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EDITORIAL

- 1145 Understanding leukemic hematopoiesis as a complex adaptive system

Thomas X

REVIEW

- 1150 Common stemness regulators of embryonic and cancer stem cells

Hadjimichael C, Chanoumidou K, Papadopoulou N, Arampatzi P, Papamatheakis J, Kretsovali A

- 1185 Therapies targeting cancer stem cells: Current trends and future challenges

Dragu DL, Necula LG, Bleotu C, Diaconu CC, Chivu-Economescu M

Contents

World Journal of Stem Cells
Volume 7 Number 9 October 26, 2015

ABOUT COVER

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Understanding leukemic hematopoiesis as a complex adaptive system

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Abstract

Normal and abnormal hematopoiesis is working as a complex adaptive system. From this perspective, the development and the behavior of hematopoietic cell lineages appear as a balance between normal and abnormal hematopoiesis in the setting of a functioning or malfunctioning microenvironment under the control

of the immune system and the influence of hereditary and environmental events.

Key words: Acute leukemia; Complex adaptive system; Stem cells; Hematopoiesis; Treatment

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Core tip: Complex adaptive systems are self-organizing systems involved everywhere. In bone marrow, cell lineages are working as a complex adaptive system. While the standard leukemia stem cell theory postulates that rare stem cells with self-renewal capacities are able to give rise to partially differentiated progenies, leukemia appears as a breakdown of the existing biological order under the influence of genetic and environmental factors, more than only the presence of abnormal cells.

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Complex adaptive systems are self-organizing systems involved everywhere in all disciplines^[1]. They have in common the emergence of self-organization on the macro-scale from micro-scale interactions of the agents contributing to the system. Complex adaptive systems share common traits of which: (1) simple rules of interaction potentially leading to self-organization when a group of individuals achieve a certain size; (2) the complexity is only at a macro level, individuals are ignorant of the overall organization since simple rules regulate local interactions between individuals and their environment; (3) local self-organization fails to emerge; and (4) interactions between agents or agents and their

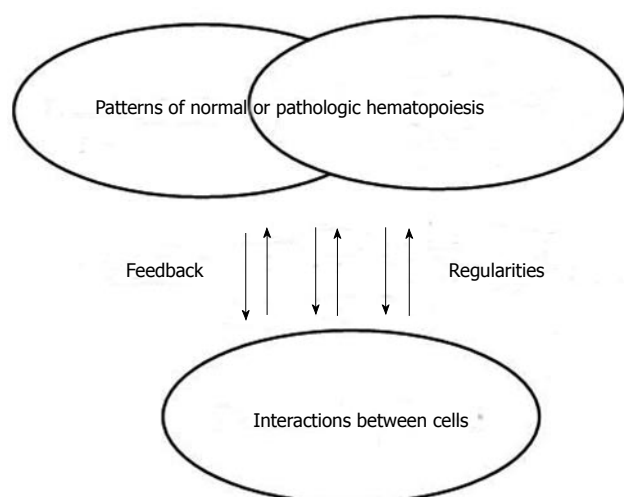


Figure 1 Normal and pathologic hematopoiesis as complex adaptive system.

environment form negative feedback loops leading to adapted responses, maintaining the complexity of the system. Although hematopoiesis is classically regarded as a linear process, stem cell processes and cell lineages that arise from them, are working within the framework of an adaptive complex network^[2,3]. Bone marrow cells share many of the above defining characteristics. They only respond to local signals and interactions through feedback loops, have no action on the entire organism and can work independently of the whole hematopoietic system.

Despite intensive studies and analyses to complete the picture, malignant hemopathies did not always behave as expected. It is apparent that pathologic hematopoiesis (like normal hematopoiesis) is working according to a very different set of rules to cause and effect than those commonly admitted. Complexity theory, which has emerged in all disciplines and can be also considered for hematopoiesis, is based on relationships, emergence, patterns and iterations (Figure 1). All cells present in the hematopoietic system should be considered as involved in that system. All interactions and connections between those cells are not predictable and not planned. A pattern will emerge from cell interactions, will feedback on the hematopoietic system and will inform the interactions of the various agents leading to the establishment of a new balance.

Our understanding of the pathogenesis of acute leukemia has evolved over the years. Leukemic hematopoiesis appears to retain some semblance of its normal counterpart, and also works as a complex adaptive system. Actually, the first one derives from the second one, and both normal and abnormal systems can coexist at variable functional, qualitative, and quantitative intensities under the influence of inner and outer factors (Figure 2). Most systems are not unique, but are within other systems and participate to those bigger systems. In the standard leukemia stem cell theory, rare stem cells with self-renewal capacities

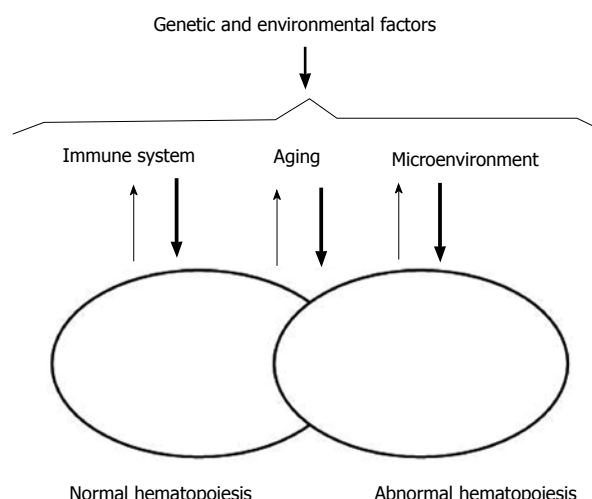


Figure 2 Factors influencing interactions between normal and abnormal hematopoiesis.

can give rise to partially differentiated progenies which will constitute the bulk of leukemia^[4,5]. However, this view of leukemia hematopoiesis reveals only dominant pathways and do not take into account the alternate pathways representative of a complex adaptive system. It is based on linear relationships which imply precise solutions. Actually, a hierarchy of command and control does not exist. Nothing is planned or managed. The hematopoietic system is continually self-organizing to find the best fit with the microenvironment. Cells interact through the process of emergence and feedback apparently at random with non-linear relationships and only approximate solutions. Patterns emerge from these interactions and will influence the behaviour of these cells within the system and the hematopoietic system itself.

The ways in which the cells interact to one another is critical to the survival of hematopoietic cell lineages. Patterns are formed from these connections as well as the feedback. Connections between the cells appear generally more important than the cells themselves. The bulk leukemia cells are supposed to possess limited proliferative potential and to have few capacities for initiating or maintaining the disease. Eradicating these cells is therefore not enough to cure the disease, while drug-resistant leukemia stem cells will re-populate the bone marrow and cause leukemia relapse^[6,7]. The potential emerging patterns are numerous, even if the rules governing the overall function of the system remain quite simple. The total number of mutations potentially found in leukemia cells is quite large. However, few mutations commonly occur in coding regions and are relevant to disease pathogenesis. Any leukemic cell has relatively few of these recurring mutations^[8,9]. Small changes in an initial condition can have significant effects. In a hematopoietic stem cell, clonal expansion starts with the occurrence of an "initiating" mutation. Such a mutation might be a translocation or a coding sequence mutation. Within the expanding clone, new

mutations can occur, completing the transformation process. Different sub-clones may develop within this founding clone. The situation may also evolve under the influence of disease evolution. New mutations may randomly occur as a matter of time. They can also occur as a result of the treatment mutagenic potential. Clonal evolution is common at the time of relapse and occurs through the acquisition of new mutations, either in the dominant primitive clone or in a subclone of the founder clone^[10]. Therapy itself can contribute to the acquisition of therapy-resistant mutations. A dominant sub-clone may evolve, possibly because of varying sensitivities to therapy or the influence of therapy on the microenvironment and on residual normal hematopoiesis^[10].

Other factors may influence the balance between normal and abnormal hematopoiesis.

Hematopoietic cells are part of their microenvironment. Cell populations present in the bone marrow niche are able to regulate stem cells. Leukemia stem cells depend on the same signals as their normal counterpart^[11]. In response to infection or bone marrow stress, the cellular composition of the microenvironment and therefore the production of cytokines are able to change fundamentally^[12]. Hematopoietic cells will have to adapt and to respond to all changes susceptible to occur in this environment to ensure the best fit. They will also modify their microenvironment by causing inflammation and by inducing an adaptive and innate immune response in the bone marrow. Furthermore, as the environment has already changed they will need to change again as a constant process. Leukemia-specific effector T cells infiltrating bone marrow may potentially target leukemia stem cells and participate to their eradication. However, mechanisms that evolved to protect normal hematopoietic stem cells and to regulate demand-adapted responses can protect leukemia stem cells against an attack from the immune system and favor their progression. Quiescence warrants the genetic integrity of stem cells as frequent chromosomal replications may introduce oncogenic DNA mutations and also protect stem cells from uncontrolled proliferation. Hematopoietic stem cells can undergo asymmetrical division. This non-exhaustive proliferation capacity can be initiated by all stress situations, of which infections and cytotoxic chemotherapy, and respond to signals from immune cells^[13]. Self-renewing hematopoietic stem cells persist for long periods of time, allowing the accumulation of genetic damage and malignant transformation. They can therefore serve as the cell-of-origin for leukemia stem cells^[14]. However, more mature progenitor cell types can serve as leukemia-initiating cells in some cases^[15]. Although not excluding a hierarchical model, this sustains the stochastic model hypothesis. Here, every malignant cell, given that it enters a permissive situation, has the ability to self-renew and to recapitulate the disease phenotype^[16].

During leukemogenesis, leukemia stem cells can "hijack" the niche and the signaling molecules from

normal hematopoietic cells. Molecular changes in the bone marrow niche contribute to leukemia development. In the niche, leukemia stem cells may be more therapy resistant and may therefore potentially provide important cellular markers of clinically relevant minimal residual disease^[17]. Immune cells are involved in the regulation of hematopoietic stem cell homeostasis and emergency hematopoiesis^[18]. CD4⁺ T cells are particularly important in maintaining hematopoietic stem cell function^[19]. T regulators represent one-third of all CD4⁺ T cells in the bone marrow. They provide an immune-privileged niche, protecting hematopoietic stem cells from immune destruction^[20]. Monocytes and macrophages are also important cells serving as regulators of hematopoietic stem cell egress from the bone marrow^[21]. The immune system interacts with hematopoietic stem cells *via* direct cell-cell interactions and *via* soluble factors. Leukemic cells express can interact with T cells through the molecular repertoire of major histocompatibility molecules and co-stimulatory ligands. Cytotoxic T lymphocytes directed against leukemia antigens have been detected^[22]. However, an activated immune system can also contribute to the leukemia progression by CD27 signaling on leukemia stem cells.

Hematopoietic stem cells are not spared by the aging process, in which DNA damage, telomerase shortening, oxidative stress, and poor homing efficiency have been reported. This could explain some of the differences between childhood and adult leukemias. Such genetic and epigenetic damage together with decreased immune system efficacy, vulnerability of older patients with a decreased function of various organs, and environmental factors can result in the development of malignant hemopathies in elderly patients^[23,24]. Furthermore, two obvious contributory factors are the dominant determinant of the country-to-country variability of leukemia incidence: heredity and environment.

Hematopoiesis permanently fluctuates between equilibrium and chaos. In a state of equilibrium, the hematopoietic system will slowly fail because its internal dynamics is not able to allow enough responses to its microenvironment. Similarly, a system in chaos will also fail, but that is because it will cease to function as a system. The most productive state always remains at the edge of chaos. This situation is the one that generates a maximum of variety and creativity, and a maximum of new possibilities for the evolution of the system. Leukemia is a disease of chaos, a breakdown of the existing biological order within the bone marrow, more than only the presence of abnormal cells. The disorder derives directly from malfunctioning of the controls that are normally present. We are still far from being able to understand with any precision how any single human leukemia arises. The process of leukemia formation is a complex one of multiple steps involving multiple alterations of cells and their physiologic control mechanisms. These steps may occur rapidly and

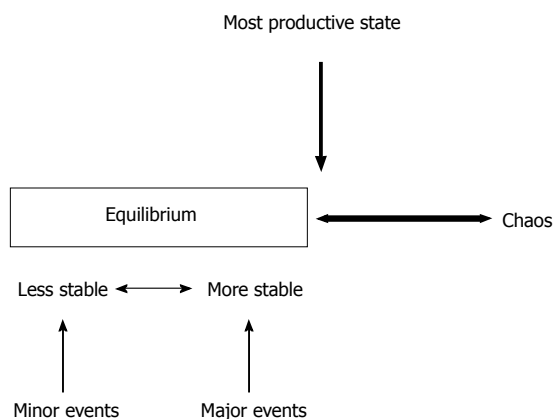


Figure 3 Hematopoiesis between equilibrium and chaos.

therefore may not be “rate-limiting”, while others may require more time to complete. Multistep leukemia progression can be depicted as a form of Darwinian evolution. Darwinian evolution involves expansions of cells that are endowed with advantageous genotypes and thus phenotypes. Recent whole genome and exome sequencing studies have begun to illustrate the extent of molecular heterogeneity in acute leukemia. The biologic behavior of leukemia cells is the sum of all the acquired genetic changes. Leukemia stem cells, rather than the bulk leukemia cell population, may be the objects of genetic alteration and clonal selection. The clonal descendants of the “favourable” mutated cell dominate by the cells that lack this mutation, resulting in a clonal expansion.

The development and the behavior of hematopoietic cell lineages appear therefore as a balance between normal and abnormal hematopoiesis in the setting of a functioning or malfunctioning microenvironment under the control of the immune system and the influence of hereditary and environmental events. Multiple equilibrium states may exist. Having leukemia cells does not mean having leukemia. All states seem possible, either stable or transitory, going from normal and functional hematopoiesis to the presence of abnormal cells easily controlled by the immune system, or to pre-leukemic stages without any consequences on normal hematopoiesis, or pre-leukemic stages with inhibition of normal cell lineages without major expansion of abnormal cells, or to the development of an abnormal clone without major infiltrative and proliferative abilities, or to the development of an aggressive leukemia clone taking absolute control of the bone marrow leading to an overt disease. The number of events necessary to affect the equilibrium depends on its stability. In this setting, massive events are required to modify a stable state, while a less stable equilibrium will be changed by minor events. The system, that will emerge, will be the one that will be better than its competitors. Then, the system will trade off increased efficiency every time in favor of greater effectiveness. The strength of a system is proportional to the variety within the system.

Contradictions are used by the hematopoietic cells, as by all complex adaptive systems. They are required to create new possibilities to co-evolve with their microenvironment (Figure 3).

Better understanding pathologic hematopoiesis as a complex adaptive system should have consequences in a therapeutic point of view and should modify our vision to consider disease control. Current therapeutic successes using aggressive treatment approaches, such as intensive chemotherapy or stem cell transplantation, have been conceived based upon the hierarchical concept of hematopoiesis with a “black or white” initial response to therapy and long-term survival defining “cure”, relying on a definitively fixed organization of hematopoiesis. Instead of this traditional reductionist approach, a moving status with a permanent adapting condition of hematopoietic cells according to inner and outer factors as suggested in a complex adaptive system is probably more realistic and could represent the common rule. Indeed, in real life, the concept of morphologic complete remission appears not anymore as an absolute requisite for therapeutic decision-making. The development of more accurate techniques to evaluate residual disease and a better knowledge of intra-cellular mechanisms leading to the introduction of targeted therapies should help the change our way to conceive leukemia therapy. Novel therapeutics should authorize more individualized therapeutic approaches. As it has already been the case in chronic leukemias since the introduction of tyrosine kinase inhibitors, a concept of acute leukemia therapy moving to an effective control of the disease compatible with a normal life instead of a supposed “complete” eradication of malignant cells seems a more accurate and more realistic goal.

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Common stemness regulators of embryonic and cancer stem cells

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Abstract

Pluripotency of embryonic stem cells (ESCs) and induced pluripotent stem cells is regulated by a well characterized gene transcription circuitry. The circuitry is assembled by ESC specific transcription factors, signal transducing molecules and epigenetic regulators. Growing understanding of stem-like cells, albeit of more complex phenotypes, present in tumors (cancer stem cells), provides a common conceptual and research framework for basic and applied stem cell biology. In this review, we highlight current results on biomarkers, gene signatures, signaling pathways and epigenetic regulators that are common in embryonic and cancer stem cells. We discuss their role in determining the cell phenotype and finally, their potential use to design next generation biological and pharmaceutical approaches for regenerative medicine and cancer therapies.

Key words: Embryonic stem cells; Cancer stem cells; Pluripotency; Self-renewal; Tumorigenicity

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Core tip: Accumulating experimental evidence has revealed the existence of common stemness regulators for embryonic and cancer stem cells. In this review, we highlight current results on biomarkers, gene signatures, signaling pathways and epigenetic regulators that determine the phenotype of these two types of stem cells. We also discuss how this knowledge may promote the design of next generation biological and pharmaceutical tools for regenerative medicine and cancer therapies.

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INTRODUCTION

Embryonic stem cells (ESCs) have two unique properties, self-renewal and pluripotency^[1]. Mouse ESCs (mESCs) are isolated from day 3.5 blastocyst and possess ground state pluripotency whereas human ESCs (hESCs) are isolated from late blastocyst and correspond to the epiblast stem cells of the mouse^[2,3]. The pluripotency of ESCs is determined by the concerted action of signaling pathways that respond to external stimuli, intrinsically expressed transcription factors and complexes that govern the epigenetic state. The extended transcriptional network of ESCs is centered on the triad of master regulators of pluripotency Oct4, Sox2 and Nanog^[4]. In the last decade the introduction in somatic cells of transcription factors (Oct4, Sox2, Klf4, c-Myc), microRNAs and small molecules allowed the generation of induced pluripotent stem (iPS) cells^[5]. Due to their ability to give rise to any type of differentiated cells and tissues, both ES and iPS cells offer many opportunities for modeling human diseases and development of regenerative medicine^[6].

ESCs and tumor cells share many common properties exemplified by rapid proliferation, similar metabolic requirements and inhibition of differentiation. Pluripotent ESCs have inherent tumorigenic potential and they generate benign tumors and teratomas when injected in immunodeficient mice^[7]. Reprogramming of somatic cells into pluripotency by oncogenes like Myc and Klf4 suggest a strong link between pluripotency and tumorigenicity^[8,9]. Currently, growing experimental evidence has revealed that tumors contain a variable number of cells that have self-renewal and partial differentiation capacities^[10-12]. Because these cells share these properties with the adult tissue stem cells from which they are likely derived they were termed cancer stem cells (CSCs)^[10-12]. The procedures of somatic cell reprogramming and CSC establishment are both dependent on transitions between epithelial and mesenchymal states (EMT/MET)^[13,14]. In addition, CSCs from epithelial tumors also exhibit ESC-like signatures^[15,16] that include the oncogene c-Myc and factors important for pluripotency such as Sox2, Dnmt1, Cbx3 and HDAC1^[16].

The CSC model^[10-12] ultimately links Cancer with Stem cell biology and provides a common framework that is proposed to account for all the properties of stem cells, regardless of their early or late developmental origin in normal or pathological states. In this review, we analyze common regulatory mechanisms of embryonic

and CSCs focusing on biomarkers, signaling pathways, transcription factors and epigenetic complexes. This information can elucidate the risks stemming from the tumorigenic potential of pluripotent ESC and iPS cells used in tissue regeneration therapies. Additionally, knowledge about their stem cell properties is valuable for the eradication of CSCs that are responsible for therapy resistance, tumor invasion and metastasis.

GENERAL PROPERTIES AND MARKERS FOR EMBRYONIC/PLURIPOTENT AND CSC

Three types of markers for the identification of either ESCs or CSCs are utilized: cell surface molecules, signaling pathway markers and transcription factors^[17]. However, cell surface molecules are mostly used as biomarkers since they can be assessed on intact living cells.

ESC biomarkers

All pluripotent stem cells express on their surface glycan epitopes that show species-specific and differentiation stage-specific expression, the stage specific embryonic antigens (SSEA-1, 3 and 4). SSEA-1 (CD15/Lewis x) is expressed on mESCs and embryonic carcinoma cells (ECCs) but is absent from hESCs^[18,19]. Upon mESC differentiation SSEA-1 expression decreases whereas SSEA-4 expression is induced. Undifferentiated hESCs express SSEA-3/4 but following differentiation they are both silenced and SSEA-1 is induced^[20-22] (Table 1).

Human ESC, embryonic carcinoma and germ tumors are characterized by the expression of tumor rejection antigens (TRA-1-60 and TRA-1-81). These are proteoglycan epitopes that reside on the 200 kDa form of podocalyxin (SC-podocalyxin)^[23,24]. Because they are not expressed on somatic cells, TRAs are useful markers for the isolation of human iPS cells during reprogramming^[25].

Cluster of differentiation antigens (CDs) are membrane proteins that function in diverse processes such as cell adhesion, communication and differentiation. Various family members are expressed in mESCs, hESCs and ECCs. Their expression usually changes following differentiation^[26]. CD324 (E-cadherin), CD31 (PECAM-1), CD24, CD90 (Thy-1), CD9, CD59, CD133 and CD326 (EpCAM) are present on the membrane of ESCs and ECCs although their expression levels can vary depending on the cell line and culture conditions^[17]. Among CDs the epithelial marker CD326 (EpCAM) is the more closely correlated with the undifferentiated state and is rapidly lost upon differentiation^[27] (Table 1). Interestingly, some of the above CDs are also used as CSC markers, as will be discussed below.

Among these markers of stemness, Cripto-1 (CR1, TDGF-1) represents an important component of a critical core pathway that is used by ESCs (Table

Table 1 Biomarkers of pluripotency in mouse and human embryonic stem cells

Biomarker	Role in mESC	Role in hESC
Oct4	Pluripotency ^[200]	Pluripotency ^[4]
Sox2	Pluripotency ^[215]	Pluripotency ^[4]
Nanog	Pluripotency ^[227]	Pluripotency ^[4]
Klf4	Pluripotency ^[253,255]	Pluripotency ^[253]
c-Myc	Pluripotency ^[190]	Pluripotency ^[190]
SSEA1/CD15	Pluripotency ^[118,19]	Not expressed
SSEA 3, 4	Not expressed	Pluripotency ^[20,22]
TRA-1-68	Not expressed	Pluripotency ^[24]
TRA-1-81	Not expressed	Pluripotency ^[24]
Cripto-1	Pluripotency ^[28,474]	Pluripotency ^[28,474]

hESC: Human embryonic stem cell; mESCs: Mouse embryonic stem cells; SSEA: Stage specific embryonic antigens; TRA: Tumor rejection antigens.

1). This growth factor acts during embryogenesis as a TGF- β ligand, co-receptor and as an oncogene. Moreover, Cripto-1 is involved in PI3K/Akt and MAPK pathways in a SMAD-independent manner and it enhances the Wnt and Notch pathways acting as a chaperone for low-density lipoprotein receptor-related protein 5 (LRP5) and Notch respectively^[28]. Cripto-1 is crucial for early embryonic development and is expressed in both mouse and human ESCs resulting in maintenance of stem cell pluripotency. Additionally, Cripto-1 regulates ESC fate choices by repressing the neural and enhancing the cardiomyocytic differentiation^[29]. Recently, it was shown that Cripto-1 performs an essential role in the etiology and progression of several types of human tumors, where it is expressed in a CSC subpopulation and facilitates epithelial-mesenchymal transition (EMT)^[28]. Intracellular transcription factors such as Oct4, Sox2, Nanog, Klf4 that are specifically expressed in undifferentiated stem cells are also important biomarkers. Their importance for pluripotency is examined in detail below.

CSC biomarkers in solid tumors

Breast cancer was the first type of solid tumor where CSCs were identified. Cells exhibiting the EPCAM⁺ ESA⁺CD44⁺CD24^{-/low}Lin⁻ phenotype were able to propagate breast tumors when injected even in a low concentration into the mammary fat pad of immunodeficient mice^[30]. Further studies identified a subpopulation of CD44⁺CD24⁻ cells expressing Aldehyde dehydrogenase (ALDH) capable of propagating tumor, by injection of as little as 20 cells in immunodeficient mice^[31]. CD44 is a cell membrane protein that binds hyaluronan (HA) and has a role in cell-cell and cell-extracellular matrix (ECM) interactions. Studies have implicated CD44 in breast cancer cell adhesion, migration, invasion and metastasis^[32]. Additionally, it protects cells from apoptosis which is an important characteristic of CSCs^[33]. HA-CD44 interaction promotes multiple cascades, activating gene transcription of stem cell-related factors in many different tumors, such as ovarian, breast, head and

neck cancer^[33]. Like CD44, CD24 is a widely expressed glycosylated cellular adhesion protein. Low expression of CD24 in breast CSCs was shown to enhance their growth ability and metastatic potential, through a chemokine receptor CXCR4 response^[34]. Additional surface markers for breast CSCs are: CD13, also a marker for brain and colon CSCs, α 6-integrin, CD61, CD29 and CD49.

The ALDH family of enzymes catalyzes the oxidation of aldehydes into carboxylic acids in a NADP⁺ dependent manner^[35]. ALDHs play a crucial role in retinoic acid biosynthesis, metabolism of cyclophosphamides and clearing toxic byproducts of reactive oxygen species^[36]. High ALDH activity was measured in human adult hematopoietic and breast stem cells, murine neural stem cells, as well as leukemia, breast, colon, head and neck CSCs^[35]. Emerging evidence supports the significance of ALDH as a biomarker for adult and CSCs of different origin, including pancreatic, prostate, ovarian, lung, head and neck squamous cell carcinoma^[37].

The first evidence for the existence of CSCs in brain tumors was provided by Singh *et al.*^[38]. Brain CSCs express the cell surface marker CD133 and lack the expression of neural differentiation markers. CD133 is a transmembrane glycoprotein expressed in mouse and human ESCs, as well as in different types of adult stem/progenitor cells, including hematopoietic and neural stem cells, endothelial precursors, mesenchymal progenitors, kidney, mammary glands and pancreatic, colorectal, testis, prostate, ovarian, lung and melanoma CSCs^[39]. The exact function of this protein remains unknown, but it was proposed to act as an organizer of the cell membrane topology^[40]. Limitations on the exclusive use of CD133 as a brain CSC marker arise from a study by Beier *et al.*^[41] who have reported that CD133 negative cells from glioblastoma sphere cultures are able to propagate tumors in immunodeficient mice. Moreover, CD133 expression was shown to depend on culture conditions and hypoxia levels. Additional markers shared by normal and cancer brain stem cells are nestin, Sox2, Musashi-1 and Bmi-1^[42]. Several studies have indicated that multidrug resistance transporters (MDR) such as ATP binding cassette (ABC) transporters and breast cancer resistance protein (BCRP/ABCG2) increase drug efflux from the cells^[43,44].

Stage-specific embryonic antigen 1 (SSEA1), also known as CD15, is a well characterized marker of undifferentiated mouse and differentiated human ESCs. It was first identified in neural embryonic progenitors and represents a putative brain CSC marker. SSEA1 expression correlates with increased cell proliferation, decreased differentiation and apoptosis^[45]. CD15⁺ cells exhibit high tumorigenicity. Another potential brain CSC marker is Nestin, an intermediate filament protein that was first identified as a neural stem cell marker^[46]. It is expressed in many different brain tumors and is involved in stemness, cell growth, invasion and migration^[47]. Finally, brain, like other CSCs

from breast and lung are resistant to chemotherapy and radiotherapy because of the expression of MDR1 transporter on their cell surface^[48].

Colorectal CSCs also express CD133^[49] and the surface molecule Epithelial Specific Antigen (ESA/EpCAM), while lacking expression of intestinal differentiation markers such as cytokeratin 20 (CK20). EpCAM is an epithelial adhesion molecule involved in proliferation, differentiation, migration and signaling^[50,51]. Another adhesion protein, CD166, was proposed as a biomarker in colorectal CSCs^[51]. CD166 is present in a wide variety of normal tissues, as well as in different cancers including breast, lung, prostate and melanoma^[52]. CD166 is used as a positive prognostic marker for survival in colorectal cancer despite the contradictory studies regarding its timeframe of expression during tumorigenesis. Other recently identified potential markers include CD24, CD29 and Lgr5. CD29 (β 1-integrin), a transmembrane receptor for extracellular proteins activates signaling cascades responsible for proliferation, differentiation, migration, survival or death^[53]. Finally, Leucine-rich repeat-containing G protein coupled receptor 5 (Lgr5), a receptor for R-spondins is characterized by a large extracellular and seven transmembrane domains. It binds R-spondin proteins which activate Wnt/ β -catenin signaling as a Wnt pathway co-receptor^[54,55]. Lgr5, a Wnt pathway target itself, is expressed in various stem cell types. High Lgr5 expression levels are associated with high vimentin and low miR-200c expression followed by increased invasiveness and lymph node metastasis^[56].

Pancreatic CSCs are characterized as CD44⁺CD24⁺-EpCAM⁺^[57], like CSCs from other solid tumors, such as ovarian cancer^[46]. On the contrary, breast CSCs exhibit low CD24 expression. Tyrosine kinase c-Met and CD133 have emerged as additional pancreatic CSC markers^[58,59]. Indeed, solely CD133⁺ cells, were shown to induce tumor formation in high frequency^[59]. Moreover, concomitant expression of CXCR4 promotes metastasis and represents a useful target for antitumor drugs^[59,60].

Prostate CSCs are characterized by high expression of CD44 and CD133, as well as the existence of ALDH1A1 and ABCG2 transporter on their cell surface, which confer chemoresistance. A2 β 1 integrin was also proposed as a prostate CSC marker, in addition to the lack of differentiation markers, such as PSA^[61].

Ovarian CSC markers are CD133, CD44 and CD24^[62]. Additional markers are EpCAM, ALDH1 and CD117 or c-kit proto-oncogene^[62]. The c-KIT receptor is activated by autophosphorylation upon stem cell factor (SCF) binding and is involved in cell proliferation, apoptosis, differentiation and adhesion^[46]. Moreover, CD117 was associated with chemotherapy resistance^[63]. Finally, other molecules that are significant for ovarian CSCs are MyD88 for chemoresistance, Lin28 and Oct4 for cancer stemness and dedifferentiation^[35].

Melanoma CSCs were recently found to be positive for CD20, CD133 and the ABC transporters, ABCG2 and

ABCB5^[64]. Additional markers are CD133 and CD166^[64]. CD133⁺ melanoma cells, which overexpress CD166 and nestin, exhibit tumor-propagating ability and high expression levels of genes responsible for tumor initiation and metastasis^[65]. CD271, which was proven to be important for maintenance of stem-like properties and tumorigenicity of melanoma cells^[66] is considered as the most convincing biomarker for melanoma CSCs. In addition, CXCR6 is implicated in their asymmetric division^[67] and Oct4 that is induced upon hypoxia is capable of promoting melanoma cell dedifferentiation into CSCs^[68].

Lung CSCs express CD133 as well as CD24, CD34, CD44, CD87 and ALDH1^[35,69]. CD133⁺ cells show increased expression of Oct4 protein and the ABCG2 transporter^[70]. Finally, Bmi-1 which is expressed in human small cell lung cancers could be applied as a potential lung CSC marker due to its role in self-renewal^[71].

Hepatocellular CSCs express CD133^[72]. CD133⁺ cells exhibited increased expression of Oct4, Notch, Wnt/ β -catenin, Hedgehog and Bmi-1, genes implicated in self-renewal, pluripotency, proliferation and differentiation^[73]. EpCAM, CD90 (Thy-1), CD44, CD13, ALDH1, ABCG2, CD117 and AFP represent additional hepatic CSC markers^[74].

The head and neck squamous cell carcinoma share the same biomarkers as most of the above described CSCs, including CD44, CD133, ABCG2, ALDH1, c-Met, Bmi-1 and Lgr5^[75-77].

In conclusion, putative CSCs have been characterized and enriched from many types of solid tumors using various cell surface markers. These CSC biomarkers offer important biological diagnostic and therapeutic tools (Table 2).

CONVERGENCE OF SIGNALING PATHWAYS IN EMBRYONIC AND CSC

Pluripotency of ESCs is regulated by core transcription factors as well as key signaling pathways, including LIF/Stat3 in the case of mESCs, Wnt, Hedgehog, Notch, FGF and TGF- β for both mESCs and hESCs^[78]. One hallmark of CSCs is their self-renewal capacity driven by developmental pathways^[13,79,80]. Below, we will outline the common signaling mechanisms in self-renewal, differentiation and pluripotency of mES, hES and CS cells in solid tumors.

Jak/Stat signaling

Binding of cytokines to their cognate receptors induces the activation of Jak kinases and the phosphorylation, dimerization and nuclear shuttling of signal transducers and activators of transcription (STATs). The Jak/Stat signaling pathway is activated by the cytokine LIF (leukemia inhibitory factor) in mESCs and is required for their self-renewal and pluripotency^[81]. In contrast, LIF does not support the pluripotency of human ESC.

Table 2 Biomarkers of cancer stem cells

Cancer type	Biomarkers
Breast	CD44 ^[32,33] ALDH ^[31,35] EpCAM ^[30] CD13 ^[35] A6-integrin ^[35] CD61 ^[35] CD29 ^[35] CD49 ^[35] ABCG2 ^[43,44] Nanog ^[204] Klf4 ^[263,264]
Brain	CD133 ^[39] Nestin ^[42] Sox2 ^[42] Musashi-1 ^[42] Bmi-1 ^[42] ABCB1 ^[48] ABCG2 ^[48] ABCC1 ^[48] SSEA1 ^[45] Nanog ^[236] Sox2 ^[42,225] Klf4 ^[269]
Colorectal	CD133 ^[49] EpCAM ^[50,51] CD166 ^[51] CD24 ^[51] CD29 ^[51] Lgr5 ^[56] Klf4 ^[266]
Pancreatic	CD44 ^[57] CD24 ^[57] EpCAM ^[57] c-Met ^[58] CD133 ^[59] CXCR4 ^[59,60] Sox2 ^[221] CD44 ^[61] CD133 ^[61]
Prostate	ALDH1A1 ^[61] ABCG2 ^[61] A2β1 ^[61] Sox2 ^[61] CD133 ^[62] CD44 ^[62] CD24 ^[62] EpCAM ^[62] ALDH1 ^[62] CD117 ^[62] c-kit ^[62] CD117 ^[63] MyD88 ^[35] Lin28 ^[35] Oct4 ^[35]
Ovarian	Nanog ^[232,238] Sox2 ^[219] CD20 ^[64] CD133 ^[64] ABCG2 ^[64] ABCB5 ^[64] CD133 ^[64] CD166 ^[64] CD271 ^[66] CXCR6 ^[67] Oct4 ^[68] Sox2 ^[224]
Melanoma	

Lung	CD133 ^[69] CD24 ^[35] CD34 ^[35] CD44 ^[35] CD87 ^[35] ALDH1 ^[35] ABCG2 ^[70] Bmi-1 ^[71] Nanog ^[234] Sox2 ^[223]
Hepatocellular	CD133 ^[72] EpCAM ^[74] CD90 ^[74] CD44 ^[74] CC13 ^[74] ALDH1 ^[74] ABCG2 ^[74] CD117 ^[74] AFP ^[74]
Head and neck (HNSCC)	CD44 ^[75] CD133 ^[76] ABCG2 ^[76,77] ALDH1 ^[77] c-Met ^[77] Bmi-1 ^[77] Lgr5 ^[77]

Numerous studies have shown the ability of STAT3 to promote tumorigenesis when it is aberrantly activated^[82]. Most importantly Stat3 has been recently shown to regulate the survival and proliferation of colon^[83] prostate^[84] and breast^[85] CSCs.

Wnt/β-catenin signaling

The Wnt/β-catenin branch of Wnt signaling-also referred to as the “canonical” Wnt-pathway- is important for proper embryonic development and adult tissue homeostasis. There are more than 30 extracellular Wnt-ligands, which bind to the receptor complex Frizzled and LRP5/6 (member of the LDL receptor family)^[86]. In the absence of Wnt signals, the scaffolding proteins Axin and adenomatous polyposis coli (APC) anchor the intracellular signaling protein β-catenin to a “destruction” complex involving Glycogen-activated kinase-3 (GSK-3). Wnt ligand-receptor binding initiates a series of events resulting in inhibition of the destruction complex and β-catenin cytoplasmic accumulation^[87]. Its concentration-dependent nuclear translocation and interaction with the T-cell factor/lymphocyte enhancer binding factor (Tcf/Lef) family leads to transcription of proliferative genes such as c-Myc and cyclinD1^[88,89].

Many members of the Wnt signaling pathway are implicated in stem-cell proliferation and activity. In mESCs, Wnt promotes self-renewal^[90]. Studies using small-molecule inhibitors have highlighted GSK-3 as a critical regulator of pluripotency for both mouse and human ESCs^[91]. β-catenin is dispensable for mESC maintenance, however, in its absence, the positive effect of GSK3 inhibition on self-renewal is abolished^[92] and β-catenin mutant mice display embryonic neural progenitor defects^[93]. Double mutants of GSK3 and β-catenin promote exit from pluripotency and

induction of neuroectoderm differentiation^[94]. From the mechanistic point of view, accumulating evidence indicates that the key pathway effector Tcf3 is acting as a repressor of Oct3/4, Sox2 and Nanog, whereas β -catenin inhibits this repression by converting Tcf3 into activator^[95]. Additionally, active β -catenin interacts with Oct4 and enhances its activity in a Tcf-3 independent manner^[96]. In hESCs, there is an ongoing debate about the contribution of Wnt signaling in self-renewal or differentiation^[91,97]. Long-term inhibition of Wnt did not affect survival of hESCs, whereas activation of Wnt signaling resulted in activation of mesoderm differentiation. In fact, differential activity of Wnt signaling associates with distinct lineage-specific differentiation potential of hESCs^[98].

Wnt signaling also regulates the activity of somatic SCs including those in the skin, blood, brain and the mammary gland, whereas aberrant signaling results in neoplasia^[94]. Mutations in key mediators of the Wnt pathway have been observed in approximately 90% of all colon cancers^[78]. Wnt activity was found to be enhanced in the CD133⁺ stem-like cell population of colorectal cancer and in Lgr5⁺ (a Wnt family member) intestinal crypt stem cells, which were identified as the origin of adenocarcinomas^[94,99]. Following transplantation in a mouse model for lineage tracing, Lgr5⁺ cells expand clonally repopulating all other adenomas^[100]. Myofibroblast-secreted factors were linked through β -catenin-dependent transcription to colon CSC clonogenicity and were shown to restore the CSC phenotype in more differentiated tumor cells, both *in vitro* and *in vivo*^[101]. These findings indicate a Wnt-dependent role of the tumor microenvironment in colon carcinogenesis.

Wnt is also involved in mammary gland tumorigenesis, since Wnt signaling expands mammary gland stem cells in early tumorigenic lesions of MMTV-Wnt1 transgenic mice^[102]. The mammary gland CSCs pool is sustained through recruitment of Wnt ligands by periostin, a component of the extracellular matrix of fibroblasts. Infiltrating tumor cells induce its expression in the stroma of secondary organs, such as lung, to allow colonization. Inhibition of its function prevents metastasis^[103].

Finally, although the role of Wnt in brain CSCs is still unclear, autocrine activation of Wnt/ β -catenin signaling by the orphan nuclear receptor Tlx, that induces glioma formation when overexpressed, was shown to enhance the proliferation of murine neural stem cells^[104]. Moreover, a recent study linked low oxygen levels in the hippocampus with neurogenesis through HIF-1 α enhanced Wnt signaling^[105]. Interestingly, brain tumor formation is promoted by hypoxia^[94] and HIFs were identified as key players in stemness and malignancy maintenance of colon cancer cells^[106].

Hedgehog and Notch signaling

Hedgehog (Hh) signaling is implicated in many developmental processes, such as in proliferation

and cell-fate specification of neural stem and neural crest stem cells. In lack of Hh pathway activity, many target genes like the Hh receptor Patched (PTC) and the Gli family of transcription factors are actively repressed. Secreted Hh-transmembrane PTC binding results in releasing PTC-repression on Smoothened (SMO), a G-protein-coupled receptor that activates downstream intracellular components^[107]. Despite the presence of functional pathway components, SHh (Sonic Hedgehog) ligand appears to be dispensable for maintaining pluripotency and promoting proliferation of undifferentiated hESCs. However, addition of exogenous SHh to embryoid bodies generated by hESCs promotes differentiation towards neuroectodermal^[108]. On the contrary, SHh was reported to stimulate mESC proliferation through the concerted effect of activated Gli1, increased intracellular Ca²⁺ levels, PKC (protein kinase C) and epidermal growth factor receptor (EGFR) activation^[109].

In adults the Hh pathway is mainly inactive, but it has been reported to participate in tissue maintenance and repair. Its deregulated activity has been linked to cancer development, however little is known about its role in CSCs^[110]. Hh signaling downstream effectors Gli1/2 and Bmi-1, a transcriptional repressor of the polycomb group and central regulator of self-renewal in normal stem cells, were recently shown to control proliferation and pluripotency of breast CSCs and normal human mammary stem/progenitor cells^[111]. Active Hh signaling has also been identified in glioblastoma CSCs. Inhibition of the pathway by chemical molecules or siRNAs leads to loss of their tumorigenic potential^[112,113]. Hh signaling is furthermore preferentially activated in colon carcinoma CSCs derived from primary clinical specimens, whereas its hindrance negatively regulates cancer cell proliferation and induced apoptosis of colon CSCs^[114]. Finally, a number of recent studies have implicated Hh signaling in EMT and metastasis, for example in pancreatic cancer cell lines inhibition of Hh results in EMT-inhibition and blocks metastasis^[115].

Notch signaling is a crucial regulator of cell to cell communication during embryogenesis, cellular proliferation, differentiation, and apoptosis^[79]. The Notch pathway consists of a membrane-tethered receptor (Notch 1-4), that undergoes proteolytic cleavage by ADAM-type metalloproteases and γ -secretase upon ligand binding (membrane-associated Delta-like, Jagged in mammals). Following cleavage, the released intracellular Notch domain shuttles to the nucleus to form a transcriptional complex with recombining binding protein suppressor of hairless (RBPJ, also known as CBF1) and mastermind-like proteins, ultimately activating genes of the hairy and enhancer of split-related family^[78,79].

Despite the presence of functional pathway components during early embryonic development, Notch signaling is dispensable for the maintenance of hESC or mESC pluripotency and constitutively activated Notch does not alter the stem cell phenotype^[116-118].

Inhibition of pathway components by siRNAs or by a chemical compound against γ -secretase activity (GSI) interfered with hESC proliferation without driving their differentiation^[119]. However, a number of studies indicate a decisive role for Notch in ESC fate determination. Activated Notch signaling was shown to promote neural commitment of both hESCs and mESCs^[117]. In undifferentiated hESCs it is required to form the progeny of all three embryonic germ layers except for trophoblast cells^[120]. In mESCs, there appears to be no *in vivo* requirement for the Notch pathway, until after all three germ layers have formed^[121].

Notch signaling is important for tissue maintenance in many organs, including the skin, blood, intestine, liver, kidney, central nervous system, bone and muscle^[121]. It promotes the maintenance of the neural, myogenic and intestinal stem cell pool in both *Drosophila* and mouse^[122].

Deregulation of Notch has been reported in several cancer types and is progressively linked to CSC self-renewal^[78]. Notch pathway components are characterized by higher expression level in pancreatic CSCs. Their inhibition using either GSI or Hes1 shRNA reduced CSC numbers and tumorsphere formation. Conversely, Notch activation increased pancreatic CSC self-renewal. *In vivo* treatment of orthotopic pancreatic tumors in NOD/SCID mice with GSI blocked tumor proliferation and reduced the CSC population^[123]. Notch signaling is also activated and plays a crucial role in promoting CSC survival, proliferation and tumor initiation (but not progression) in colon cancer. An antibody against Notch ligand DLL4 inhibited tumor growth in a xenograft mouse model^[124,125].

In medulloblastoma, increased Notch and Hh signaling have been linked to the maintenance of a stem-like cell population. Pharmacological depletion of Notch signaling inhibits medulloblastoma growth in mouse xenografts^[126]. In this context, Notch was proposed to interact with Hh signaling to promote oncogenesis^[127]. Additional pathway interactions were found in human breast epithelial cells, where oncogenic conversion is driven by increased Wnt signaling *via* Notch-dependent mechanism^[128]. Deregulation of Notch signaling is an early event in pre-invasive ductal carcinomas. Reduced mammosphere forming efficiency of *in situ* ductal carcinoma in the presence of Notch inhibitors suggested that Notch regulates breast CSC self-renewal^[129]. In normal breast tissue, Notch1 was proposed to regulate progenitor-to-luminal differentiation, whereas Notch4 stem-to-progenitor cell transitions. Interestingly, inhibition of Notch4 and, to a lesser extent, Notch1 signaling results in decrease of the stem-like cell population and of tumorsphere formation in primary breast cancer samples and cell lines and in limited tumor formation *in vivo*^[130]. Furthermore, Notch4 (but not Notch1) activation inhibits mammary epithelial cell differentiation and promotes mammary carcinogenesis in mice^[80,131].

TGF- β signaling

The TGF- β pathway plays a major role in development. Depending on the downstream effector molecules, it can be classified into the Smad1/5/8, the Smad2/3 and the Tab/Tak pathways. Secreted TGF- β ligands bind to the extracellular domain of Ser/Thr kinase type I and type II TGF- β trans-membrane receptors (TGF- β R) thereby phosphorylating and activating latent cytoplasmic SMAD transcription factors^[132]. Among 42 known ligands in humans, bone morphogenetic proteins (BMPs) and growth differentiating factors bind type I receptors (GDFs) activate Smad1/5. Activin and Nodal trigger phosphorylation of Smad2/3 through TGF- β R I/II. Activated Smads form a higher-order protein complex with Smad4, which then translocates to the nucleus to modify gene transcription. Inhibitory cytoplasmic Smad6/7, as well as molecules secreted by neighboring cells like Lefty, further increase the regulatory complexity of the pathway^[78].

During embryonic development cell fate determination, such as mesoderm and primitive streak formation in the mouse, as well as neural induction and mesoderm specification in *Xenopus* are affected by the TGF- β pathway^[133]. Both the Smad1/5/8 and the Smad2/3 branches are involved in ESC pluripotency/differentiation. Activin/Nodal/Smad2/3 signaling is important for sustaining self-renewal and pluripotency of mouse and human ESCs^[134-136], whereas BMP/Smad1/5/8 signaling promotes self-renewal in mESCs^[137,138] and differentiation in hESCs^[139-141]. The partly divergent signaling outcomes observed in mouse vs human ESCs are most likely due to the different developmental stages from which they are derived, hESCs being more similar to mouse epiblast stem cells (EpiSCs)^[3,142].

In mESC culture, concerted BMP/LIF signaling sustains pluripotency through the induction of inhibitor of differentiation (Id) proteins, and by inhibiting two major differentiation pathways, namely extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) at the same time^[137,138,143]. Furthermore, it was recently reported that mESC self-renewal is endogenously activated by autocrine loops of Activin/Nodal^[135].

In hESC culture, Activin A, which is secreted by mouse embryonic fibroblast feeder layers, suppresses BMP signaling and hESC differentiation, while stimulating the expression of pluripotency factors (e.g., NANOG, OCT4, FGF2/8, NODAL)^[144,145]. Nodal is secreted by hESCs themselves, reinforcing their pluripotent state by an autocrine mechanism. A regulatory loop is formed by simultaneous secretion of the Nodal inhibitor Lefty. Upon differentiation Nodal expression is rapidly down-regulated^[134,136,146]. Pluripotency is further sustained by downstream pathway effectors Smad2/3 which bind to and trans-activate Nanog expression in undifferentiated hESCs^[147,148]. Smad2/3 phosphorylation levels decrease upon early hESC differentiation^[134]. Smad3 alone was

also shown to form a complex with Oct4 and directly regulate many Oct4 targets^[149]. On the contrary, SMAD1/5 phosphorylation levels increase upon hESC differentiation^[134,150], a finding consistent with the ability of BMP4 to initiate differentiation to trophoblasts *in vitro*^[139].

The TGF- β pathway is also involved in EMT during embryonic morphogenesis. Interestingly, some epithelial cells acquire thereby self-renewing characteristics reminiscent of stem cells^[151]. In carcinomas, EMT can lead to metastasis and high-grade malignancy^[152].

TGF- β signaling plays a complex, context-dependent and tissue-specific role in cancer development and CSC proliferation. While inhibiting the onset of carcinogenesis the pathway may promote invasion and metastasis at later disease stages^[153].

Skin epithelia lacking TGF- β II exhibiting enhanced integrin/focal adhesion kinase (FAK) signaling were prone to age-dependent squamous cell carcinoma development^[154]. Moreover, tumor regression occurred in cancer cells lacking integrin/FAK signaling^[155]. CSCs isolated from the tumor/stromal interface of TGF- β II null squamous carcinoma formed less differentiated, highly aggressive and metastatic skin cancers. Interestingly, FAK depletion counterbalanced the TGF- β II-null phenotype^[156]. These data support an important role for TGF- β in counterbalancing the integrin/FAK-dependent tumorigenic effects in squamous cell carcinoma by down-regulating CSC proliferation and expansion^[133].

Breast cancer cells respond to TGF- β by exhibiting stem-like properties. A recent study revealed that chemotherapy relapse of triple-negative breast cancer (TNBC) might involve expansion of CSCs caused by activated TGF- β and IL-8 signaling in The resistant CSCs are prone to TGF- β pathway inhibitors^[157].

In malignant gliomas, TGF- β signaling also appears to exert agonistic effects on tumorigenesis through the Nodal/Activin branch of the pathway, increasing self-renewal of glioma stem cells (GSCs) by enhancing LIF/STAT signaling^[158]. Furthermore, TGF- β -Sox4-Sox2 signaling appears to be important for the maintenance of stemness of GSCs^[159]. On the other hand, the activation of the BMP branch of the pathway by BMP4, initiated neural differentiation and blocked tumor growth in a mouse xenograft model^[160]. In addition, epigenetically silenced BMP signaling was proposed to desensitize glioblastoma stem-like cells to normal differentiation cues and to promote their proliferation^[161].

The differential CSC responses to TGF- β cues underlie a serious dilemma over the clinical use of TGF- β agonists/antagonists^[133].

Fibroblast growth factor signaling

Most fibroblast growth factor ligands (FGF1-22) function in a classical autocrine or paracrine manner. Ligand-receptor binding results in autophosphorylation and dimerization of the intracellular region of a tyrosine kinase trans-membrane receptor (FGFR1-4)^[162]. The signal is further relayed through four main pathways:

RAS-RAF-MAPK (ERK), PLC γ -PKC, PI3K-AKT and JAK/STAT. Emerging evidence suggests that FGFRs also traffic to the nucleus, activating entirely different downstream molecules^[163].

Mutations of FGF pathway components result in pre-implantation lethality of the early mouse embryo^[162]. Autocrine FGF-induced ERK1/2 signaling is dispensable for mESC pluripotency but requisite for their differentiation into neural and mesendodermal lineages^[164,165]. Ying *et al.*^[166] further suggested that addition of LIF and BMP to culture media promotes mESC self-renewal solely by compensating for the pro-differentiation effects of FGF4. mESCs are comprised of heterogeneous populations. Cells primed for differentiation towards the primitive endoderm express FGF5 and Brachyury. FGF4 signaling on the other hand additionally maintains the primed state towards germ layer differentiation^[162,167].

In stark contrast to mESCs, hESCs require exogenous FGF2 to sustain self-renewal and the capacity to give rise to somatic lineages^[168,169]. The combined use of FGF2 and Activin is the most effective in maintaining hESCs and EpiSCs self-renewal. Recent studies suggest that spontaneous extra-embryonic differentiation, to which both hESCs and EpiSCs are prone, may be blocked by FGF^[3,162]. FGF2 seems to influence the pluripotent state of hESCs on several levels. It activates NANOG expression in cooperation with Activin signaling through SMAD2/3^[147] and synergizes with Noggin to repress trophoblast-inducing BMP signaling^[140,170]. FGF/ERK signaling leads to phosphorylation of c-Myc, c-Jun and c-Fos. Its inhibition leads to a decrease in the expression of core pluripotency factors Nanog, and Oct4^[171-173]. Interestingly, ERK and GSK-3 inhibition have successfully supported the reprogramming procedure for the generation of human iPS cells^[174]. Furthermore, conversion of hESCs to an mESC-like phenotype was achieved by the concerted action of ERK and p38 inhibitors with LIF^[175]. A number of recent studies raise the possibility of differential and sometimes opposing functions of FGF in hESCs, depending on the downstream effector signaling cascades^[172,176].

Exogenous FGF2 is also required for growth and maintenance of CSCs isolated from different human carcinomas (*e.g.*, brain, breast) in tumorspheres, but the mechanisms of FGF action remain to be elucidated^[177,178]. A recent study revealed that the expansion of a functional breast CSC pool in response to estrogens is induced through a paracrine FGF/FGFR/Tbx3 signaling cascade, which is also functional in epithelial stem cells from the normal mammary gland^[179]. Guthridge and colleagues transformed NIH3T3 cells with FGF4 and found out, that HSP-90, p63, LAMP-1 and CyclinD1 were massively activated^[180]. The obtained results suggest a link between FGF4, CSC expansion and tumorigenesis^[181]. Indeed, Cyclin D1 is considered to be a marker for cancer onset and progression, while in stratified epithelial tissues p63 is thought to regulate stem cell characteristics^[182,183].

In conclusion, emerging evidence indicates a complex crosstalk between signaling pathways in development, adult tissue homeostasis and cancer. A better understanding of the pathway interplay and how it controls the biology of ESCs, SCs and CSCs will be essential for the advance of regenerative medicine and for developing effective cancer therapies.

COMMON TRANSCRIPTIONAL REGULATORS OF EMBRYONIC/PLURIPOTENT AND CSC

Regulatory networks in pluripotency

Transcription factors Oct3/4 (Pou5f1), Sox2 and Nanog constitute the “core pluripotency network” that regulates pluripotency of both mouse and human ESCs. They bind synergistically to their own promoter/enhancer elements establishing an auto-regulatory circuit^[4,184]. This master pluripotency network, including additional factors such as Sall4, Klf4, and Stat3 binds to and regulates the expression of two distinct groups of genes in ESCs: genes related to self-renewal (active) and genes related to differentiation (silenced)^[185]. The latter group of genes is co-occupied by the epigenetic silencing complexes Polycomb (PRC1 and PRC2)^[186]. A second important multi-protein complex is centered on the oncoprotein Myc (Myc network). Both networks are mutually regulating each other and interact physically and functionally with chromatin remodeling and modification complexes^[187,188]. The Myc complex binds near the transcription start site (TSS), whereas the core pluripotency complex binds to upstream promoters and enhancers^[189-191]. The master pluripotency factors (Oct4, Sox2, Nanog) form super-enhancers, clusters of enhancer elements that recruit Mediator and determine cell identity^[192,193].

Oct4, Sox2, Klf4 and Myc were the initial factors with the potential to reprogram somatic cells into pluripotency^[188,194]. The individual role of these factors in pluripotent and CSCs will be examined below. Oct4 in ESCs and CSCs Oct4 belongs to the POU family of homeodomain proteins and is encoded by the *Pou5f1* gene. Its expression has been identified in undifferentiated ESCs, embryonic carcinoma cells (ECCs), pluripotent epiblast and embryonic germ cells (EGCs)^[195-197]. Nichols *et al.*^[198] reported that Oct4 expression is essential for the maintenance of ESC properties. They showed that Oct4-deficient embryos did not form a pluripotent inner cell mass and differentiated to trophectoderm^[199]. Moreover, inhibition of Oct4 in mESCs led to the upregulation of trophectoderm genes (*Cdx2*), while its overexpression caused differentiation into primitive endoderm and mesoderm^[200]. Under serum free culture conditions Oct4 overexpression in ESCs promoted neuroectoderm formation and subsequent neuronal differentiation^[201]. Oct4 cooperates with Sox2 to regulate the expression level of genes important for self-renewal and pluripotent

phenotype of ESCs (e.g., Nanog). On the other hand, when the Oct4 or Sox17 expression levels increased, Sox2 was replaced by Sox17 and targeted genes that trigger the endodermal expression program^[202]. These results indicated that the precise levels of Oct4 determined the ESC fate and that Oct4 is a master player in sustaining stem cell self-renewal.

Numerous studies have indicated that Oct4 plays a crucial role in tumorigenesis and tumor metastasis. It was shown to be upregulated in many human cancers such as bladder, seminoma, prostate and breast cancer^[15,203-206]. Hu *et al.*^[207] reported that in murine lung carcinoma cells and human breast cancer MCF7 cells, ablation of Oct4 expression leads to apoptosis of CSC-like cells through the Oct4/Tcl1/Akt1 pathway and inhibition of tumor growth. Another study confirmed that the reduction of Oct4 in lung cancer cells blocked the clonogenicity and tumor invasion^[70]. Chiou *et al.*^[208] enriched oral CSCs by sphere formation and concluded that these cells highly expressed Oct4 and had similar characteristics of stem cells and malignant tumors. A year later, Rentala *et al.*^[209] reported that the expression of Oct4 in prostate CSCs maintained their stem cells properties. Moreover, it has been revealed that ectopic expression of Oct4 into normal primary breast epithelial preparations generated cell lines which form triple-negative breast carcinomas in nude mice^[210]. Recently, Wang *et al.*^[211] demonstrated that cervical cancer cells expressed higher Oct4 levels than normal cervix cells. They proposed that Oct4 promotes tumor formation *in vivo* and inhibits apoptosis by the activation of miR-125b expression^[211]. In addition, Oct4 has been suggested to regulate stemness of head and neck squamous carcinoma CSCs. The overexpression of Oct4 activated Cyclin E leading to tumor growth and tumor invasion through slug expression^[212].

Sox2 in ESCs and CSCs

Sox2 is a member of the Sox (SRY-related HMG box) family that consists of transcription factors with a single high-mobility group box DNA-binding domain and also belongs to the SOXB1 subgroup^[213]. Sox2 is expressed in the inner cell mass (ICM) and extraembryonic ectoderm of pre-implantation blastocysts^[214]. Sox2 deficient blastocysts could not form a pluripotent ICM. Moreover, Sox2-deficient mESCs differentiated primarily into trophectoderm, while the Oct4 overexpression rescued the pluripotency of Sox2-null mESCs^[215]. As a result, Sox2 is critical for the maintenance of Oct4 expression and hence the stem cells' properties. Furthermore, Masui *et al.*^[215] identified a synergistic function of Sox2 and Oct4 for the activation of Oct-Sox enhancers, leading to the regulation of various pluripotency genes, including Nanog, Oct4 and Sox2. Overexpression of Sox2 in ESCs led to their differentiation^[216,217]. This effect was due to the repression of pluripotency genes *Sox2*, *Oct4*, *Nanog*, *Fgf4* and *Utf1*^[216] and the induction of neuroectoderm, mesoderm and trophectoderm^[217].

To date, many reports have demonstrated the

involvement of Sox2 in cancer biology and especially in CSCs. Sox2 is critical for osteosarcoma cell self-renewal and antagonizes the pro-differentiation Wnt pathway which can also affect negatively the expression of Sox2^[218]. In addition, it is implicated in the promotion of cell migration and invasion in ovarian cancer, through regulating fibronectin 1^[219]. Studies in gastric cancer showed that inhibition of Sox2 results in reduction of spheres formation and in increase of apoptotic sphere cells^[220]. The contribution of Sox2 in pancreatic CSCs was suggested by the fact that it regulates stemness *via* the control of genes of G1/S transition and EMT^[221]. In prostate CSCs, the inhibition of EGFR signaling led to the decrease of Sox2 expression and self-renewal of prostate CSCs. Moreover, knockdown of Sox2 reduces the ability of prostate CSCs to grow under anchorage-independent conditions^[222]. Similar findings have been extracted from non-small cell lung cancer studies. Singh and colleagues inhibited the expression of Sox2 and noticed a 2.5-fold reduction in sphere formation^[223]. Additionally, EGFR/Src/Akt signaling influenced Sox2 protein expression, due to the decreased levels of Sox2 during the EGFR or SRC inhibition^[223]. In melanoma CSCs, Sox2 is highly expressed and interact with Hedgehog-GLI (HH-GLI) signaling^[224]. In more detail, Santini *et al*^[224] showed that knockdown of Sox2 decreases the melanoma sphere formation and self-renewal of melanoma CSCs. Two HH-GLI signaling transcription factors, GLI1 and GLI2, have the ability to bind the proximal promoter of Sox2 and thus the HH-GLI signaling regulates Sox2. Finally, Favaro *et al*^[225] proved that Sox2 is required for CSC maintenance in a high-grade oligodendroglioma mouse model.

Nanog in ESCs and CSCs

Nanog is the third member of the core pluripotency network in undifferentiated ESCs^[226-228]. It is a homeodomain containing transcription factor, which was discovered through a functional screening for pluripotency factors which allowed the maintenance of ESC properties, in the absence of the LIF-STAT3 pathway^[227,228]. Chambers *et al*^[228] also added that Nanog expression is high in Oct4-null embryos, whereas its overexpression does not counteract the differentiation program of ESCs prompted by Oct4 deletion. In the absence of Nanog, embryos do not form a pluripotent ICM^[227,229], although Nanog-null mESCs can be established^[227,228]. Intriguingly, these Nanog-deficient mESCs although disposed to differentiation, could still be maintained in the undifferentiated state^[227,228]. In 2005, Hyslop *et al*^[230] showed that Nanog down-regulation in human ESCs promotes differentiation towards extraembryonic lineage, as shown by the upregulation of endodermal- and trophoblastic-characteristic genes. This suggests a pivotal role for Nanog in the maintenance of pluripotency in human embryonic development. Oct4/Sox2 heterodimers bind to the octamer/Sox elements within the Nanog proximal promoter and regulate

Nanog expression in ESCs^[231]. Moreover, Nanog, Oct4 and Sox2 cooperate with signaling pathways mediators resulting in delivering signals directly to the genes regulated by the core factors^[191]. Sites co-occupied by the three core regulators generally have enhancer activity, while the transcription of the respective genes requires the recruitment of at least one of the trio^[191].

Nanog expression was investigated in several types of cancer, including lung, breast, oral, kidney, gastric, cervix, brain, ovarian and prostate cancer^[232-237]. In particular, high expression levels of Nanog are related to a poor prognosis for ovarian serous carcinoma, colorectal, and breast cancer patients^[238-240]. In oral squamous cell and lung adenocarcinoma, Nanog and Oct4 high levels were linked to advanced cancer stage and shorter patient survival^[208,234]. Several groups demonstrated that Nanog expression is much higher in CSCs than in non-stem cancer cells in many types of cancer^[233,241-246]. In colorectal cancer, Nanog-positive CSCs constitute approximately 2% of the total cancer cell population^[241]. In addition, a direct connection between the surface markers of CSC and Nanog has not been clarified yet, but there are many studies which demonstrated that cancer cells expressing these markers would have higher levels of pluripotency genes^[247]. For instance, CD133⁺ or CD44⁺ cancer cells express significant lower levels of Nanog compared to CD133⁺ or CD44⁺ cells, respectively^[248,249]. Moreover, functional studies in various cancer types showed that Nanog induces CSC-like characteristics. Jeter *et al*^[242] demonstrated that NANOGP8 overexpression in prostate cancer increased clonogenicity and tumor regenerative ability^[247]. Nanog activation leads a small population of colorectal cancer cells to acquire a stem-cell like phenotype^[247]. Furthermore, Han *et al*^[250] proved that Nanog binds to Cyclin D1 promoter region and regulates proliferation and cell cycle of breast cancer cells^[247,250]. Recently, Siu *et al*^[251] reported that increased expression of Nanog in ovarian cancer controls cell proliferation, migration and invasion through E-cadherin and FoxJ1 deregulation^[247,251]. These results propose that Nanog may constitute a CSC marker and play a vital role in cancer progression^[251].

Klf4 in ESCs and CSCs

Following the identification of KLF4 as a critical transcription factor for reprogramming, more attention was given to its actions. Klf4 belongs to the Kruppel-like transcription factor family and has a central role in cell cycle regulation, somatic cell reprogramming and pluripotency^[194,252-254]. Klf4 is highly expressed in mESCs and its expression decreases strongly upon differentiation^[255]. The inhibition of Klf4, using RNAi, leads to the differentiation of ESCs^[252,254], while Klf4 ectopic expression postpones differentiation, enhances the expression of Oct4 and promotes self-renewal^[256]. Klf4, in conjunction with Oct4 and Sox2 drives the expression of Lefty1^[257] and Nanog^[258]. In addition, the expression of Klf4 is regulated by STAT3 and Nanog^[252].

being a direct target of both transcription factors^[252,259]. In a recent report, Aksoy *et al.*^[260] described that Klf4 reduction induces differentiation towards visceral and definitive endoderm, concluding that Klf4 inhibits endoderm differentiation in mESCs.

It is not surprising that Klf4 plays a key role in maintaining CSC populations. It is known that telomerase activity is sustained by Klf4 *via* telomerase reverse transcriptase in both CSCs and hESCs, suggesting that Klf4 is important for the long-term proliferative potential of these cells^[261]. Moreover, Hoffmeyer *et al.*^[262] reported that β -catenin regulates Tert expression *via* the interaction with Klf4, supporting a connection between stem cells and oncogenesis. In 2011, Yu *et al.*^[263] reported for the first time that Klf4 is crucial in maintaining breast CSCs and inducing cell migration and invasion. Klf4 is expressed at high levels in CSC populations in mouse primary mammary tumor and human breast cancer cell lines. The inhibition of Klf4 in MCF-7 and MDA-MB-231 decreases the ability of breast CSCs to self-renew and form mammospheres and tumors *in vivo*^[264]. On the other hand, it was shown that Klf4 suppresses metastasis in MDA-MB-231 cells by maintaining the expression of E-cadherin and inhibiting EMT^[265]. Klf4 is highly expressed in colorectal CSCs and its knockdown leads to the decrease of spheres formation, migration, invasion and EMT. These results prove the essential role of Klf4 for maintaining colorectal CSCs^[266]. Furthermore, Wellner *et al.*^[267] reported that Klf4 is induced by ZEB1 through the repression of stemness inhibitor miR-203, and controls/enhances pancreatic and colorectal cancer cells ability to initiate tumor development. Moreover, repression of Klf4 by miR-7 inhibits metastasis of human breast CSCs in nude mice^[268] whereas inhibition of Klf4 by mir-152 suppresses the generation of glioblastoma SCs^[269].

These results propose that Klf4 has important roles not only in stem cell self-renewal and cell motility, but also in CSC and carcinoma cell invasion and metastasis.

Myc in ESCs and CSCs

Myc family which includes three important members - c-Myc, N-Myc and L-Myc - acts as an essential regulator in cell growth, proliferation, differentiation and apoptosis and it is thought to be crucial for stem cell pluripotency and proliferation^[270-272]. Myc is directly regulated by LIF/STAT3 signaling and its constitutive activity renders ESC self-renewal independent of LIF. In contrast, the overexpression of Myc dominant negative form induces differentiation^[273]. Although the individual inactivation of c-Myc and N-Myc has no effect on pluripotency, their simultaneous deletion destabilizes the pluripotent state leading to primitive endoderm and mesoderm differentiation^[274]. Moreover, the overexpression of either c-Myc or N-Myc restore pluripotency of ESCs^[274], supporting the idea that c-Myc and N-Myc perform redundant roles in maintaining pluripotent stem cell identity. Recently, Chappell *et al.*^[275] showed that Myc represses MAPK signaling and results in inhibiting

differentiation.

Several genome-wide analyses have been performed in order to determine how Myc regulates ESC pluripotency. These studies showed that Myc binds to and possibly regulates the transcription of at least 8000 genes in ESCs^[191,276-278]. The Myc-centered complex in ESCs is binding to the TSS and includes also E2F Max and NuA4 HAT complex^[189].

Myc deregulation and elevation have been observed in a wide range of human malignancies, associated with aggressive and poorly differentiated tumors^[279]. It is well-known that Myc is involved in the regulation of 15% of genes in the human genome^[280] and regulates important pro-tumorigenic factors including KRAS and AKT, and tumor-suppressors PTEN and p53^[281,282]. Some reports also showed that Myc-centered protein interaction networks in ESCs are enriched in some cancers, especially in the CSCs, conferring metastatic potential and poor outcome^[16,183]. These findings suggest that the Myc network is responsible for the similarities between ESCs and cancer cells. Wang *et al.*^[283] determined that glioma CSCs expressed high levels of Myc, which is crucial for growth, proliferation and survival. Furthermore, glioma CSCs with low levels of Myc did not generate neurospheres *in vitro* or tumors after xenotransplantation in the brains of immunodeficient mice^[283]. Salcido *et al.*^[284] found that Myc is expressed at high levels in CSC population of small-cell lung cancer. Additionally, it has been demonstrated that Myc expression is significantly upregulated in tumor spheres formed by rhabdomyosarcoma cell lines^[285]. In a recent study, it has been indicated that silencing of Myc using promoter targeting siRNA, decreased prostate CSC maintenance and tumorigenicity and induced senescence in the prostate CSC subpopulation^[286]. The clarification of the role of Myc in hepatic CSCs biology came from Akita work in 2014. They revealed a direct link between c-Myc expression levels and CSC properties and they have further mechanistically demonstrated that c-Myc modulates the hepatic CSC phenotype in a p53-dependent manner^[287].

Collectively the result of accumulating research suggests that ESCs and CSCs share critical transcription factors (Table 3). It is clear that pluripotency factors Oct4, Nanog, Sox2, Klf4 and Myc play a crucial role in cancer development and contribute to cancer treatment. However, further investigation of their role in determining the CSC phenotypes, will provide the exact regulatory mechanisms and possibly new regulatory factors relating to tumorigenesis and metastasis.

COMMON EPIGENETIC REGULATORS IN ESCS AND CSC

Cell epigenetic state has been recognized as an important factor in diverse developmental and differentiation processes *via* global or gene specific regulatory mechanisms. Genome wide analyses and

Table 3 Common signaling pathways, transcription factors, non-coding RNAs and epigenetic regulators of embryonic stem cells and cancer stem cells in solid tumors

ESCs		CSC type
Signaling pathways		
Wnt/ β -catenin	Self-renewal in mESCs/hESCs ^[91] Differentiation in hESCs ^[98]	Brain ^[104] Breast ^[103] Colon ^[99] Lung ^[475] Prostate ^[476]
Hedgehog	Self-renewal in mESCs ^[109] Differentiation in hESCs ^[108]	Brain ^[112] Breast ^[111] Pancreas ^[115]
Notch	Differentiation in mESCs and hESCs ^[117]	Brain ^[126] Breast ^[128] Colon ^[125] Pancreas ^[123]
TGF- β	Activin/Nodal promote self-renewal in mESCs and hESCs ^[134,135] BMP promotes self-renewal in mESCs ^[138] and differentiation in hESCs ^[141]	Brain ^[158] Breast ^[157] Skin ^[133]
FGF	Differentiation in mESCs ^[164] Self-renewal in hESCs ^[169]	Bladder ^[477] Brain ^[177] Breast ^[178]
Transcription factors		
4-Oct	Self-renewal and pluripotency ^[198]	Breast ^[207,210] Lung ^[70,207] Oral ^[208] Prostate ^[209] Cervical ^[211] Head and neck ^[212]
Sox2	Self-renewal and pluripotency ^[215]	Osteosarcoma ^[218] Ovarian ^[219] Gastric ^[220] Pancreatic ^[221] Prostate ^[222] Lung ^[223] Melanoma ^[224]
Nanog	Self-renewal and pluripotency ^[226-228]	Breast ^[250] Oral ^[208] Lung ^[234] Colorectal ^[238,241] Prostate ^[242] Ovarian ^[251]
Klf4	Self-renewal and pluripotency ^[254,256]	Breast ^[263-265,268] Colorectal ^[266,267] Pancreatic ^[267] Brain ^[269]
c-Myc	Self-renewal and pluripotency ^[189,191,270]	Brain ^[283] Lung ^[284] Rhabdomyosarcoma ^[285] Prostate ^[286] Hepatic ^[287]
DNA methylation regulators		
DNMT1	Differentiation ^[293]	Colon ^[336] Breast ^[338]
TET2	Differentiation ^[315,322]	Breast ^[344]
Chromatin modifications regulators		
EZH2	Self-renewal and pluripotency ^[186]	Breast ^[393] Pancreas ^[393] Brain ^[395] Prostate ^[396] Bone ^[397]

BMI-1	Self-renewal and pluripotency ^[186]	Prostate ^[400] Esophageal ^[401] Head and neck ^[402] Cervical ^[403] Colorectal ^[404] Laryngeal ^[405] Ovarian ^[406] Salivary adenoid cystic carcinoma ^[399]
Suz12	Self-renewal and pluripotency ^[186]	Breast ^[407] Colon ^[408]
MLL1	Self-renewal and pluripotency ^[186]	Brain ^[409]
MicroRNAs		
Let-7	Differentiation ^[430,431]	Breast ^[434] Prostate ^[448] Breast ^[437]
MiR-200 family	Differentiation ^[267]	
MiR-34a	Differentiation ^[429]	Brain ^[444] Prostate ^[448] Pancreatic ^[451] Gastric ^[450] Colon ^[452] Brain ^[445] Breast ^[440]
MiR-145	Differentiation ^[424]	
Long non-coding RNAs		
LncRNA-RoR	Self-renewal ^[418]	Breast ^[461]

CSC: Cancer stem cell; ESCs: Embryonic stem cells; mESCs: Mouse ESCs; hESCs: Human ESCs.

knockout studies provide new information about the role of epigenetic processes in self-renewal and cancer initiation and permit the development of "epigenetic" therapy as a cancer treatment option^[288,289].

This part of the review highlights our current view of the most important common epigenetic regulators associated with DNA methylation, histone modifications as well as long-non coding RNAs and miRNAs in ESCs and CSCs, their significance in normal development and their deregulation in tumorigenesis.

DNA methylation regulators in ESCs and CSCs

DNA methylation is generated by DNA methyltransferases (DNMTs), enzymes that add methyl groups on cytosines. The most studied members of the mammalian DNMT family include DNMT1, DNMT3a and DNMT3b. DNMT1 is thought to be responsible for the replicative maintenance of the DNA methylation, while DNMT3A and DNMT3B function as *de novo* methyltransferases^[290]. Nevertheless, recent evidence shows that DNMT1 may also be required for *de novo* DNA methylation^[291] and that DNMT3a and DNMT3b can also participate in the maintenance of the DNA methylome^[292]. These findings emphasize the need to clarify the exact role of each DNMT and their potential crosstalk.

Restriction enzyme digestion-mediated and Methylation DNA Immunoprecipitation-ChIP (MeDIP-ChIP) analyses of global DNA methylation show that the DNA methylation levels are reduced in mouse ESC compared to the somatic cells and that methylations on promoter regions lie primarily outside of CpG islands^[293-295]. A methylation analysis of CpGs by Bibikova *et al.*^[296]

reported that the methylation levels of over 370 genes in 14 hESC lines were lower than those of mESCs^[294].

Two groups, Lister *et al.*^[297] and Laurent *et al.*^[298] have compared the methylation maps of hESCs and human fibroblast cell lines and observed significantly higher levels of non-CpG methylation present in hESCs that may be due to differences in methylation regulatory mechanisms between un- and differentiated cell types. Laurent *et al.*^[298] observed that CpA methylation was the most frequent type of non-CpG methylation in hESCs, and that this modification was lost upon differentiation. Non-CpG methylation is also present in mESCs and is reduced from 8% to 4.3%, six days after induction of differentiation^[299]. This non-CpG methylation is more abundant within gene bodies than promoter regions and is catalyzed by DNMT3a and DNMT3b requiring also the presence of DNMT3L^[300]. Methylation profiles of iPS cells are highly similar to the ones of ESC, showing that this is a unique characteristic of pluripotent cells^[297,301]. However its functional role remains unclear.

Deletion of Dnmt1 or 3b in mice results in embryonic lethality^[302], while Dnmt3a^{-/-} mice die within 4 wk after birth showing that these enzymes are essential for normal development^[303]. DNMT1 overexpressing ESCs when injected in blastocysts resulted in embryonic lethality, resembling the effect of DNMT1 deficiency^[304]. DNMT1, DNMT3a and DNMT3b are expressed in hESCs^[305]. Mouse knockout experiments have shown that deletion of DNMTs does not affect ESCs self-renewal but deregulates cell specification, suggesting that global methylation may be dispensable for the undifferentiated state but is critical for differentiation. More specifically, DNMT1^{-/-} EBs contain a large number of Oct4 positive pluripotent cells indicating that methylation is required for proper cell differentiation^[293]. In Dnmt3a^{-/-} Dnmt3b^{-/-} mouse ESCs, only 0.6% of CpGs are demethylated^[293], suggesting that these molecules have limited contribution on global DNA methylation. Furthermore, Pawlak *et al.*^[306] showed that Dnmt3a and Dnmt3b are dispensable for nuclear reprogramming.

Although many observations implied the reversibility of DNA methylation, only recently were identified the TET (ten eleven translocation) enzymes that actively demethylate DNA. The mammalian TET family has three members, TET1, TET2 and TET3 that catalyze 5mC oxidation and generate the 5mC derivatives 5hmC, 5fC or 5caC (5-hydroxymethylcytosine, 5-formylcytosine, 5-carboxylcytosine)^[307-310]. 5hmC is the first intermediate toward DNA demethylation and its variable amounts in different cells and tissues implies a distinct regulatory role^[311]. Recent reports, studying the genomic distribution of 5hmC in mouse and human ESCs, provide evidence that this modification may function as a specific epigenetic mark in gene expression regulation^[312-314].

The expression levels of TET1 and 2 are high in undifferentiated ESCs and decline upon differentiation, in parallel with an increase of TET3^[315]. Further studies have shown that TET1 adds 5hmC on promoter regions and TSSs whereas TET2 activity is detected

in the coding regions^[316]. TET1 activity in ESCs is associated with the demethylation and expression of pluripotency related genes as well as repression of Polycomb targeted developmental regulators^[317]. Moreover, TET proteins were found to participate in various gene expression regulatory complexes *via* interaction with Sin3A co-repressor^[318], Polycomb Repressing Complex 2 (PRC2)^[317] and the O-linked N-acetylglucosaminetransferase, (Ogt)^[319,320].

Although earlier studies have suggested a role for TET1 and TET2 in ESC self-renewal^[321], it was recently clarified that these enzymes are in fact required for proper differentiation of ESCs^[322]. TET1 and TET2 are regulated by the Oct4-Sox2 complex in ESCs and TET1 knock down promotes the differentiation toward endoderm/mesoderm and trophoblast pathways^[315]. A Tet1/2/3 triple knock-out mouse ESC line was unable to generate embryoid bodies, teratomas and could not give rise to healthy chimeras^[322].

A current model proposes that the accumulation of epigenetic and/or genetic changes in a normal adult stem cell generates eventually a heterogeneous tumor population that contains a subset of "CSCs" that are responsible for its long term maintenance^[323]. Regardless of the hierarchical or stochastic nature of the CSCs, studying their epigenome is of paramount importance both for understanding the origin and evolution of cancer as well as applying novel epigenetic therapies. As a result, a growing number of studies has recently been directed in the elucidation of common or distinct epigenetic mechanisms that govern the self-renewal program of ESCs and CSCs.

Cancer epigenome exhibits global DNA hypomethylation and specific promoter hypermethylation^[324,325]. DNA hypomethylation promotes cancer development by increasing genomic instability and activating growth-promoting genes such as R-Ras^[326]. On the contrary site-specific hypermethylation favors oncogenesis by repressing tumor suppressor genes, other genes encoding transcription and DNA repair factors as well as PRC target genes^[327,328]. The above studies provide compelling evidence for deregulated DNA methylation in cancer but do not address distinct cell subpopulations. In this line, Yasuda *et al.*^[329] studied 10 tumor suppressor genes (TSGs) in bulk and CSC enriched MCF7 breast cancer cells based on their ability to form tumor-spheres and found lower DNA methylation levels and H3K27m3 marks in the latter population. Ikegaki *et al.*^[330] proved that epigenetic modifiers can affect the expression of stemness genes and contribute to the establishment of CSCs. They showed that short-term treatment of Neuroblastoma cell lines with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AdC) and/or the histone deacetylase inhibitor 4-phenylbutyrate, enhances their CSC phenotype^[330].

The expression levels of DNMTs are elevated in several cancer types^[331-333]. DNMT3b has been shown to play a crucial role in *de novo* hypermethylation of promoter CpG islands. In line with the role of DNMTs in

cancer development, sustained overexpression of the murine *dnmt1* gene in NIH3T3 cells results in cellular transformation^[334]. Conversely, reduction of DNMT1 by an antisense DNMT-RNA reversed the transformed phenotype of the Y1 tumor cell line^[335].

Morita *et al.*^[336] compared colorectal HCT116 WT and its *dnmt1* knockout derivative and showed that the latter has reduced stem cell markers and contained less CSCs, as assessed by tumor formation following xenotransplantation. Again no difference in DNMT3a and DNMT3b was detected^[336]. The importance of DNMT1 in CSC self-renewal was further confirmed by Trowbridge *et al.*^[337] using MLL-AF9 induced mouse leukemia. Additionally, a recent paper about the lyotropic reagent chloroquine reports that it can eliminate CSCs in a TNBC population through reduction of DNMT1 and Jak2 expression^[338]. The above studies demonstrate the role of DNMT1 in the maintenance of the CSC properties and *in vivo* tumorigenicity.

Concerning the effects of DNA demethylase, 5hmC levels are decreased in a broad range of cancer cells^[339]. TET1 has been found as a fusion partner of MLL in a subset of patients with acute myeloid leukemia^[340,341]. TET2 has been reported as one of the most frequently mutated genes in hematopoietic cancer types, but many mutations appear in sites that don't affect its enzymatic activity^[342]. In agreement with this, it was recently found that enzymatically inactive TET1, acting as a transcriptional co-activator for Hif1a, is required for EMT^[343]. Additionally, work by Song *et al.*^[344] has shown that TET2 is involved in breast cancer stemness and metastases due to the silencing of miR-200.

Investigating the methylation signature of CSCs permits identification of modifiers that can target their stemness properties, leading to increased tumor sensitivity to chemotherapy. Current DNMT inhibitors used in cancer therapy, such as Decitabine (5'-aza-2' deoxycytidine) act through incorporation into DNA therefore causing adverse side effects^[345]. Less hazardous alternatives include use of small molecule inhibitors such as SGI-1027^[346] and dietary phytochemicals^[342].

Chromatin modifiers in ESCs and CSCs

It is the complex interplay of DNA methylation with the posttranslational modifications of the histone tails that determines the transcriptional activity of a particular locus^[347]. The effect of histone modifications on diverse cellular functions, has caused intense interest in studying the chromatin modifying enzymes. These epigenetic modifiers include a variety of factors, such as histone methyltransferases (HMTs), demethylases (HDMs), acetyltransferases (HATs) and deacetylases (HDACs)^[348,349]. All the above mentioned factors participate in the regulation of chromatin structure that in turn governs gene transcription.

ESCs are characterized by permissive chromatin structure and consequently higher transcriptional activity compared to differentiated cells. Generally,

histone marks associated with active transcription are more abundant in ESCs and become reduced upon differentiation^[350,351], whereas repressive marks appear in higher levels in differentiated cells. Another interesting feature most commonly found in ESCs are bivalent domains, which are defined by the presence of the active H3K4me3 mark alongside the repressive H3K27me3 mark and is believed to hold genes in a "transcription-ready" state^[352,353]. However a recent study by Denissov *et al.*^[354] challenges the prevailing view by showing that not all bivalently marked genes in mESCs lose their differentiation responsiveness upon loss of H3K4me3. Thus, the role of bivalency and its association with pluripotency remains an open question.

Self-renewal in ESCs necessitates the action of chromatin repressive complexes in order to inhibit expression of differentiation-promoting genes. The best studied silencers of differentiation pathways in pluripotent cells are the PcG proteins, which are organized into two multiprotein complexes PRC1 and PRC2^[355]. PRCs are highly expressed in ESCs and bind mainly to CpG-rich promoters of developmentally regulated genes^[186,356].

The PRC2 complex has three core protein subunits: The enhancer of zeste homology (EZH2) component that catalyzes di- and trimethylation of H3k27, Embryonic ectoderm development (Eed) and suppressor of zeste (Suz12). PRC2 triggers gene silencing by recruiting PRC1, histone deacetylases and DNA methyltransferases^[357]. The PRC1 complex composition is highly variable and the canonical complexes include CBX (polycomb), polycomb group factor (PCGF), human polyhomeotic homolog (HPH) and RING, the E3-ligase that catalyzes the monoubiquitination of histone H2A on lysine 119^[358].

Depletion of PRC2 results in embryonic lethality in mice^[359], while mESCs lacking Eed, Suz12 or Ezh2 show loss of H3K27me2/3, retention of self-renewal capacity and *in vitro* differentiation defects^[360-363]. On the contrary, inactivation of PRC1 in mice leads to deficiencies concerning later developmental stages^[364]. RING1b (subunit of PRC1 complex) deficient mESCs show a slight deregulation of some genes and loss of differentiation potential^[365], whereas mESCs double mutated for Ring1a/Ring1b lose also the ability to self-renew^[366]. In summary, PcG proteins seem to be required for proper ESC cell fate transition but not for their self-renewal. More information is required about their partners to fully understand their regulatory role in ESCs^[367]. Moreover, it was shown that PRC1 and PRC2 can occupy distinct genomic sites and act independently^[368].

The maintenance of the expression program that determines pluripotency requires the presence of both repressive and activatory chromatin modifiers. A complex that counteracts the repressing effect of Polycomb is the Trithorax/MLL^[369]. Trithorax group (TrxG) complex contains a histone K4 tri-methyltransferase

(Set1a/b, MLL1-4), a subunit that recognizes the H3K4me3 mark, tryptophan-aspartate repeat protein 5 (WDR5), absent-small-homeotic-2-like (Ash2L), retinoblastoma-binding protein 5 (RbBP5) and dumpy-30 (DPY-30)^[369,370].

Both activating H3K4me3 and repressive H3K27me3 and H3K9me3 marks are removed by histone demethylases belonging to the Jumonji domain-containing protein family (Jmjd)^[371]. Jmjd proteins connect ESC core transcriptional network with chromatin modulation. More specifically, Jmjd2c participates in stem cell maintenance by reversing H3K9me3 marks at the Nanog promoter, consequently protecting it from silencing, whereas *Jmjd1a/2c* gene expression is positively regulated by Oct4^[372]. The diverse role of Jmjds is underlined by Pasini *et al.*^[362], who reported the involvement of JARID2 in the recruitment of PRC2 by differentiation-related genes in ES cells.

Another chromatin modifier called LSD1/KDM1 factor, is a histone demethylase that suppresses gene expression by removing methylation groups from H3K4. LSD1 has been found to colocalize with NuRD at the enhancer of pluripotency genes and down-regulates their expression upon differentiation^[373]. Finally, Suv39H1, Suv39H2 and G9a methylases, generate H3K9me3 repressive mark important in ESCs^[374].

Histone acetyltransferases (HATs) and the equivalent histone deacetylases (HDACs) constitute another critical category of chromatin modifiers acting as co-activators or co-repressors respectively^[375]. Although deacetylation is associated with gene silencing, ChIP-sequencing studies show that HDACs also colocalize with acetyltransferases at transcriptionally active loci, probably to reset acetylation levels after gene activation^[376].

HDAC1 and HDAC2, the most studied HDACs in ESCs, have been shown to be dispensable for mESCs self-renewal^[377]. However, HDAC1 knockout- ESCs show differentiation defects. HDAC1 and HDAC2 are usually part of large complexes with repressive action like NuRD, CoREST and Sin3^[378]. Alteration of the histone acetylation pattern interferes with stem cell pluripotency and differentiation^[376] as well as reprogramming^[379]. Most importantly inhibitors of HDACs are used as facilitators of reprogramming^[380]. Noteworthy, NuRD complex, which couples chromatin remodeling capacity and histone deacetylation activity^[381], has been shown to act as negative regulator of pluripotency associated genes by fine tuning their expression levels and sensitizing cells to differentiation stimuli^[382]. The distinct repression targets of PRCs and NuRD may explain why reprogramming efficiency is increased by overexpression of PRCs components^[383], but depletion of NuRD proteins^[384].

Finally, small molecule drugs targeting histone demethylases or DNA demethylases are also valuable tools for reprogramming since they can substitute for transcription factors^[385].

The information related to chromatin modifiers

in ESCs has facilitated the elucidation of their role in tumorigenesis. In addition to DNA hypo-methylation, reduced histone acetylation but enhanced histone methylation is an epigenetic feature characteristic of cancer cells^[386,387].

Polycomb HMT proteins are commonly upregulated in cancer^[388]. EZH2, the best studied PcG protein has been found to promote tumor growth by inhibiting pro-differentiation pathways and enhancing cell cycle progression^[389]. Furthermore HDMs, which reverse the action of HMTs, like LSD1, have also been implicated in oncogenesis^[390]. Increased HDAC activity usually characterizes cancer cells^[391]. Resetting normal acetylation levels through treatment with HDAC inhibitors (HDACi) has lowered tumorigenicity, suggesting HDACs as attractive targets for cancer epigenetic therapy^[392].

The role of chromatin regulators in CSCs has recently started to be under study with Polycomb Group (PcGs) proteins to be in the spotlight. EZH2 proved to be essential for the maintenance of breast and pancreatic CSCs^[393]. Intriguingly, EZH2 promotes NFκB signaling in ER-negative breast cancer cells^[243], that in turn has been shown to contribute to the generation of CSCs through a positive feedback loop involving IL6^[394]. In previous studies, silencing of EZH2 in glioblastoma CSCs significantly delayed intracranial tumor formation, demonstrating the necessity for EZH2 in CSC-driven tumorigenesis^[395], whereas treatment of prostate cancer cells with the PRC2 inhibitor DZnep (3-Deazaneplanocin A) inhibited CSC spheroid formation and decreased CSC frequency^[396]. Furthermore, EZH2 seems to contribute to the stemness phenotype of Ewing tumors by suppressing endothelial and neuroectodermal differentiation^[397]. Gupta *et al.*^[398] developed a model of phenotypic transitions to study stochasticity in regulating cell-state equilibrium in cancer cells and EZH2 was used as one of the main phenotypic markers for the CSC population.

Increasing number of studies implicate BMI-1, another PcG member, in cancer stemness. Its expression has been found elevated in many CSC populations such as in salivary adenoid cystic carcinoma^[399], prostate^[400], esophageal^[401], head and neck^[402], cervical^[403], colorectal^[404], laryngeal^[405] and ovarian^[406] CSCs. In addition it has been reported that depletion of Suz12 - a component of PRC2 complex - results in the blockade of mammospheres formation^[407] and increased apoptosis in colon CSCs^[408]. These findings highlight Suz12, as an essential regulator of CSCs.

Downregulation of the histone methyltransferase MLL1 reduces CSC self-renewal and tumorigenicity suggesting a role in CSCs^[409]. Additionally, HDMs like LSD1 are crucial for the biology of CSCs too. Wang *et al.*^[410] proved that inhibition of LSD1 inhibits the proliferation of pluripotent cancer cells but not that of normal somatic or non-pluripotent cancer cells. Except for histone methylation, histone acetylation is also essential for CSCs as a recent study showed that inhibition of HDACs limits the population of CSCs of

head and neck cancer^[411].

In conclusion, epigenetic regulators such as members of the Polycomb and Trithorax complexes, histone demethylases and histone deacetylases are essential for ESC pluripotency and at the same time can promote cancer stemness (Table 3). DNA methyltransferases are indispensable for both cell types and have been already reported as important targets for iPS generation and cancer chemotherapies (Table 3). Finally, DNA demethylases (TET 1, 2, 3) are critical targets for iPS generation, whereas their functions in CSCs await further clarification (Table 3).

MicroRNAs in ESCs and CSCs

The crucial role of microRNAs in mouse and human ESCs has been identified using Dicer and DGCR8 knockout mice. Dicer deletion resulted in embryonic lethality in mice^[412], while DGCR8-deficient mouse ESCs retained self-renewal capacity^[413].

Among the first families of microRNAs identified, miR-290-295 (miR-371 family, human homologous) and miR-302-367 clusters, which include the majority of miRNAs in mouse and human ESCs. Common characteristics of the two clusters is the promoter binding of the core pluripotency transcription factors (Oct4, Sox2 and Nanog)^[414] and the decrease of their expression during differentiation^[415]. Reintroduction of these miRNAs into Dicer1-knockout^[416] and DGCR8-knockout^[413] mice rescued proliferation and normal ESC self-renewal, respectively. It was found that these two clusters maintain the self-renewal by targeting retinoblastoma like 2 (Rb12), a repressor of DNA methyltransferases (Dnmt3a and Dnmt3b). The latter methylates CpG islands and epigenetically silences Oct4^[416,417]. In addition, these miRNAs were shown to regulate the G1/S transition of the ESC cell cycle, by repressing directly or indirectly the expression of the G1/S transition inhibitors (p21, Lats2, Rb1, Rb12 and Rb11)^[418,419].

Other miRNAs acting on the hESC cell cycle were studied by Qi *et al.*^[420], showed that miR-195 and miR-372 promote the transition of G2/M and G1/S, by suppressing the G2/M checkpoint kinase WEE1 and CDKN1A respectively^[420]. Furthermore, miR-92a and 92b were identified to target the *CDKN1C* gene and CDKN2B (known as p57), promoting in this way the G1/S transition^[421].

Beside their function in maintaining pluripotency, miRNAs play important role in the differentiation of ESCs. Tay *et al.*^[422,423] demonstrated that miR-134, miR-296 and miR-470 bind to the coding regions of Oct4, Sox2 and Nanog and suppress their expression and thus the self-renewal state. Similarly, miR-200c, miR-203 and miR-183 target Sox2 and Klf4^[267], while miR-145 was shown to repress human OCT4, Sox2 and KLF4 by binding to their 3'UTR, suggesting its role to regulate pluripotency^[424]. In another study, induction of differentiation caused the increase of miR-21 expression levels revealing its crucial role in stem cell

differentiation by targeting Nanog, Sox2 and Oct4^[425]. Moreover, the expression of miR-22 was also detected in high levels during ESC differentiation^[426]. Landgraf *et al.*^[427] demonstrated that miR-26a, miR-99b, miR-193, miR-199a-5p, and miR-218 are able to suppress the self-renewal of ESCs but the mechanism remains unclear. Another example of miRNAs that regulate the differentiation of ESCs is the miR-125 and miR-181 clusters^[428]. A recent study showed that miR-125 and miR-181 families suppress Cbx7, the primary Polycomb ortholog of the PRC1 complex, in undifferentiated ESCs. Their overexpression leads to the differentiation of ESCs via regulation of Cbx7^[428]. Furthermore, it was found that miR-34a, miR-100, and miR-137 are required for the differentiation of ESCs, and that they function in part by targeting Sirt1, Smarca5 and Jarid1b mRNAs^[429].

Let-7 is another miRNA family which has been widely involved in the establishment of the differentiated cell fate. Melton *et al.*^[430] identified that silencing of pluripotency and self-renewal of ESCs can be caused by the introduction of let-7 into DGCR8-knockout ESCs. In addition, let-7 binds to 3'UTR, inhibits expression of several stemness factors (c-Myc, Sall4n, Lin28) and induces ESCs differentiation^[430]. Interestingly, Lin28 forms a negative feedback loop with let-7, resulting from its blocking function during let-7 biogenesis at the Dicer processing step^[431,432]. Let-7 can also target the G1/S transition activators (cdc25a, cdk6, cyclinD1 and cyclinD2) that increase susceptibility of G1 phase cells to pro-differentiation signals and promote differentiation of ESCs^[431,432].

MiRNAs also play important roles in regulating CSC properties such as cell cycle exit and pluripotency, pro-survival and antistress mechanisms, EMT, migration and invasion, which contribute to tumor metastatic potential.

Because of the early discovery and better understanding of breast CSCs, miRNA studies are more advanced in this cancer type^[433]. Let-7 regulates the properties of CSCs and its overexpression results in the reduction of mammosphere and tumor formation, metastasis and cell proliferation. Moreover, let-7 diminishes the expression of HMAG2, c-Myc, and RAS and controls CSC properties^[434]. Another miRNA that is repressed in breast CSCs is miR-30. The inhibition of miR-30 induces metastasis and self-renewal of breast CSCs^[435] and the transfection of both let-7 and miR-30, causes a more complete reduction of mammospheres formation and CSC abilities in breast CSCs^[435].

Iliopoulos *et al.*^[436] showed the importance of an inflammatory positive feedback loop involving NF-κB, Lin28, let-7 and Il6 in the epigenetic maintenance of the cell transformation state following Src oncogenes activation. The same group using miRNA profiling defined a set of 22 miRNAs that are differentially expressed in normal vs CSCs that included the let-7 and the miR-200 families^[407]. Interestingly, Shimono *et al.*^[437] also identified that miR-200 cluster is down-regulated in breast CSCs and miR-200c reduces the

tumor formation. In addition, members of this family have the ability to regulate the breast CSC properties by repressing the BMI-1^[437] and a subunit of a polycomb repressor complex, SUZ12^[407]. Furthermore, several studies showed that miR-200 family and miR-205 modulate EMT, which is crucial for metastasis and tumor invasion. It was found that the expression of these miRNAs is decreased in cells undergoing TGF-beta induced EMT and their overexpression inhibits the EMT process. These miRNAs modulate the expression of EMT activators, ZEB1 and ZEB2, causing the inhibition of EMT program^[438]. In contrast, ZEB1 and ZEB2 activate EMT by forming a double negative feedback loop with the miR-200 family, resulting from their binding to promoter regions of miR-200 family members^[439]. As previously mentioned, miR-22 directly suppresses the expression of the TET family members (TET1-3), which are implicated in the demethylation of miR-200 promoter. In other words, miR-22 inhibits the activity of miR-200 cluster and promotes EMT and metastasis^[344]. Polytaichou *et al.*^[440] identified that miR-15/16, miR-103/107, miR-145, miR-335, and miR-128b inhibit breast CSC function and growth by directly inhibiting the expression of Suz12, Bmi1, Zeb1, Zeb2, and Klf4.

MiR-9/9*, miR-17 and miR-106b are the mostly represented miRNAs in the CSC population in glioblastoma. It was demonstrated that knocking down of miR-9 and miR-17 causes a reduction of neurosphere formation^[441]. Another study showed that miR-128 reduces glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo*^[442]. Furthermore, miR-128 overexpression modulates the properties of glioma^[443] and prostate CSCs^[400] by inhibiting BMI-1. Li *et al.*^[444] examined the role of miR-34a in brain tumor cells and human gliomas and they demonstrated that miR-34a causes cell cycle arrest, apoptosis or xenograft tumor repression, regulating the glioblastoma CSCs. In CD133⁺ CSCs of glioblastoma, miR-145 causes the inhibition of tumor formation as well as the reduction of CSC properties by targeting Sox2 and OCT4^[445]. miRNA expression profiles of glioblastoma stem cells and non-stem cells revealed that miR-451 is downregulated in the glioma CSCs. Transfection with the above miR leads to the inhibition of tumor growth of glioma CSCs and neurospheres formation^[446]. Another miRNA which is important for CSCs of brain tumors is miR-199b-5p. It was shown that miR-199b-5p blocks Notch signaling and inhibits the self-renewal capacity of medulloblastoma cells by targeting HES-1, a transcription factor of the Notch signaling pathway^[447].

Using prostate CSCs, Liu *et al.*^[448] performed miRNAs expression profiling and found that miR-34a, let-7b, miR-106a and miR-141 are downregulated whereas miR-301 and miR-452 are increased. They also showed that overexpression of miR-34a inhibits prostate CSC metastasis by targeting CD44, while knocking-down of miR-34a promotes tumor migration and development^[448]. MiR-320 was also investigated for

its role in prostate CSCs. Hsieh *et al.*^[449] illustrated that β -catenin is directly targeted by miR-320. Moreover, gene expression profile of miR-320-overexpressing prostate cancer cells revealed a diminished expression of both the genes involved in the Wnt/ β -catenin pathway and the markers of CSCs^[449].

As mentioned before, miR-34a does not play pivotal role only in glioma and prostate CSCs, but also in pancreatic and gastric CSCs^[450,451]. Overexpression of miR-34a in these two cancer types reduces sphere formation and tumor regeneration^[450,451]. Bu *et al.*^[452] reported that miR-34a inhibits colon CSC self-renewal and suppresses tumor formation. Particularly, miR-34a targets Notch-1 resulting in inhibition of Notch signaling which is frequently crucial in colorectal cancer^[452].

LncRNAs in ESCs and CSCs

A number of studies investigated the role of lncRNAs in self-renewal and differentiation of mouse and human ESCs. By performing genome-wide screening, Sheik Mohamed *et al.*^[453] identified four lncRNAs residing proximally to the genomic binding sites of Oct4 and Nanog. Two of them, the AK028326 and the AK141205 have been shown to be the direct targets of Oct4 and Nanog, while their overexpression or inhibition resulted in dramatic changes in the mRNA expression levels of the two core transcription factors, revealing their involvement in the regulatory network^[453]. Chakraborty *et al.*^[454] introduced a new technique combining knock-down and localization analysis of noncoding RNAs (c-KLAN) to study lncRNAs. By inhibiting Panct-1, a non-coding transcript, the amounts of Oct4 and Nanog mRNA were decreased, whereas the expression of differentiation markers increased (Gata6, Fgf5, and T-Brachyury)^[454]. To further examine the role of lncRNAs in ESCs, Wang *et al.*^[418] demonstrated the lncRNA-RoR function as a critical regulator of self-renewal and differentiation in hESCs. lncRNA-RoR prevents the suppression of Oct4, Sox2 and Nanog by miRNAs (miR-145) and forms a feedback loop with the core transcription factors and microRNAs^[418].

Concerning hESCs, the lncRNA_N1 lncRNA_N2 and lncRNA_N3 are important regulators of neuronal differentiation fate^[455].

LncRNAs have also been investigated in cancer. Their role is not well understood in CSCs, but recent studies have correlated some lncRNAs with CSC activity, based on their ability to promote metastasis^[103]. Gutschner *et al.*^[456] developed a metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) knockout model in human lung tumor cells, which confirmed the role of MALAT1 as a biomarker for lung cancer metastasis and revealed its ability to regulate genes associated with lung cancer metastasis^[456]. Furthermore, MALAT1 was implicated in cervical cancer by regulating gene expression (caspase-3, -8, Bax, Bcl-2, BclxL), while in a more recent study it was shown that MALAT1 promotes cell migration and proliferation of cervical

cancer cells^[457,458]. HOTAIR is another lncRNA which has been widely studied for its role in cancer. Gupta *et al.*^[459] demonstrated that HOTAIR is a strong metastasis and tumor invasiveness biomarker in primary breast tumors. This function is due to the recruitment of PRC2 that silences metastasis suppressor genes^[459]. Moreover, other studies suggest that HOTAIR is a potential biomarker for the existence of lymph node metastasis in hepatocellular carcinoma (HCCs)^[460]. As discussed earlier, lncRNA-RoR is an important ESC self-renewal regulator. Recently, it has been shown that its expression was negatively regulated by the NRF2 transcription factor in mammary stem cells^[461]. In particular, the inhibition of NRF2 led to an increase of both mammosphere formation in breast cancer cells and lncRNA-RoR levels supporting an involvement of lncRNA-RoR in tumorigenicity^[461].

To summarize, the above studies demonstrate an essential role of ncRNAs in stemness of ESCs and CSCs (Table 3). A better understanding of ncRNA biology will ultimately provide further insights into the molecular mechanisms of tumorigenesis and lead to the development of new therapeutic strategies against cancer.

DISCUSSION

In recent years there is a tremendous expansion of research focused on the biology of stem cells. As understanding of the differences and similarities of stem cells of various normal or abnormal developmental origins increases, so is the appreciation of their great complexity. In this review we have presented an overall comparison between embryonic and CSCs in terms of biomarkers (Tables 1 and 2), signaling pathways (Figure 1, Table 3), transcriptional and epigenetic regulators (Table 3).

Cell surface markers are very important for the characterization and separation of stem cells. Pluripotent (ES, iPS) cell markers are very useful for fast and effective enrichment when undifferentiated cells are required (positive selection). In addition, such markers are valuable in order to eliminate the pluripotent cells from their differentiated descendants (negative selection) before transplantation. Concerning cancer, a small proportion of cells that bears stem cell features - CSCs - are endowed with self-renewal and differentiation capacity and enhanced tumorigenicity. These cells are refractive to conventional therapies. In line with the above, promising therapies can be based on targeting CSC *via* their specific surface markers, as it has been shown in experimental models and clinical trials. An obvious disadvantage of such an approach is that while various markers are currently reported to mark or enrich for CSCs, no exclusive CSC markers are so far available to ensure high cell specificity of targeting. Table 2 shows combinations of markers that are used in order to identify CSCs from various cancer types. Among them, the major category are cell adhesion or

communication molecules CD44, Esa/EpCAM, CD166, CD129 and receptor-associated proteins such as Lgr5, c-met, c-kit. The enzymatic activity of ALDH can be a useful tool for separating CSCs, however a detailed identification of the active isoforms that are expressed in various cancer types is required^[462]. Most importantly, some ALDH isoforms can have functional roles in CSCs since they are directly involved in retinoic acid signaling and stemness. Another category of various CSC markers are the ABC transporters (Table 2) that actually render CSCs resistant to therapies. A recent promising method for CSC isolation is based on measurement of the intrinsic auto-fluorescence of epithelial CSCs due to the accumulation of riboflavin in cytoplasmic complexes containing ABCG2 transporters^[463]. Some CSC markers are also common with those of ESCs such as CD133, SSEA1, EpCAM and the pluripotency transcription factors Oct4, Sox2 and Nanog. Brescia *et al.*^[464] have demonstrated that silencing CD133 expression in human GBM neurospheres disrupts the self-renewal and tumorigenic properties of the neurosphere cells, indicating that CD133 could potentially be used as a therapeutic target in these tumors. Transcription factors regulating pluripotency could be particularly useful as biomarkers since they are not expressed in tissue stem cells. However, pluripotency factors are intimately cross-regulated and difficult to assess unless convenient assays are used. The importance of these common markers in tumor aggression and metastasis awaits further investigation.

Cell differentiation during embryonic development and cell transformation that leads to oncogenesis share common signaling pathways, suggesting that deregulation of embryonic signaling pathways in adult tissues might result in tumor progression by transforming adult stem and progenitor cells. Many signaling pathways are involved in the stemness of both pluripotent (ES/iPS) and CSCs (Figure 1, Table 2). Wnt, TGF/BMP and FGF are important regulators of ESC self-renewal and differentiation and are able to enhance the CSC population. On the other hand, Hedgehog and Notch pathways have great importance for CSCs and only marginal role in ESCs. Chemical inhibitors of the Wnt, Notch and Hh pathways have been proven valuable in combating CSC populations from diverse solid tumors^[79]. Agents that regulate Wnt signaling such as the CBP/ β -catenin antagonist ICG-001, that forces β -catenin to bind p300 instead of CBP, are tested in pre-clinical trials for the eradication of CSC^[464]. Gamma-secretase inhibitors are able to block the Notch signaling pathway resulting in inhibition of CSC proliferation and tumor regression^[79]. Cyclopamine and synthetic small molecules that antagonize Hh pathway are used alone or in combination with conventional antitumor agents for the inhibition of pancreatic and glioblastoma CSCs^[465].

Additional research on the regulation of normal stem cell response to external stimuli will offer alternative therapeutic means for cancer treatment.

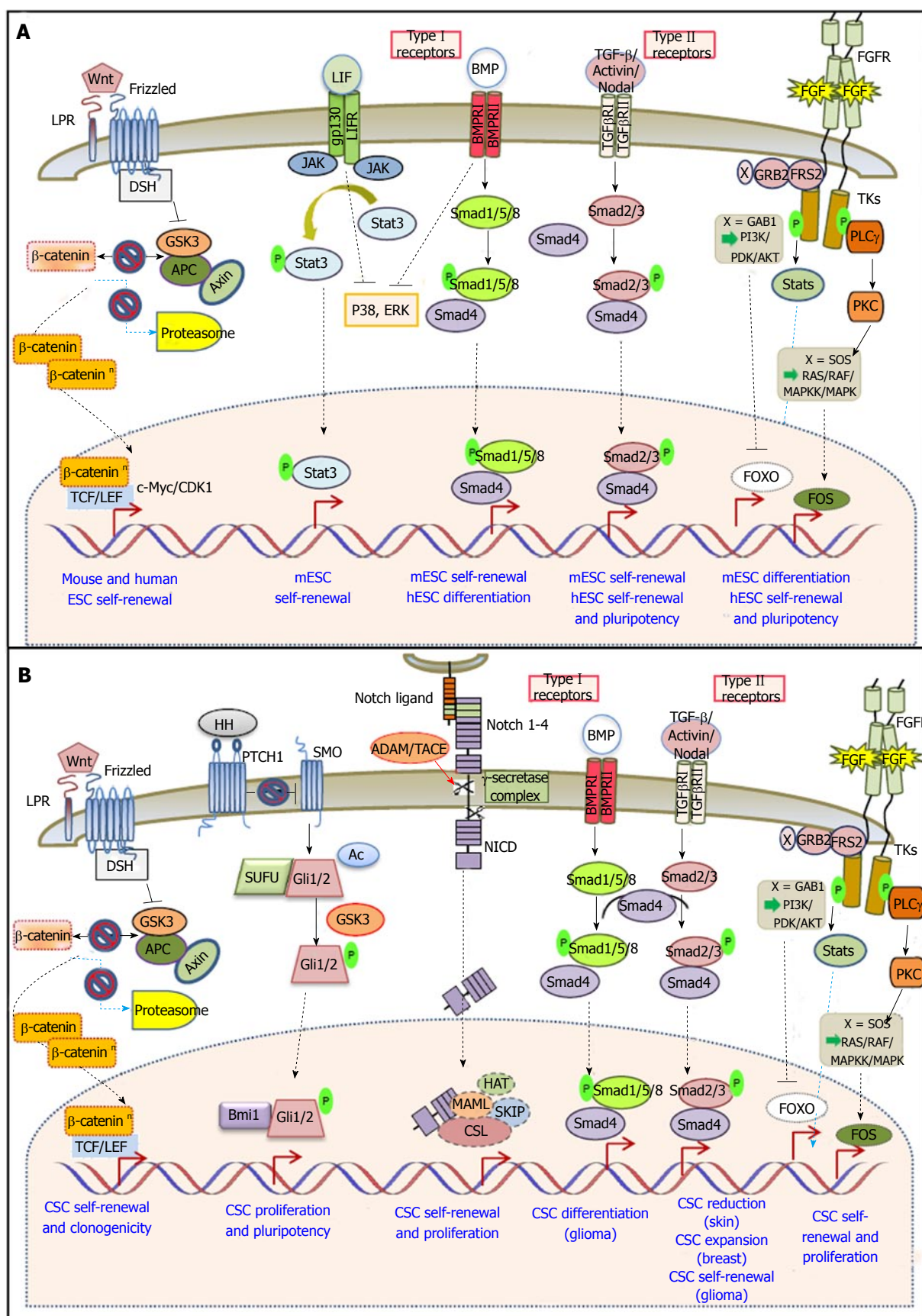


Figure 1 Core signaling pathways in embryonic stem cells and cancer stem cells. A: Mouse ESCs require concerted LIF/BMP signaling to sustain self-renewal and pluripotency, whereas hESC pluripotency depends on the activity of the TGF- β and the FGF pathway. Wnt signaling is important for pluripotency in both species; B: Wnt, Hedgehog, Notch and FGF signaling pathways are associated with CSC self-renewal, while BMP signaling is implicated in CSC differentiation. The TGF- β /Activin/Nodal pathway has a controversial role in CSCs depending on cancer type. ESCs: Embryonic stem cells; CSCs: Cancer stem cells; hESC: Human ESCs; APC: Adenomatous polyposis coli; Dsh: Dishevelled; FGF: Fibroblast growth factor; GSK3: Glycogen synthase kinase 3; JAK: Janus-family tyrosine kinase; LRP: Lipoprotein receptor-related protein; PKC: Protein kinase C; SUFU: Suppressor of fused homolog; TCF/LEF: Tcell factor/Lymphocyte enhancer binding factor.

Emerging evidence suggests that ESCs and CSCs may depend on common critical transcription factors (Table 3). In addition to the Myc oncogene that orchestrates a complex on its own (Myc-complex), CSCs also express members of the ESC core pluripotency complex such as Oct4, Sox2 and Nanog^[466]. Although Oct4, Sox2 and Nanog expression positively correlates with the development of CSCs, the role of Klf4 is highly context-dependent^[467]. While it promotes the self-renewal of various CSCs, it can inhibit EMT signaling pathway^[260,265] and attenuate lung and liver metastases^[468]. Pluripotency transcriptional factors may provide advantageous targets for the elimination of CSCs. For this purpose it would be important to characterize their regulatory circuits in CSCs to the same precision as it was previously attained in ESCs. This knowledge may provide novel approaches for combating cancer^[247,469].

In addition to transcription factors, critical regulators of gene expression in embryonic and CSCs are non-coding RNAs (ncRNAs). Interestingly, ncRNAs, which are important for ESC pluripotency such as lncRNA-RoR, miR-145, miR-200, miR-34a and let-7, can affect the generation of CSCs (Table 3). Accumulating evidence indicates that individual miRNAs and lncRNAs behave as tumor suppressors or oncogenes. Among them, the miR-200 family in combination with the TGF- β signaling are major determinants of the EMT transition that correlates tightly with the appearance of CSCs^[433,434]. Therefore, further investigation of their function in ESCs and CSCs may lead to clinical applications of non-coding RNAs in cancer diagnosis, treatment, and prognosis.

Epigenetic alterations are very important for the inheritance of the cellular phenotype properties. By governing chromatin state transitions and gene expression regulation, epigenetic processes have a crucial role in cell differentiation and tumorigenesis. Most importantly differentiated cancerous cells are driven into stemness *via* a profound epigenetic reprogramming that sustains the malignant phenotype^[470,471]. Although there is already accumulating information regarding epigenetic alterations and therapeutic use of epigenetic modifiers in cancer, less is known about their role in CSCs. Muñoz *et al.*^[471] recently reviewed the role of epigenetic effectors (such as DNMT, EzH2, BMI1 and MLL1) in leukemia and solid tumors and proposed that epigenetic targeting of CSCs may contribute to therapy. The advent of somatic cell reprogramming to pluripotency (iPS) offered a new way to test the effect of extreme epigenetic changes in tumor cells. Can cancer cells be reprogrammed to pluripotency and then induced to differentiate? Although not abundant, recent results in this area suggest a cancer context dependent response to the iPS process^[472]. In those cases, pluripotency was able to dominate over the cancerous phenotype leading to "normalization" although the neoplastic identity reappeared when cancer iPS cells were coaxed to differentiate to their lineage of origin^[472]. Thus reprogramming could offer a new way to track the history of tumor progression.

In conclusion, studies in the fields of stem cells, iPS and CSCs provide strong evidence of cross-complementing benefits. Insight of the tumorigenic properties of stem cells and their differentiated descendants is required before their use in cell replacement therapies^[473]. Understanding the biology and gene circuits shared by both normal stem cells and CSCs, is important for efficient cancer treatment. Cancer heterogeneity is contributed by both clonal and CSC components that are responsible for tumor aggressiveness, invasion and resistance to therapies. An emerging concept in cancer biology is that primary tumors may be composed of evolving and variable clones, each containing biologically distinct stem cells. As cell line models suffer from limited preservation of heterogeneity and newer models of patient derived xenografts are much more labor intensive and costly, alternative, *ex vivo* cancer models are important. As a result, use of the iPS approach may be useful. The hypothesis of normalizing cancer cells by epigenetic reprogramming has been recently under testing but its limits are not defined yet. Mixed initial results may reflect the heterogeneity of the experimental systems utilized, and various oncogenic and genome aberrations that may not be reversed by pluripotency. However, this is a new promising area that may generate diverse iPS-cancer clones from primary tumors. Thus even if the iPS process cannot "cure" cancer, it may provide, as with other human diseases, novel experimental models for studying cancer biology and drug discovery.

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Therapies targeting cancer stem cells: Current trends and future challenges

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Abstract

Traditional therapies against cancer, chemo- and radio-therapy, have multiple limitations that lead to treatment failure and cancer recurrence. These limitations are related to systemic and local toxicity, while treatment failure and cancer relapse are due to drug resistance and self-renewal, properties of a small population of tumor cells called cancer stem cells (CSCs). These cells are involved in cancer initiation, maintenance, metastasis and recurrence. Therefore, in order to develop efficient treatments that can induce a long-lasting clinical response preventing tumor relapse it is important to develop drugs that can specifically target and eliminate CSCs. Recent identification of surface markers and understanding of molecular feature associated with CSC phenotype helped with the design of effective treatments. In this review we discuss targeting surface biomarkers, signaling pathways that regulate CSCs self-renewal and differentiation, drug-efflux pumps involved in apoptosis resistance, microenvironmental signals that sustain CSCs growth, manipulation of miRNA expression, and induction of CSCs apoptosis and differentiation, with specific aim to hamper CSCs regeneration and cancer relapse. Some of these agents are under evaluation in preclinical and clinical studies, most of them for using in combination with traditional therapies. The combined therapy using conventional anticancer drugs with CSCs-targeting agents, may offer a promising strategy for management and eradication of different types of cancers.

Key words: Cancer stem cells; Targeted therapy;

Anticancer drugs; Biomarkers; Signaling pathways; Apoptosis

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Core tip: Cancer stem cells (CSCs) play important roles in tumor formation, metastasis and cancer relapse. In this article, we review the literature on the recent progress in developing anti-cancer stem cell strategies based on improved understanding of CSCs properties and molecular features. These novel therapeutic systems are designed with the aim of eradicating CSCs, by targeting surface specific markers and altering signaling pathways or mechanisms involved in CSCs maintenance and drug resistance, and also to disturb microenvironmental signals that sustain CSCs growth, with specific aim of impede CSCs regeneration and cancer relapse.

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INTRODUCTION

Tumor progression is explained by two models: The clonal (stochastic) evolution model and cancer stem cell model. The first one sustain that all transformed cell within a tumor have carcinogenic potential, with unlimited proliferation capacity, and the disease healing requires as therapy the elimination of all tumor cells^[1,2]. This hypothesis is supported by several studies demonstrating that a large number of cancer cells sustain tumor growth when are transplanted into histocompatible mice and the relevant results in this area depends on the xenotransplantation model used. Thereby, modifying xenotransplantation assay conditions can increase the detection of all tumorigenic cells^[3,4]. The second cancer evolution model sustain that tumors evolve from a small population of cells with self-renewal ability and high resistance to chemotherapy and radiotherapy. These cells were called CSCs or cancer initiating cells, due to their capacity to auto-regenerate, proliferate and induce tumor formation. Among their properties, resistance to standard oncology treatments is responsible for ineffectiveness of traditional cancer therapies and lead to tumor recurrence and metastasis^[5].

The CSCs model gained wide acceptance over the last years, based on continuous observations. Recent data suggests that CSCs might evolve from normal stem cells, progenitors or more differentiated cells

with whom they share many resemblance like: Self-renewing, differentiation to progenitor cells, expression of surface markers, common signaling pathways and a close association with microenvironment^[2].

There are two hypothesis regarding CSCs formation: (1) transformation of normal stem cells or progenitor cells into CSCs, process that occurs through multiple gene mutations as result of genetic and epigenetic instability^[6]; and (2) tumor cells progressively acquire stem cell properties through reversal of ontogeny based on oncogene-induced plasticity^[7].

Several studies suggest that epithelial-mesenchymal transition (EMT) process characterized by the repression of epithelial markers (*e.g.*, E-cadherin) and up-regulation of mesenchymal markers (*e.g.*, vimentin, fibronectin and N-cadherin) can also generates cells with stem-like properties^[8]. Mani *et al.*^[9] using immortalized human mammary epithelial cells demonstrated that EMT induction results in the enrichment of cells with stem-like properties, an increased expression of stem-cell markers and an increased capacity of cells to form mammospheres. Gupta *et al.*^[10] showed that E-cadherin inhibition and EMT induction, respectively leads to an increase number of CSCs in breast cancer cell populations, characterized by an increased resistance to chemotherapy.

CSCs were first described in acute myeloid leukemia, in which a population of CD34⁺CD38⁻ was noticed to possess stem cells capacities of proliferation, self-renewal and differentiation, and to reconstitute a heterogeneous cell population in nonobese diabetic/severe combined immunodeficiency mice^[11]. Later, CSCs were identified in various solid tumors including glioma^[12], as well as breast^[13], head and neck^[14], lung^[15], pancreatic^[16], liver^[17], stomach^[18], colon cancer^[19], *etc.*

Current review presents basic information about CSCs and discusses targeted therapeutic strategies developed for cancer eradication.

CSC: DEFINITION, CHARACTERISTICS, MARKERS

CSCs are auto-regenerating cells, able to proliferate and differentiate through symmetrical and asymmetrical cell divisions, with tumorigenic potential and specific surface markers useful for CSC identification and isolation. Additionally, several other properties like sphere forming capacity in serum-free medium or soft agar, dye exclusion ability based on over-expression of drug-efflux pumps (ATP binding cassette or multidrug resistance transporters), enzymatic activity of aldehyde dehydrogenase 1, are used to identify CSCs. However, the most important property of CSCs can be verified only by *in vivo* assay: Tumorigenicity in animal model, maintained even after serial transplantation.

The surface markers for CSCs vary according to

Table 1 Cell surface antigens present on cancer stem cells

Cancer type	Surface antigens	Ref.
Leukemia	CD34 ⁺ CD38 ⁺ CD47 ⁺ CCL-1 ⁺ CD96 ⁺ TIM3 ⁺ CD32 ⁺ CD25 ⁺	[11,20-24]
Brain	CD133 ⁺	[12]
Head and neck	CD44 ⁺ CD24 ⁺	[14]
Breast	ESA ⁺ CD44 ⁺ CD24 ⁺	[13,16]
Pancreatic	ESA ⁺ CD44 ⁺ CD24 ⁺	[13,40]
Lung	CD133 ⁺ CD44 ⁺	[15]
Liver	ESA ⁺ CD133 ⁺ CD90 ⁺ CD44 ⁺ CD24 ⁺	[17]
Gastric	CD44 ⁺	[18,38]
Colorectal	ESA ⁺ CD133 ⁺ CD166 ⁺ CD44 ⁺ CD24 ⁺	[19,26]

tumor type. Main surface markers for CSCs from solid tissue are CD133, CD44 and CD24. To these, several other more specific markers might be added, according to tumor tissue origin (Table 1). Thus, the phenotype epithelial specific antigen (ESA⁺) together with CD44⁺-CD24⁺ was described in pancreatic CSCs^[16], while ESA⁺CD44⁺CD24^{low} was the phenotype identified in breast CSCs^[13]. For liver CSCs the following combination ESA⁺CD133⁺CD90⁺CD44⁺CD24⁺ was proposed^[17]. For hematological malignancy CD34 and CD38 are main surface antigens^[11]. Subsequently novel markers have been found to be highly expressed on leukemia stem cells than normal hematopoietic stem cells. These include CD47^[20], C-type lectin-like molecule-1 (CLL-1)^[21], CD96^[22], TIM3^[23], CD32 and CD25^[24].

Although recent studies have contributed to a better understanding of CSCs surface molecules, the picture is not complete. It is often observed that CSCs do not express the same markers, or that normal cells also express these surface antigens. Therefore, it is not possible yet to certainly isolate CSCs, but only to identify a CSCs-enriched population. Consequently, identification of CSCs must be based on additional functional assays such as the ability to form spheres in serum-free medium and to initiate tumor growth after serial transplantation in immunocompromised animal models, based on their self-renewal capacity. However, these assays also have limitations due to microenvironment. Thus, the *in vitro* assay may not detect quiescent stem cells that are not capable to develop spheres due to lack of additional extrinsic signals needed for their activation. Moreover, in this type of assay there is a selection pressure imposed by specific culture conditions in presence of exogenous growth factors. Serial transplantation as well might have limitations, since considerable number of cells is required to induce tumor growth *in vivo*, due to insertion in a foreign microenvironment deficient in specific signals for survival and development^[25].

Therefore, to specifically address CSCs in further experiments, it is necessary to sort cells based on surface markers and subsequently to assess their functional abilities by *in vitro* and *in vivo* specific assays.

THERAPIES TARGETING CANCER STEM CELLS

Traditional therapies against cancer, chemo- and radiotherapy, have multiple limitations that result in treatment failure and cancer recurrence. These limitations are related to systemic and local toxicity because the agents are not selective enough and may affect also healthy tissue. An additional restriction is drug resistance due to CSCs specific properties like: Slow rate of division, high expression of drug-efflux pumps, high capacity for DNA repairing, and also to microenvironment characteristics: hypoxia and acidosis. Therefore, targeting CSCs became essential in treating cancer and preventing tumor relapse.

Recently, multiple strategies have been conceived with the specific aim of destroying CSCs and their niche. These include targeting specific surface markers, modulation of signaling pathways, adjustment of the microenvironment signals, inhibiting of drug-efflux pumps, manipulation of miRNA expression, induction of CSCs apoptosis and differentiation. A summary of these therapeutic strategies is presented in the Figure 1. At the moment, some of them are successfully used in clinic, mainly in combination with traditional therapies, and others are still under evaluation.

Targeting surface markers

The surface markers used for identification and isolation of CSCs are also important targets for therapy. Immunotherapy that involves antibodies targeting CSCs specific markers is often used as an adjunct to chemotherapy, radiotherapy and surgery. The most important CSCs associated markers together with strategies for targeting them are mentioned below.

CD133 (prominin-1) is a cell surface glycoprotein widely expressed on CSCs in solid tumors such as glioma^[12], lung^[15], liver^[17] and, colorectal cancer^[19,26]. Cancers with large CD133 subpopulation have a drug resistant phenotype and poor prognosis. For this reason, several strategies for anti-CD133 therapy have been generated. The polymeric nanoparticles loaded with paclitaxel (CD133NPs) targeting CD133 were tested on colorectal adenocarcinoma Caco-2 cells and proved to efficiently decrease the cell number and colonies formed. On the other hand, in xenograft model, the CD133NPs improved therapeutic efficacy compared to free paclitaxel treatment^[27]. Another anti-CD133 antibody constructed through the fusion with pseudomonas exotoxin 38, inhibited the progression of tumor growth after multiple intraperitoneal injections of drug over a period of 4-6 wk in xenografted mice with ovarian cancer. The elimination of CD133⁺ ovarian cancer cells resulted in long-term disease free tumor survivors^[28]. These studies suggest that anti-CD133 therapy might be associated with drug delivery, forming antibody-drug conjugates that enhance the effect on

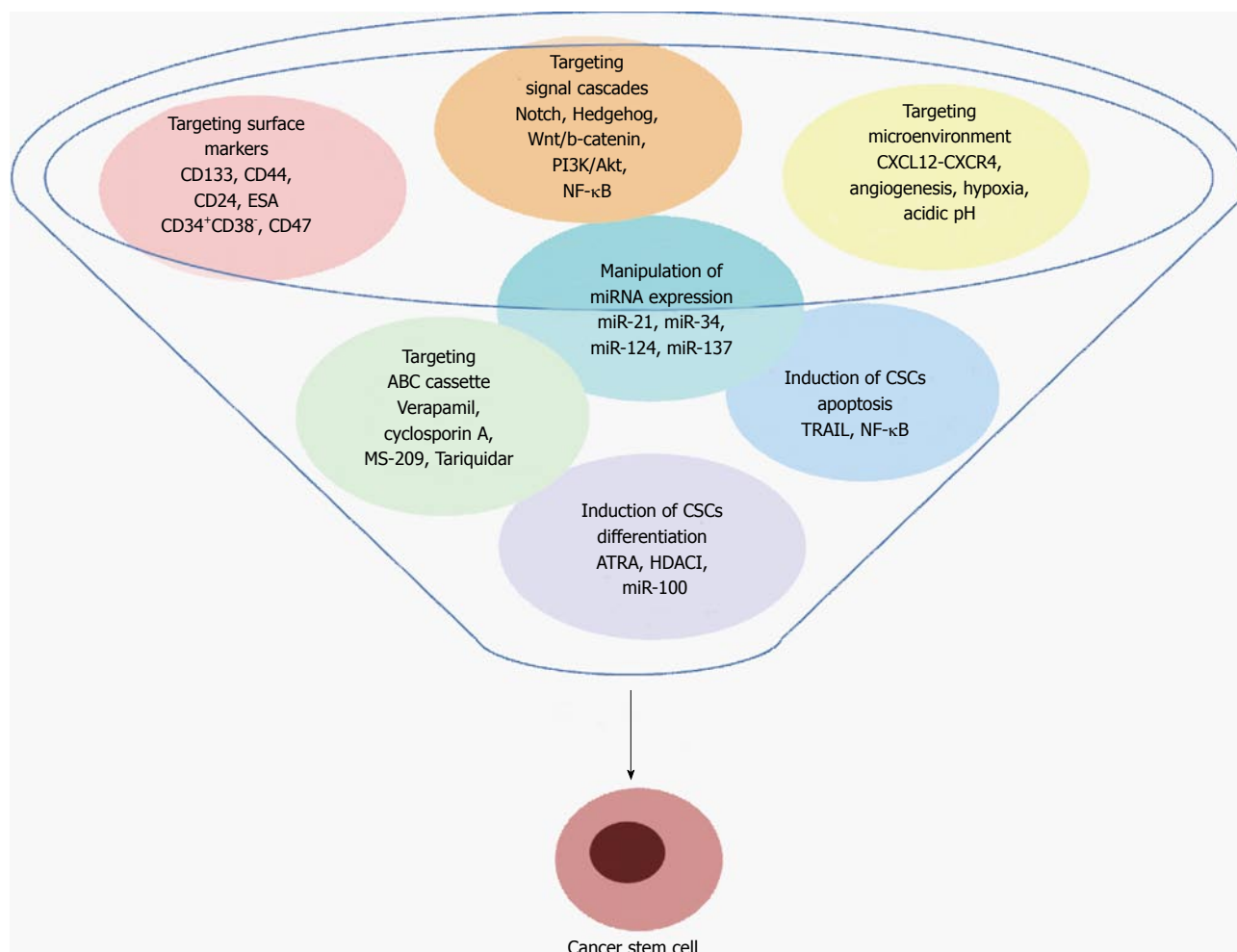


Figure 1 Therapies targeting cancer stem cells. Numerous therapies aiming to eradicate cancer stem cells have been developed during last year's. Here we highlighted most common seven approaches: targeting surface biomarkers, signaling pathways that regulate cancer stem cells (CSCs) self-renewal and differentiation, drug-efflux pumps involved in apoptosis resistance, microenvironmental signals that sustain CSCs growth, manipulation of miRNA expression, and induction of CSCs apoptosis and differentiation, with specific aim of hamper CSCs regeneration and cancer relapse. ATRA: All trans retinoic acid; HDACI: Histone deacetylase inhibitors; PI3K: Phosphatidylinositol 3-kinase; NF-κB: Nuclear factor kappa B; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand.

CD133⁺ CSCs and eliminate them. Anti-CD133⁺ cell therapy was also tested on sarcoma CSCs, reducing proliferative capacity and resulting in decreased sarcoma tumor-initiating ability^[29]. Similar results were obtained for pancreatic and hepatic CSCs^[30,31].

However CD133 expression is not restricted to CSCs. Shmelkov *et al.*^[32] demonstrated that both CD133⁺ and CD133-metastatic colon cancer cells can initiate tumors. Also, Beier *et al.*^[33] demonstrated that CD133⁺ and CD133-glioblastoma cancer cells meet stem cell criteria, but they might reflect two biologically distinctive glioblastoma subtypes. The authors concluded that primary glioblastomas might develop either from different cells of origin or from related cell types that further acquired different molecular alterations.

CD44 is a transmembrane protein that mediates cell to cell adhesion and cell to extracellular matrix interactions, being a receptor for hyaluronic acid, selectin, collagen, osteopontin, fibronectin and laminin^[34]. CD44 is involved in cell proliferation, survival, migration, differentiation, apoptosis, self-renewal, niche preparation, epithelial-mesenchymal transition, and resistance

to apoptosis^[35,36]. It was found overexpressed in many tumor cells: breast^[13], bladder^[37], gastric^[38], prostate^[39], pancreas^[40], ovar^[41], colorectal^[26,42], hepatocellular^[43], head and neck^[44], acute myeloid leukemia (AML) CSCs^[45], etc. Targeting CD44 with monoclonal antibodies appears as a good strategy to eliminate CSCs. Treatment with anti-human CD44 monoclonal antibody can induce myeloid differentiation in patient-derived AML blasts, inhibits homing to the microenvironmental niche and alters stem cell fate^[46]. Marangoni *et al.*^[47] used an anti-human CD44 monoclonal antibody in combination with doxorubicin and cyclophosphamide to prevent relapse of aggressive breast cancer. Moreover, a mouse IgG1 anti-human CD44 receptor that inhibits CD44-STAT3 signaling pathways was used on human pancreatic cancer stem-like cells or MiaPaCa-2 cells and was found to decrease *in vitro* tumor sphere formation of CSCs, inhibiting pancreatic tumor growth, metastasis and tumor recurrence in xenografted nude mice^[48]. Similar results were obtained in colorectal, lung, bladder, larynx and breast cancers^[49,50].

CD47 is a transmembrane protein, receptor for

thrombospondin family members and for signal regulatory protein alpha (SIRP α)^[51]. It was found widely expressed on AML CSCs^[20] and almost all human solid tumor cells^[51,52]. Two anti-CD47 mAbs such as B6H12.2 and B6H12 were developed as strategy for cancer therapy. In a xenograft mouse model, B6H12.2 antibodies prevented the engraftment of human AML cancers stem cells and completely eradicated them^[20]. Administration of B6H12.2 prevented and inhibited growth of tumors derived from glioblastoma, ovarian, breast, colon, bladder cancer^[52], human non-Hodgkin lymphoma^[53], acute lymphoblastic leukemia^[54] and multiple myeloma CSCs^[55]. B6H12, a fully humanized anti-CD47, effectively inhibits aggressive leiomyosarcoma growth and metastasis in xenograft mice model^[56].

Other antibodies approved by FDA for the treatment of solid and haematological tumours, *e.g.*, rituximab (anti-CD20), cetuximab (anti-EGFR), trastuzumab (anti-HER2), bevacizumab (anti-VEGF-A), ipilimumab (anti-CTLA-4), pembrolizumab (anti-PD-1) are currently used in immunotherapy against tumor cells^[57].

Targeting signal cascades

One of the mechanisms by which CSCs manage to avoid or to survive cancer treatments seems to be represented by signals generated within the tumor microenvironment, due to dysregulation of signaling pathway networks^[58]. Like normal stem cells, CSCs use signaling pathways that are essential for self-renewal, proliferation and differentiation in order to preserve stem cell properties but the final result is carcinogenesis. Many studies have focused on signaling pathways dysregulation in CSCs attempting to find new strategy for cancer therapy; this line of research is promising mainly because many cancers present up- or down-regulation of the same signaling cascades. In this regard, CSCs can be identified by surface markers but also by the signals they send in tumor microenvironment^[59]. The major signaling pathways involved in the regulation of self-renewal and differentiation of normal and cancer stem cells are Notch, Hedgehog, Wnt/ β -catenin, NF- κ B, phosphatidylinositol 3-kinase (PI3K)/Akt, PTEN; sustained by aberrant activation of these pathways, CSCs have the capacity to initiate cancer and promote recurrence after the surgical removal of tumor^[60].

Notch signaling cascade is one major pathway involved in numerous critical cellular processes, including stem cell maintenance, progenitor cell proliferation and differentiation, and determination of cell fate during embryonic development^[61,62]. Notch signaling involvement in carcinogenesis and tumor progression seems to depend on tissue/cell-type. Thereby, Notch signaling was identified as oncogenic due to an increased activation in T-cell acute lymphoblastic leukemia, medulloblastoma, colorectal cancer, non-small cell lung carcinoma, hepatocellular carcinoma, melanoma, and breast cancer while in myeloid malignancies, head and neck squamous cell carcinoma, Notch displays tumor

suppressor functions^[63,64]. Notch signaling pathway comprises four Notch receptors (Notch 1, Notch 2, Notch 3 and Notch 4) and five ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1 and Jagged 2); depending on the cellular type, many studies have been focused on activation or inhibition of these proteins^[65,66]. *In vitro* and *in vivo* studies showed that human lung cancer lines presented an overexpression of Notch3; downregulation of Notch signaling, using a specific inhibitor was associated with decrease of tumor cell proliferation, induction of apoptosis, and inhibition of *in vivo* growth. Notch3 was also found to be overexpressed in resected human lung cancers and was associated with poor overall survival^[67,68]. Moreover, activation of Notch4 and Notch1 was noted breast CSCs, and inhibition of these receptors, especially Notch4, leads to decreased activity of breast CSCs *in vitro*, and reduced tumor development *in vivo*^[69]. In hepatocellular carcinoma, inhibition of Notch signaling pathway also reduced the invasion of tumor cells by downregulation of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor^[70]. In colorectal cancers, Notch1 signaling seems to have a dual role; it can increase the tumor progression but also counteracts β -catenin signaling whose activation is an important factor in human colorectal carcinogenesis^[71]. Many studies reported an overexpression of Notch signaling proteins in head and neck squamous cell carcinoma, sustaining a pro-oncogenic role of this signaling but recent exome sequencing analyses reported loss-of-function mutations in the *Notch1* gene in a significant proportion of patients with this malignancy^[72].

Notch signaling inhibitors tested in clinical trials include inhibitors of γ -secretase complex involved in Notch activation and antibodies against DLL-4 and Notch-1, -2 or -3 receptors^[43]. Results for phase 1 clinical trial using BMS-906024, one of these γ -secretase inhibitors, for patients with relapsed T-cell acute lymphoblastic leukemia showed at least 50% reduction in BM blasts in 8 of the 25 patients (32%)^[73].

Recently was reported the development of an antibody OMP-59R5 (tarextumab) which alone or together with chemotherapeutic agents can inhibit Notch2 and Notch3 function. The antitumor effect of OMP-59R5 was observed on xenograft tumors representing different types of epithelial cancers like breast, small-cell lung, ovarian, and pancreatic; and was associated with down-regulation of Notch target genes in tumor cells and with suppression of Notch3, HeyL, and Rgs5, expression in tumor stroma^[74].

Hedgehog is another pathway that plays an important role in stem cells maintenance and embryonic development, being involved in various cellular processes such as proliferation and differentiation; this cascade was also aberrant activated in several human tumors, including glioblastoma, breast cancer, pancreatic adenocarcinoma, multiple myeloma and chronic myeloid leukemia^[75,76]. Furthermore, Hedgehog signaling

contributes to development and maintenance of CSCs and acquisition of epithelial-to-mesenchymal transition, highlighting the involvement of Hedgehog cascade in cancer cell invasion, metastasis, chemotherapeutic resistance and tumor recurrence^[77]. Canonical Hedgehog signaling involves three ligands: Sonic (SHH), Indian (IHH), and Desert (DHH) with different expression depending on cell type; SHH is widely expressed, mostly during embryogenesis, IHH is found in hematopoietic cells, bone, and cartilage, while DHH is expressed in the peripheral nervous system and testes. Hedgehog signaling implies Hedgehog ligands binding to Patched receptor, Smoothened activation, and activation of transcriptional effectors that belong to the GLI family^[78,79]. Aberrant activation of Hedgehog signaling may be due to gene mutations, resulting in ligand-independent pathway activation, or by interaction with other molecular signaling pathways, such as Ras/Raf/MEK/Erk, PI3K/Akt/mTOR, and Notch. Thus, there are many studies sustaining that the combination therapies targeting more than one signaling pathway improves antitumor efficacy and survival in animal models^[80]. There are also several preclinical studies showing that in several cancers, Hedgehog signaling inhibition using specific inhibitors of Smoothened leads to the blocking of drug resistance, relapse, and metastasis. In basal cell carcinoma and medulloblastoma patients, Smoothened inhibitors such as vismodegib, BMS-833923, saridegib (IPI-926), sonidegib/erismodegib (LDE225), PF-04449913, LY2940680, LEQ 506, and TAK-441 were used as monotherapy with promising results^[81]. A recent study reported a significant negative association between GLI1 and GLI2 expression and overall survival, and also an increase of DHH plasma levels in acute myeloid leukemia patients. By *in vitro* and *in vivo* experiments the authors demonstrated that GLI1/2 inhibition induces apoptosis, and reduces proliferation, and colony formation in acute myeloid leukemia cells, and also increases the survival in murine models^[82]. Another study focused on biliary tract cancer showed that inhibition of Hedgehog and mTOR signaling pathways with rapamycin and vismodegib specific inhibitors results in decrease of Nanog and Oct-4 expressions and also in decrease of CSCs and ALDH-positive cells proliferation^[83]. In pancreatic cancer, the combination of focal radiation with Hedgehog inhibitors reduces lymph node metastasis, sustaining the involvement of this signaling pathway in carcinogenesis^[84].

Wnt/ β -catenin signaling pathway is one of the most deregulated pathways in many cancers, including leukemia, colon, breast and skin cancers. For instance, in human colon carcinoma, mutations in adenomatous polyposis coli (APC) result in aberrant activation of Wnt signaling and induce transformation of epithelial cells^[60]. There are also studies that support the involvement of Wnt signaling in self-renewal and maintenance of stem cells and CSCs, in several tissues like skin, intestine and

mammary gland^[85]. A minor subpopulation of breast tumor-initiating cells associated with drug resistance was identified in human breast cancer; *in vitro* and *in vivo* study showed that suppression of Wnt signaling inhibited sphere- and colony-formation of primary breast tumor cells and blocked tumor growth in murine models^[86].

Wnt/ β -catenin signaling pathway includes Wnt ligands, Frizzled receptors and a complex, composed of APC, Axin1, Glycogen synthase kinase 3- β , and CK1 (casein kinase 1) which stabilizes β -catenin^[87]. Wnt/ β -catenin inhibitors include small-molecule inhibitors (e.g., nonsteroidal anti-inflammatory drugs and natural compounds: aspirin, indomethacin, vitamins A and D derivatives) and biologic inhibitors (monoclonal antibodies, small interfering RNAs and recombinant proteins)^[2].

OMP-54F28 is an example of Wnt signaling inhibitor with interesting results in clinical trials, in several advanced solid tumors, such as ovarian, pancreatic and hepatocellular cancers^[88]. OMP-18R5 - an antibody targeting several Frizzled receptors - reduces tumor cell proliferation and tumor-initiating cell number in lung, breast, colon, and pancreatic tumors^[89]. *In vitro* study on lung CSCs showed that β -catenin nuclear transfer inhibitor PP significantly decreased colony formation and downregulated pluripotent stem cell signaling pathway, being a promising therapeutic approach in lung adenocarcinoma patients^[90]. The studies performed until now showed that Wnt inhibitors and modulators can eliminate drug-resistant CSCs and tumor-initiating cells but more studies are needed regarding the safety of this therapeutic approach, given the essential role of Wnt signaling in tissue homeostasis and repair^[91].

PI3K/Akt/mTOR signaling pathway is an important cascade of phosphorylation reactions, comprising several key molecules involved in carcinogenesis processes: PI3K - presents activating mutations in several human cancers; Akt - overexpression/activation of this protein kinase is associated with tumor metastasis and invasion; mTOR - protein kinase critical for cancer cell growth, cell proliferation, survival and angiogenesis^[59].

Many studies suggest the involvement of this signaling pathway in maintaining CSCs features. In breast cancer, inhibition of PI3K or Akt activity reduced generation and growth of CD44/CD24 mammospheres, leading to stem cell/mesenchymal phenotype loss and recovery of epithelial-like markers^[92]. In prostate cancer, PI3K/Akt/mTOR pathway deregulation is associated with CSCs quiescence and maintenance; moreover, prostate CSCs seem to present resistance to selective mTOR inhibitors^[93]. Another study showed that combination between PI3K/mTOR inhibitor (BEZ235) and radiotherapy increased radiosensitivity and apoptosis, and also reduced CSCs marker expressions in prostate cancer radioresistant cell lines^[94]. In endometrial tumors, PTEN/PI3K/Akt/mTOR pathway aberrant activation by miRNAs is a common event,

being involved in epithelial-mesenchymal transition and CSC maintenance^[95].

PTEN (phosphatase and tensin homolog) is a tumor suppressor and an inhibitor of PI3K and ERK activities, being one of the most inactivated tumor suppressor genes in cancers; many tumors present loss of PTEN function by mutations, deletions, transcriptional silencing, or protein instability affecting important cell processes such as survival, proliferation, energy metabolism and cellular architecture. Loss of PTEN activity was also linked to CSC development and proliferation in several cancers, including prostate, lung, intestinal, and pancreatic cancer^[95]. Moreover, PTEN loss and Akt activation lead to increase activity of β -catenin in hematopoietic stem cells while Notch signaling activation results in reduced PTEN expression in human T-cell acute lymphoblastic leukemia^[96].

Nuclear factor kappa B (NF- κ B) is a transcription factor constitutively activated in several tumors, being also associated with self-renewal and expansion of CSCs^[97]. NF- κ B interacts with many apoptosis-related proteins, including Bcl-xL, Bcl-2, survivin, cellular inhibitors of apoptosis (cIAPs), TRAF and cell cycle regulatory components, and NF- κ B aberrant expression was related to cancer development and progression, chemoresistance, chronic inflammation and autoimmune diseases^[60].

In hepatocellular carcinoma cell lines, one of the most activated signaling pathways is NF- κ B cascade; NF- κ B inhibition using SN50, suppressed tumor cell growth and the authors suggested that targeting NF- κ B signaling might specifically inhibit CSCs populations and it could be considered a new therapeutic strategy for hepatocellular carcinoma patients with poor prognosis^[97]. Pancreatic ductal adenocarcinoma characterized by a pronounced hypoxic tumor microenvironment presents epithelial-mesenchymal transition and stem-like features related to NF- κ B signaling activation. An *in vitro* study demonstrated that inhibition of NF- κ B with triptolide can reverse epithelial-mesenchymal transition and reduced migration, self-renewal activity, stem cell-related signaling^[98]. NF- κ B signaling is also considered an important therapeutic target in breast cancer; HER2-NF- κ B-HER2 pro-survival pathway seems to be activated in breast CSCs upon radiation therapy^[99].

Numerous studies have focused on identifying the molecular mechanisms and signaling pathways characteristic for CSCs, in solid and hematological malignancies. Notch, Hedgehog, Wnt/ β -catenin, NF- κ B, PI3K/Akt, and PTEN cascades present aberrant activation in cancers and they have been associated with high proliferative and metastatic capacity, self-renewal and differentiation of CSCs and also multi-drug resistance, being considered attractive targets for CSCs specific eradication. However, more studies are required to demonstrate the safety of these targeted therapies, considering the crucial role of these signaling pathways for normal stem cell maintenance^[97].

Targeting microenvironment

The tumor microenvironment provides necessary signals for CSCs maintenance, regulation of self-renewal and homeostatic processes such as angiogenesis, hypoxia and weakly acidic pH.

Interaction between CSCs and tumor stroma is ensured by CXCL12-CXCR4 axis. CXCL12 [also called stromal-derived factor-1 (SDF-1)] is a chemokine that binds its receptor CXCR4 and it is involved in migration, invasion and survival of normal and malignant cells^[100]. CXCR4 is highly expressed on several stem cells mainly haematopoietic, but also on endothelial, neural and embryonic stem cells, facilitating their response to gradients of CXCL12 produced in case of tissue damage and hypoxia. On the other hand, CXCL12 ligand has a constitutive expression on stromal cells in many tissues. It has an increased level in bone marrow, lymph nodes, lung and liver, and a low level in small intestine, skin skeletal muscle^[101].

Many studies have emphasized that under physiological conditions CXCL12-CXCR4 axis plays a crucial role in processes like normal development, tissue regeneration and repair. With respect to tumor growth, it was shown that CXCL12-CXCR4 axis ensures a close contact between cells and tumor stroma and consequently activates various signals related to cell growth, metastasis and chemoresistance^[102]. In human breast cancer CXCL12 generated by stromal fibroblasts exerts two types of effects on tumor growth: an endocrine effect by promoting angiogenesis *via* recruiting endothelial progenitor cells into the tumor mass, as well as a paracrine effect by acting directly on tumor cells through CXCR4^[103].

The important roles of CXCL12-CXCR4 axis in cancer led to an intense research for developing drugs that inhibit signaling through this axis. A series of inhibitors have been investigated, targeting either CXCR4 or CXCL12. Among CXCR4 inhibitors, AMD3100, also known as plerixafor, induces the rapid mobilization of hematopoietic stem and progenitor cells into the blood in mice and humans^[104], inhibits growth of intracranial glioblastoma and medulloblastoma xenografts by increasing apoptosis and decreasing the proliferation of tumor cells^[105], and can reduce the intraperitoneal dissemination of epithelial ovarian cancer^[106]. On the other hand, in a recent study it was shown that treatment with the AMD3100 diminished metastatic growth, but it didn't affect significantly the frequency of metastases or overall survival in a murine model of metastatic human non-small cell lung cancer^[107].

CTCE-9908 is another CXCR4 antagonist that proved to be effective in reducing both tumor growth and metastasis in xenograft mouse models of inflammatory breast cancer^[108] and also in an orthotopic model of esophageal carcinoma^[109]. A recent study highlighted the role of CTCE-9908 in decreasing the tumor invasivity and angiogenesis in prostate cancer^[110].

One of the most studied CXCL12 inhibitor is NOX-A12

that seems to suppress CXCL12-induced chemotaxis of chronic lymphocytic leukemia cells and to cause chemosensitization^[111]. In addition, Liu *et al.*^[112] observed that this compound had the potential to improve tumor response in glioblastoma multiforme animal model after irradiation.

CXCL12-CXCR4 axis can also promote angiogenesis. Liang *et al.*^[113] have shown that CXCR4 induces an increased expression of vascular-endothelial growth factor (VEGF) at both the mRNA and protein levels through the activation of PI3K/Akt pathway.

Tumor angiogenesis is another mechanism promoted by microenvironment that has been related with CSCs survival and tumor growth, since targeting VEGF can lead to normalization of the vasculature, decrease in tumor growth and disruption of the CSCs niche^[60,114]. Inhibitors against the VEGF/VEGFR-system are already in clinical use. Among the approved drugs that target VEGF/VEGFR-system are bevacizumab, anti-VEGF blocking antibody, and pazopanib, sorafenib and sunitinib, VEGFR-2 pathway inhibitors. Moreover, a series of compounds are tested in clinical trials or they are just in experimental phase^[115]. From those, fruquintinib (HMPL-013), a small molecule inhibitor very potent and highly selective against VEGFR family, is currently in phase II clinical studies^[115].

Another feature of the tumor microenvironment is hypoxia that is regulated by inducible transcription factors HIF-1 and HIF-2. Many studies correlated tumor hypoxia with tumor growth, cancer progression, metastasis, resistance to chemo- and radiotherapy. Therefore targeting HIF activity might represent an effective method of inhibit tumor metastasis and improve the outcome of chemo- and radiotherapy^[116,117].

In breast cancer, inhibition HIF activity in BrCa cells by using RNA interference or digoxin treatment prevent primary tumor growth and also reduce breast cancer dissemination in lungs by down-regulating the expression of angiopoietin-like 4 (ANGPTL4) and L1 cell adhesion molecule (L1CAM) 21860410. In a recent study, Gillespie *et al.*^[118] investigated the inhibition of HIF-1 α by using small interfering RNA in an orthotopic mouse model for glioblastoma. *In vivo* treatment reduced tumor growth and increased survival.

Another important therapeutic target can be represented by acidic extracellular pH, a major feature of tumor tissue that is the result of cancer cells increased metabolic activity and of the poor vascular perfusion of tumors^[119,120]. As it was shown in previous studies, tumor acidity can offer a selective advantage of cancer cells over the normal tissues and contribute to drug resistance^[119,121].

The acidity of tumor environment can be managed either by using delivery drugs that have specificity for acid environment, or can be reversed directly with systemic buffers or indirectly by using inhibitors for pH-regulatory pathways like carbonic anhydrase IX (CAIX).

Developing compounds that are tumoral acidic pH-

responsive is a dynamic area of research; liposomes, micelles, polymers and inorganic nanoparticles have been tested for anticancer therapy^[122]. An improved drug-delivery method is to encapsulate drugs into silica matrices like camptothecin and doxorubicin^[122,123].

Sodium bicarbonate is one of the systemic buffers that can be used to alkalinize the microenvironment. In intraductal tumors it have been shown that sodium bicarbonate can prevent or slow down the transition from in situ to invasive cancer^[124].

One approach, more captivating is to regulate cellular pH with drugs that inhibit CAIX, a hypoxia and HIF-1-inducible protein, overexpressed in many cancers. Data from phase III clinical trial suggest that CAIX score can be also used as statistically prognostic biomarker for survival in patients with high-risk nonmetastatic renal cell carcinoma (ccRCC)^[125].

Targeting the tumor microenvironment might affect communication with stroma and self-regulating process, the vasculature as the main nutrients supply, and essential features like oxygen level and acidity needed by CSCs, all these being fundamentals for CSCs maintenance. However these strategies are not specific enough and might affect also normal stem cells and their stroma. Consequently, a more particular approach is needed that addresses only tumor stroma.

Targeting ATP-binding cassette transporters

The ability to isolate and characterize CSCs from different tumors facilitated a more specific investigation of the therapeutic possibilities based on another distinctive feature of the CSCs, chemoresistance^[126]. CSCs identification by augmented efflux of Hoechst 33342 dye through ATP-binding cassette (ABC) transporters (defined as side population, SP cells) in flow cytometry analysis, proved to be very useful in isolating hematopoietic stem cells, and a variety of CSCs from solid tumors, including ovarian, breast, colon and hepatic cancers^[127-131]. Aberrant expression of ABC transporters is a major mechanism of chemoresistance in cancers cells, including cancer CSCs^[132] but may be generated by other mechanisms involved directly or indirectly in the process, from transcription to protein expression. All the mechanisms involved in ABC transporter modulation might be potential targets for overcoming chemoresistance in CSCs.

ABC transporter proteins are members of the ABC superfamily that have as most important physiologic role prevention of the accumulation of xenobiotics and toxic compounds in normal cells. These efflux pumps consist of a single or multiple sets of transmembrane domains and nucleotide binding domains. Nucleotide binding domains hydrolyze ATP giving power for the efflux while a variety of structurally unrelated substrates, including drugs, sugars, proteins, and metabolites are "pumped-out" through the transmembrane domains.

Among the numerous members of the ABC-transporters described to date, only few were well docu-

mented to be expressed in human CSCs: Multidrug resistance 1 (MDR1) or P-glycoprotein (Pgp)/ABCB1, multidrug resistance protein 1 (MRP1)/ABCC1, and breast cancer resistance protein (BCRP)/ABCG2/MXR/ABCP. These proteins differ in structure and in substrate specificity. MDR1 is a protein that confers cross-resistance to many antitumor agents, including anthracyclines, mitoxantrone, epipodophyllotoxins, and taxanes. ABCC1 is structurally similar to ABCB1 but shares only 15% amino acid sequence identity; it also generates resistance to anthracyclines, mitoxantrone, and epipodophyllotoxins but differs from MDR1 in the level of resistance to taxanes. BCRP also confers resistance to mitoxantrone, but anthracyclines resistance was found to depend on mutations at the codon 482^[133].

The new concept of targeting ABC transporters considers inhibitors as "CSC sensitizing agents". Clinical studies have attempted to overcome drug resistance by combination therapies in which a cytotoxic drug was given along with an ABC-transporter inhibitor. However, ABCB1 inhibitors have shown very limited effectiveness in clinical trials despite the debate on the fact that the clinical trials on the first generation of inhibitors have not been targeting CSCs but only the reduction of the tumors that express a particular drug transporter (usually ABCB1). If the stem cells are considered the principal culprits of drug resistance, the efficacy of ABC inhibitors would be better evaluated monitoring the relapse instead of tumor size reduction^[134]. However, ABC inhibitors would be most effective if are combined with an anticancer agent that specifically targets the stem cells.

Three generations of ABC transporter blockers were investigated until now and the fourth generation, based on natural compounds, is under development^[135].

First generation of inhibitor drugs were already in use for different conditions and also were able to block MDR1 (ABCB1), such as calcium channel inhibitors like verapamil, immuno-suppressants like cyclosporin A, anti-arrhythmics and neuroleptics like quinidine, reserpine, and yohimbine, and antiestrogens like tamoxifen and toremifene. The efficacy of these drugs was limited by their toxicity, which urged the development of drugs with less important side effects as first generation compounds.

Second generation of MDR1 modulators like R-verapamil, elacridar (GF120918), dofequidar (MS-209), valspodar (PSC833), biricodar (INCEL, VX-710), or timcodar (VX-853) were derived from first generation P-gp modulators. Although second generation modulators are less toxic than first generation modulators, they still induce important side effects due to non-selective inhibition of multiple cell ABC transporters and unpredictable pharmacokinetic interactions^[136].

Third generation modulators such as Zosuquidar (LY335979), oc144093 (ONT-093), laniquidar (R101933), and tariquidar (XR-9576), which are more selective inhibitors of ABCB1, ABCC1, ABCG2, are still in different phases of investigation as CSCs sensitizing

agents.

Although the expression of ABC transporters could render CSCs resistant to drugs, it is not the unique cause of resistance by ABC proteins, as multiple levels of regulation between transcription and protein expression might be susceptible to modifications. Future trends of drug development should consider such levels of regulation of ABC transporters that might be speculated in cancer therapy. One such direction should evaluate, for example, the potential of using miRNAs targeting specific RNA that results in its degradation. MiR-328, miR 519c, miR-520h, miR-487a, miR-181a are only few of the miRNAs reported to be involved in ABC transporter regulation.

Recently, was observed that the presence of side population (SP) cells in tumors is highly dependent on the driving genetic alterations of the tumor. For example, hepatic tumors driven by Myc, but not Akt and RAS, had a significant number of SP cells that show the same properties as chemoresistant tumor-initiating CSCs. These studies show that even the common mechanisms of chemoresistance may change under genomic alterations conditions that are very frequent in cancer. There are increasing evidence that small-molecule tyrosine kinase inhibitors (TKIs), such as AST1306, lapatinib, linsitinib, masitinib, motesanib, nilotinib, telatinib and WHI-P154, can inhibit ABC transporters, suggesting that these TKI might have also a direct effect on ABC proteins inhibitors^[137]. Effect of Akt, PI3K and mTOR inhibitors on ABC transporters trafficking and/or expression has been well documented^[138]. Particularly, LY294002, a derivative of quercetin, is able to inhibit PI3Ks, mTOR and ABCG2 and has been recently proposed as a new candidate to target efficiently ABCG2-expressing CSCs that depend on Akt signaling for survival. ABCG2 expression and the SP might be regulated by PTEN through the PI3K/Akt pathway, which was proposed as a potentially effective strategy for targeting CSCs^[139].

Using nanomedicines as delivery vehicles can enhance the therapeutic response in resistant tumors by bypassing efflux pumps or by increasing the concentration of drugs in CSCs at a given time point^[140].

Simultaneous targeting of genomic alterations that are responsible for different types of cancer and resistance phenotypes together with new strategies of delivery and retention of the drugs inside the CSCs, will allow the development of more selective therapy.

Manipulation of miRNA expression

MicroRNAs (miRs) are small non-coding RNAs (20 to 24 nucleotides in length) that negatively regulate post-transcription by binding to the 3'UTR of target messenger RNA, having a broad range of effects over self-renewal, differentiation and division of cells^[141]. MiRNA are involved not only in maintaining normal cell functions, but also might modify several signaling pathways that could transform stem cells into cancer stem cells. Aberrant expression of miRNA in cancer

stem cells was noted by many studies. MiRNAs differentially regulate the key properties of CSCs, including cell-cycle exit and differentiation, prosurvival and antistress mechanisms and EMT, migration and invasion, which increase tumor initiation and metastatic potential^[142]. They can act either as oncogenes or tumor suppressors.

Targeting oncogenic miRNAs can be achieved by antisense oligonucleotide inhibition. Studies have shown that miR-21 has been found to be frequently up-regulated in different CSCs^[143]. Thus, knockdown of miR-21 inhibits cell proliferation, migration and tumor growth in breast cancers^[144,145], ovarian^[146], and lung cancer^[147].

Several studies have exposed the potential of miRNA based therapeutics as a novel strategy towards cancer stem cells. MiR-34 is a target of tumor suppressor gene *p53* and is down-regulated in many cancers^[148]. Liu *et al.*^[149] showed that miR-34a, was underexpressed in CD44⁺ prostate cancer cells and increased expression of miR-34a in CD44⁺ prostate cancer cells inhibited clonogenic expansion, tumor regeneration, and metastasis. Moreover, Shi *et al.*^[150] demonstrated that transfection of synthetic miR-34a in CD44⁺ non-small cell lung cancer cell lines inhibited clonogenic expansion, and tumor regeneration *in vivo*. Consistently, forced expression of miR-124 and miR-137 in human derived glioblastoma stem cells leads to loss of their self-renewal and oncogenic capacity, leaving normal stem and precursor cells unharmed^[151].

Abnormal miRNA expressions lead to the initiation, development, and progression of cancer. Thus, miRNA based therapy that can correct abnormal transcripts at CSCs level show great potential for cancer cure.

Induction of CSCs apoptosis

Apoptosis is a critical mechanism that mediates death and survival through a complex signaling mechanism. Escape from this system is the precondition for cancer initiating cells. Usually, the apoptotic mechanisms are impaired during cancer development and progression.

Induction of apoptosis in CSC holds great promise for cancer therapy. Therefore, many compounds have been developed to target intrinsic and extrinsic apoptotic pathways. For example, activating of the death receptors [CD95 and trimeric human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)], part of extrinsic apoptotic pathway, leads to caspase-8 activation. Once activated, caspase-8 either directly cleaves and activates effector caspase-3 or, alternatively, processes Bid into the active fragment tBid, which translocates to mitochondrial membranes to initiate mitochondrial outer membrane permeabilization^[152]. Treatment with TRAIL in combination with various anticancer agents was reported to be effective in removing cancer stem cells. Thus, co-treatment with cisplatin was described to be very efficient in reducing triple negative breast cancer stem cells through inhibition of Wnt signaling

and increasing of apoptosis^[153]. Likewise, combined treatment with TRAIL and cytarabine or daunorubicin, has been shown to suppress the growth of acute myeloid progenitors^[154]. Furthermore, TRAIL in addition to Bortezomib, a proteasome inhibitor, was recently shown trigger apoptosis in glioblastoma stem cells^[155]. Moreover, mesenchymal stem cells (MSC) engineered to express TRAIL through transduction with a lentiviral vector, were used to activate apoptosis in of squamous and lung cancer stem cell population. Experiments were done by injecting TRAIL-secreting MSC subcutaneously into nude mice that hold tumors. Results showed that MSC migrate and localized near the cancer site and inhibited tumor growth through apoptosis induction^[156].

Another target in inducing CSCs death might be NF- κ B, a transcription factor linked to the control of apoptosis signaling. Mainly, NF- κ B inhibits apoptosis and promotes cell proliferation, inflammation, tumor promotion, angiogenesis and metastasis^[157-159]. In breast cancer, pharmacological inhibition of NF- κ B with small-molecules like parthenolide, pyrrolidinedithiocarbamate and its analog diethyldithiocarbamate preferentially target breast cancer stem cells. These results underline that NF- κ B activity is critical to maintain the survival of tumor-initiating cancer stem cells^[160]. Moreover, inhibition of NF- κ B by the proteasome inhibitor MG-132 together with the anticancer drug idarubicin induced apoptosis preferentially in the leukemic stem cells population but considerably less in normal hematopoietic stem cells^[161].

Targeting CSC population by reactivation of apoptosis programs has been shown in preclinical studies to offer the possibility to eradicate cancers. Future challenges should include the increasing of specificity and efficiency in targeting cancer stem cells and avoid toxicity of normal tissue stem cells.

Induction of CSCs differentiation

Traditional anti-cancer therapies successfully manage differentiated cancer cells but did not affect CSCs. This observation leads to another method to restrain cancer progression, induction of CSCs differentiation that became favored over self-renewal programs, diminishing CSC population. Many studies are currently in progress proposing various differentiation agents like: retinoic acid, histone deacetylase inhibitors, miRNAs, tyrosine-kinase and signaling pathways inhibitors.

Retinoic acid and its analogs (ATRA) are currently used to treat acute promyelocytic leukemia. Retinoic acid regulates several chromatin remodeling factors due to its interaction with retinoid receptors^[162]. Campos *et al.*^[163] demonstrated that ATRA induced differentiation of glioma CSCs, and showed that the anti-tumor effect is present both *in vitro* and *in vivo* experiments. Moreover, Ginestier *et al.*^[164] showed that modulation of retinoid signaling may promote self-renewal or induce differentiation of breast CSCs, and suggested that ATRA may be considered as targeted therapy for breast CSC

population.

Other agents that can affect cancer stem cell differentiation are histone deacetylase inhibitors (HDACI). To date, HDACI are used as differentiation therapy in several hematologic malignancies. Recently, this type of therapy was proposed for breast CSCs^[165] and head and neck CSCs^[166]. In the first study HDACI (abexinostat) induced CSC differentiation. Moreover, in the second study, HDACI (suberoylanilide hydroxamic acid and trichostatin A) altered the CSCs phenotype and also induced cell cycle arrest and apoptosis.

Petrelli *et al.*^[167] showed that miR-100 favors breast CSCs differentiation, converting a basal like phenotype into luminal. It induces the expression of a functional estrogen receptor and renders basal-like BrCSCs responsive to hormonal therapy. Vaz *et al.*^[168] suggest that pancreatic differentiation 2 (PD2) is a novel CSC maintenance protein, loss of which renders the CSCs more susceptible to drug-induced cell death. Knockdown of PD2 protein in CSCs decreased cell viability, enhanced apoptosis and diminish expression of CD133 and MDR2. Dong *et al.*^[169] demonstrated that long-term culture of glioblastoma cancer stem cells with imatinib mesylate, a tyrosine-kinase inhibitor, induced cell differentiation, confirmed by decreased expression of stem cell markers, CD133, Oct-3/4, nestin, and Bmi1, and increased expression of GFAP (astrocyte marker) and class III β -tubulin isotype (Tuj1, neuron marker), associated with reduced ability to form aggregates and colonies *in vitro* and tumorigenicity *in vivo*. Another strategy targeting signaling pathways in order to induce CSCs differentiation is directed against hyperactivated Akt/mTOR and the inhibited wild-type p53 pathways in glioma CSCs. Daniele *et al.*^[170] uses two inhibitors (FC85 and ISA27) that blocked proliferation and promoted the differentiation of GSCs by reducing Akt/mTOR signalling and reactivating p53 functionality.

Inducing CSC differentiation combined with CSC-targeted therapies and traditional therapies may bring real benefits in depleting cancer cells by increasing the efficacy.

CONCLUSION

In this review we have summarized recent advances in identification of CSCs molecular markers that might become targets for developing new therapies, aiming to hamper CSCs regeneration and cancer relapse. These novel therapeutic strategies are designed to target directly CSCs or to interrupt signals from the microenvironment that regulate important properties like self-renewal, differentiation and apoptosis resistance.

All these alternative therapies are very promising, but some of them not specific and might affect also healthy tissue since CSCs niche is similar and close to normal stem cell niche that can also be affected by compounds. There are also many ways by which CSCs could evade treatment. They reside in an area of low

oxygen and vascularity, preventing efficient delivery of the drugs.

Future challenges should include the increasing of specificity and efficiency in targeting CSCs, avoiding toxicity of normal tissue stem cells, and also new strategies of delivery and retention of the drugs inside the CSCs. These new therapies should increase the efficacy of existing drugs against aggressive cancers, and thus should prevent tumor relapse and enhance patient survival.

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