CSCs have been detected in both solid and non-solid tumors, including leukemia, lung, brain, head and neck, breast, and colon and pancreatic cancers [35–41]. CSCs are resistant to chemotherapies or radiations, indeed, while conventional anticancer drugs might successfully destroy differentiated cancer cells, they will not be effective against CSCs. Likewise, although a transitory control of neoplastic disease may be achieved with current therapies, a large number of patients may not respond to the treatment, experiencing pharmacological resistance from the onset of the disease and/or following therapies. Furthermore, tumor relapse after an apparent disease-free period might occur, together with the metastatic dissemination to secondary tumor sites. As all these biological behaviors have been attributed to the action of CSCs, which resist to conventional therapies, it appears evident that identifying and eradicating CSCs represent a challenging but promising target to fully ablate cancer [42].

But how recognize CSCs from the heterogeneous tumor tissue as well as from noncancer stem cells? The identification of CSCs is classically based on the evaluation of cell surface antigens like CD34, CD44, and CD133 by flow cytometry. Indeed, leukemia stem cells show a CD34⁺/CD38⁻ surface marker phenotype [24], while an ESA⁺CD44⁺CD24⁻/low lineage has been identified to characterize breast cancer stem cells [35]. An enzyme-based assay for the detection of the aldehyde dehydrogenase activity (ALDH) is also frequently used to identify CSCs [43,44].

Furthermore, a transition between two phenotypic status in breast CSCs has been revealed, indicating that in response to different environmental conditions, CSCs may switch from a more proliferative epithelial-like state characterized by increased ALDH activity and a mesenchymal-like state characterized by expression of CD44⁺/CD24⁻ and a more quiescent and invasive behavior [45]. Hence, the expression of surface and cytoplasmic markers is not a static property of CSCs and may deeply vary as a consequence of different environmental milieu. In addition, not all CSCs express the markers, which can also be expressed in cancer cells not classifiable as CSCs. The surface antigen expression is not only selective and may largely vary during tumor progression and after long-term culture, but also for the high degree of heterogeneity existing between patients and also within the same tumor [46]. Because of the unspecificity and fluctuating trend in the expression of current markers, call for more research is required to selectively identify CSCs.

When?

The metabolic transition from quiescent to proliferative CSCs

The most accredited hypothesis for the origin of CSCs sets its rise in mutational events occurring in normal stem/progenitor cells [4,6,7]. Such genetic deregulations appear to be involved in the transformation of normal quiescent stem cells, with a strict regulated metabolism, to a constitutively activated phenotype characterized by elevated metabolic activity and plasticity. Classically, CSCs have been considered quiescent, with a slow

capability to enter the cell cycle [47,48]. One of the evidence supporting this idea might dwell in the evidence that conventional anticancer therapies, which preferentially target fast cell cycle entering cells, are ineffective in killing CSCs. Indeed, such therapeutic strategies eradicate only the tumor bulk, which can be regenerated by the activation of CSCs, whose re-enter in the cell cycle after a period of latency might be responsible for disease relapse and metastasis. Dormant CSCs share many biological features and stemness-associated factors with their normal counterpart, which has been shown to maintain a low metabolic activity that favors cell dormancy [47,48]. For instance, dormant hematopoietic stem cells (HSCs) preferentially utilize the glycolytic pathway to get energy from the hypoxic niches [49,50], producing very low levels of reactive oxygen species (ROS), which reflects the low metabolic activity [50–52]. Despite this quiescent phenotype characterized by limited energy metabolism, a single HSC might be awoken from the dormant state in response to damage, in order to reconstitute the entire bulk of blood cells. In this context, it should be noted that an active proliferation does not necessarily inhibits stem cell properties, as demonstrated by the evidence that HSCs are characterized by an elevated proliferation rate [53]. The transition from a quiescent to a proliferative state defines the intrinsic HSCs potential to rapidly divide and regenerate the hematopoietic tissue. Such phenotypic transition is suggestive of the highly plastic metabolic profile of HSCs, which allow them to respond to environmental stress by activating a biochemical program that supports cell proliferation. Extending these findings, Chen et al. [54] have demonstrated that the repression of mitochondrial biogenesis and activity is required for maintaining HSCs in a quiescent state. In particular, a disruption in quiescence and long-term functions of the HSCs was generated by targeted mutation in *TSC1* (tuberous sclerosis complex 1), a negative regulator of the mTOR complex. In such conditions, the authors observed a massive increase in HSC proliferation, RNA synthesis, mitochondrial biogenesis, ROS production and up-regulation of genes involved in oxidative function in the HSCs [54]. These data suggest that in HSCs, the plastic transition from a dormant to a proliferative state relies on the metabolic shift from a glycolytic to an oxidative phenotype. Supporting these findings, low mitochondrial respiration and poor ROS production seem to be crucial events for maintaining stem cell quiescence [55]. Although HSCs may represent a useful model to study the resembling metabolic state of CSCs, recent investigations have highlighted that CSCs may display a broader repertoire of biochemical behavior in response to different environmental conditions; indeed, it is more difficult to univocally identify the changes in energy supply that support CSC transition from a quiescent to a proliferative state. Overturning the existing paradigm on CSC metabolic properties, which were mainly suggested to rely on glucose rather than mitochondrial oxidative metabolism [56–60], accumulating evidence has suggested that together with an increased glycolytic pathway activation, CSCs also utilize OXPHOS, fatty acid oxidation, and glutaminolysis [18,19]. In this regard, it has been recently demonstrated that in lung and ovarian cancer models, CSCs with high telomerase activity (hTERT-high) show the most energetically activated phenotype, characterized not only by enhanced glycolysis but also by increased OXPHOS [61]. In particular, by performing cell fractionation, the authors demonstrated that in hTERT-high cells, the increase in mitochondrial function is paralleled by enhanced mitochondrial mass, together with increased capacity for stem cell activity, cell proliferation, and migration [61]. Likewise, all these enhanced biological responses were effectively inhibited by classical modulators of energy metabolism like glycolysis and OXPHOS inhibitors, and interestingly by drugs that interfere with mitochondrial biogenesis [61]. Of note, the authors identified two different cellular subpopulations of hTERT-high cells, one proliferative and the other non-proliferative, suggesting that non-proliferative hTERT-high cells might be involved in tumor dormancy [61].

As the biochemical and energy machinery deeply influence the properties of CSCs, as well as their transition from a quiescent to a proliferative state, it is very likely that targeting the metabolic events at the basis of this phenotypic transition may represent a potential strategy to halt metastatic dissemination, tumor recurrence, and refractoriness to treatment.

Translating these findings in a therapeutic scenario, two approaches have been proposed to halt tumor relapse by inhibiting CSCs quiescence: (i) induce CSCs to enter the cell cycle and then target them with conventional anticancer therapies and (ii) keep cells in a dormant state. These two approaches may be triggered using metabolic strategies; however, the actual therapeutic benefit of waken-up or hibernation strategies is still controversial and potentially limited to specific patients. Indeed, hibernation strategies might be more appropriate for elderly patients, as the treatment might be administered for the rest of the patient's life, with the risk to select resistant clones. On the other hand, wake-up strategies might be more beneficial to younger patients, as these therapies aim to eradicate CSCs, hence avoiding such long-term risk [48]. Although further studies are necessary to validate the use of wake-up or hibernation therapies, it appears clear that targeting CSC

metabolism and the biochemical-driven transition between proliferation and quiescence might be a useful approach to hamper tumor relapse.

Where?

Metabolism in the CSC niche

In an effort to investigate the mechanisms that favor CSC maintenance and propagation, great research interest has been addressed toward the identification of the peculiar microenvironment which serves as niche for CSCs. Similar to normal stem cells, CSCs reside in a peculiar niche where multilevel interactions control biochemical reactions and biological responses essential for maintaining CSC population [62]. Tumor-specific factors contribute to create a cancer stem cell niche, characterized by an intricate network of biochemical and paracrine cross-communications involving activated fibroblasts, endothelial cells, macrophages, immune cells, and adipocytes. Furthermore, environmental local factors like hypoxia, as well as growth factors, cytokines, and extracellular matrix have been shown to strongly contribute to the activation of self-renewal pathways, such as the Wnt/ β -catenin, Notch, and Hedgehog pathways. The cross-communications between CSCs and the surrounding niche have also been shown to affect the plasticity of cancer cells, which might reversibly acquire a stem-like state, mainly by activating dedifferentiation programs like the epithelial–mesenchymal transition (EMT) [63]. For their relevant role in regulating the paracrine and biochemical interactions with cancer cells, and for the ability to support the perivascular niche that provides energy fuels and oxygen, we will detail the biochemical features of stromal and endothelial cells, which both contribute to maintain a proper niche for CSCs. Furthermore, we will recapitulate the most emerging findings on the metabolic responses to low oxygen availability in the CSC niche.

Cancer-associated fibroblasts

Within the CSC niche, cancer-associated fibroblasts (CAFs), which are regarded as key components of the tumor microenvironment, have been shown to undergo a metabolic reprogramming, with a more stricter reliance on aerobic glycolysis than oxidative phosphorylation [64]. To sustain their enhanced proliferative and migratory capability, as well as their active secretion of cytokines and growth factors, CAFs gain energy from the activation of autophagic programs [65–67]. Indeed, autophagy-derived substrates from catabolic CAFs have been shown to support the energy needs of pancreatic ductal adenocarcinoma, taking over on glucose and TCA-cycle metabolite requirements [68]. Furthermore, CAFs actively use glutamine to fuel the CSC niche and support tumor development and progression. Likewise, the simultaneous depletion of glutamine pathways in both tumor and stromal compartment has been shown to ablate tumorigenicity in a mouse model of ovarian cancer [69]. The strict dependence from glutamine utilization has been indicated by the evidence that CAFs recruit additional carbon sources for glutamine synthesis when this fuel is scarcely abundant [69]. Indeed, in ovarian cancer, CAFs have been shown to use both branched-chain amino acids and aspartate to provide the nitrogen supply for glutamine synthesis [69]. Importantly, CAFs may adjust their metabolic strategies in order to support inflammation-driven tumorigenesis. In this regard, Valencia et al. [70] demonstrated using both *in vitro* and *in vivo* approaches that metabolic reprogramming triggered by p62 deficiency in the tumor stroma triggers prostate tumorigenesis driven by IL-6. In particular, loss of p62 in the stromal compartment was associated with decreased glucose uptake, GLUT1 expression, lactate secretion, and decreased flux through the oxidative PPP. Furthermore, p62 KO cells displayed lower glutamine metabolism, as evidenced the reduction of the glutamine transporters SLC7A5 and SLC1A5, as well as glutaminase-1. Likewise, such perturbations in glutamine metabolism also led to reduced GSH levels and to the subsequent accumulation of ROS, which in turn mediated by IL-6 production [70]. On the other hand, p62 was found to induce mTORC activation and c-Myc induction leading to survival and expansion of ROS-containing HCC (hepatocellular carcinoma)-initiating cells [71]. These *in vitro* findings were corroborated by the evidence that p62 is up-regulated during preneoplasia and required for HCC induction in mice [71]. Taken together, these data suggest that the same mediator might drive different biological outcomes by regulating compartment-specific biochemical events involving the oxidative stress response in the tumor microenvironment [70,71]. In this scenario, CAFs have been shown to induce a pro-oxidant environment from which cancer cells efficiently escape by activating survival pathways and mechanisms of anoikis resistance [72,73]. Adding to this, cancer cells themselves worsen the oxidant status because of their increased metabolic activity caused by aberrant growth factors and cytokines signaling and excessive functionality of ROS-producing enzymes, such as nitric oxide synthases, cyclooxygenases, and

lipoxygenases [74]. To effectively cope with oxidative stress, CSCs have developed an extremely efficient antioxidant system, mainly relying on the redox buffer glutathione, whose maintenance is dependent on glucose metabolism through the PPP cycle [75]. Indeed, targeting the aberrant antioxidant response may inhibit clonogenicity and radioresistance, as demonstrated by using pharmacological depletion of ROS scavengers in CSCs [76].

Endothelial cells

Relatively few investigations have attempted to clarify the metabolic features of CSCs residing in specialized perivascular niche, and their cross-talk with endothelial cells (ECs) for survival and cell renewal. In head and neck squamous cell carcinomas, IL-6 secreted by tumor-associated endothelial cells activates STAT3 transduction pathway and promotes tumorigenicity [77], as evidenced by transplanting primary human head and neck cancer stem-like cells into IL-6 knockout mice. In addition, tumor formation is inhibited when ALDH^{high}/CD44^{high} cells are co-injected with endothelial cells stably transduced with shRNA IL-6 or using tocilizumab, which targets IL-6 receptor [77], thus suggesting that IL-6 might play a pivotal role in the cross-talk between ECs and CSCs within the niche. The ability of IL-6 signaling to activate aerobic glycolysis might, at least in part, explain the molecular mechanisms involved in maintaining the substantial glycolytic and quiescent phenotype of ECs [78,79]. Indeed, the pharmacological blockade of glucose utilization by 2-deoxy-D-glucose (2-DG) is toxic to ECs [80,81]. Interestingly, CSCs expressing vessel markers display the ability to form tumor-associated blood vessels [82]. Further investigating the interdependence of endothelial cells and CSCs, co-culture strategies have disclosed the essential role of endothelial cells in providing factors involved in CSC renewal and survival [83]. Moreover, the antiangiogenic drug bevacizumab has been shown to reduce a subpopulation of brain cancer cells with stem-like features [83]. Of note, glioblastoma stem-like cells (GSCs) were able to activate an angiogenic response characterized by the secretion of the angiogenic mediator vascular endothelial growth factor (VEGF) and the induction of endothelial tube formation. The ability of CSCs to support tumor-associated angiogenesis was further strengthened by the observation that GSCs are able to differentiate to both endothelial and tumor cells via a CD133⁺/CD144⁺ progenitor [84,85].

Considering that ECs are not only crucially involved in the formation of new blood vessels, but also in providing a specific niche essential for CSC biology, it is not surprising that ECs energy requirements may be fulfilled using different sources [86]. As mentioned above, glucose represents the favorite metabolic source to ECs, probably for its ability to produce ATP faster than OXPHOS. Not surprisingly, angiogenic stimuli like VEGF have been shown to support the angiogenic switch and vessel sprouting mainly by stimulating glycolytic enzymes like phosphofruktokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3), which regulates one of the rate-limiting steps of glycolysis, the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6P2) by 6-phosphofruktokinase-1 (PFK-1) [78]. Corroborating these findings, *in vitro* and *in vivo* studies have shown that the efficiency of angiogenesis is reduced when the glycolytic pathway is inhibited by PFKFB3 knockdown in ECs [78].

The pentose phosphate pathway (PPP), important for nucleotide synthesis and redox homeostasis, has been shown to support energy metabolism in ECs. For instance, the PPP has been shown to provide NADPH, which serves as a cofactor for endothelial NO synthase, a pivotal actor in maintaining CSC phenotype in perivascular niche through the activation of Notch signaling [85].

In addition, glucose-6-phosphate dehydrogenase (G6PD) gene, which has been shown to mediate the VEGF-dependent activation of ECs [87], has also been involved in the transformation of NIH 3T3 cells and tumor induction in nude mice [88].

Although ECs scarcely utilize the OXPHOS pathway to get ATP [86], their mitochondria have been implicated in triggering signaling pathways relevant to the maintenance of the perivascular niche. In this regard, many studies have demonstrated that in ECs, VEGF increases mitochondria biogenesis and stimulates mitochondrial metabolism [89,90], which are recently emerging as relevant biochemical responses implicated in CSC propagation [18]. As VEGF signaling deeply affects the vascular niche in controlling stemness features in diverse types of cancer [91,92], it would not be surprising that at least part of these effects could be mediated by metabolic-driven and mitochondria-dependent cell responses.

Hypoxia in the CSC niche

Mounting evidence indicates that hypoxia represents one of the most important features of the CSC niche [93]. In solid malignancies, the rapidly growing mass often outpaces the local blood supply, thus generating hypoxic

microenvironments. In addition, the dysfunctional biology of tumor vasculature delivers an aberrant blood flux, which contributes to lower the oxygen availability in the tumor mass. It should be mentioned that although oxygen has a higher diffusion rate compared with glucose, the solubility of oxygen is lower than that of glucose, which explains why glucose-based metabolism takes over in cancer contexts.

Hence, to overcome the lack of oxygen, the metabolic needs of CSCs are mainly fulfilled by glucose, which acts as an alternative energy substrate in the hypoxic niche [93]. Such adaptive responses undertaken by cells in low oxygen conditions are mainly mediated by the transcription factor HIF, which mediates the expression of a large number of genes involved in relevant biological functions, including the regulation of cell metabolism [94]. HIF-1 is a highly conserved heterodimeric factor, consisting of an oxygen-regulated HIF-1 α subunit and a constitutively expressed HIF-1 β subunit; the transcriptional complex HIF-1 binds to the consensus sequence 5'-RCGTG-3', located in the promoter of HIF-1-regulated genes, thus activating changes in the cell transcriptional machinery [94]. Many glycolytic enzymes are included among the HIF-1 target genes, in normal and CSC cells, thus corroborating that hypoxic conditions favors a glycolytic phenotype [94]. In particular, in hypoxic mouse embryonic stem cells, HIF-1 α was shown to mediate the transcription of glucose transporters and all the glycolytic enzymes [95]. HIF-1 α action in tumors has been associated with stem cell features, as evidenced in breast, hematological, prostate, bladder, and central nervous system malignancies [96–99]. Furthermore, HIF-1 α has emerged as a relevant activator of the EMT program, which drives the acquisition of CSC markers, together with a higher migratory and therapy-resistant phenotype [100]. Likewise, chemoresistant pancreatic cancer cells showed CSC and EMT phenotypes, together with a strict reliance on glycolysis and low ROS levels, as demonstrated using the glycolysis inhibitor 2-DG [101]. Extending these findings, the chemoresistant behavior of lung cancer has been associated with increased HIF-1-mediated expression of the glucose transporter GLUT1 and the transmembrane protein CAIX, which neutralizes intracellular acidosis [102], thus suggesting that targeting the glycolytic phenotype and normalizing the intracellular pH levels might be an effective strategy in counteracting the resistance to chemotherapeutic regimen.

In this context, carbonic anhydrases have been shown to play a pivotal role in the expansion of the cancer stem-like population, as targeting CAIX activity effectively inhibits the expansion of breast CSCs during hypoxia [103]. Interestingly, CAIX up-regulation activates the EMT process and drives the acquisition of stemness features through the Notch1 and Jagged1 pathway [104]. Furthermore, a CAIX signature characterizes the differential response of breast cancer cell to hypoxia, identifying a subpopulation of cells with CSC markers and elevated self-renewal capacity, thus suggesting that CAIX expression may be used to enrich for CSCs in the hypoxic niche [103].

Within the CSC niche, catabolic reactions are mainly characterized by glycolysis and ketogenesis, which supports the anabolic requirements of CSCs and favors the acquisition of stemness properties [104–109]. In addition, non-glycolytic stem-like cells may use fuels coming from more differentiated glycolytic cells in breast cancer, indicating that a reverse Warburg metabolism may support CSC energetic requirements [17].

Additional evidence indicates that alternate catabolites including ketones and lactate might serve as metabolic fuels and a driver of stemness, recurrence, metastasis, and poor clinical outcome in breast cancer [110,111].

Why?

Oncogenic mediator pathways involved in the metabolic reprogramming of CSCs

Many hypotheses have been proposed to clarify why metabolic reprogramming occurs in cancer cells toward dedifferentiation and acquisition of stem traits. In this regard, specific genetic background, epigenetic modifications, and developmental and oncogenic pathways seem to be mechanistically involved in promoting metabolic phenotypes involved in CSC maintenance and dissemination. Therefore, the characterization of such genetic aberrations and their link with metabolic alterations would provide a causative connection explaining the involvement of the energy machinery in CSC biology.

For instance, it has been recently demonstrated that elevated telomerase expression, which is generally observed in normal and cancer stem cells, is a signature of stemness associated with elevated glucose and mitochondrial-dependent metabolic activity, in both ovarian and lung cancer stem cells [61].

Moreover, mutations in mitochondrial DNA (mtDNA) have been associated with the onset of diverse types of cancer, including colorectal, prostate, bladder, renal cancer, and hematological diseases [112], suggesting that maintaining an intact mitochondrial function is essential for tumor development. In line with these observations, new haplotypes in the mitochondrial ATP synthase subunit 6 correlate with acute lymphoblastic

leukemia, further corroborating that metabolic dysfunctions and alterations of genes involved in the energy metabolism might play a pivotal role in tumorigenesis [113].

An oxidant environment, which often results from elevated OXPHOS activity in highly proliferative cells, has been indicated as the main driver of mitochondrial genome aberrations involved in tumorigenesis. CSCs respond to changes in the redox status by regulating their differentiation as well as their propagation. Indeed, the formation of 3D tumor spheres from breast cancer cells exposed to hypoxia is inhibited by scavenging mitochondrial ROS [114]. Accordingly, administration of antioxidants like *N*-acetyl-cysteine (NAC) has been shown to counteract glioma stem cell (GSC) activation and orthotopic tumor formation [115]. A useful approach to characterize the factors wakening up cancer cells toward a metabolic-dependent stemness program could be that of combining genomic analyses with functional CSC assays. For instance, it has been recently demonstrated that some pluripotency genes like NANOG trigger CSC maintenance and promote tumorigenicity *in vivo* by inducing a functional reprogramming of mitochondrial metabolism in HCC [116]. Lee et al. [117] recently shaped the oncometabolites and transcriptomic profile of breast cancer stem-like cells, and found that the Wnt pathway regulates nicotinic acid adenine dinucleotide phosphate (NAADP) levels, which in turn promote CSC survival. In addition, non-coding genomic sequences may be implicated in CSC action, as demonstrated by the evidence that an miR-122-mediated regulation of the glycolytic enzyme PK4 inhibits stem phenotypes in CD133 (+) HCC cells [118]. Recently, the transcriptomic profiling of miRNA in breast tumor spheroid-enriched CSCs has indicated that many miRNAs controlling several metabolic processes are differentially regulated in CSC-like cells compared with adherent MCF7 cells, as evidenced by NGS combined and gene ontology analysis [119].

Collectively, these data indicate that the primitive mutations and subsequent genomic alterations, together with additional factors like microRNA and developmental pathways, are accountable for the metabolic activation of CSCs toward tumor development and progression. Based on the above observations, investigating the genomic heterogeneity of cancer cells and the subsequent variety of metabolic pathways might be useful to characterize the biology of CSCs to identify their Achilles' heels. Genome-wide analysis of single-cell RNA sequencing might represent a useful strategy to combine information from diverse metabolically integrated cell subpopulations, in order to disclose their stem capacity and tumorigenic potential.

The metabotstemness theory: metabolism as an emerging character in the CSC tale

Growing evidence indicates that genome alterations might, at least in part, account for the acquisition of stem features that drives tumor initiation and progression. Adult stem cells (ASCs), which are characterized by innate self-renewal and multi-potency capacity, can accumulate many oncogenic mutations for a period of time long enough to support transformation and the development of malignant disease. Supporting this hypothesis, somatically acquired genetic lesions occurring in rare multipotent stem cells and progenitors have been shown to drive the evolution of myelodysplastic syndromes toward the leukemic transformation [120]. ASCs and committed progenitors have therefore been suggested to represent the cells of origin in most tumors, for their ability to self-renew and accumulate oncogenic mutations. CSCs that come from mutations in ASCs are placed at the top of a hierarchical organization, while the disorganized repertoire of cells constituting the tumor bulk could be regarded as the anomalous outcome of an aberrant developmental process. Nevertheless, breast, lung, pancreatic, prostate, and liver cancer do not derive from the direct transformation of normal tissue stem or progenitor cells, although in all these tumor types, cells with stem-like features have been detected. In this context, differentiated cancer cells may be reprogrammed to acquire CSC properties, because of the high plasticity that characterizes the components of the tumor microenvironment. Of note, the transition to a malignant and aggressive cancer phenotype seems to be correlated to the capability of generate CSCs from non-CSC populations, in response to transcriptional, epigenetic, or external stimuli [121]. For instance, hypoxia, lack of nutrients, inflammation, and therapy-induced stress may increase the plastic potential of differentiated cancer cells toward a stem-like status. For the above observations, targeting cancer cells reprogramming might be a useful tool to normalize the aberrant differentiating program characterizing the oncogenic lesions. In this complex scenario, it has been recently proposed that the altered differentiation that supports malignant transformation may be controlled and orchestrated by specific cellular 'metabotypes', which drive the acquisition of stemness features [122]. The term 'metabotstemness', recently coined by Menendez and Alarcón, actually delineates the stem properties to be dependent on cell metabolism, which in turn may switch on and off the stem

potential of tumor cells (Figure 1) [122]. By regulating epigenetic and transcriptional networks involved in self-renewal, the metabolotypes can drive normal and cancer cells toward a CSC-like status. While certain metabolic events may act as early drivers of transcriptional and epigenetic reprogramming, additional second metabolic hits may long-term arrange and dictate the stemness properties within the tumor tissue. Hence, specific metabolic dynamics may govern the genetic reprogramming that forces the acquisition of stem traits from noncancer or differentiated cancer cells. Supporting these hypothesis, cellular metabolism in tumor tissues has been recently included among the well-acknowledged hallmarks of cancer [12]. Cancer-associated metabolic changes, rather than being considered as secondary biochemical events triggered by the increased anabolic requirements of tumor cells, might be then considered as crucial operators involved in regulating the kinetics of stemness reprogramming and the balance between non-CSC and CSC-like states during tumor initiation and evolution [12,122]. Remarkably, the hierarchical metabolic frame that functionally integrates modifications in the transcriptional genetic machinery to regulate CSC function is a hot topic under investigation. First of all, metabolic-triggered reorganization in chromatin structure and epigenetic changes may systematically drive the transition from a non-CSC to a CSC-like state in response to specific biochemical settings [123]. Indeed, many metabolic signals have been shown to affect chromatin organization, adding to the classical notion that hormones and growth factors are the main activators of transcriptional responses [123]. In support of this model, several transcription factors involved in stemness like *c-Myc* have been shown to globally act on chromatin rearrangement and to indirectly exert a regulatory role on metabolic fluxes [124,125]. Moreover, the epigenetic regulation of crucial enzymes has been implicated in the activation of key metabolic pathways involved in reprogramming toward stemness. For instance, the methylation in the promoter of fructose-1,6-bisphosphatase (FBP1), one of the key enzymes in the gluconeogenesis, maintains the glycolysis pathway active to increase CSC-like properties and tumorigenicity potential in basal-like breast cancer [126]. On the other hand, post-transcriptional modifications in stem-related genes have been shown to affect cell metabolism, thus evidencing a bi-directional interaction between epigenetics and metabolic responses [122]. It should be mentioned that most of the cofactors necessary for epigenetic DNA modifications derive from metabolic pathways such as glycolysis, TCA cycle, OXPHOS, and fatty acid oxidation; this is the case for *S*-adenosyl-*L*-methionine (SAM), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), and acetyl Coenzyme A (reviewed in ref. [123]). Furthermore, mutations in genes codifying for metabolic enzymes have been shown to trigger the production of aberrant metabolites with oncogenic functions. Likewise, diverse oncometabolites play a causal role in epigenetic reprogramming involved in tumor development and progression. In this context, mutations in TCA cycle enzymes and overproduction of oncometabolites like *R*(-)-2-hydroxyglutarate, fumarate, and succinate have been associated with epigenetic DNA rearrangements, transcriptional dysfunctions, and benign or malignant neoplasms [127–129]. Moreover, it has been proposed that Isocitrate dehydrogenase (IDH) mutations, by promoting hypermethylation of genes involved in differentiation, may shift the balance between undifferentiation and differentiation toward a pluripotency status, ultimately leading to the increase in the number of cells with stem-like features [123]. Parallel, a flexible epigenetic scenario requires an intact metabolic function of IDH, which would affect the DNA methylation status to effectively permit a metabolotype-driven reprogramming process [122]. Looking at cancer as a disease of differentiation and reprogramming initiated and supported by aberrant metabolotypes would allow to characterize the biochemical orchestrators involved in intratumor biological plasticity and complexity, providing a new framework for identifying novel cancer hallmarks and eradicating CSCs.

Metabolic features of CSCs

Because of the complex spectrum of different microenvironmental conditions they survive in, CSCs are most likely supposed to get energy from different sources, according to substrate availability. Indeed, evidence for a glucose- and oxidative-based metabolism has been widely provided. In addition, amino acids like glutamine and lysine may serve as an alternate fuel for CSCs. Normal cells mainly generate ATP using mitochondria and the TCA cycle coupled with OXPHOS to catabolize acetyl-CoA produced from glycolysis and fatty acid oxidation [18]. Different from normal cells, cancer cells increase the glycolytic flux also in aerobic conditions, through the well-described Warburg effect. In these conditions, although the generation of ATP is less efficient, its production rate is higher, rendering ATP immediately available for the huge anabolic requirement of cancer cells [18]. Furthermore, the catabolism of glucose via glycolysis provides intermediates for nucleotides and amino acid biosynthesis. Hence, the switch from oxidative to glycolytic metabolism efficiently provides cancer cells with the ability to survive in harsh environmental conditions characterized by poor oxygen and enable

cancer cells to proliferate, migrate to distant sites, and invade secondary tissues [18]. The metabolism of CSCs has been shown to differ from non-CSCs, whose phenotype has been, at least in part, paralleled to that of normal stem cells [22], which primarily use glucose. Indeed, in induced pluripotent stem cells, metabolic reprogramming toward glycolysis and evidence of mitochondrial involution parallel the acquisition of pluripotent markers [130]. Nevertheless, several studies have reported that OXPHOS and mitochondria may play a pivotal role in CSC metabolism, together with secondary pathways like fatty acid oxidations, PPP pathway, and glutaminolysis [22].

Evidence that CSCs get energy from glucose

Many studies have well established the importance of glucose for CSC maintenance and propagation in diverse cancer cells including brain, breast, lung, liver, nasopharyngeal cancer, osteosarcoma, and glioblastoma [20,59,131–133]. By using a panel of cancer cell lines, Liu et al. [20] recently demonstrated that a subpopulation of cells with stem-like properties mainly rely on glucose as a primary fuel. Indeed, glucose was able to increase the number of cancer stem-like cells, in which many glycolytic enzymes were up-regulated and lactate production was elevated. Likewise, the inhibition of glycolysis was shown to reduce the number of CSCs and interfere with their tumor-forming ability *in vivo* [20]. An exacerbation of glycolysis and acquisition of stem features were observed when the activity of the mitochondrial complex I was inhibited for loss of FBPI [126]. In addition, the overexpression of FBPI, which stimulates the gluconeogenic pathway and inhibits glycolysis, has been shown to reduce the number of cancer cells with stem properties in basal-like breast cancer cells and reduce tumor spheroid formation *in vivo* [132]. Further extending these findings, Shen et al. have recently demonstrated that a subset of hepatic cancer cells CD133⁺ preferentially activate aerobic glycolysis and inhibit the gluconeogenic pathway, compared with CD133⁻ cells [131]. The CSC-like subpopulation was shown to have an increased glycolysis rate and glycolytic capacity, up-regulation of the glycolytic enzymes GLUT1, HK2, PDK (pyruvate dehydrogenase kinase), and PGAM1, together with down-regulation of the gluconeogenic enzymes G6PC and PEPCK [131]. These data suggest that CSCs from diverse tumors use glycolysis as the main catabolic pathway and inhibit anabolic *de novo* synthesis. This has been reported also for colorectal cancer (CRC), where a peculiar metabolic signature has been recently revealed in CSCs. In particular, the authors implemented a transcriptomic study of five microarray datasets from the GEO database of CD133⁺ and CD133⁻ cell subpopulations isolated both from CRC cell lines and patients [134], together with high-resolution unbiased metabolomics. This allowed portraying the metabolic behavior of CSCs, which was characterized by enhanced expression of genes and metabolites from the glycolytic pathway and TCA cycle, with down-regulation of the fatty acid biosynthesis [134]. High-throughput data from proteomic and targeted metabolomics analysis were recently used to investigate the metabolic phenotype of breast cancer cells grown as spheroids or in adherent conditions. A shift from mitochondrial oxidative phosphorylation toward fermentative glycolysis was revealed in cancer stem-like cells, as evidenced by the increased activity of the pyruvate kinase M2 isoform, lactate dehydrogenase and glucose 6-phosphate dehydrogenase [56]. In an attempt to identify genes and pathways relevant to glioblastoma CSC survival, Goidts et al. [135] utilized RNA interference (RNAi) to screen the complete human kinome and phosphatome, identifying many genes involved in metabolism and in particular the glycolytic enzymes PFKFB4, PDK1, and PKM2, which were found relevant for the maintenance of brain CSCs.

Altogether, these observations are supportive of the remarkable role of glucose as a main fuel in CSCs, and indeed, oxidative pathways could be reasonably disadvantaged because of the poor oxygen availability in the hypoxic CSC niche. In this context, TICs isolated from human glioblastoma xenografts have been shown to use glycolysis for ATP generation and prefer low oxygen conditions to maintain their stemness properties and tumor-forming capacity [136]. The contribute of hypoxia in CSC expansion has been extensively investigated and associated with glucose dependence, particularly in the quiescent phenotype of CSCs. Recently, Mahase and co-workers reviewed the possibility that multiple mechanisms including CSC propagation and metabolic alterations may account for the resistance to antiangiogenic drugs in the clinical management of glioblastoma patients. Indeed, the generation of intratumor hypoxia following administration of antiangiogenic agents has been shown to increase the subpopulation of cells with stem properties in lung and breast cancer, as well as in glioblastoma [137–139]. The increase in ALDH⁺ population has been attributed to HIF-1 α [138,139], which, as previously discussed, enables the transcription of genes involved in glucose control and ATP production. Likewise, glioma cells residing in perinecrotic areas aberrantly express the first enzyme in the Embden–Meyerhoff/glycolytic pathway named hexokinase-2, whose overexpression is involved in glioblastoma cell

proliferation and aerobic glycolysis [140]. Furthermore, HIF-1 α -mediated action includes the increase in PDK1, thus inhibiting pyruvate dehydrogenase activity and TCA cycle entry [139,140].

Evidence that CSCs get energy from OXPHOS: focus on mitochondria

An overwhelming amount of studies indicates that CSCs preferentially use mitochondrial respiration and oxidative metabolism, in contrast with the old paradigm of a main glycolytic phenotype for CSCs. Indeed, evidence of a reduced glycolytic flux and increased mitochondrial-driven ATP production have been provided by diverse independent authors. For instance, in CSCs isolated from ovarian cancer patients, an up-regulation of enzymes involved in mitochondrial OXPHOS and fatty acid oxidation has been revealed [141]. Accordingly, the metabolic features of spheroids generated from both ovarian and cervical carcinoma were recently analyzed and compared with the same cells cultured in adherent conditions. Interestingly, the authors found a reprogrammed metabolism through the TCA cycle in spheroid cancer stem cells, with respect to non-CSCs [142]. Using a similar experimental approach, Gao et al. recently FACS sorted CSCs from small cell lung cancer cell, in order to analyze their metabolic status. CSCs were found to possess a higher dependence on OXPHOS and mitochondrial function when compared with their non-stem counterpart [143]. CSCs isolated from glioma have been demonstrated to consume less glucose, produce less lactate, and maintain high ATP levels from oxidative phosphorylation [144]. A similar trend for the use of mitochondria respiration over glycolysis has been reported in CD133⁺ human glioblastoma cells, with a mechanism depending on the insulin-like growth factor 2 mRNA-binding protein (IMP2) [145]. In particular, the authors demonstrated that CD133⁺ glioblastoma cells have enhanced expression of IMP2, which is involved in regulating OCR (oxygen consumption rate), mitochondrial mass, and the expression of several stemness markers, including CD133, SOX2, OCT4, and NANOG [145]. As IMP2 directly interacts with several mitochondrial complex genes to orchestrate the assembly of mitochondrial complexes I and IV, it could be assumed that the enhanced IMP2 expression detected in glioblastoma CSCs may serve for the increased OXPHOS requirements in these cells [145]. Additionally, Viale et al. analyzed the population of dormant cells surviving the ablation of the oncogene RAS in a mouse model of pancreatic cancer. These dormant cells were shown to exhibit stemness features and rely on oxidative phosphorylation and mitochondrial activity, rather than glycolysis and glutaminolysis [146].

Of note, the metastatic potential of cancer cells has been associated with the activity of the transcription co-activator peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PPARGC1A, also known as PGC-1 α) [147]. Clinically, PGC-1 α has been shown to couple oxygen consumption, OXPHOS, and mitochondrial biogenesis with enhanced migratory and invasive capability of cancer cells, as revealed by using human invasive breast tumor samples [148,149]. Supporting the role of PGC1 α in CSCs maintaining and propagation via mitochondrial activity, its overexpression has been detected in circulating tumor cells, as well as in breast cancer stem cells, where its inhibition reduces stemness properties [148,149]. On the basis of these observations, an intact mitochondrial activity and function seems to be necessary for the CSC biology. In this scenario, mitochondria biogenesis has recently emerged as a key feature of CSCs, who display increased mitochondrial mass and membrane potential, higher generation of mitochondria-derived ROS, and enhanced oxygen consumption, when compared with the differentiated cells in the tumor bulk [141,149–154].

A recent report has supported the role of mitochondrial dynamics in brain tumor-initiating cells (BTICs), which exhibit higher mitochondrial fission mediated by dynamin-related protein 1 (DRP1) [155]. Interestingly, DRP1, which controls mitochondrial fission by pinching off the membrane stalk between two forming daughter mitochondria, was correlated with poor prognosis in glioblastoma, hence suggesting that targeting mitochondria in BTICs may represent a useful approach to halt disease progression [155]. Of note, an efficient maintenance of the mitochondrial network and a proper fragmentation and segregation of mitochondrial population have been correlated with the propagation of stem-like cells in breast epithelium [156]. It should be mentioned that in order to contrast tissue aging and promote renewal, stem cells asymmetrically divide into one daughter cell that retains stemness properties and another cell that is subjected to a differentiation program. By using photo-activated marker proteins to analyze the fate of old and young organelles during stem cells asymmetrical division in human breast epithelium, Katajisto et al. recently demonstrated that stem cells sort mitochondria by age. In particular, stem cells apportion aged mitochondria asymmetrically between daughter cells, with cells receiving younger mitochondria to be destined to maintain stem traits [156]. Hence, stem-like cells can exclude older mitochondria, with a highly efficient mechanism including mitochondria spatial segregation. Of note, disruption of such tightly regulated processes during mitochondrial fission may cause loss of stem trait in the progeny cells [156]. Extending these findings, it has been demonstrated that the activation of several oncogenic

pathways like MAPK are involved in mitochondrial fragmentation, which may be regarded as an early step involved in cell reprogramming toward pluripotency [157]. Likewise, in breast cancer cells, c-Myc has been shown to promote mitochondrial fusion through the engagement of YAP/TAZ signaling to drive clonogenic growth, which is a distinguishing feature of cells with stem properties [158]. Interestingly, in human mammary epithelial cells, a mitochondrial retrograde signaling pathway has been shown to activate an EMT-like reprogramming, toward altered morphology and increased migratory and invasive capacity [159].

On the basis of these observations, mitochondrial functions and energetic dynamics may be involved in CSC dissemination; as the maintenance of a healthy mitochondrial population is essential for keeping and propagating the stem traits, targeting these organelles in a therapeutic setting might represent a useful strategy to eradicate CSCs.

Additional metabolic fuels for CSCs

CSCs from liver have been shown to use fatty acid oxidation, as demonstrated by the metabolic analysis of CD133⁺/CD49f⁺ cells sorted from HCC [116]. An increase in lipid content and Wnt/b-catenin activity was observed in CD133⁺ cells isolated from CRC patients [160]. Genes associated with fatty acid oxidation were found up-regulated in CSCs isolated from ovarian cancer patients [141]. Likewise, the block of fatty acid oxidation by etomoxir, a carnitine palmitoyltransferase-1 inhibitor, has been shown to inhibit spheroid formation in breast cancer *in vitro* and decrease tumor growth *in vivo* [114].

Conversely, inhibition of fatty acid synthesis by Soraphen A, cerulenin, and resveratrol has been shown to decrease the expression of CSC markers and sphere formation efficiency [161–163]. However, further studies are required to clarify the role of lipid metabolism in CSC biology, particularly in response to specific changes in the tumor niche.

Focusing on the role of pathways other than glycolysis and OXPHOS, CSCs have been shown to boost the PPP, particularly during hypoxia and reoxygenation [164]. Indeed, the expression of crucial PPP enzymes is increased by acute oxygenation and decreased upon hypoxia, which triggers the expression of glycolytic genes. Such inverse correlation between the activation of glycolysis and the PPP pathway in a differential oxygenated microenvironment may reflect a glycolysis-mediated cell migration upon hypoxia and PPP-mediated cell proliferation during acute oxygenation [164].

Finally, glutamine metabolism also plays a remarkable role in CSCs from several tumors including lung and pancreatic and ovarian cancer [142,165]. Glutamine metabolism appears to be essential in c-Myc-overexpressing cells, suggesting that a pluripotency gene profile selects for glutamine dependence [166]. The inhibition of glutamine availability has been shown to reduce the stemness gene signature and sensitize pancreatic CSCs to radiation therapy, both *in vitro* and *in vivo* [165]. Nicely fitting with these observations, a parallel study performed in a mouse model of systemic metastasis has shown that the inhibition of glucose metabolism via L-DON (a glutamine analog) is able to inhibit the liver, lung, and kidney metastatic dissemination [167].

Looking at metabolism to identify and target CSCs

As the study of CSC biology has indicated their metabolic features as relevantly involved in survival and functionality, the idea of identifying a subpopulation of cancer cells with stem properties based on a distinguishing metabolic profile has recently emerged. In addition, novel therapeutic approaches could be used to push cancer out of race, simply by hitting the biochemical energy reactions allowing CSC maintenance and dissemination. Classically, the phenotypic identification of stem cells relies on the use of flow cytometry coupled with a functional stem cell assay. However, the sole use of surface and cytoplasmic markers does not seem to be an effective strategy, because of many limitations due to technical problems, inter- and intratumor heterogeneity, and lack of high specificity, as discussed above [46]. In this context, the identification of metabolic markers could integrate the information coming from the acknowledged *bona fide* CSC markers, thus allowing a more reliable identification of the cancer cells with stem properties. In this context, evidence that increased mitochondrial mass, a surrogate marker for elevated mitochondrial biogenesis, can be used to identify cells with increased self-renewal capacity in diverse cancer types has been provided [61,114,143,153]. In particular, tracking mitochondrial mass via fluorescent probes has been described as a simple and efficient tool to identify CSCs, independent of their glycolytic- or OXPHOS-dependent metabolic phenotype [143,149,153]. By performing a metabolic fractionation of MCF7 breast cancer cells via MitoTracker, a fluorescent probe selectively staining mitochondria, it has been recently demonstrated that high mitochondrial mass enriches for anabolic CSCs.

Functional validation of these findings has been provided by performing mammosphere assays, while an unbiased proteomic approach has allowed to establish that mitochondrial proteins are among the most strongly up-regulated in MCF7 cells overexpressing WNT1 and FGF3, which are main drivers of the propagation of mammary cancer stem cells [143].

Enrichment in mitochondrial content has been associated with higher DNA repairing capacity in human breast cancer stem cells, suggesting that an increased mitochondrial mass may enable CSCs to efficiently cope with the action of certain anticancer drugs [153]. Recently, Moschoi et al. [168] have demonstrated that AML cells increase their mitochondrial mass by transferring mitochondria from bone marrow stromal cells, a unidirectional process exacerbated upon exposure to chemotherapeutic agents. Such transfer occurs also in the leukemia-initiating cells and progenitors, where it has been shown to provide survival advantage and long-term culture potential [168]. These findings add to previous studies showing asymmetrical apportioning of young mitochondria into stem cells, suggesting that dynamic mitochondrial movement and mitophagy can account for the stem features of cancer cells and provide a useful tool for their identification.

In addition, the identification of rare CSCs within the heterogeneous tumor mass could be achieved looking at the mitochondrial membrane potential, a parameter to measure the mitochondrial functional status. Indeed, the membrane potential of mitochondria has been associated with differentiation programs, as well as the malignant progression of neoplastic disease [169]. Recently, a broad molecular ‘tool-kit’ for the identification of CSCs in breast cancer has been assembled [170]. Based on the assumption that breast CSCs are characterized by (i) increased mitochondrial biogenesis mediated by PGC-1 α ; (ii) increased generation of ROS; and (iii) increased NADH levels, it has been assessed that a subpopulation of MCF7 cells with increased PGC1 α activity, ROS production, and NADH autofluorescence shows higher stemness features, determined by mammosphere-forming efficiency [170]. Furthermore, the hypothesis that CSC propagation is promoted by ROS-driven mitochondria biogenesis, oxidative metabolism, and a functional glycolytic pathway was proved by using specific inhibitors to target these metabolic processes [170].

mtDNA is often genetically altered in cancer, and hence, selectively targeting certain mitochondrial responses may be useful to disrupt CSC function, without off-side effects on healthy tissues. Indeed, several pharmacological drugs are currently being tested *in vitro* and *in vivo*, as well as in preclinical and clinical studies for cancer treatment.

The ability of salinomycin to target one of the most important stemness pathway like Wnt/b-catenin has been correlated with its inhibitory action on diverse cancer cell lines; furthermore, salinomycin has been shown to induce mitophagy and mitoptosis together with depletion of ATP levels [171]. The mitochondria inhibitor VLX600, which acts by reducing mitochondrial respiration, has been shown to target quiescent cancer cell populations, inducing tumor growth inhibition *in vivo* [172]. A specific inhibitor of the ERR α -PGC1 signaling pathway, which triggers mitochondrial biogenesis, impairs breast CSC survival and propagation, and both effects can be reversed using L-acetyl-carnitine as a mitochondrial fuel [149].

Encouraging preclinical data from a large number of studies have revealed that the repurposing of previously FDA-approved drugs may efficiently target mitochondria and halt CSC dissemination. One of the most striking ideas is that of inhibiting mitochondria by using antibiotics. This strategy is well constructed on the evidence that mitochondria have evolved from endosymbiotic α -proteobacteria belonging to Rickettsia gender [173]. As such, diverse antibiotics have been shown to halt CSC propagation by inhibiting mitochondrial processes. For instance, salinomycin, bedaquiline, tetracyclines, glycyliclones, and erythromycines have been shown to effectively eradicate the CSC population by interfering with mitochondrial functionality (Figure 2) [174–176].

Additional inhibitors of mitochondrial respiration recently identified for inhibiting CSC dissemination include the antielmintic drugs niclosamide, nitazoxanide, closantel, and pyrivinium pamoate, and the antimalarial drug atovaquone which has been shown to induce a Warburg-like effect in breast cancer cells, by inhibiting OXPHOS and activating aerobic glycolysis [176–179].

In this context, the pharmacological manipulation of glycolysis to halt CSC propagation has been proved to be an effective strategy in diverse tumor types, including pancreatic adenocarcinoma, glioblastoma, and ovarian and breast cancer [173–175]. Accordingly, the metabolomic analysis of diverse cancer cell lines treated with metformin, which is an anti-diabetic drug retrospectively linked to cancer prevention and CSC disruption, revealed the inhibition of glycolysis, the co-ordinate decrease in the TCA cycle, and the inhibition of nucleotide synthesis, associated with the inhibition of mammosphere-derived breast CSC formation and decreased *in vivo* tumorigenic potential [180]. Additionally, in CD133⁺ pancreatic cancer cells, metformin induces an energy crisis that drives cell death due to the inhibition of mitochondrial complex I and the impossibility to switch to

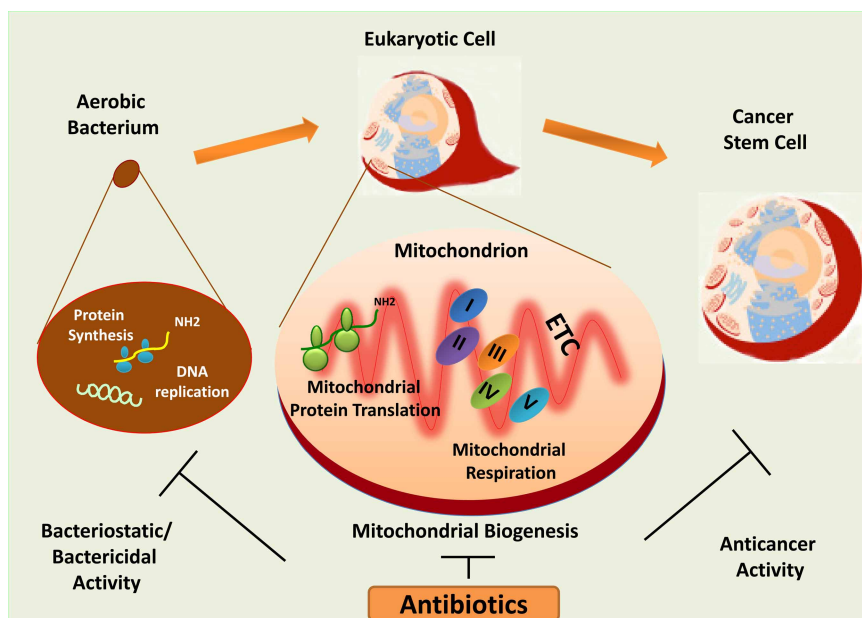


Figure 2. Targeting CSCs with mitochondria-interfering agents.

The endosymbiotic hypothesis for the origin of eukaryotic mitochondria suggests that mitochondria evolved from engulfed aerobic bacteria. The symbiont bacterium was able to conduct cellular respiration, thus providing a remarkable evolutionary advantage to the host cell, which mainly relied on glycolysis and fermentation. Supporting this endosymbiotic hypothesis, mitochondria possess their own circular DNA and conserve efficient and independent transcriptional and translational machinery. High similarity has been evidenced between mitochondrial ribosomes and bacterial ribosomes. Likewise, many antibiotics have been shown to interfere with mitochondrial protein translation as an off-target effect. Based on these observations, the repurposing of FDA-approved antibiotics could be an effective and safe strategy to halt the propagation of CSC, which are severely damaged by mitochondrial dysfunction.

glycolysis [151,180]. Indeed, CSC metabolism shows a highly plastic profile which allows to fulfill the energy requirements, according to the most suitable environmental condition. Evidence for this metabolic flexibility comes from studies performed in diverse tumor types like glioma, brain, and breast CSCs, which efficiently gain energy from glycolysis when OXPHOS is blocked [126,144,181]. On the contrary, CSC survival is impeded when this metabolic malleability is lacking. This is the case for pancreatic CSCs resistant to K-ras oncogenic ablation, which is responsible for alterations of the metabolic program and subsequent inability to switch to glycolysis when OXPHOS is inhibited [146].

On the basis of the observations above, targeting the metabolic flexibility in CSCs holds promise to be an effective strategy for eradicating neoplastic disease. This is nicely supported by the evidence that leukemia cells are more sensitive to the action of glycolysis inhibitors after treatment with tigecycline, which disrupts mitochondrial protein synthesis [182]. Furthermore, it has been recently demonstrated that breast cancer cells chronically treated with doxycycline, which impairs mitochondrial function, energetically depend on glycolysis, whose pharmacological disruption halts CSC survival (Figure 3) [183].

The future of targeting metabolism to eradicate CSCs

Consistent data provide a solid platform to include metabolism among the hallmarks of cancer and to consider the metabolic profile of CSCs as a relevant target in anticancer therapy. The unique metabolic features of CSCs allow to design specific pharmacological approaches to selectively inhibit CSCs and preserve the function of normal stem cells.

Clearly, a unifying concept on which energy reactions are used by CSCs in response to a plethora of environmental stressful conditions including hypoxia, radiotherapy, and chemotherapy would provide a useful tool toward the identification of the metabolic Achilles' heels to be considered as preferential therapeutic targets.

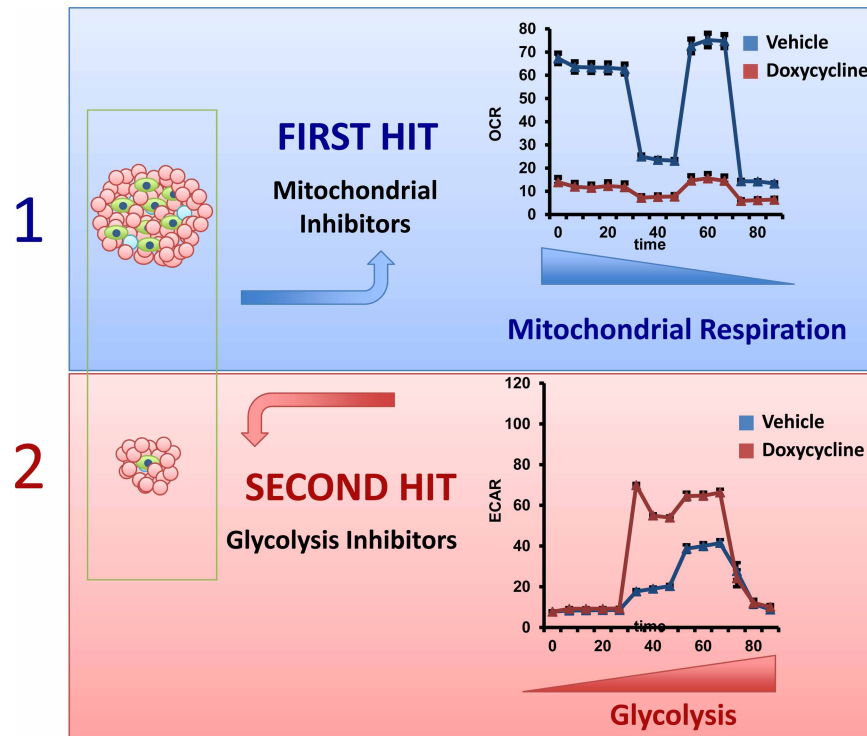


Figure 3. Two 'metabolic' hit strategy for the eradication of CSCs.

Exploring the metabolic plasticity of CSCs may provide unique possibility for therapeutic intervention based on targeting key energy processes. For instance, the prolonged treatment with a mitochondria-interfering agent like doxycycline drastically impairs OCR and mitochondrial respiration in MCF7 breast cancer cells. Such impairment in mitochondrial activity represents a first metabolic hit that constrains cellular metabolism toward glycolysis, as evidenced by the increased extracellular acidification rate (ECAR). The use of a glycolysis inhibitor may therefore act a second metabolic hit that efficiently targets CSCs by halting their biochemical machinery. This approach reverses CSC metabolic plasticity toward an inflexible biochemical phenotype that can be efficiently targeted with specific metabolic-oriented pharmacological intervention.

One of the first difficulties in gaining such integral information comes from inter-tumor heterogeneity; nevertheless, contrasting outcomes have been achieved analyzing the metabolic profile of CSCs within the same cancer type. Looking at the possible reasons for these differences, it is emerging that the technique used to isolate and cultivate CSCs could be accountable for the diverse responses detected in analyzing their metabolic behavior. For instance, the analysis of metabolic pathways in ovarian CSCs revealed a preferential use of glycolysis from *in vivo* studies, whereas an OXPHOS-dependent phenotype was detected from *in vitro* studies [141,184]. However, it should be mentioned that in the first case, the experimental system was represented by a murine model of ovarian cancer, while in the second case CSCs from human primary cell cultures were used, suggesting that species-dependent differences could also potentially play a role in the observed contrasting effects. A lack of suitable microenvironment is the main pitfall in studying CSCs from established cancer cell lines *in vitro*; indeed, the importance of the tumor niche in driving the biological and biochemical behavior of CSCs has been largely discussed. In this context, an increased stemness gene signature and a switch to OXPHOS have been detected in breast CSCs under the influence of fuels released by glycolytic stromal cells [109]. According to these observations, the investigation of the metabolic phenotype of CSCs should be performed trying to use an experimental model that highly recapitulates the intratumor features and using fresh patient or animal samples.

It is worth mentioning that understanding the metabolic background of CSCs, its specific regulators and effectors would be extremely helpful to design therapeutic approaches selectively targeting CSCs, without affecting the functions of normal stem cells, essential for tissue homeostasis.

Novel metabolism-based approaches are currently being added to the classic strategies used to characterize and target CSCs; on the basis of the identification and inhibition of crucial network of regulators involved in

CSC survival and propagation, high-throughput data analysis and large-scale drug screening have been performed in order to identify and eradicate CSCs using their metabolic singularities. In particular, -omic technologies and high-throughput screening have allowed to get relevant information regarding the metabolic status of CSCs, providing a detailed picture of the biochemical profile of CSCs. The integration of metabolomic data with gene expression and/or proteomic studies has provided a more comprehensive knowledge of CSC biology in a metabolic perspective.

For instance, gene expression profiling using Affymetrix microarrays representing over 47 000 transcripts and variants allowed the investigators to determine the importance of mevalonate metabolism in regulating breast CSC phenotype; the subsequent pharmacological inhibition of the geranylgeranyl transferase 1, which blocks the metabolic mevalonate pathway, reduced the subpopulation of breast CSCs both *in vitro* and *in vivo*, as demonstrated using primary breast cancer xenografts [185]. Although CSCs represent a very rare subpopulation of tumor cells characterized by elevated instability in culture, high screening tools are still applicable to identify selective CSC inhibitors, as demonstrated by Gupta et al. The authors screened a collection of 16 000 chemicals from commercial libraries and collections of natural compounds in a genetic model of mesenchymally transdifferentiated breast cancer cells. By inducing an EMT-like phenotype, the authors enriched for CSC population, whose abundance was shown to be 10-fold higher compared with the wild-type cells. Next, the authors identified the chemical identities of three compounds (salinomycin, etoposide, and abamectin), which exhibited strong selectivity for the stem population and ability to reduce the expression of stem markers [174]. By using a platform of induced cancer stem-like cells as a functional assay system, a large-scale drug screening was performed on 6000 compounds, allowing to identify the antimalarial artesunate as a selective inhibitor of CSC survival through the induction of mitochondrial dysfunction [186].

Considering that the inhibition of mitochondria, which evolutionary derive from the engulfment of aerobic bacteria, impairs CSC biology, a novel mitochondria-targeted approach has been proposed to halt stemness. Based on their binding to the 3D structure of the mammalian mitochondrial ribosome, 880 compounds have been identified through high-throughput screening and computational chemistry. The first 10 compounds selected for their efficacy in inducing mitochondrial dysfunction and ATP depletion [187].

Altogether, these studies provide evidence that high-throughput strategies combined with large-scale drug screening may be used to identify selective inhibitors of CSCs toward cancer eradication.

Several drugs targeting metabolic pathways have been enrolled in human randomized controlled trials, after the promising results obtained in cell and animal models, as well as in preclinical models [187,188]. Nevertheless, to date, none of these drugs have shown encouraging results, probably for their effects on the tumor bulk. A biotechnology company, named MetaboStem from the Catalan Institute of Oncology, has currently aimed at specifically hitting the metabolic vulnerability of CSCs using the drug MS-001 in a preclinical trial.

Conclusions

CSC have been regarded as the cells of origin of cancer and are crucially involved in metastatic dissemination, radioresistance and chemoresistance, and disease recurrence. Mounting experimental evidence and clinical studies indicate that metabolism is not a mere player in the tumor bioenergy machinery, but it actually orchestrates stemness by enabling cell reprogramming in response to a large repertoire of environmental conditions within the stem niche. Recently, targeting the peculiar metabolic features of CSCs has hold promise to prevent disease progression and recurrence and efficiently eradicate cancer. High-throughput data combined with large-scale drug screening represent the state of the art for the characterization of the metabolic peculiarity of CSCs and the identification of selective pharmacological targets. In this scenario, the repurposing of FDA-approved drugs represents a concrete and inexpensive opportunity to extend the pharmacological and biological properties of existing compounds, and in the meanwhile gain a better understanding of CSC action in cancer. Nevertheless, a deeper focus on the metabolic plasticity of CSCs and their ability to switch to different metabolic pathways in response to certain environmental stressors like hypoxia or chemotherapies would provide a better strategic platform to hit this biochemical malleability. Furthermore, the evaluation of the metabolic fuels, intermediates, and pathways involved in maintaining the stemness traits and implicated in CSC survival in harsh conditions could unveil novel metabolic Achilles' heels to be used in a therapeutic setting. A drug-controlled process aimed at forcing CSCs to adopt a certain metabolic profile could be an effective approach to prevent the metabolic adaptability of CSCs. Indeed, targeting this drug-induced metabolic inflexibility would definitely compromise CSC survival.

Abbreviations

2-DG, 2-deoxy-D-glucose; ALDH, aldehyde dehydrogenase activity; AML, acute myeloid leukemia; ASCs, adult stem cells; BTICs, brain tumor-initiating cells; CAFs, cancer-associated fibroblasts; CAIX, carbonic anhydrase 9; CRC, colorectal cancer; CSC, cancer stem cells; DRP1, dynamin-related protein 1; ECAR, extracellular acidification rate; ECs, endothelial cells; EMT, epithelial–mesenchymal transition; ERR α -PCG1, estrogen-related receptor alpha–peroxisome proliferator-activated receptor gamma, coactivator 1; FBP1, fructose-1,6-bisphosphatase; GSCs, glioblastoma stem-like cells; HCC, hepatocellular carcinoma; HIF-1, hypoxia inducible factor-1; HSC, hematopoietic stem cells; IDHs, isocitrate dehydrogenases; IMP2, insulin-like growth factor 2 mRNA-binding protein; mtDNA, mitochondrial DNA; mTORC, mammalian target of rapamycin complex; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PDAC, pancreatic ductal adenocarcinoma; PDK1, pyruvate dehydrogenase kinase 1; PFK-1, 6-phosphofructo-1-kinase; PFKFB3, phosphofructokinase-2/fructose-2,6-bisphosphatase 3; PGC1 α , peroxisome proliferator-activated receptor gamma co-activator 1-alpha; PPARGC1A, also known as PGC-1 α , peroxisome proliferator-activated receptor gamma, co-activator 1 alpha; PPP, pentose phosphate pathway; ROS, reactive oxygen species; TCA, tricarboxylic acid; TICs, tumor-initiating cells; VEGF, vascular endothelial growth factor.

Author Contribution

E.M.D.F. wrote the first draft of the review article, which was then edited by M.P.L. and F.S.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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