Metastatic Cancer Stem Cells: New Molecular Targets for Cancer Therapy

G.J. Leirós and M.E. Balañá*

Fundación Pablo Cassará, Instituto de Ciencia y Tecnología Dr. Cesar Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Ciudad de Buenos Aires. Argentina

Abstract: The cancer stem cell (CSC) hypothesis, predicts that a small subpopulation of cancer cells that possess "stem-like" characteristics, are responsible for initiating and maintaining cancer growth. According to the CSC model the many cell populations found in a tumour might represent diverse stages of differentiation. From the cellular point of view metastasis is considered a highly inefficient process and only a subset of tumour cells is capable of successfully traversing the entire metastatic cascade and eventually re-initiates tumour growth at distant sites. Some similar features of both normal and malignant stem cells suggest that CSCs are not only responsible for tumorigenesis, but also for metastases. The CSC theory proposes that the ability of a tumour to metastasize is an inherent property of a subset of CSCs. The similar biological characteristics shared by normal stem cells (NSCs) and CSCs mainly implicate self-renewal and differentiation potential, survival ability, niche-specific microenvironment requirements and specific homing to metastatic sites and may have important implications in terms of new approaches to cancer therapy in the metastatic setting. There are several agents targeting many of these CSC features that have shown to be effective both *in vitro* and *in vivo*. Although clinical trials results are still preliminary and continue under investigation, these new therapies are very promising. The identification of new therapeutic targets and drugs based on CSC model constitutes a great challenge.

Keywords: Aberrant differentiation, cancer stem cells, metastasis molecular targets, metastatic process, mobilisation and metastasis, self-renewal ability, stem cell markers.

INTRODUCTION

Metastasis, the spread of cancer cells from its primary site to other places in the body, is the principal cause of death among cancer patients, due mainly to the ineffectiveness of current therapies once metastases begin to form. Its prevention and management are therefore among the key goals in clinical and basic cancer research.

Most cancers are collections of phenotypically mixed cell populations with variable proliferative potential and with highly variable abilities to survive, grow, and metastasize. There is growing evidence that tumours display a hierarchy similar to normal tissues, and represent a mixture of cells with notable morphologic, genetic and functional differences. The cancer stem cell (CSC) hypothesis [1], predicts that a small subpopulation of cancer cells that possess "stem-like" characteristics, are responsible for initiating and maintaining cancer growth. According to the CSC model the morphologically different populations of tumour cells might represent cells of different differentiation stages. The importance of cancer stem cells (CSCs) in tumour-initiation has been firmly established in leukaemia and recently reported for a variety of solid tumours. Many studies are showing that a variety of cancers follow the cancer stem cell model, where

markers distinguishing CSCs from non-tumourigenic cancer cells have been identified.

The role of CSCs in multistage cancer progression, particularly with respect to metastasis, has not been well-defined. The cellular and molecular mechanisms of cancer metastasis are still poorly understood. Metastasis is considered a highly inefficient process from the cellular point of view and only a subset of tumour cells are capable of successfully traversing the entire metastatic cascade and eventually re-initiate tumour growth in distant sites. Some similar characteristic features of both normal and malignant stem cells suggest that CSCs are not only responsible for tumorigenesis, but also for metastases. The CSC theory proposes that the ability of a tumour to metastasize is an inherent property of a subset of CSCs.

The many similarities in the biology of normal stem cells (NSCs) and CSCs, including an unlimited capacity for self renewal; the promotion of migration using the α-chemokine stromal-derived factor 1 (SDF-1)/chemokine receptor 4 (CXCR4) axis; the requirement for a specific 'niche' to grow; the increased resistance to apoptosis and the molecular mechanisms operating in resistance to radio-chemotherapy, may have important implications in terms of new approaches to cancer therapy in the metastatic setting [2].

This review will focuses on the CSC hypothesis and the metastatic process, describing the many connections between metastatic and stem cells.

It will discuss, as well, the recent advances in the development of therapeutic agents targeting metastatic CSCs characteristics.

*Address correspondence to this author at the Fundación Pablo Cassará, Instituto de Ciencia y Tecnología Dr. Cesar Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Saladillo 2468 (C1440FFX). Ciudad de Buenos Aires- Argentina;
Tel:/Fax: (5411) 4687-2542 / 4686-3687;
E-mail: mbalana@fundacioncassara.org.ar

THE CANCER STEM CELL THEORY

The traditional view proposed a stochastic model of cancer development [3] that dictates that cells of many different phenotypes in cancer are capable of proliferating extensively and forming new tumours. The initiating step, produced by a tumourigenic agent, affects the genetic material of the cell. If the cell does not repair this damage, then promoting factors may progress the cell toward a malignant phenotype. The period of tumour promotion may be a very slow process.

There is growing evidence that tumours display a hierarchy similar to normal tissues. It has long been known that histological analysis of the tumours shows that they are composed by cells of different morphologies and not all of the cancer cells are equally capable of generating tumours. The morphologically different populations of tumour cells might represent cells of different differentiation stages. Benign tumours only have alterations in the control of cell proliferation, which explains the formation of the tumour mass. Malignant tumours also have alterations in the normal programmes of cell differentiation. According to these observations, the tumour mass is formed as a combined result of alterations in proliferation that have to be coupled with a block in the normal differentiation programme.

The CSC theory [4] states that malignant transformation occurs in the adult stem cell and gives rise to a cancer stem cell [5]. Tumour tissues have long been known to be composed of heterogeneous populations of cancer cells. In several types of human cancer, only a phenotypic subset of cancer cells has tumourigenic capacity; these cells possess indefinite potential for self-renewal and sustain the growth of a new tumour when injected in immunocompromised animals.

This small fraction of cancer cells is currently defined as "cancer stem cells" (CSC) [6] and is proposed to persist in tumours as a distinct population and cause relapse and metastasis.

The main properties of these cells are:

1) Tumourigenic capacity recreating the full phenotypic heterogeneity of the parent tumour in contrast to other non-tumourigenic cancer cells.

2) Expression of a distinctive profile of surface markers allowing its isolation from non-tumourigenic cells in a reproducible manner by means of immunoselection procedures.

3) Generation of tumours through the stem cell processes of self-renewal and differentiation into multiple cell types.

CSCs can thus only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumour. Alternative terms in the literature, such as ''tumour-initiating cell'' and ''tumourigenic cell'' also describe putative CSCs.

As stem cells are long-lived, the CSC theory explains how a cell would survive long enough to acquire the appropriate number of genetic changes. This has given rise to the concept that tumours are composed of both CSCs, which have a large proliferative capacity, and a daughter population of cells, with a limited proliferative potential.

According to the CSC model a small number of tumourigenic cells is responsible for metastases; CSCs have the unique capability of successfully traversing the entire metastatic cascade and eventually re-initiate tumour growth in distant sites going through an aberrant process of differentiation that give rise to phenotypically diverse cancer cells with a limited capacity to divide.

EVIDENCES SUPPORTING THE CSC THEORY

The pathways that regulate the self-renewal of NSCs would be deregulated in CSCs resulting in the continuous expansion of self-renewing cancer cells and tumour formation.

Many studies are showing that a variety of cancers follow the CSC model, where markers distinguishing CSCs from non-tumourigenic cancer cells have been identified.

In the 1970s the possible relationship between hematopoietic stem cells and human leukaemia was proposed. Diseases as diverse as chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML), essential thrombocythemia, and polycythemia vera were all characterized by the expansion of a monoclonal population of cells that contained multiple lineages of differentiated mature blood elements [7-10].

In the early 1990s, with modern research tools for investigating the behaviour of defined cell populations, Dick and colleagues started to understand whether the functional hierarchy observed in normal haematopoiesis was conserved in blood tumours and provided the first evidence of the existence of CSCs [11, 12]. The authors isolated a subpopulation of leukemic cells with a specific cell surface phenotype (CD34$^+$ CD38$^-$) characteristic of early human haematopoietic progenitors, that possesses the differentiate and proliferative capacities and the potential for self renewal expected of a leukemic stem cell. Only these cells generated acute myelogenous leukaemia (AML) when injected into immunodeficient hosts like the NOD/SCID (nonobese-diabetic/severe combined immunodeficient) mice. More differentiated tumour cells that had lost their self-renewal and differentiation capacity were unable to initiate tumours. The tumours generated were histologically similar to the donor, indicating that the CSCs are able to regenerate the complete tumour tissue, in a similar way as how stem cells can regenerate a whole tissue from just a single cell.

Several studies in solid tumours indicate that the concept of stem cells in cancer might have broader implications beyond the field of haematopoiesis. Further evidence comes from histology; many tumours are very heterogeneous and contain multiple cell types native to the host organ. Heterogeneity is commonly retained by tumour metastases. So, the cell that produced them possesses the multi-differentiate potential, characteristic of stem cells [13].

The CSC hypothesis has been validated with similar experimental approaches in many solid tumours. There is mounting evidence that solid tumours originate from undifferentiated stem cell-like cells coexisting within a heterogeneous tumour mass that drive tumour formation, maintain tumour homeostasis and initiate metastases. Data have been provided to support this theory in brain [14], breast [15],

colon [16, 17], ovary [18], pancreas [19], prostate [20, 21] cancers and melanoma [22, 23].

METASTASIS AND THE CSC THEORY

Among the most menacing properties of malignant cancer cells is their capacity to metastasize. Metastasis is a complex series of steps in which cancer cells move from their primary tissue of origin and seed in a different anatomical compartment, where they sustain the growth of a secondary tumour lesion. It requires cancer cells to become motile so they can detach from the original tumour, migrate to blood vessels and travel through the bloodstream, attach to new sites and form micrometastases, develop a blood supply, and finally form macroscopic, clinically relevant metastases [24, 25]. Metastasis is a highly inefficient process and only a subset of tumour cells is capable of successfully traversing the entire metastatic cascade. Metastatic inefficiency is due primarily to the regulation of cancer cell growth in secondary sites [26, 27].

Many cancers show an organ-specific pattern of metastasis and several theories exist to explain the metastatic specificity. In 1889 Paget introduced the "seed and soil" hypothesis [28, 29] proposing molecular mechanisms to explain the specific metastatic sites chosen by cancer cells to survive and proliferate in secondary sites. Both the cancer cell ('seed') and the factors in the organ environment ('soil') contribute to the organ-specific pattern of metastasis.

In 1928 James Ewing postulated a mechanical hypothesis [30] for metastasis: blood-flow patterns carrying cells from the primary tumour determine which organ the cells travel to first, accounting entirely for the unequal distribution of metastases . As cancer cells are much larger than blood cells, they would be forced to arrest in the capillary bed of the first organ they encounter in the circulation, forming metastases. The fact that the lung, which is the first organ traversed by most breakaway tumour cells, has a high incidence of metastases supported this "mechanical" hypothesis.

Another concept, the homing theory, suggests that organs distant to sites of primary malignancy actively attract specific types of tumour cells to 'home to' and arrest in a particular organ via expression of adhesion receptors or by secretion of soluble chemotactic factors [31]. Identification of adhesion receptors on endothelial cells in vascular beds of distal organs that specifically trap circulating malignant cells supports the active arrest view of the homing theory [32].

It is possible that both theories of dissemination were partly correct; if the organ containing the first capillary bed downstream from the primary site has a metastatic microenvironment suitable to sustain tumour growth, most of the metastases will be there. Thus, the primary method of dissemination may be mechanical or dependent on chemotactic factors and the formation of a tumour will depend if the organ is permissive.

Until recently according to the most spread model of metastasis, these lesions arise from monoclonal expansions of very specific, individual tumour sub-clones that emerge relatively late in primary tumour development. These cells would accumulate several stochastic mutations during the progression process that would provide them with selective advantages to migrate from primary tumours and colonize distant tissues. From this point of view, the metastatic cells should be substantially different from primary tumour ones both in genotypic and phenotypic features. Nevertheless this model of tumour progression drives to conceptual and experimental inconsistencies [33, 34]. This model can not explain the cellular heterogeneity observed at the metastatic lesions and the fact that in both epithelial primary tumour and metastasis a similar concentric epithelial differentiation is easily detected [35]. Overall transcriptional profile defined by gene-expression microarrays also showed that the relevant transcriptional signatures may be detected in early-stage primary tumours that are destined to metastasize. DNA microarray studies showed that primary breast tumours that developed metastasis differed from those that remained localized [36] and that the transcriptional profiles among primary breast tumours and autologous metastasis were quite conserved [37, 38]. These results were also observed in other tumour types, like in colorectal cancer [34]. Other microarray studies have also shown the existence of signatures that allow predicting the preferential metastasis sites [39, 40]. These data suggest that the metastatic capacity of tumours might be acquired at much earlier tumorigenesis stages than previously assumed.

A new metastatic model might help us to find explanations to the experimental evidence exposed above. This model is based on the existence of metastatic Cancer Stem Cells (mCSCs)[6]. These cells would correspond to a subset of CSCs with an inherent capability to metastasize and that would be present from early stages in primary tumours.

The disruption of epithelial-cell homeostasis correlates with the loss of epithelial characteristics and the acquisition of mesenchymal proteins and a migratory phenotype. The idea that this phenomenon, first identified in embryonic development, in which polarized epithelial cells are converted into motile cells, is implicated in the progression of primary tumours towards metastases has gained favour in recent years. According to this "migrating cancer stem cell" (MCC) concept [35] the epithelial to mesenchymal transition (EMT) of tumour cells is considered to be crucial in malignant progression. This phenotype increases motility and invasiveness necessary to expand the primary tumour, as well as to colonize new target organs during the metastatic process [41-43]. Various factors responsible for mediating EMT at the molecular level have been described: Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF)[41], Transforming Growth Factor β (TGF-β) [42], Wnt/β-catenin, Notch and Hedgehog signalling pathways [41, 43-46]. Repression of E-cadherin by transcriptional regulators such as Snail or Twist emerges as one critical step driving EMT.

Many of these molecules have also been associated with stem cell maintenance contributing to the growing evidence supporting that the CSC are responsible for tumour initiation and progression.

Metastasis re-express E-cadherin, reduces the expression of nuclear β-catenin and thereby recapitulates the differentiated phenotype of its primary tumour [35], indicating that EMT process must be reversible. Therefore the EMT in disseminating tumour cells is only transient and is reversed by a

mesenchymal to epithelial transition (MET) in established metastasis [47, 48].

A recent review that analyzes the potential roles of TGFβ on breast metastasis [49] postulated, based on relevant experimental data, a model that assumes the existence of a primitive multipotent tumour cell population that is estrogen and progesterone receptor negative (ER^{-}, PR^{-}), CD44^{+}, CD24^{low}, ESA^{+}, KRT5^{+}, ALDH1^{+}, TGFβ-receptor 2 positive (TGFBR2^{+}) and possibly expresses mesenchymal markers. As true stem cells, when these cells divide they can give two identical daughters or alternatively precursors of a differentiated progeny like epithelia luminal cells (ESA^{+}, KRT8/18^{+}, CDH1^{+}, ER^{+}, PR^{+}, CD44^{-}, CD24^{+}, TGFBR2^{-}). This model proposes that exposure to TGFβ enriches the multipotent tumour initiating cells that express mesenchymal markers, while treatment with TGFβ signalling inhibitors leads to enrichment with non-clonogenic differentiated luminal cells. Therefore the constitutive activation of TGFβ signalling would contribute to the CSC enrichment enhancing the metastatic potential, drug resistance and disease progression.

All these inductor factors may be secreted by tumour infiltrating cells. In this way, in the typical colorectal cancer the micro-environmental signals would prevail over genetic alterations as inducers of EMT [35] explaining the reversal of EMT and the observed epithelial differentiation in many metastases.

COMPARISONS OF NORMAL STEM CELLS AND METASTATIC CELLS PATTERNS OF BEHAVIOUR

Aggressive tumour cells share many characteristics with embryonic progenitors. Thus, the parallelism established between NSCs and mCSCs biological behaviour may help to identify new targets for therapeutic intervention.

Among the properties shared by both NSCs and highly malignant tumour cells the more relevant are: an unlimited capacity for self-renewal; the requirement for a specific microenvironment to grow (niche), resistance to apoptosis, migration using the SDF-1/CXCR4 axis and increased capacity for drug resistance.

The Niche Environment

NSCs require a specific microenvironment or niche in order to grow and survive [50]. The stem cell niche is a specific anatomic location identified in many different tissue types that regulate how the stem cells participate in tissue generation, maintenance and repair [51]. It provides protection to stem cells and exclusion from molecules that may cause differentiation or mutation, and also play a role in determining cell fate. Niche-forming cells can be stimulated by growth factors to produce ligands that act on stem cell receptors such as Notch to initiate stem cell mitosis or to specify differentiation [52].

The existence of mCSCs is not sufficient to generate metastasis and other previous events are also needed. Metastatic cells, like NSCs, require a particular niche to grow [53]. The tumour microenvironment has a major role in modulating the metastatic capacity of most cancers. Many studies revealed that the tumour exploits the supporting cells to increase metastatic potential [54].

In vivo studies in mouse models have highlighted the importance of the niche environment during tumour initiation and development. The acquisition of the malignant phenotype by stem cells, may be in part a reflection of the cellular environment [54, 55].

Recent studies have suggested that by virtue of their cytokine and chemokine repertoire, tumours may have the ability to prepare the microenvironment of distant organs to receive disseminating cells and allow their proliferation.

The formation of metastatic deposits is intimately linked with the bone marrow. It was demonstrated [56] that the primary tumour may signal the bone marrow to mobilise cells to various sites in the body to create a specific environment for metastasis. Bone marrow-derived haematopoietic progenitor cells (BMDCs) that express vascular endothelial growth factor receptor 1 (VEGFR1) home to tumour-specific pre-metastatic sites and form cellular clusters before the arrival of tumour cells. These VEGFR1^{+} haematopoietic cells alter the local microenvironment, which leads to activation of integrins and chemokines (such as SDF-1 also referred to as CXCL12) that promote attachment, survival, and growth of tumour cells. When VEGFR1 function was abolished using antibodies or by the removal of VEGFR1^{+} cells from the bone marrow of wild-type mice, the formation of these pre-metastatic clusters was abrogated and tumour metastasis prevented. Specific up-regulation of fibronectin and clustering of BMDCs co-expressing matrix metalloproteinases (MMP9) and VLA-4 (α4β1 Integrin) in distant tissue sites before tumour cell arrival are proving to be indispensable for the initial stages of metastasis. The arrival of BMDCs to distant sites represents early changes in the local microenvironment, termed the "premetastatic niche," which dictate the pattern of metastatic spread [57].

Other studies have shown that the tumour microenvironment facilitates metastatic spread by eliciting changes in the phenotype of cancer cells; bone-marrow-derived human mesenchymal stem cells mixed with non-metastatic human breast carcinoma cells increase the metastatic potency of cancer cells when introduced in immunocompromised mice [58]. The breast cancer cells stimulate *de novo* secretion of the CCL5 chemokine from mesenchymal stem cells, which acts in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis.

Local oxygen concentrations can directly influence stem cell self renewal and differentiation. One attractive hypothesis is that stem cells, particularly in long-lived animals, might benefit from residing in hypoxic niches where oxidative DNA damage may be reduced. Hypoxic effects on NSCs and CSCs have been described [59]. These effects include the activation of pathways that induce the dedifferentiation of cancer cells, the maintenance of stem cell identity and increased metastatic potential. Hypoxia may contribute to tumour progression by specifically impacting these pathways in CSCs [60]. Regions of hypoxia arise in tumours due to rapid cell division and aberrant blood vessel formation. The hypoxia inducible factor (HIF) mediates transcriptional responses to localised hypoxia in normal tissues and in cancers and can promote tumour progression by altering cellular metabolism and stimulating angiogenesis. Lysyl oxidase (LOX) and its regulator, HIF, may also be factors that influence the

tumour microenvironment or niche to favour metastasis. HIFs have been shown to activate specific signalling pathways such as Notch and the expression of transcription factors such as Oct4 that control stem cell self renewal and multipotency. Application of the CSC theory may suggest that a hypoxic environment may support the initiation of cancer in adult stem cell populations [61]. Hypoxia is clinically associated with metastasis and poor patient outcome due to more aggressive metastasis. Patients with high LOX-expressing tumours have shown short metastasis free disease and poor prognosis. Secreted LOX is thought to be responsible for the invasive properties of hypoxic human cancer cells through FAK (Focal adhesion kinase) activity and cell matrix adhesion [62]. Interestingly, inhibition of LOX eliminates metastasis in mice without effect on the primary tumour. This suggests that hypoxic environments and the induced expression of LOX and HIF may be required to create a niche permissive for metastatic growth.

Cancer cells may disseminate to distant sites but never develop into true metastases despite remaining detectable in remote tissues [35]. It would seem plausible within the CSC theory that the tumour microenvironment actively contributes to the growth and invasion of metastatic tumours, and that non-CSCs may actually contribute to the creation of the CSC niche. In metastasis, the niche could be supporting the establishment and expansion of CSCs either through normal or deregulated signalling.

In the stem cell niche of adult somatic tissues, the balance between proliferation-inhibiting and proliferation-promoting signals is the key to homeostatic regulation of stem cell maintenance versus tissue regeneration. CSCs may alter the niche by dominant proliferation-promoting signals or intercept the molecular machinery used by NSCs for homing to or mobilizing from the niche, for invasion and metastasis [63]. Exploring this process and the underlying molecular mechanisms will provide important insight into understanding cancer cell metastasis and will benefit developing treatments aimed at destroying CSCs without adversely affecting NSCs self-renewal.

Mobilisation and Metastasis. The SDF-1/CXCR4 Axis

Studies on haematopoietic stem cells have shown that normal bone marrow stem cells possess the capacity to mobilise and migrate into the circulation to distant sites in response to tissue damage and stress with complex, coordinated homing mechanisms being involved [64]. Bone marrow stem cells display plasticity allowing them to differentiate into a variety of cell types [65, 66].

There are many similarities between the mechanisms governing the migration of NSCs and the metastatic dissemination of tumour cells. The more relevant is the interaction between the G-protein-coupled seven-span transmembrane receptor CXCR4 and its ligand, SDF-1. Chemokines are believed to cooperate with adhesion receptors in determining where tumour cells arrest and extravasate. Secretion of SDF by host tissue stromal fibroblasts is suggested to promote chemotaxis of tumour cells expressing CXCR4 and to determine, at least in part, the localization of metastases of certain tumour types [31, 67]. SDF-1 induces motility, chemotactic responses, adhesion, secretion of MMPs and secre-

tion of angiopoietic factors such as VEGF in cells that express CXCR4. SDF-1 also modulates the activity of cell surface integrins contributing to cell adhesion [68].

CSCs also express CXCR4 on their surface and, as a result, the SDF-1/CXCR4 axis is also involved in directing their metastases to organs that highly express SDF-1 as lymph nodes, lungs, liver and bones. Different cancers are found to express several chemokine receptors, and their corresponding ligands are expressed at sites of tumour metastases, which may help to explain the organ-specific nature of metastatic growth. The responsiveness of $CXCR4^+$ normal and malignant stem cells to an SDF-1 gradient may be regulated positively by several small molecules related to inflammation, which enhance incorporation of CXCR4 into membrane lipid rafts and may be blocked by small CXCR4 antagonist peptides resulting in inhibition of the metastatic ability [67, 69].

In breast cancer, CXCR4 expression correlates with the CSC content, and thus the aggressiveness of cancer cell lines. CXCR4-high-expressing tumour cells were most efficient in the formation of a large tumour and organ-metastasis in SCID mice than CXCR4-low-expressing tumour cells [70].

Human pancreatic cancer tissue contains CSCs defined by CD133 expression that are exclusively tumourigenic and highly resistant to standard chemotherapy. This population could be further subdivided into two subsets based on the expression of the CXCR4 molecule ($CD133^+/CXCR4^{neg}$ and $CD133^+/CXCR4^+$). Both subpopulations were equally capable to sustain tumour growth. However, only the subpopulation of migrating $CD133^+/CXCR4^+$ cancer stem cells resulted essential for tumour metastasis. Depletion of the cancer stem cell pool for these migrating CSCs virtually abrogated the metastatic phenotype of pancreatic tumours without affecting their tumourigenic potential [71]. This study, for the first time, demonstrated the role of CXCR4 in tumour metastasis by using a model where multiple phenotypic cancer cell subpopulations coexist in a dynamic equilibrium and where the tumourigenic and metastatic properties of distinct cell subsets, including CSC, can be tested independently.

In neuroectodermal cancers, the role of the SDF-1/CXCR4 axis in metastasis dissemination has also been established, and potential therapeutics targeting CXCR4 have been proposed [72].

Consequently, strategies aimed at modulating the SDF-1-CXCR4 axis could have important clinical applications in oncology to inhibit metastasis of cancer stem cells.

Resistance to Apoptosis and Protection from Cellular Damage

NSCs and CSCs express higher levels of antiapoptotic proteins than differentiated cells and can resist apoptosis by a number of mechanisms [73]. In pancreatic tumours, for example, Hedgehog signalling remains active in cell lines established from primary and metastatic pancreatic adenocarcinomas and the inhibition of Hedgehog signalling by cyclopamine induced apoptosis and blocked proliferation, suggesting that this pathway may have an early and critical role in the genesis of this cancer [74].

Stem cells must also resist early senescence in order to maintain the stem cell pool, Hedgehog pathway and BMI-1 play important roles in regulating self-renewal of normal and tumourigenic human mammary stem cells [75]. Furthermore, despite their limitless self-renewal capacity, NSCs are relatively quiescent and divide infrequently unless activated [76]. Similarly, CSCs and metastatic cells may cycle through long periods of quiescence and since most chemotherapeutic anticancer agents are designed to target rapidly dividing cells, this may be one mechanism by which CSCs and metastatic cells escape cytoxicity from these drugs [77-79].

The protection of adult stem cells from damage or death due to toxins is a critical function of an organism; NSCs have a number of unique properties that help protect them from cellular insult and ensure their long lifespan. One of the principal mechanisms for protecting stem cells is through the high expression of multifunctional efflux transporters from the ATP-binding cassette (ABC) gene family that facilitate rapid efflux of toxins and drugs, but these genes get turned off in committed mature cells [78, 80]. These same transporters that include ABCB1, which encodes P-glycoprotein, and ABCG2, which encodes a protein called breast cancer resistance protein (BCRP), also play a role in multidrug resistance of tumour cells. ABCC1, ABCB1 and ABCG2 represent the three principal multi-drug resistance (MDR) genes overexpressed in tumour cells [81-83]. Thus, drug resistance could be an inherent feature of CSCs and provides one mechanism in which CSCs could survive to cytotoxic or targeted therapies and lead to tumour regrowth or relapse [77, 80].

Another mechanism intrinsic to the biology of both NSCs and CSCs that could be responsible for their DNA stability is proposed by the 'immortal DNA strand' hypothesis, postulated by Cairns (1975) [84, 85]. According to it, stem cells undergo asynchronous DNA synthesis segregating the newly synthesized DNA strands to the differentiating daughter cell. The parental "immortal" DNA strand always segregates with the new stem cell keeping stable through the stem cell generations, thus helping to protect the stem cell population from DNA damage [89-91]. Any errors that may have occurred during DNA replication would not stay in the stem cell compartment, but be passed on to transit cells with a limited life span.

Similarly, CSCs are believed to be resistant to radiation therapy by preferentially upregulating their DNA proofreading mechanisms in order to avoid cellular death due to DNA damage. The intrinsic radiosensitivity of CSCs varies between tumours [86]. Experimental evidence suggests that CSC content may differ between tumours even of the same histopathological type, and that a higher proportion of CSCs correlates with higher radioresistance [87-89]. Evidence for the ability of CSCs to contribute to radioresistance has been provided in human gliomas. CSCs contribute to glioma radioresistance through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. CD133-expressing tumour cells preferentially activate the DNA damage checkpoint in response to radiation, and repair radiation-induced DNA damage more effectively than CD133-negative tumour cells [90].

CSC MODEL AND THERAPEUTIC APPROACHES FOR METASTASIS

Traditional therapies can be useful in the reduction of tumour mass, both in primary and metastatic lesions, but usually fail to eradicate all tumour cells and consequently the disease relapses are frequent. Many of these classical therapies are mainly based on drug and radiation toxicity associated to cell replication. As it was reported in AML, there is a small number of quiescent leukemic progenitors [91], representing putative dormant CSCs, that have their cell cycle arrested and therefore are resistant to these toxic effects. Another clinical relevant property of CSCs would be their ability to pump drugs out of the cell as happens in NSCs [92]. Previous attempts to inhibit the ABCB1 transporter resulted in limited clinical success, but new ABCG2 specific inhibitors may lead to better results [78]. The relative resistance to DNA damage, the efficiency in DNA repair [90, 93] and the intrinsic high levels of anti-apoptotic molecules [4] also make the CSC a very resistant target for the conventional cancer therapies.

If the CSC model is correct, a rare subset of tumour cells drives cancer formation and therefore this cell population should be an important target for novel cancer therapies. Since many pathways involved in maintaining NSCs are the same that are deregulated in CSCs, therapies against these pathways may also be potentially lethal for NSCs. However, several potential differences between normal and cancer stem cell physiology might be explored and eventually used as therapeutic targets.

Novel therapies against metastasis might consider several aspects of the disease. One aspect is relative to the own CSCs and their self-renewal, differentiation and survival pathways, and another one corresponds to the pre-metastatic niche formation and CSCs homing to the metastatic sites (Table **1**).

Self-Renewal and Differentiation Pathway Targets

Wnt signalling has been shown to be required for self-renewal of tumour-initiating cells in several cancers including CML [94] and squamous cell carcinomas [95]. Wnt inhibitors have already been discovered including Secreted Frizzled-Related Proteins (SFRPs) and Dickkopf proteins (DKKs) which act at the cell surface inhibiting Wnt signalling through its receptors [96]. The Wnt signalling can also be blocked upstream by neutralization of Wnt molecules by specific monoclonal antibodies [97] or downstream by small molecules that antagonize the oncogenic transcription factor TCF/βcatenin protein complex [98]. Other two signalling pathways have been involved in self-renewal and differentiation processes, the Hedgehog and Notch pathways. Inhibition of Hedgehog pathway is also a potential therapeutic target both by antibodies against Hedgehog [99] as well as by small-molecule inhibitors of the Hedgehog co-receptor smoothened homologue (SMO) [100]. The inhibition of Hedgehog signalling has shown to kill CML tumour initiating cells [101]. It was demonstrated that Hedgehog signalling also regulates the self-renewal of both normal and neoplastic mammary stem cells and that its regulation involves a polycomb gene BMI-1 activity [102]. This suggests that Hedgehog signalling, acting through BMI-1 is able to regu-

Table 1.   CSC Model and Therapeutic Approaches for Metastasis

CSCS Characteristics Targeted		Type of Cancer	Therapeutic Approach
Self-renewal and differentiation pathway	Wnt signalling in self-renewal of tumour-initiating cells	CML [94] Squamous cell carcinomas [95]	• Secreted Frizzled-Related Proteins (SFRPs) and Dickkopf proteins (DKKs) inhibiting Wnt signalling through its receptors [96] • Neutralization of Wnt molecules by specific monoclonal antibodies [97] • Small molecules antagonizing the oncogenic transcription factor TCF/βcatenin protein complex [98]
	Hedgehog pathway	CML tumour initiating cells [101] Neoplastic mammary stem cells [102] [103]. Human pancreatic cancer [104].	• Antibodies against Hedgehog [99] • Small-molecule inhibitors of the Hedgehog co-receptor smoothened homologue (SMO) [100] • Specific inhibitors : cyclopamine [102] • Orally bioavailable small-molecule inhibitor (IPI-269609) [104]
	Notch pathway	Colorectal cancer [106] Brain tumours [107]	• γ-secretase inhibitors can inhibit Notch cleavage and activation [105] • Antagonist antibodies against individual Notch receptors [108]
	Tumour suppressor genes (PTEN)	Leukemia	• Rapamycin (mTOR inhibitor), in PTEN-deleted cells depleted leukemia-initiating cells and restored normal HSC function [110]
	Specific development pathways inducing differentiation	Glioblastoma	• Bone Morphogenetic Proteins (BMPs) in Preclinical model: differentiation of CD133+ initiating glioblastoma cells into astrocyte-like cells [111]
		Basal-like breast cancers	• TGFβ antagonists: differentiation of stem cell population into luminal, non-proliferating and non-metastatic phenotype[49]
Survival pathways	Apoptosis of CSCs	AML CML	• Proteosome inhibitor MG-132 and the anthracycline idarubicin: Induction of apoptosis of CSCs without affecting the survival of normal HSCs [113] • Nuclear factor κB (NF-κB) inhibition [113] • Inhibition of promyelocytic leukemia protein (PML) by arsenic: sensitization to pro-apoptotic stimuli [114]
	Inhibition of DNA damage response	Glioma Breast tumours	• Inhibitor of Checkpoint Kinase I (CHK1) and CHK2: reversion of radioresistance in CD133+ glioma-initiating cells [90] • Pharmacological depletion of reactive oxygen species scavengers: transformation of tumour-initiating cells in radiosensitive cells [93]
	Drug efflux pumps ABCB1 and ABCG2		• Inhibitors of ABCB1 and ABCG2 [117]
Niche formation	Recruitment of BMDCs at the future metastasis sites	Breast, lung, prostate, and colon cancers [118-121]	• VEGFR1+ cells, VLA4 and Opn molecules: identification and prevention of the onset of metastasis
mCSCs homing	Chemotactic and invasive responses through CXCR4 or CCR7 signalling	Human breast cancer [31] Pancreatic cancer [127].	• CXCR4 antagonists [128]

late the self-renewal of normal and malignant mammary stem cells [103]. This process is blocked by specific inhibitors such as cyclopamine [102]. A new orally bioavailable small-molecule inhibitor of Hedgehog signalling (IPI-

269609) inhibited systemic metastasis in orthotopic xenografts established from human pancreatic cancer cell lines but had minimal effect on primary tumour volume [104]. The only discernible phenotype observed within pri-

mary tumour was a significant reduction of tumour-initiating population of pancreatic cancer. These data indicate that this antagonist of Hedgehog signalling targets CSCs in both primary and metastatic tumours.

Some γ-secretase inhibitors can inhibit Notch cleavage and activation [105]. One of these showed to induce a rapid and massive conversion of colorectal proliferative crypt cells into post-mitotic goblet cells [106] and other one showed to deplete tumour-initiating cells in brain tumours [107]. Antagonist antibodies against individual Notch receptors are also been studied [108].

Molecules encoded by tumour suppressor genes such as PTEN seem to be also involved in signalling pathways associated to self-renewal capacity [109]. PTEN deletion in adult haematopoietic cells has shown to promote myeloproliferative disease whereas induced depletion of Haematopoietic Stem Cells (HSCs) [110] and mTOR inhibition by rapamycin, in these PTEN-deleted cells, not only depleted leukemia-initiating cells but also restored normal HSC function [110]. These mechanistic differences between NSCs and CSCs may be used as therapeutic targets in order to deplete cancer cells without normal stem cell damages.

If the inhibition of one or all of these signalling pathways are sufficient to differentiate CSCs, inhibit their proliferation or kill them is still unclear.

The targeting of a specific development pathway that induces differentiation could be a more general strategy for eliminating CSCs. It has been shown that CD133^{+} initiating glioblastoma cells can be differentiated into astrocyte-like cells by Bone Morphogenetic Proteins (BMPs) in a preclinical model [111].

The treatment of basal-like breast cancers with TGFβ antagonists may have anti-tumour effects by converting the putative stem cell population into luminal, non-proliferating and non-metastatic phenotype[49]. On the contrary, this treatment might antagonize with anti-estrogen therapy in estrogen-dependent luminal cancers, but this tumour cell type is probably committed to epithelial differentiation. However, when these cells become estrogen-independent, the use of TGFβ pathway antagonist treatment would be very useful in preventing metastasis and reducing CSC pool at primary tumour [112].

Survival Pathways Targets

It has been observed that the treatment of AML with the proteasome inhibitor MG-132 and the anthracycline idarubicin induces apoptosis whereas does not affect the survival of normal HSCs [113]. Nuclear factor κB (NF-κB) is constitutively active in CSCs, but not in normal HSCs, and therefore its inhibition contributes to apoptosis of these CSCs [113]. Inhibition of promyelocytic leukemia protein (PML) by arsenic trioxide disrupted the maintenance of CML-initiating cells, induced the differentiation and progression through the cell-cycle of these otherwise quiescent tumour cells and sensitized them to pro-apoptotic stimuli [114].

DNA damage response is preferentially activated in glioma-initiating cells. The radioresistance of CD133^{+} glioma-initiating cells can be reversed with an inhibitor of Checkpoint Kinase I (CHK1) and CHK2 [90]. Pharmacological depletion of reactive oxygen species scavengers transforms tumour-initiating cells in radiosensitive cells [93]. These preliminary studies show the potential of inhibiting DNA damage response as therapeutic target.

A very important characteristic of NSCs and CSCs is the high expression of the drug efflux pumps ABCB1 and ABCG2 [115, 116]. Therefore the targeting of these pumps would be very useful to kill the CSCs in cooperation with chemotherapy. Inhibitors of ABCB1 and ABCG2 pumps have been studied as anticancer agents both in preclinical animal models and patients [117].

Pre-Metastatic Niche Formation Targets

As mentioned above, BMDCs are recruited to pre-metastatic sites by secreted factors present in cancer cell conditioned media [56]. BMDCs express VLA4, a known natural receptor of Osteopontin (Opn). Opn is a secreted protein known to be over-expressed in several neoplasms and is associated with tumorigenesis, tumour invasion, and metastasis in breast, lung, prostate, and colon cancers [118-121]. Increased levels of circulating Opn were also reported in many malignancies (including breast, and prostate cancers) and were associated with poor disease prognosis [122-124]. All these data suggest that Opn secreted by tumour cells recruits BMDCs at the future metastasis sites in order to restructure the local microenvironment that allows metastatic cell growth. Therefore, VEGFR1^{+} cells, VLA4 and Opn molecules may be relevant therapeutic targets in order to identify and prevent the onset of metastasis.

mCSCS Homing Targets

Repair of ischemic injuries involves the selective recruitment of circulating or resident progenitor cells. HIF-1, a central mediator of tissue hypoxia, induces SDF-1 expression in ischemic areas [125]. The SDF-1 expression by endothelial cells attracts circulating stem and progenitor cells expressing its specific receptor (CXCR4) to areas of tissue damage. Increasing evidence [126] suggests that epithelial tumour cells exploit mechanisms that normally regulate the leukocyte trafficking and homing described above. CXCR4 and CCR7 are highly expressed in primary human breast cancer and its metastases [31]. Their respective ligands SDF-1 and CCL21 are over-expressed in organs that represent the first destination of breast cancer metastasis. In breast cancer cells, signalling through CXCR4 or CCR7 mediates actin-polymerization and pseudopodia formation, and subsequently induces chemotactic and invasive responses [31]. It has also been reported that pancreatic CSCs contain a sub-population of migrating CSCs characterized by CXCR4 expression [127]. CXCR4 antagonists appear to have antitumour activity in animal metastatic models [128]. These data indicate the potential clinical efficacy of these antagonists in metastasis treatments.

CLINICAL APPROACHES

Among several agents that act on self-renewal process, small molecules that target in Hedgehog pathway such as SMO antagonist cyclopamine [129] showed to be effective to lead basal cell carcinomas to regression. In addition, an

orally active small molecule that targets SMO appears to have anti-tumour activity in locally advanced or metastatic basal-cell carcinoma in a Phase I Clinical Trial [130]. It is advancing to phase II trials for metastatic colorectal cancer and other advanced epithelial tumours [131]. These and other agents targeting self renewal signalling with therapeutic potential have already been extensively reviewed [105, 132].

As mentioned above an important characteristic of CSCs is the presence of high expression levels of drug efflux pumps. A second generation of ABCB1 specific inhibitors showed promising activity in a Phase I clinical trial including patients with treatment-resistant locally-advanced or metastatic cancers [133].

There are some TGFβ signalling antagonists in early phases of clinical development [49]. Among them the TGFβ2 antisense (Antisense Pharma GmbH -AP 12009) and the monoclonal antibody (mAb) against all TGFβ (Genzyme Inc-GC1008) are outlined. Phase I/II clinical trials with these TGFβ signalling antagonists are actually being carried-out [134, 135].

There are several antibodies that target cell surface markers on tumour initiating cells in clinical trials [105]. One of these surface markers is the Epithelial Cell Adhesion Molecule (EpCAM) that is expressed on tumour initiating cells from breast [15], colon [17] and pancreatic cancer [19]. There is evidence that EpCAM also contributes to the migration of gastric cancer cells, suggesting that EpCAM-targeting therapy might be a promising strategy in metastatic gastric cancer [136]. Nevertheless until now the clinical trials with EpCAM mono-specific antibodies, either murine or humanized, have shown limited efficacy [137], suggesting that immune tolerance or Antibody-Dependent Cellular-mediated Citotoxicity (ADCC) stimulated by these mAb are not efficient to kill tumour cells. To overcome these limitations bispecific antibodies target to both EpCAM and CD3 were used in PhaseI/II clinical trials in order to put into proximity cancer cells and T lymphocytes [138]. This immunotherapy prevented the accumulation of ascites and efficiently eliminated tumour cells with an acceptable safety profile in ovarian cancer patients.

Although clinical trial results are still preliminary and continue under investigation, these new therapies are very promising (Table **2**). The identification of new therapeutic targets and drugs based on CSCs model constitute the next challenge.

The evaluation of therapeutic success is also of great importance and will require the identification and estimation of remaining CSC population after treatment. As mentioned above many specific markers have already been described for CSCs in some tumour types, but CSC specific markers for other types of tumours will need further studies. NSCs and CSCs populations from different tissues express ABC drug transporters such as ABCG2 which can also be used to identify them. Nevertheless the identification of new CSC specific markers continues to be an urgent task.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent advances in cancer and stem cell biology research have increased the understanding of tumour initiation and progression. The cancer stem cell hypothesis states that CSCs are a small subpopulation of self-renewing cancer cells responsible for initiating and maintaining cancer growth. These CSCs remain in patients after conventional therapy has been completed, providing a logical explanation to its therapeutic failure. The resistance to apoptosis and protection from cellular damage, the persistence of stem cells in their niche and a state of quiescence are among the CSC properties that explain the failure of currently available treatments.

The hypothesis predicts that effective tumour eradication will require agents targeting CSCs. The many connections between stem cells and cancer are providing clues about the origins of cancer and will ultimately yield new approaches to fight this disease. Stem cell characteristics offer promising targets for the development of novel cancer therapies. However, it is important that agents directed against CSCs discriminate between them and NSCs.

Metastasis is the result of cancer cell adaptation to a new tissue microenvironment. Success of secondary tumour growth is then determined by the nature of the host response

Table 2.   **Ongoing Clinical Trials.**

Therapeutic Drugs	Type of Cancer	Clinical Trials
Orally active small molecule targeting SMO	Locally advanced or metastatic basal-cell carcinoma	Phase I [130]
	Metastatic colorectal cancer and other advanced epithelial tumours	Phase II [131]
Second generation of ABCB1 specific inhibitors	Treatment-resistant locally-advanced or metastatic cancers	Phase I [133].
TGFβ2 antisense (Antisense Pharma GmbH -AP 12009) Monoclonal antibody (mAb) against all TGFβ (Genzyme Inc-GC1008).	Pancreatic carcinoma (stageIV) Colorectal carcinoma Advanced malignant melanoma (MM) Renal cell carcinoma (RCC)	Phase I/II [134, 135]
EpCAM mono-specific antibodies (limited efficacy) Bi-specific antibodies targeting EpCAM and CD3	Breast cancer Colon cancer Pancreatic cancer Metastatic gastric cancer	PhaseI/II [136, 137] [138]

and the tumour cells ability to subvert it in a way that allows them to proliferate and survive. As the role of the host tissue stroma in promoting tumour cell growth and dissemination has been established, tumour-host interaction mechanisms appear to be interesting therapeutic targets. The fact that early cancer harbours metastatic potential should allow to design new preventive treatments of metastatic disease and to have a better comprehension of the mechanisms whereby cancer cells spread. Many drugs that can interfere with CSCs characteristics are under clinical investigation. However, to evaluate the efficacy of stem cell- targeted treatments, stem-cell markers should be included as correlates of response, and for many types of cancers they are not yet defined. Moreover, because chronic treatment of patients with cancer is likely to be necessary for the eradication of CSCs, the estimation of the effects of anti-cancer drugs on normal adult stem cells is also of major importance before their use in cancer therapies. Overall, strategies targeting CSCs appear very promising for progress in cancer treatments and consequently, for decreasing patient mortality.

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ABBREVIATIONS

CSC	=	Cancer Stem Cell
NSC	=	Normal Stem Cell
SDF-1	=	Stromal cell-Derived Factor 1
CXCR4	=	Chemokine Receptor 4
CML	=	Chronic Myeloid Leukaemia
AML	=	Acute Myeloid Leukaemia
NOD/SCID	=	Non-Obese-Diabetic/Severe Combined Immunodeficient
ESA	=	Epithelial-Specific Antigen
MMIC	=	Malignant-Melanoma-Initiating Cells
mCSCs	=	Metastatic Cancer Stem Cells
MCC	=	Migrating Cancer Stem Cell
EMT	=	Epithelial to Mesenchymal Transition
TGF-β	=	Transforming Growth Factor β
MET	=	Mesenchymal to Epithelial Transition
HGF	=	Hepatocyte Growth Factor
EGF	=	Epidermal Growth Factor
ER	=	Estrogen Receptor
PR	=	Progesterone Receptor
TGFBR2	=	TGFβ-Receptor 2
BMDCs	=	Bone Marrow-Derived Hematopoietic Progenitor Cells
VEGFR1	=	Vascular Endothelial Growth Factor Receptor 1
MMP9	=	Matrix Metalloproteinase 9
VLA-4	=	α4β1 Integrin
HIF	=	Hypoxia Inducible Factor
LOX	=	Lysyl Oxidase
MMPs	=	Matrix metalloproteinases
ABC	=	ATP-Binding Cassette
BCRP	=	Breast Cancer Resistance Protein
MDR	=	Multi-Drug Resistance
SFRPs	=	Secreted Frizzled-Related Proteins
DKKs	=	Dickkopf Proteins
SMO	=	Hedgehog Co-Receptor Smoothened Homologue
HSCs	=	Hematopoietic Stem Cells
BMPs	=	Bone Morphogenetic Proteins
NF-κB	=	Nuclear Factor κB
PML	=	Promyelocytic Leukaemia Protein
CHK	=	Checkpoint Kinase
Opn	=	Osteopontin
EpCAM	=	Epithelial Cell Adhesion Molecule
ADCC	=	Antibody-Dependent Cell-mediated Cytotoxicity

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