Metabolism-Redox Interplay in Tumor Stem Cell Signaling

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Abstract

Cancer stem cells (CSCs) are defined as a subpopulation of cells within the heterogeneous tumor mass endowed with the ability to self-renew and differentiate into non-CSCs. Over-activation or abnormal functioning of intracellular pathways that control normal stem cells, participate in, or contribute to the origin, survival, and maintenance of CSCs. In addition, expression of genes involved in the stemness also depends on epigenetic processes controlling by intermediary metabolites – mainly derived from glycolysis – or can be achieved by the

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S. Chakraborti et al. (eds.), *Handbook of Oxidative Stress in Cancer: Mechanistic Aspects*, https://doi.org/10.1007/978-981-15-9411-3_114

action of reactive oxygen species (ROS) – produced by cellular metabolism – establishing a relationship between metabolism and ROS as a central axis in the CSCs control. In fact, metabolic adaptation is one of the hallmarks of cancer, being aerobic glycolysis the main metabolic change in cancer that moves from oxidative phosphorylation toward lactate production as a way of obtaining energy, even under normal oxygen concentrations. In this sense, data form CSCs indicates that some of them preferably use aerobic glycolysis, while others preferentially use mitochondrial oxidative metabolism. This indicates that CSCs are not a fixed population, but that their metabolic phenotype can be modified depending on the needs. Metabolic factors would be the key for transcription and signaling pathways programs necessary so that intrinsic or environmental factors can direct a particular cell toward a CSC state. In this chapter we review the role of redox state in the regulation of intracellular pathways controlling CSCs and the metabolic plasticity in this tumor subpopulation, thus establishing a point of interconnection between stemness, ROS, and metabolism.

Keywords

Cancer stem cells · Metabolism · Epigenetics · Reactive oxygen species · Intracellular signaling pathways

Introduction

Self-renewal and pluripotency are the two fundamental characteristics that define a normal stem cell. Thus, every stem cell must be able to undergo both symmetric cell division, giving rise to an identical daughter cell, and asymmetric cell division, giving rise to all the differentiated cell linages needed to populate a specific tissue (Cahan and Daley 2013). Due to the high cellular heterogeneity of the tumors and their recurrence after treatments, is has been suggested that tumors could develop from a small subpopulation of cells within the tumor that share these stem cell properties. This was the foundation of the cancer stem cell (CSC) hypothesis. Thus, CSCs have been thoroughly investigated in recent decades and have been proposed to be responsible for the tumor recurrence and the metastases. CSCs have been identified in hematological and solid tumors, including breast, brain, thyroid, melanoma, colon, pancreas, liver, prostate, lung, head and neck, ovary, and stomach cancer (Turdo et al. 2019), establishing a large number of different biomarkers for identification (Table 1). Thus, CSCs are defined as a subpopulation of cells within the heterogeneous tumor mass endowed with the ability to self-renew and differentiate into non-CSCs, which is reflected by their ability to reproduce the tumor of origin when transplanted into immunocompromised mice. CSCs are also considered responsible for metastatic spread and chemoresistance. In this way, they evade conventional treatments, including radio and chemotherapy, being responsible for minimal residual disease and cancer relapse. In fact, CSCs are characterized by more pronounced levels of drug transporters, improved DNA damage repair mechanisms, and the ability to escape

Table 1 Biomarkers reported to characterized CSCs

Marker	Tumor type	Relationship with metabolism
CD133+	Breast cancer; colon cancer; gastric cancer; glioblastoma; head and neck cancer: liver cancer: lung cancer; ovarian cancer; pancreatic cancer; prostate cancer	Decrease hexokinase II expression; promotes hypoxia
CD44+	Breast cancer; colon cancer; gastric cancer; head and neck cancer; liver cancer; lung cancer; liver cancer; lung cancer;	Promotes glycolysis via PKM2 suppression.
CD24+	Breast cancer; colon cancer; gastric cancer; liver cancer; pancreatic cancer	Induced by hypoxia
CD123+	Leukemia (AML); Breast cancer; non-small lung cancer; ovarian cancer; pancreatic cancer; prostate cancer	Promotes glycolytic enzymes activity
CD49+	Prostate cancer; breast cancer; glioblastoma	Not specified
CD34+	Leukemia (AML)	Not specified
CD90+	Liver cancer; lung cancer	Not specified
$ALDH^{high}$	Breast cancer; colon cancer; gastric cancer; glioblastoma; liver cancer; lung cancer; ovarian cancer; pancreatic cancer; prostate cancer	Converts acetaldehyde to acetate; maintains low ROS
EpCAM	Breast cancer; colon cancer; pancreatic cancer	Not specified
ESA	Breast cancer; colon cancer; pancreatic cancer	Not specified
$ABCG2^{high}$	Liver cancer; lung cancer; prostate cancer; pancreatic cancer; melanoma; head and neck cancer; glioblastoma	Induced by hypoxia

(Snyder et al. 2018; Turdo et al. 2019)

cytotoxic chemotherapy by maintaining a quiescent state (Turdo et al. 2019). Furthermore, in some tumors, chemotherapy has been shown to stimulate the division of CSCs, thus favoring tumor repopulation (Chen et al. 2012).

Mechanistic studies have indicated that dysfunction of various developmental-related signaling pathways may cooperate in the dysregulation of the self-renewal and differentiation that characterized CSCs (Matsui 2016). In addition to the involvement of these pathways in the regulation of CSCs, the evidence also suggests that stem cell properties can be acquired as a consequence of mutations and metabolic changes occurring in normal stem cells or differentiated cancer cells that move up the cancer cell hierarchy for their expression of pluripotent genes, making them more susceptible to epigenetic reprogramming. In this sense, many of the CSCs biomarkers identified have some role in cellular metabolism (Table 1). These metabolic changes, capable of inducing this reprogramming in CSCs in the context of a premalignant tumor, are collectively called "metabostemness"(Menendez and Alarcon 2014). This epigenetic reprogramming can also be achieved by the action of reactive oxygen species (ROS), mainly produced by cellular metabolism, establishing a relationship between metabolism and ROS as a central axis in the CSCs behavior control.

In this chapter we review the relationship between the main intracellular pathways controlling CSCs and the metabolic plasticity in this tumor subpopulation, focusing on the role that cellular redox state plays in the control of both

aspects, thus establishing a point of interconnection between stemness, ROS, and metabolism.

Cancer Stem Cell Signaling

It is well-known that over-activation or abnormal functioning of the intracellular pathways that control normal stem cells, participate in, or contribute to, the survival and maintenance of CSCs. The proper functioning of a normal stem cell requires fine control of these signaling pathways, which mainly include the pathways governed by JAK/STAT, Sonic/Hedgehog, WNT, Notch, PI3K/AKT, and NFkB (Matsui 2016) (Fig. 1). These pathways, highly regulated in normal stem cells, do not actually represent independent and linear intracellular pathways, but rather interlaced networks of signaling mediators that feed each other.

The **Sonic/Hedgehog pathway** (Fig. 1a) includes three ligands that are secreted (Sonic, Desert, and Indian), its Patched receptor, the transmembrane protein Smoothened, and three transcription factors (Gli 1-3). In the absence of ligand, the receptor acts by repressing the activity of the transmembrane protein, thus keeping the transcriptional activity inactive. The binding of the ligand to the receptor inhibits this repressive action, thus allowing the transcription of the target genes. Aberrant expression of members of this signaling pathway has been described for several tumors. In fact, an increased expression of some of its members has been described in the CSCs subpopulation, so that its inhibition results in a loss of stem cells properties (Merchant and Matsui 2010).

The **WNT pathway** (Fig. 1b) is a complex but highly conserved pathway in evolution that includes 19 ligands and more than 15 receptors. It includes two different, although not independent, pathways known as the canonical and the noncanonical pathway, dependent or independent of the transcriptional activity of β-catenin, respectively. In the absence of ligand, β-catenin levels are kept low by the action of a multiprotein complex responsible for its degradation (consisting of the axin, APC, casein 1, and GSK3B proteins). In the canonical pathway, when the ligand binds to the receptor, this complex is sequestered and anchored to the receptor-associated proteins so that the stabilization and thereby the transcriptional activity of β-catenin is allowed. The noncanonical pathway is activated by family receptors but does not involve the participation of β-catenin. Its function is essential to regulate the release of calcium from the endoplasmic reticulum (ER) to control intracellular calcium levels. Mutations in some of the components of the pathway are very frequent in tumors. In fact, depletion of some of its components in glioma cells inhibits their growth, produces their differentiation, and reduces their tumorigenic capacity (Borcherding et al. 2015).

The **Notch pathway** (Fig. 1c) includes several ligands and receptors, all of them transmembrane proteins, so that activation of the pathway takes place when a ligand expressed in one cell binds to a receptor expressed in the adjacent cell leading to the proteolytic excision of the receptor's cytoplasmic domain and the release of the intracellular domain. This intracellular domain translocates to the nucleus acting as a

transcription factor. This pathway is essential for the regulation of CSCs, although its role may be different, acting as a tumor promoter or suppressor in different cellular contexts (Ranganathan et al. 2011).

The **PI3K/AKT pathway** (Fig. 1d) activation is triggered after the binding of ligands to tyrosine kinase receptors. This binding triggers phosphorylation and activation of AKT kinase which can then mediate the activation of several effectors including mTOR. It is a highly conserved signaling pathway and is involved in numerous cellular processes such as proliferation or survival. It also plays an important role in the regulation of normal stem cells, participating in the self-renewal of embryonic cells or in the expansion and differentiation to different lineages of hematopoietic stem cells (Xia and Xu 2015). The main inhibitor of the pathway is PTEN phosphatase, which is usually inactivated by mutations in a large number of tumors. In fact, the loss of function of this phosphatase in hematopoietic stem cells is capable of triggering the development of myeloproliferative diseases and leukemia (Xia and Xu 2015). The pathway is over-activated in CSCs, participating both in the maintenance of these cells and in their capacity to stimulate neovascularization, acting as initiators of new vessel formation by promoting the secretion of pro-angiogenic factors (Xia and Xu 2015).

The **JAK/STAT pathway** (Fig. 1e) is initiated by the binding of several ligands – interleukins, interferon, hormones, and growth factors – to their respective receptors, inducing their oligomerization and the recruitment of JAK family proteins to their intracellular domains where they are phosphorylated and activated. These active proteins, in turn, induce phosphorylation and activation of STAT family proteins that can thus be translocated to the nucleus, acting as transcription factors. The pathway regulates numerous cellular processes in a large number of different tissues, including the maintenance of embryonic stem cells, hematopoiesis, or neurogenesis. Furthermore, aberrant activation has been described in CSCs of several tumors, including breast cancer, glioblastoma, prostate cancer, and hematological tumors (Stine and Matunis 2013).

The **NFκB family transcription factors** (Fig. 1f) are composed of dimers of five different proteins (p65, c-Rel, RelB, p50, and p52), which are normally inactive in the cytosol due to their binding to IkB proteins. NFκB can be activated by two signaling pathways, the classical and the alternative one. Classical activation, which can be triggered by numerous stimuli, is initiated by phosphorylation of IkB proteins, mediated by IKKα/IKKβ heterodimer, which leads to their proteolytic degradation, allowing the transcription factor release and translocation to the nucleus. In the alternative pathway, IKKα/IKKα homodimer phosphorylates protein p100, given as a result the formation of the mature p52 subunit (Karin 1999). It is actually a very complex pathway, extensively studied for its involvement in inflammation and the immune response, although it is also involved in other functions such as proliferation, survival, or cell differentiation. Unlike the other pathways, its role in regulating normal stem cells has not been widely studied, although there are data that indicate that the loss of their activity produces inhibition of self-renewal, as well as a decrease in the number of normal hematopoietic stem cells and abnormal differentiation into different lineages. Although there are some studies that indicate its participation in

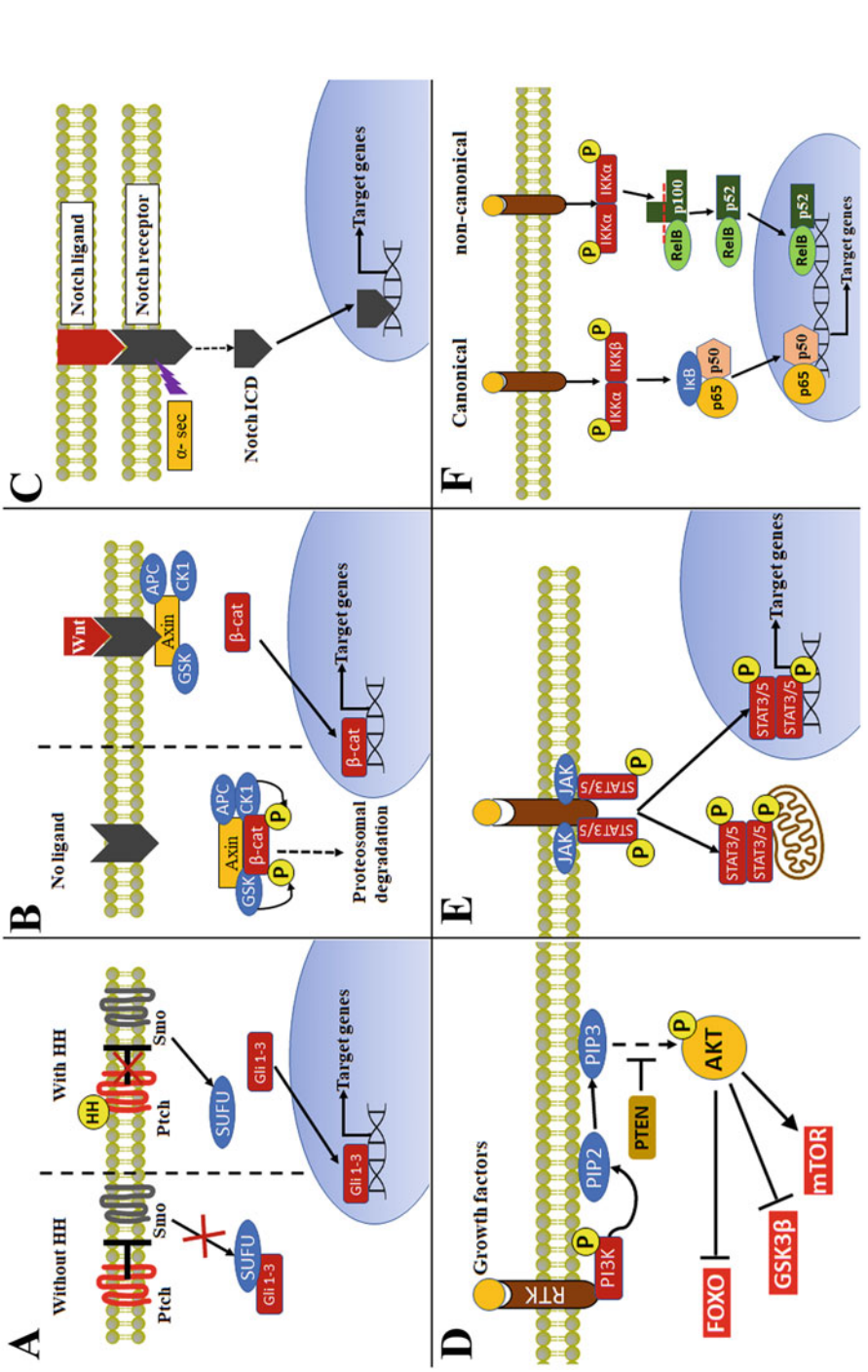

Fig. 1 **Cancer stem cell signaling pathways.** (**a**), ***Sonic/HH pathway***. Upon ligand (HH) binding to Patched receptor, repression of Smoothened is released allowing the transcriptional activity of Gli transcription factors. (**b**), ***WNT pathway*** (canonical). In the absence of ligand, β-catenin levels are kept low by the

self-renewal or the tumorigenic capacity of cancer cells – mainly in breast cancer – the relationship with the maintenance of CSCs is not yet entirely clear. Anyway, activating mutations of this pathway have been described for many types of cancer (Rinkenbaugh and Baldwin 2016).

As already mentioned, these are not independent linear pathways. There are studies that demonstrate the necessary cooperation of the Notch and WNT pathways in maintaining the undifferentiated state of intestinal tumor stem cells, or the cooperation of the pathways in the development of epidermal or pancreatic tumors and the increase in resistance to treatments in metastatic breast cancer, favoring the survival of tumor stem cells and thus tumor repopulation (Matsui 2016).

Cancer Stem Cell Metabolism

Genetic alterations, with mutations in oncogenes and tumor suppressor genes, and environmental modifications, such as hypoxia, converge in one of the traits that define tumor cells and that are in the spotlight for the design of new therapeutic strategies: metabolic reprogramming. In fact, metabolic adaptation is considered one of the hallmarks of cancer (Hanahan and Weinberg 2011). The main metabolic change in cancer is aerobic glycolysis or the Warburg effect, that moves from oxidative phosphorylation (OXPHOS) as a way of obtaining the necessary energy toward lactate production, even when there are normal oxygen concentrations, hence the name aerobic glycolysis. This allows to redirect metabolic intermediaries toward macromolecule biosynthesis pathways (much needed in highly proliferative cells such as tumor cells) (Jang et al. 2013). In this way, some intermediaries are diverted toward the pentose phosphate pathway (PPP), for the production of nucleotides and NADPH (necessary for the correct maintenance of the cellular redox state) and others toward the formation of glycerol 3 phosphate for lipid synthesis and toward the serine

Fig. 1 (continued) action of a multiprotein complex responsible for its degradation. After ligand binding to the receptor, the complex is anchored to the receptor-associated proteins allowing the release and stabilization of β-catenin and so the transcription of target genes. (**c**), *Notch pathway*. Activation of the pathway takes place when a ligand expressed in one cell binds to a receptor expressed in the adjacent cell leading to the proteolytic excision of the receptor's intracellular domain and its translocation to the nucleus. (**d**), *PI3K/AKT pathway*. Upon activation of tyrosine kinase receptors, AKT is phosphorylated and activated. AKT kinase is then able to phosphorylate several effectors such as FOXO, mTOR, or GSK3β. (**e**), *JAK/STAT pathway*. After ligand binding to the receptors, JAK family proteins are recruited to their intracellular domains where they are phosphorylated and activated. These active proteins in turn induce phosphorylation and activation of STAT family proteins that can thus be translocated to the nucleus, acting as transcription factors or to the mitochondria. (**f**), *NFκB pathway*. Classical activation (canonical) is initiated by phosphorylation of IkB proteins, mediated by IKKα/IKKβ heterodimer, which leads to their proteolytic degradation, allowing the transcription factor release and translocation to the nucleus. In the alternative pathway (noncanonical), IKKα/IKKα homodimer phosphorylates protein p100 given as a result the formation of the mature p52 subunit

synthesis for protein synthesis as well as nucleic acids, ATP and reducing power. Lactate is finally secreted out of the cell, generating acidification of the microenvironment that can benefit the aggressiveness of the cancer (Jang et al. 2013).

Normal stem cells have a greater glycolytic metabolism compared to differentiated cells derived from them. However, there are relatively few studies and quite a few discrepancies in relation to the metabolism used by CSCs. Some of them indicate that they preferably use aerobic glycolysis, while others show that they preferentially use mitochondrial oxidative metabolism (Peiris-Pagès et al. 2016). Thus, there is abundant literature that supports aerobic glycolysis as the main bioenergetic source in CSCs of various tumor types such as breast, colon, ovarian, or glioblastoma. In fact, it has been described that hypoxia in the tumor niche is a key determinant for the glycolytic metabolism in breast CSCs (Peiris-Pagès et al. 2016). Furthermore, the regression of the mitochondria toward a more immature state induces the epithelial-mesenchymal transition and the acquisition of stem properties (Guha et al. 2014). Similarly, glioblastoma CSCs generally use aerobic glycolysis as an energy source, showing a preference for hypoxic niches and a decrease in oxidative metabolism (Zhou et al. 2011). Same has also been described in CSCs derived from osteosarcoma, ovarian carcinoma, or colon cancer (Menendez et al. 2013).

However, other authors have also found CSCs that preferentially use OXPHOS, such us breast CSCs (Peiris-Pagès et al. 2016; Snyder et al. 2018). In fact, the inhibition of complex I of electron transport chain (ETC) partially inhibits the stemness in breast cancer. A marked oxidative profile in CSCs has been also described for glioblastoma, where CSCs can move from one to the other type of metabolism. Same occurs in ovarian or pancreatic cancer, where the dependence of CSCs on OXPHOS, as well as overexpression of genes that regulate mitochondrial function, have been described. However, the treatment of pancreatic CSCs with a mitochondrial inhibitor such as metformin is not effective against a proportion of the CSCs subpopulation, which can use either oxidative metabolism or aerobic glycolysis, suggesting the existence of cellular subgroups with great metabolic plasticity (Sancho et al. 2015).

These different data, even in the same tumor type, suggest that CSCs must have a more complex biochemical, molecular, and metabolic behavior than their non-tumor counterparts, showing great metabolic plasticity. Thus, metabolic type of CSCs would depend on the characteristics of the niche in which they are located (Sancho et al. 2016). At this point, it seems clear that CSCs can use both aerobic glycolysis and OXPHOS, depending on the state of differentiation, tumor microenvironment, or expression of certain oncogenes. There are several possible causes that could explain these divergent results, even within the same tumor type (Snyder et al. 2018).

On the one hand, due to this high plasticity, cells can be collected at different metabolic stages depending on the different laboratory protocols or in different niches of the tumor with different microenvironment, i.e., necrotic areas or hypervascularized areas. Thus, hypoxia promotes dedifferentiation and the maintenance of stem properties, increasing surface markers such as CD133 and at the same time, through the stabilization of hypoxia inducible factor (HIF1α), promotes aerobic glycolysis. Unlike what happens in normal stem cells, in which the niche maintains

the balance between self-renewal and differentiation, the tumor microenvironment necessary for the maintenance of CSCs is altered, with the signals that favor the proliferation (Li and Neaves 2006). The importance of the tumor niche in the formation and maintenance of CSCs has been well documented, demonstrating the influence of fibroblasts and epithelial cells residing in the niche, as well as the hypoxia that prevails within it (Li and Neaves 2006).

On the other hand, the possible different origin of CSCs must be taken into account. While this is unclear, it is now believed that CSCs could originate from transformation of differentiated tumor cells that move up in the cancer cell hierarchy or could derive from transformation of normal stem cells. Menendez et al. (Menendez et al. 2013; Menendez and Alarcon 2014) have proposed a very interesting hypothesis about the origin of CSCs, so that the generation of these cells by transformation of normal stem cells would depend on epigenetic processes controlled by intermediary metabolites that would regulate the expression of genes involved in the stemness. Therefore, cellular metabolism and nutrient availability would play a key role in activating enzymes that will modify histones and DNA and that will later lead to the different gene expression that will originate tumor cells with stem properties. This does not imply that the only origin of CSCs occurs always from transformation of normal stem cells since differentiated tumor cells can also be reprogrammed and acquire characteristics of stem cells by activating pathways not yet fully understood. These cells, which can be reprogrammed, would present different cellular states depending on genetic, epigenetic, metabolic, and extrinsic factors (tumor niche).

Thus, the reprogramming of the bioenergetic state of the tumor cell is considered as the metabolic change that defines the origin of the cancer (Menendez et al. 2013). Metabolic factors would be the key for transcription and signaling pathways programs necessary so that intrinsic or environmental factors can direct a particular cell toward a CSC state. Indeed, when transcription factors, oncogenes, or oncomiRNA are used to convert differentiated somatic cells to induced stem cells (iPSCs), these cells spontaneously form teratocarcinomas into nude mice (Blum et al. 2009), demonstrating the close relationship between reprogramming toward stem cells and tumorigenicity. These iPSCs recapitulate all the features of metabolic reprogramming that have been observed in tumor cells, including the appearance of immature mitochondria and low levels of oxidative stress. Therefore, the bioenergetic characteristics of the cells change, going from the use of OXPHOS to aerobic glycolysis. When they differentiate again, the cells reacquire the mitochondrial bioenergetic profile. This means that the ability to develop an anabolic or Warburg-like metabotype would represent a crucial early molecular event that would suppose an a priori barrier to the transformation process of differentiated somatic cells to CSCs. In short, factors present in the tumor niche, such as different cell types, hormones, growth factors, oxygen levels, and metabolites, can regulate epigenetic activity and gene transcription, leading to reprogramming that leads to the transformation of differentiated tumor cells in CSCs. On the other hand, subsequent metabolic changes may be responsible for the characteristics of CSCs within a tumor, with tumor metabolism being one of the hallmarks of cancer (Hanahan and Weinberg

2011). Thus, tumor metabolism has gone from being an important event in the development and progression of cancer, to being probably the determining factor.

As mentioned above, epigenetic reprogramming plays a key role in metabolic changes and in the origin and maintenance of CSCs. Metabolic adaptations that occur after tumor transformation cause epigenetic changes that in turn regulate tumor metabolism and contribute to tumor progression. Thus, some glycolytic enzymes such as pyruvate kinase M2, GAPDH, or LDH translocate to the nucleus to perform non-metabolic functions such as regulating gene transcription and epigenetic modifications (Yu et al. 2018). Similarly, enzymes that participate in other metabolic processes (lipid or nucleotide synthesis) such as ACLY or ACSS2 for the synthesis of ACo A for histone acetylation or that participate in the synthesis of SAMs for DNA methylation have been localized to the nucleus in various tumors (Yu et al. 2018). On the other hand, the enzymes that catalyze DNA and histone modifications use metabolites and coenzymes that come from glycolysis, TCA, and other metabolic pathways, for its catalytic reactions (Yu et al. 2018).

Because of the epigenetic modifications caused by metabolic adaptations, the methylation state of DNA or the activity of histones are modified, which in turn regulate metabolic plasticity in tumor cells. Thus, increased expression of histone demethylases observed in several tumors are recruited into the promoters of various genes involved in glycolysis, causing demethylation of histones and activation of their transcription. Similarly, mutations in various histone deacetylases (HDACs) increase aerobic glycolysis or glutamine metabolism (Miranda-Gonçalves et al. 2018).

Although the role of epigenetic regulation in CSCs is not yet fully resolved, there are important data that indicate a clear interrelation between the epigenetic state and the origin and maintenance of the stem cell properties. Thus, H1.0 linker histone has been shown to be critical in the self-renewal of CSCs of various tumor types. When the gene that encodes it is repressed due to the methylation of the promoter, genes related to stem properties are expressed, which correlates with the aggressiveness of the tumors (Wainwright and Scaffidi 2017; Li et al. 2019). Chromatin remodeling by the EZH2, BMI1, and SUZ12 polycomb-group proteins, which leads to the silencing of genes through modifications of histones, is a specific trigger for stemness (Wainwright and Scaffidi 2017; Li et al. 2019). On the other hand, suppression of gene expression by histone demethylase LSD1 is essential for the proliferation of pluripotent tumor cells, while it is not relevant in the proliferation of non-pluripotent tumor cells or normal somatic cells (Wainwright and Scaffidi 2017; Li et al. 2019). Genes related to pluripotency and self-renewal have also been shown to be hypomethylated in CSCs (Wainwright and Scaffidi 2017). Epigenetic regulation may also be responsible for changes in cellular metabolism that lead to the acquisition of stem properties. As an example, inhibition of fructose 1-6 biphosphatase 1 (FBP1) expression (specific enzyme of gluconeogenesis) by methylation of the promoter, induces aerobic glycolysis, decreased consumption of oxygen and ROS production, which results in an increase in cancer-stem like properties and tumorigenicity in breast cancer cells (Dong et al. 2013). Thus, the interrelation between metabolism and epigenetic status contributes to the plasticity of CSCs and to tumorigenicity.

As will be discussed later, metabolic adaptations are not only regulated at the epigenetic level but many of the signaling pathways involved in the CSCs regulation also participate in the control of cellular metabolism. For example, the PI3K/AKT pathway, which establishes a point of convergence for many of the essential pathways for CSCs, stimulates aerobic glycolysis, which can ultimately affect intracellular ROS levels and tumorigenesis.

Thus, it seems clear that CSCs are not a fixed population, but that their metabolic phenotype can be modified moving from aerobic glycolysis to OXPHOS by the action of many factors of the microenvironment such as growth factors, inflammatory signals, or by interaction with stromal cells, for example.

Redox Regulation in Cancer Stem Cells

Reactive oxygen species (ROS) can be endogenously generated by various oxidases and peroxidases in different cellular compartments such as cell membranes, peroxisomes, or the ER, although the main endogenous source is the mitochondria through the ETC. Initially considered as by-products of cellular metabolism that were harmful to cells, it is currently well known that low or moderate levels of ROS promote cell proliferation and survival acting as second messengers, while only high levels can cause cytotoxicity and trigger cell death. Thus, ROS are involved in the physiological regulation of many biological processes related to cell development at different levels, from gene expression, signal transduction to protein–protein interactions (Martin and Barrett 2002). Maintaining a fine balance between production and removal is therefore essential. To do this, cells have powerful and complex antioxidant systems that include the enzyme superoxide dismutase (SOD), catalase, peroxyredoxins (PRX), thioredoxins (TRX), glutathione peroxidase (GPX), and glutathione reductase (GR). The GPX enzyme breaks down hydrogen peroxide into two water molecules using glutathione (GSH), one of the most abundant antioxidant molecule in cells (Martin and Barrett 2002).

Tumor cells have higher ROS levels than their normal counterparts. This increased ROS levels favors tumor promotion and progression by increasing proliferation, survival, and metastasis. Similarly, there are increasing evidence suggesting an important role for ROS and redox signaling in the functioning of CSCs (Lee et al. 2019). In acute lymphoblastic leukemia, the population of $CD44^+$ cells with low levels of ROS has been found to be a tumor-initiating cells enriched subpopulation. Furthermore, there is a correlation between the frequency of CSCs and the expression levels of GPx3 (a ROS scavenger enzyme). Thus, ROS-inducing treatments such as disulfiram (an aldehyde dehydrogenase inhibitor) kill the stem cell population by inhibiting nuclear factor erythroid 2-related factor 2 (Nrf2) activity and activating the JNK pathway. Similar results have also been described in hepatocellular carcinoma, where disulfiram reduces the population of CSCs by increasing cellular ROS levels and activating the p38 MAPK pathway (Lee et al. 2019). However, although many CSCs appear to prefer a low ROS environment, this does not occur in all cases. In fact, $CD133^+$ glioblastoma CSCs have higher ROS levels than non-CSC cells (Lee et al.

2019). Similarly, breast CSCs have been reported to have higher levels of ROS than non-CSCs due to an increase in mitochondrial biogenesis (Lee et al. 2019). In other words, although a preference for low levels of ROS has been described by CSCs, this does not always occur. This divergence agrees with the discordant data regarding the basal metabolism of CSCs, which could indicate a relationship between both aspects. Thus, a preferential glycolytic metabolism would be related to the maintenance of low ROS levels, whereas a preferentially mitochondrial metabolism would be related to an increased production of ROS. In any case, maintenance of low ROS levels does not always correspond to a preference for glycolytic metabolism. In fact, leukemic CSCs have been described to have low levels of ROS but are surprisingly dependent on OXPHOS for survival and maintenance of the quiescent state (Lagadinou et al. 2013). Therefore, the use of the glycolytic pathway or the mitochondrial pathway by CSCs depending on the state they are in, quiescent or proliferative, is critical in order to maintain energy needs and redox balance, establishing a relationship between ROS and metabolic plasticity. As an example, it has been described that quiescent breast CSCs have a high metabolic rate of the PPP, which favors the generation of reducing power (NADPH), essential for the maintenance of the state cellular redox (Debeb et al. 2016).

In any case, a fine regulation of the cellular redox state is essential for the maintenance of CSCs, so that these cells have a powerful antioxidant system that is finely controlled by the hypoxic niche in which they develop, as well as by other factors such as transcription factors of the FOXO family or Nrf2, or other oxidative stress sensors such as Ataxia Telangiectasia Mutated kinase (ATM) (Wang et al. 2013).

CSCs can promote the synthesis of GSH due to the increased import of cysteine from the extracellular medium. Thus, there is a decrease in ROS that inhibit the activation of the p38/MAPK intracellular pathway, preventing differentiation and apoptosis (Ding et al. 2015). Along with GSH, thioredoxin metabolism is the other main mechanism of elimination of hydroperoxides that also plays a key role in increasing radiation resistance in CSCs (Ding et al. 2015).

Regulation of ROS levels can also be done through transcription factors such as NFκB and Nrf2. NFκB pathway participates in maintaining self-renewal in CSCs. In fact, its inhibition causes a decrease in the size of the CSCs population (Ding et al. 2015). In AML stem cells, treatment with partenolide (NFκB inhibitor) produces an increase in ROS, activation of p53 and triggers a cell death process that can be prevented by antioxidant compounds (Rinkenbaugh and Baldwin 2016). On the other hand, Nrf2 is considered the master regulator of the antioxidant response since it controls the expression of many detoxification and antioxidant genes. Maintaining a low oxidative microenvironment through the activation of Nrf2 favors the development of quiescent CSCs. If these cells suffer an increase in oxidative stress, they differentiate into proliferative cells that support higher levels of ROS and that will also have Nrf2 activated, which will allow them to continue growing and invading tissues (Ding et al. 2015).

Ataxia Telangiectasia Mutated kinase (ATM), a master regulator of DNA damage, has also been postulated as one of the main modulators of the response to

oxidative stress and mitochondrial homeostasis. Defects in ATM cause an increase in ROS in hematopoietic stem cells and the loss of self-renewal that can be reversed by treatment with antioxidant compounds (Wang et al. 2013). Furthermore, the intracellular cascade of ATM has been described to be increased in CSCs compared to normal tumor cells, and treatment with ATM inhibitors reverses resistance to radiological treatments, denoting the importance of this kinase in CSCs (Wang et al. 2013).

Finally, there is also a relationship between ROS and the epigenetic state of cells at a given time. Thus, SAM synthetase enzymes are dependent on the cellular redox state, so that an oxidizing environment reduces their activity. Furthermore, methionine synthetase, which participates in the methionine cycle for its recycling, is dependent on cobalamin (vit B12), whose oxidation inactivates the enzyme. These data suggest that the oxidative state of the tumor cell environment could lead to hypomethylation and hence the activation of oncogenes. On the other hand, it is known that ROS cause DNA damage by oxidizing guanine and producing 8-oxoguanin (8-OG), which has great mutagenic capacity. If 8-OG formation occurs on a CpG island, binding to DNMTs and thus methylation is inhibited, it leads to DNA hypomethylation in those areas (Hitchler and Domann 2012).

Intracellular Signaling – Redox State Crosstalk: Metabolism Interplay

Oncogenic transformation, mitochondrial dysfunction, and alterations in cell signaling pathways in tumors cause an increase in ROS, which at low or moderate levels are capable of modulating a wide variety of intracellular signaling pathways, transcription factors, phosphatases, or kinases such as JAK/STAT, MAPKs, PI3K/AKT, NFkB, Nrf2, FOXO, ATM, HIF1α . . . with the consequent stimulation of survival, proliferation, and differentiation (Ding et al. 2015). Furthermore, many of the intracellular pathways and transcription factors implicated in the maintenance of CSCs also participate in the control of the redox state in these cells, thus establishing a positive feedback mechanism (Ding et al. 2015). A further level of complexity should be added since it must be borne in mind that cellular metabolism is the main source of ROS and many of these intracellular pathways are in turn involved in the control of cellular metabolism.

Regulation of the **PI3K/AKT pathway** in CSCs can be mediated by ROS (Fig. 2, #1), so that they are capable of inducing AKT activation or can inhibit the activity of PTEN (main inhibitor of the pathway). In turn, activation of the pathway can regulate ROS levels in CSCs through the regulation of one of its targets, the transcription factor FOXO. This transcription factor has been described to be essential for maintaining of self-renewal in hematopoietic stem cells through the up-regulation of the expression of the antioxidant enzymes catalase and manganese-SOD. Furthermore, FOXO deficiencies increases ROS production and lose of the quiescent status of CSCs (Miyamoto et al. 2007). On the other hand, PI3K/AKT pathway is considered one of the master regulators of aerobic glycolysis. AKT

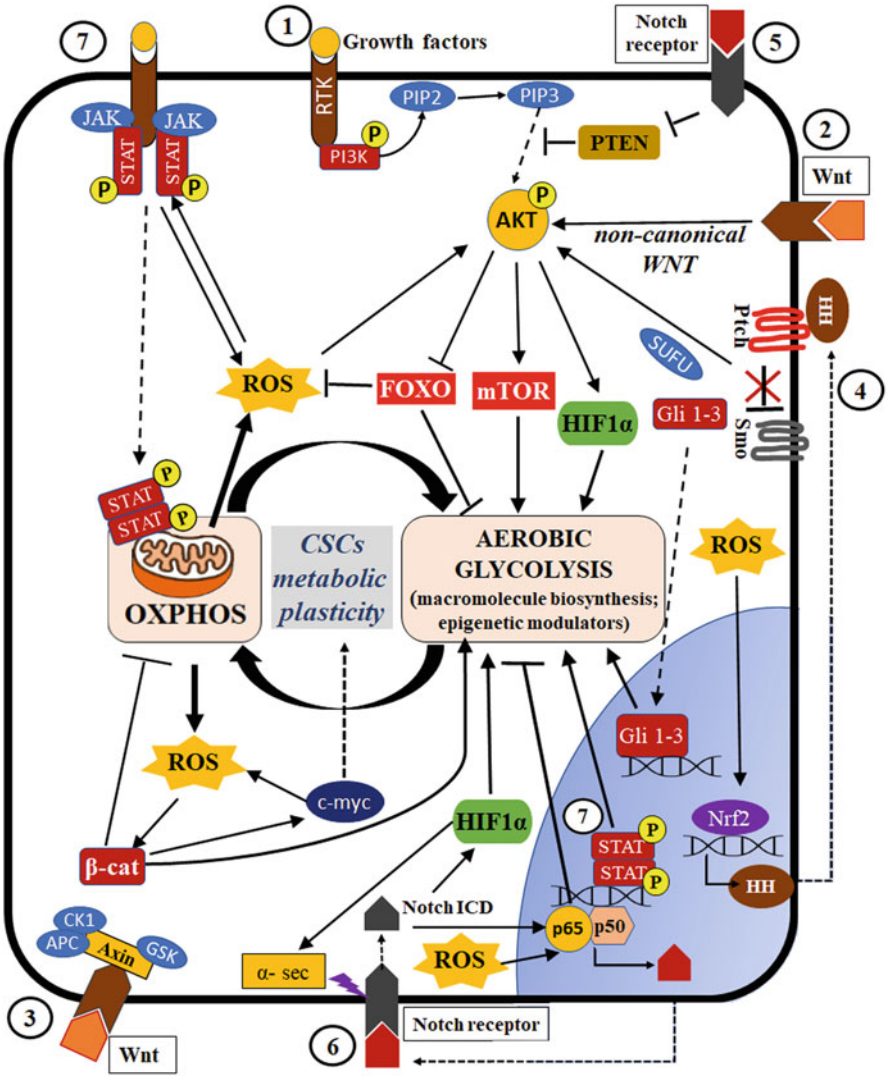

Fig. 2 **Crosstalk between CSCs signaling pathways, redox state, and metabolism. 1:** activation of PI3K/AKT pathway, that can be mediated by ROS, inhibits FOXO activity, and stimulates mTOR and HIF1α, leading to a stimulation of aerobic glycolysis. Moreover, FOXO also participates in the regulation of cellular redox state reducing ROS levels through the upregulation of antioxidant enzymes expression. **2:** the activation of PI3K/AKT pathway can also be achieved by WNT noncanonical pathway (β-catenin independent). **3:** on the other hand, β-catenin transcriptional activity can be stimulated by ROS, leading to an increase in aerobic glycolysis and an increase in c-Myc expression, which in turns plays an essential role in the metabolic plasticity of CSCs regulating several metabolic pathways such us aerobic glycolysis, glutaminolysis, or lipid synthesis. PI3K/AKT pathway can be also activated by other ROS-stimulated CSCs signaling pathways such us Sonic/HH or Notch. **4:** ROS activate transcription factor Nrf2 that induces the expression of HH triggering activation of Sonic/HH pathway. Through the transcription activity of

increases gene expression of glucose transporters; increases the phosphorylation of key enzymes in glycolysis, such as hexokinase and phosphofructokinase 2; inhibits FOXO transcription factors, which will result in changes in gene expression that favor aerobic glycolysis; and activates mTOR, which promotes the translation of messenger mRNA and synthesis of macromolecules (Elstrom et al. 2004). PI3K/AKT pathway also induces HIF1α, which is considered the master regulator of aerobic glycolysis (Elstrom et al. 2004).

Furthermore, the AKT signaling pathway represents a point of convergence with other important intracellular pathways for CSCs such as the **WNT pathway**, which can also be regulated by ROS (Fig. 2, #3). Transcriptional activity of β-catenin can be regulated by ROS (Bowerman 2005). It has been described that nucleoredoxin, an antioxidant protein of the thioredoxin family, enhances the activation of the canonical WNT pathway. In the same way, ROS enhances β-catenin-FOXO interaction, inducing a more differentiated state, decreasing tumorigenicity and pluripotency of cells (Bowerman 2005). Recent studies show that the activity of the WNT pathway plays a key role in the regulation of cellular metabolism, although this role will be different depending on the cellular context (Sherwood 2015). WNT pathway can stimulate both mitochondrial metabolism and aerobic glycolysis, at least in normal cells. A large number of genes involved in cellular metabolism have been described to be transcriptional targets of the pathway, including genes that regulate the metabolism of glucose, glutamine, or fatty acids (Sherwood 2015). In tumor cells, canonical WNT signaling stimulates aerobic glycolysis in several tumors, increasing the transcription of genes such as PDK1 or lactate transporters (Sherwood 2015) or inhibiting transcription of genes involved in the ETC, respectively (Sherwood 2015). Moreover, c-Myc is among the transcriptional targets of β-catenin. The proto-oncogene, usually dysregulated in tumor cells, coordinates various biological processes in CSCs such as cellular metabolism, redox homeostasis, self-renewal, differentiation, and growth. c-Myc has been shown to be highly expressed in CSCs from glioblastoma and necessary for the maintenance of the glycolytic

Fig. 2 (continued) Gli1-3, the pathway stimulates aerobic glycolysis. **5:** activation of Notch induces a decrease in the expression of PTEN, which in turn activates PI3K/AKT. **6:** Notch also establishes connections with other important intracellular regulators of cellular metabolism. Thus, there is a crosstalk between Notch and HIF1α. On the one hand, Notch stimulates HIF1α transcriptional activity leading to an increase in aerobic glycolysis. And on the other hand, HIF1α increases the proteolytic processing of Notch receptor, leading to the activation of the pathway. Notch activation also results in NFκB activation giving rise to positive feedback mechanisms since NFκB transcriptional activity increases Notch ligand expression. On the other hand, NFκB, which is a well-known redox sensitive transcription factor, has been classically described to inhibit aerobic glycolysis and stimulate OXPHOS, although this effect varies depending on tumor type. **7:** finally, ROS can stimulate JAK/STAT signaling pathway in a positive feedback loop since activation of the pathway increases intracellular ROS levels. Once stimulated, the pathway can exert different effects on cellular metabolism depending on the subcellular localization of activated STAT3/5. Thus, translocation to the mitochondria leads to upregulation of OXPHOS, while translocation to the nucleus upregulates aerobic glycolysis

phenotype. It stimulates the transcription of several genes involved in the regulation of glycolysis, in oxidative phosphorylation, glutaminolysis, or lipid synthesis, among others (Dang et al. 2009). This convergence between c-Myc and WNT leads to the overexpression of key proteins that stimulate glycolysis such as glucose transporters, LDH, or the enzyme PKM2 (which catalyzes the last step of glycolysis) (Dang et al. 2009). Noncanonical WNT activation also regulates glucose metabolism in tumor cells, being its crosstalk with the AKT-mTOR signaling pathway the main mechanism implicated (Fig. 2, #2). In this sense, β-catenin independent WNT activation leads to the activation of mTOR, a master regulator of cell metabolism. This regulation of mTOR occurs at the level of GSK3β, which phosphorylates and activates TSC proteins (mTOR inhibitors). Thus, noncanonical activation of WNT leads to phosphorylation and activation of AKT, which in turn phosphorylates and inhibits GSK3β, resulting in stimulation of mTOR and thereby stimulation of glycolytic metabolism (Inoki et al. 2006). This mechanism of stimulation of glycolytic metabolism mediated by noncanonical WNT activation has been described for several tumors such as prostate cancer or esophageal carcinoma.

In addition to the crosstalk with the WNT pathway, AKT also established interconnection with other stem cell pathways such as Sonic/Hedgehog and the Notch pathway (Hales et al. 2014; Sun et al. 2017).

Sonic/Hedgehog can also be activated by ROS in CSCs by an indirect mechanism (Fig. 2, #4). Thus, in hepatocarcinoma CSCs, ROS activate the transcription factor Nrf2 that directly interacts with the Sonic hedgehog (HH) promoter triggering activation of the pathway (Wing Leung et al. 2020). Through the activity of the Gli, the Sonic/HH pathway induces a metabolic change stimulating aerobic glycolysis. In fact, it has been described that in SHH-medulloblastoma (a type of tumor caused by mutation of the pathway) there is an increased expression of hexokinase 2 and pyruvate kinase M2 and an increased production of lactate, indicators of a Warburg-type metabolism. These changes are responsible, at least in part, for the maintenance of the undifferentiated and self-renewal state of CSCs in this tumor type. Furthermore, treatment with glycolysis inhibitors reduces HH-induced cell proliferation (Di Magno et al. 2014).

Activation of **Notch** produces a decrease in the expression and activity of PTEN and an increase in phosphorylation and activation of AKT (Fig. 2, #5), which is accompanied by an increase in glucose uptake and glycolysis (Hales et al. 2014). The Notch pathway also establishes connections with other important regulators of cellular metabolism (Fig. 2, #6). In this sense, there is a crosstalk between the Notch pathway and HIF1α. On the one hand, it has been described that HIF1α directly interacts with the Notch intracellular domain, participating in the transcription of Notch-dependent genes and thus blocking the differentiation of normal stem cells. In fact, there is a positive correlation between HIF1α and Notch activity levels (Gustafsson et al. 2005). On the other hand, it has also been described that the Notch pathway stimulates the transcriptional activity of HIF1α and thus aerobic glycolysis. Upregulation of HIF1α, that can be triggered by several pathways, has been demonstrated to favor self-renewal and maintain the redox balance in CSCs through the increase of the glycolytic pathway, decreasing the flow into the

mitochondria, which favors low ROS levels (Soeda et al. 2009). Moreover, Notch pathway is capable of regulating ROS levels in the CSCs population in a feedback mechanism, since ROS are in turn capable of stimulating the activity of the Notch pathway (Qiang et al. 2012).

Notch signaling also leads **NFκB** activation in a crosstalk between both since activated NFκB also upregulate the expression of Notch ligands (Fig. 2, #6), promoting the production of CSCs by activating Notch signaling pathway (Moriyama et al. 2018). On the other hand, the interrelation between NFκB and ROS is a well-described and well-known fact. ROS- dependent NFκB activation plays an important role in inducing the expression of a wide variety of factors that promote cell survival and prevent cell death in tumor cells. NFκB can activate intracellular cascades that culminate in a decrease in ROS and thus favor the development of quiescent CSCs (Rinkenbaugh and Baldwin 2016). Also, the NFκB pathway has been reported to help maintain CSCs self-renewal. Its inhibition causes a decrease in the CSCs population and also in the expression of stem cell markers such us CD44, Nanog, and Sox, among others (Rinkenbaugh and Baldwin 2016). Several cytokines regulate NFκB signaling which in turns controls the expression of a variety of other cytokines that are essential for CSCs function. Moreover, NFκB signaling has been described to play a key role in the interaction of CSCs and the microenvironment. It has to be noticed that CSCs subpopulation occupy certain niches within the tumors and that the interaction with the niche microenvironment are essential for CSCs maintenance (Rinkenbaugh and Baldwin 2016).

Furthermore, NFκB plays a key role in the metabolic adaptation of tumor cells. Activation of NFκB increases mitochondrial respiration and inhibit aerobic glycolysis in mouse embryonic cells. Thus, NFκB is controlling the balance between the use of aerobic glycolysis and mitochondrial respiration (Mauro et al. 2011). This regulation can vary depending on the tumor type, so that while in some tumors NFκB acts by activating mitochondrial respiration, in others it has been clearly described that it is capable of enhancing aerobic glycolysis by increasing the expression of glucose transporters or key glycolytic enzymes such as hexokinase 2 or pyruvate kinase M2 (Mauro et al. 2011). These variations are due in part to the pathway involved in each case, so that in general, the classical pathway would act by promoting aerobic glycolysis, while the alternative pathway would act by promoting mitochondrial respiration. On the other hand, aerobic glycolysis is in turn able to stimulate the activation of NFκB by establishing a reciprocal crosstalk.

In this regard, it is common for NFκB signaling to work in coordination with other pathways, such as those regulated by p53 or JAK/STAT3. In fact, constitutive activation of NFκB and STAT3 has been reported in glioblastoma CSCs to regulate the expression of a variety of target genes that lead to activation of the Notch pathway, again demonstrating that these intracellular pathways essential for the control of CSCs are not independent but interconnected pathways and that the final effects depend on a fine regulation of them.

JAK/STAT signaling pathway is primarily involved in inflammation, survival, and proliferation by activating transcription factors of the STAT family. Of these

proteins, STAT3 and STAT 5 constitute the most relevant members in tumors, being overexpressed in a large number of tumor types (Rane and Reddy 2000). ROS can regulate the activation of these transcription factors both positively and negatively by a direct or indirect mechanism through the regulation of tyrosine kinases or tyrosine phosphatases pathways (Fig. 2, #7). Furthermore, the cellular redox state may later be regulated as a consequence of activation of the pathway. Thus, it is well known that once activated, STAT3 can migrate to the mitochondria and stimulate the ETC, increasing the production of ROS. On the other hand, STAT3 activation and translocation to the nucleus stimulates aerobic glycolysis. In other words, STAT3 effect on cellular metabolism will depend on its subcellular location, either in the mitochondria or in the nucleus, which is ultimately determined by the residue that is phosphorylated for activation (Linher-Melville and Singh 2017). These different effects according to the subcellular location will also determine a differential action on the cellular redox state. The stimulation of the ETC leads to an increase in the production of ROS, while among the nuclear targets of STAT3 are some antioxidant enzymes such as SOD that determine a decrease in intracellular ROS (Linher-Melville and Singh 2017). Contrary to STAT3, the mitochondrial localization of STAT5 marks a shift from metabolism to an aerobic glycolytic one, mediated by overexpression of HIF2α, an isoform of HIF closely related to HIF1α that was identified in hematopoietic stem cells. Thus, HIF2α stimulates the expression of glycolytic genes. In fact, the inhibition of this HIF2α reduces the expansion and frequency of hematopoietic stem cells (Fatrai et al. 2011).

In summary, main intracellular pathways controlling CSCs can be regulated by ROS and act in a coordinated way to control key functions in this tumor subpopulation, including metabolic plasticity. This fine control allows them to move from aerobic glycolytic to mitochondrial metabolism and vice versa, in order to cover the needs of every moment (from quiescent status to a proliferative and differentiated one).

Conclusions

CSCs are highly resistant to conventional chemotherapy or radiotherapy and are mainly responsible for tumor relapse in patients. CSCs not only have the ability to initiate a tumor, but also have greater aggressiveness and ability to metastasize. Therefore, cancer-targeted treatments must be able to destroy this cell population in addition to the tumor mass. However, plasticity of CSCs represents a problem in the development of therapeutic options since multiple phenotypes within a single tumor may appear. Thus, a single given therapy will always fail to kill some of the CSCs. For that reason, approaches targeting plasticity of CSCs would be more effective (Das et al. 2020).

As previously mentioned, there is great heterogeneity in the metabolic phenotype of CSCs, even in the same type of tumor. Thus, CSCs can modify their metabolic phenotype according to their needs (Peiris-Pagès et al. 2016). This metabolic plasticity offers advantages to CSCs, including chemoresistance and ability to metastasize, making it a potential target for CSCs eradication. Oxidative

metabolism-dependent cells would respond better to mitochondrial respiration inhibitors while cells most dependent on glycolytic metabolism would respond better to glycolysis inhibitors. Several FDA approved drugs have been shown to affect CSCs in both options (Jagust et al. 2019). However, due to the great metabolic heterogeneity and plasticity present in tumors, combined treatment in which a greater variety of metabolic pathways are affected result much more effective (Jagust et al. 2019). Therapeutic strategies against CSCs targeting different pathways such as lipid, amino acid, or ketone metabolism have been also contemplated with mixed results (Jagust et al. 2019). Heterogeneous microenvironment conditions such as hypoxia, glucose deprivation, or low pH constitutes one of the main sources of metabolic adaptations in CSCs and is regulated by several factors including the HIF1-2 master regulator, making it an interesting therapeutic target for which various compounds have been developed (Das et al. 2020).

In addition to metabolic plasticity, the cellular redox state (which have a great dependence on cell metabolism), also plays an essential role in CSCs, which usually prefer a low ROS environment. To achieve low levels of ROS, CSCs rely primarily on GSH redox system. Thus, blocking GSH synthesis could be an interesting therapeutic strategy to eliminate the CSC population. Treatments against SOD or GPX have also shown an improvement in CSCs response to conventional therapies (Jagust et al. 2019). In the same way, several compounds against Nrf2, the master regulator of the antioxidant response, have been tested (Jagust et al. 2019; Kahroba et al. 2019).

Finally, the dependence of CSCs on epigenetic regulators both for the origin and for the maintenance and plasticity (Menendez et al. 2013) open the door for using epigenetic modulators as therapeutic strategies (Das et al. 2020).

In summary, targeting CSCs plasticity seems to be the key to eliminate this population. However, since this plasticity is regulated by different interconnected mechanisms, the therapeutic approach should be oriented to the development of combined therapies that target more than one CSC property at the same time.

Cross-References

- ▶ Implications of ROS in Cancer Stem Cells Mechanism of Action
- ▶ Reactive Oxygen Species-Dependent Signaling Pathways in Cancer Stem Cells
- ▶ Targeting Redox Signaling and ROS Metabolism in Cancer Treatment
- ▶ Two-Faced Role of ROS in the Regulation of Cancer Cell Signaling

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