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Carcinogenesis and environment: the cancer stem cell hypothesis and implications for the development of novel therapeutics and diagnostics

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1. ABSTRACT

Stem cell research has greatly contributed to the field of oncology with the identification and isolation of cancer stem cells from a variety of tumors. The discovery of rare subpopulations of cancer stem cells has indeed entirely changed the focus of cancer research. Normal adult stem cells and cancer stem cells can both self-renew and undergo a differentiation program that, in turn, gives rise to a high number of differentiated cells. Adult stem cells and their malignant counterparts share almost all of the same intrinsic and extrinsic factors to regulate self-renewal, differentiation and proliferation pathways. Fractions of normal and cancer stem cells are naturally more resistant to toxic injuries than any other cell type. Overall, these observations lead to the conclusion that adult stem or progenitor cells can eventually become malignant by generating cancer stem cells, which are responsible for the development and maintenance of the tumor mass. In addition, chemo-resistant cancer stem cells may cause the relapse of the disease following an apparent beneficial treatment. Indeed, the study of the biology of cancer stem cells might lead to the improvement of preventive cancer diagnosis and to the development of novel therapeutics, which must be designed to selectively target malignant stem cells without affecting normal adult stem cells.

2. INTRODUCTION

The study of the biology of adult stem cells has had a profound impact on the field of cancer research, which changed its focus entirely after the formulation of the so-called "cancer stem cell hypothesis" (1-19). The interest in adult stem cells is obviously related to their capacity to regenerate various types of tissues. Indeed, this property might have important implications in cell replacement therapy for a wide variety of maladies, such as neurological diseases, spinal cord injuries, diabetes, cardiovascular disorders and many kinds of genetic illnesses (19-22).

Stem cells, by definition, have the ability to self-renew and differentiate to give rise to a high number of progeny (Figure 1). Such a characteristic allows for the regeneration of most tissues, which can either occur constitutively, as in the blood, intestine, skin and hair, or upon injury, as in the liver following partial hepatectomy (21). On the other hand, tissue-specific adult stem cells might acquire a malignant phenotype and, therefore, initiate a tumor (Figure 1) (1, 2, 8). On these grounds, paradoxically, tissue homeostasis and carcinogenesis seem to be two sides of the same coin, as adult stem or progenitor cells might acquire a transformed cell phenotype

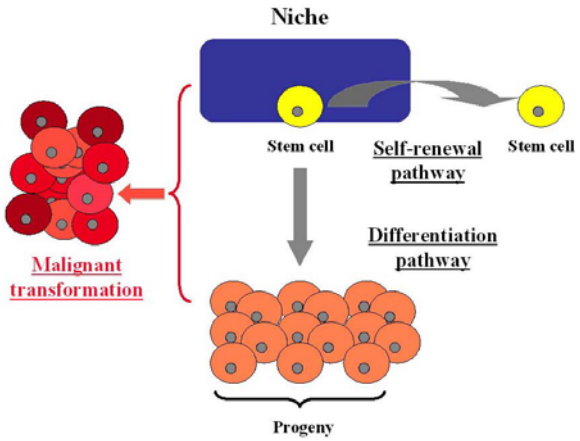

Figure 1. The niche, or microenvironment, is a crucial factor for the control of the fate of adult stem cells, which can either undergo symmetric cell division for the self-renewal program, or asymmetric cell division with self-renewal and differentiation, which, in turn, generates a high number of progeny and, ultimately, fully differentiated cells. On the other hand, the dysregulation of the factors involved in the control of the biology of adult stem cells might lead to malignant transformation, which gives rise to a very heterogeneous cancer cell population. This may happen at the stage of undifferentiated adult stem cells, intermediate amplifying cells and early progenitor cells. The latter two cell types are not shown in the figure. The malignant transformation of early progenitor cells requires additional genetic mutations to the dysregulation of the aforementioned stem cell self-renewal pathway.

via deregulation of the self-renewal signaling pathway (8, 19). Thus, a neoplasia can arise only from a very rare subpopulation of specialized cells. This notion is in contrast with the classic "stochastic model of carcinogenesis", which was predominant over the cancer stem cell hypothesis, until the emerging of the field of stem cell biology that occurred in recent years. According to the stochastic model, every cell of the body might potentially trigger a tumor, after undergoing a certain number of random genetic mutations with subsequent clonal selection (23). However, the nowadays fashionable cancer stem cell hypothesis was in fact conceived well before the massive advent of stem cell research. As surprising as it may sound, already one century and a half ago various investigators independently expressed the concept that cancer might be originated by rare cellular subpopulations with stem cell-like features (24-26). These empirical observations were reinstated and further developed in the early 1960s by Till and McCullogh (27) and then in the late 1960s by Pierce (28). Till and McCullogh claimed that certain tissue-specific adult stem cells might be the starting point of cancer (27), whereas Pierce stated that a malignancy consists of a maturation arrest of stem cells (28).

Undoubtedly, stem cell research conducted in recent years provided major support to the validation of the cancer stem cell hypothesis, which is now predominant over the stochastic model of carcinogenesis.

3. THE BIOLOGY OF ADULT STEM CELLS AND THE ESTABLISHMENT OF A MALIGNANT CELL PHENOTYPE

Both adult stem cells and their malignant counterparts have the ability to self-renew and differentiate while proliferating (Figure 1) (8, 21). In addition, normal and cancer stem cells exhibit an active telomerase expression, migratory properties, resistance to programmed cell death, or apoptosis, enhanced activity of membrane transport and, interestingly, can both growth under anchorage-independent *in vitro* conditions (8, 9, 21, 29, 30). The latter property was previously considered as one of the main hallmarks of malignant cells that grow adherently under *in vitro* conditions (29-32). Typical assays for testing anchorage independence are usually based on cell growth and proliferation on soft agar, or on polyhema-coated tissue culture plates (30). In both cases, cells cannot growth and proliferate adherently and, therefore, a number of signaling pathways are switched off, such as vitronectin and fibronectin receptors (31, 32). Under these *in vitro* conditions only transformed and stem cells have alternative intracellular pathways that promote cell survival and proliferation (31, 32).

Self-renewal of stem cells is indeed a highly regulated process, which is under the strict control of a combination of intrinsic and extrinsic factors (19, 21, 33-37). Stem cell intrinsic factors include Wnt, Hedgehog and Notch signaling pathways (19, 33-37), whereas extrinsic factors are related to the microenvironment, or niche (19, 21, 33-37), which plays a crucial role in orchestrating the interaction between extrinsic factors and stem cells. The niche consists of particular cellular substrates that can interrelate with stem cells either by cell-to-cell contact, or by secreting soluble factors, or both (19, 21, 33-37). Cell-to-cell contact engages certain membrane receptors in order to trigger stem cell intracellular signaling systems (19), whereas soluble factors comprise cytokines and/or growth factors (19, 21). The influence of the niche on the stem cell self-renewal pathway was investigated in the context of tissues of the small intestine, epidermis, hair follicle, blood and central nervous system (CNS) (8, 21). Cellular substrates of the niche vary according to the type of tissue and may comprise fibroblasts, macrophages, osteoblasts and astrocytes. Consequently, tissue-dependent differences can be observed also for microenvironmental soluble factors (8, 21). An interesting example is provided by the niche of the central nervous system, which seems to be structured into three main components: 1) astrocytes; 2) the basal lamina; and 3) molecular signaling pathways based on membrane-bound receptors, extracellular matrix and soluble factors (21). Astrocytes of the subventricular zone (SVZ) might be related to the neurogenesis induced by the niche and there is some controversy whether or not astrocytes can be, *per se*, neurogenic (21). The basal lamina of the SVZ is rich in laminin and collagen-1, which, in turn, form a substrate that connects the various components of the niche, including fibroblasts and macrophages (21). These cell types secrete soluble factors such as cytokines and growth factors, which are important components of the niche. The soluble factors include VEGF, EGF, FGF, bone

Table 1. Link between dysregulation of various types of stem cell self-renewal pathways and carcinogenesis

Main factor involved in the dysregulation of stem cell self-renewal pathway	Type of tumor
Wnt	Colon cancer Chronic myelogenous leukemia
Hedgehog	Pancreatic tumors Basal skin carcinoma Gastric cancer Breast carcinoma Prostate tumor
Notch	Human T-cell acute lymphoblastic leukemia Breast cancer Cervical cancer

Table 2 List of cellular markers associated with various types of human cancer stem/progenitor cells

Cellular marker for cancer stem/progenitor cells	Type of tumor
$CD34^{(+)}/CD38^{(+)}$	Leukemia
$CD138^{(-)}$	Multiple myeloma
$CD44^{(+)}/CD24^{(+)}$ $Oct4^{(+)}$ $CX43^{(-)}$	Breast cancer
$CD133^{(+)}/nestin^{(+)}$	Brain tumor
$CD133^{(+)}$	Colon cancer
$CD44^{(+)}/\alpha_2\beta_1^{hi}/CD133^{(+)}$	Prostate cancer
$Sca1^{(+)}$	Prostate cancer

morphogenic proteins, Shh, TGF-α, LIF and the Eph/ephrin family of signaling molecules. Thus, several membrane-bound receptors come into play to mediate the niche-regulated stem cell signaling pathways, which include the Notch family of receptors, CD15 and, of course, all the receptors for the aforementioned ligands (21).

A second interesting example of tissue-dependent factors is provided by hematopoietic stem cells, which reside in the inner surface of bones (21, 38). In this case, osteoblasts constitute one of the key components of the niche (21, 38). The loss of contact from osteoblasts causes the migration of hematopoietic stem cells towards the blood vessels situated in the center of the bone marrow cavity, where they differentiate to give rise to various blood cell types (21, 38). Another important regulator of hematopoietic stem cell trafficking from the niche to the bone marrow is the CXCR4-CXCL12 axis (39). CXCR4 is a chemokine receptor and its cognate ligand is CXCL12 (39). The latter is also termed stromal derived factor (SDF)-1 (39).

As already mentioned, dysregulations in signaling pathways of each of the aforementioned intrinsic factors can be associated with carcinogenesis, as observed in animal models and in human systems (Table 1) (8, 33-37, 40, 41). For instance, abnormalities in the Wnt-related signaling pathway were linked to human colon cancer and chronic myelogenous leukemia (8). An aberrant Hedgehog-related signaling pathway was observed in a variety of human tumors, such as pancreatic cancer, basal skin carcinoma, gastric tumors, breast carcinoma and prostate cancer (36-38). Dysfunctions of Notch-related signaling pathway were detected in human T-cell acute lymphoblastic leukemia, breast carcinoma and cervical cancer (40-42). These findings regarding the dysregulation in signaling pathways could be achieved through the identification of some cellular markers associated with human cancer stem/progenitor cells (Table 2), which have allowed for the isolation and consequent characterization of cancer stem cells from human tumors of the central nervous system, breast, prostate, colon and blood (i.e. leukemia and multiple myeloma) (8).

The cancer stem cell hypothesis envisions that a specific cellular compartment with stem cell-like properties is responsible both for triggering tumorigenesis and maintaining the tumor mass (8). On these grounds, the self-renewal property, albeit abnormal, is a major requirement to establish and maintain a malignant cell phenotype. *In vitro* transfection of AML-ETO into purified populations of hematopoietic progenitor cells eventually led to the transformation of some myeloid progenitor cells, which also gained the feature of self-renewal (43). A similar finding was observed for retroviral-mediated gene transfer of mixed lineage leukemia (MLL)-AF9 fusion protein into murine granulocyte macrophage progenitor cells (44). MLL-AF9 fusion protein derives from the translocation t(9;11)(p22;q23) and is found in human leukemias (44). Granulocyte macrophage progenitor cells acquired leukemia stem cell-like properties following the expression of retroviral-encoded MLL-AF9 and formed oligoclonal acute myelogenous leukemia (AML) in transplanted mice, which was indeed analogous to other MLL fusion leukemia models (44). Interestingly, a number of fusion proteins resulting from translocation events detected in human leukemias were shown to be able to confer leukemia stem cell-like properties to murine committed hematopoietic progenitor cells (45-47). These additional fusion proteins comprise MLL-GAS7 (45), MLL-ENL (46) and MOZ-TIF2 (47). Taken together, all the findings observed in these studies further support the notion that rare populations of cancer stem cells are accountable for the maintenance of neoplastic tissues. On the other hand, they also raise the question of whether or not cancer stem cells invariably derive from normal stem cells, as the expression of recombinant fusion proteins associated with human leukemias could transform and impart cancer stem cell-like features on murine committed hematopoietic precursor cells (44).

4. THE CANCER STEM CELL HYPOTHESIS AND VARIOUS MODELS FOR THE STUDY OF HUMAN CANCERS

Animal models and cell culture systems are both essential for the analysis of genetic and epigenetic mutations that are required to establish a transformed cell phenotype. A very critical aspect of cancer research is the relevance of animal models in replicating the pathology of human malignancies (48, 49). Indeed, transgenic mouse models have greatly contributed towards the considerable advances in better understanding the pathogenesis and development of human cancers. However, transgenic mouse systems were not able to reproduce human cancers in many circumstances. In this respect, the stem cell hypothesis for carcinogenesis may provide an interpretation of the phenomenon. In several cases, transgenic mouse technology relies upon tissue-specific promoters to express oncogenes under characterization. It was observed, however,

that various tissue-specific promoters seem to be preferentially active in differentiated cells, rather than stem cells and early progenitor cells (48). In this scenario, the expression of oncogenes in differentiated cells might not be able to trigger carcinogenesis. For instance, a report showed that the oncogenes c-Myc and c-Met could generate carcinomas in mice, provided that they were under the control of the 5' long terminal repeat (LTR) of the stem cell virus (48), which is a retroviral vector system genetically engineered to optimize transgene expression in stem cells (48). Conversely, no carcinoma was observed in mice when the expression of c-Myc and c-Met was under the control of the promoter of the mammary tumor virus (48), which is suitable for the expression of transgenes in differentiated cells, but it is not efficient in driving transgene expression in stem cells. Thus, the expression of the oncogenes c-Myc and c-Met in differentiated cells was not a relevant model for the study of tumorigenesis in human cells.

Studies on animal models and *in vitro* cell culture systems provided useful insights into the cancer metastasis process and stromal-epithelial interactions (50-52). The latter issue has very important implications both for carcinogenesis and tumor metastasis (50-52). In this context, the structural analysis of the normal adult stem cell niche has a primary role in the characterization of stromal-epithelial interactions contributing to carcinogenesis and tumor metastasis. For instance, as already discussed, the chemokine receptor CXCR4 and its cognate ligand CXCL12 are important regulators of the trafficking of hematopoietic stem cells to the bone marrow (39). On the other hand, a number of reports demonstrated that the CXCR4 and CXCL12 chemokine-receptor system plays also a crucial function in promoting the growth and metastasis of malignancies of the prostate (50) and breast (51, 52). Indeed, the detection of micrometastasis in the bone marrow at the time of diagnosis of breast cancer is correlated with poor diagnosis (53). In addition, CXCR4 expression was observed in neuroblastomas, malignant gliomas, ovarian cancer, some leukemia cell lines, Burkitt's lymphomas and multiple myeloma (50). Interestingly, the ligand CXCL12 was detected in the ascitic fluid of patients affected by ovarian cancer (50). This finding strongly suggests a possible involvement of the CXCR4-CXCL12 axis in the spreading of ovarian cancer cells to the peritoneum of patients (50).

CXCL12, or SDF-1, is also a major contributor in human breast cancer progression (52). CXCL12 is secreted by so-called carcinoma-associated fibroblasts (CAFs), which can be isolated from human breast carcinomas (52). CAFs have the ability to promote neoangiogenesis by attracting endothelial progenitor cells to the tumor site, whereas the chemokine CXCL12 induces a direct stimulation on tumor growth via its receptor CXCR4, which is expressed by breast cancer cells (52).

5. CURRENT CHEMOTHERAPEUTICS AND CANCER STEM CELLS: IMPLICATIONS FOR THE DEVELOPMENT OF NOVEL THERAPEUTICS AND DIAGNOSTICS

Based on what has been discussed so far, several lines of evidence support the notion that cancer stem cells are specialized in maintaining the tumor mass. A consequent question is whether cancer stem cells might be also responsible for the relapse of the disease following chemotherapeutic interventions in patients. A report recently demonstrated that a subpopulation of human leukemia stem cells were in fact resistant to the Abl tyrosine kinase inhibitor termed imatinib, which is a very effective agent against differentiated leukemic cells (54). The human leukemic stem cells that survived imatinib treatment regenerated the tumor, providing further evidence in support of the important role played by cancer stem cells in the progression of the disease (54).

As already mentioned, both normal and cancer stem cells are more resistant to apoptosis and have an enhanced membrane transport activity. High levels of membrane transport activity, along with resistance to apoptosis, are both required to protect resting adult stem cells of normal tissues from possible exposure to toxic agents. These properties are indeed essential to preserve a very rare cell population, which has the critical function to regenerate various tissues of the body (8, 9, 21). On the other hand, resistance to apoptosis and high levels of membrane transport activity are also a characteristic of cancer stem cells, which, therefore, become more resilient to chemotherapy than any other differentiated cancer cell.

Multiple drug resistance (MDR) is, *per se*, one of the major issues in cancer therapy. The MDR transporter was first identified in human breast cancer cells and derived from drug selection (9). A recent study investigated the expression of MDR transporter ABCG2 and stem cell markers in therapeutically naïve cancer cells isolated from lung, breast, ovarian and gastric cancers (reviewed in reference 55). This report found that roughly a 58% fraction of cancer stem cells tested positive for ABCG2. Such a finding indicates that even therapeutically naïve epithelial cancers comprise a subpopulation of chemoresistant cancer stem cells expressing the MDR transporter ABCG2 (55). A small fraction of these cells was able to develop tumors in a xenograft mouse model and were also clonogenic in the *in vitro* system. Thus, some cancer stem cells exhibit a constitutive MDR activity and serve as a reservoir of drug-resistant cells, which may cause relapse and metastasis after an apparently effective therapeutic intervention (9, 55).

Selective resistance to toxic injury was also observed in bronchioalveolar stem cells (BASCs) of a mouse model of lung cancer (49). Toxic agents used in this study were either bleomycin or naphthalene (49).

After establishing that both normal and cancer stem cells share extensive phenotypic and functional features, the main goal of cancer research consists of finding novel therapeutic approaches leading to the selective destruction of cancer stem cells, without affecting normal adult stem cells. A group of investigators convincingly demonstrated that dependence on tumor suppressor gene Pten distinguishes normal hematopoietic stem cells from leukemia stem cells in a mouse model (56). In this preclinical study, deletion of the tumor suppressor

gene Pten in somatic hematopoietic stem cells led to myeloproliferative illness in mice within days and to transplantable leukemia within weeks (56). The depletion of Pten also primed a proliferation of hematopoietic stem cells, which generated a high number of progenies lacking parental hematopoietic stem cells and, therefore, were not able to reconstitute the bone marrow of irradiated recipient mice (56). Interestingly, mTOR was the main mediator of the effects related to the depletion of Pten and rapamycin, in turn, could inhibit mTOR functions. In this respect, rapamycin was very efficient in eliminating leukemia stem cells and, remarkably, could restore the functions of normal hematopoietic stem cells in the murine system (56). This is one of the first reports on mechanistic differences between normal adult stem cells and their malignant counterparts. Even more importantly, these mechanistic differences allowed for the selective targeting of cancer stem cells in the murine system, whereas normal adult stem cells were not affected by the treatment (56).

Other strategies for the development of novel therapeutics for cancer consist of targeting the Notch-1 and Hedgehog signaling pathways (37, 57-59). Notch-1 was initially described as an essential player in maintaining the neoplastic phenotype in Ras-transformed human cells (57). In turn, the Notch-1 signaling system relies upon the processing regulated by the enzyme γ-secretase (57). The γ-secretase inhibitor GSI induced a dramatic decrease of Ras-transformed human fibroblasts proliferation both *in vitro* and *in vivo*, by blocking Notch-1 cleavage and activation (57).

Another study focused on the antagonism between Notch and Numb signaling systems (58). Such an antagonism generates a biological balance that is required for the regulation of cell proliferation and differentiation in development and homeostasis (58). In this context, Numb is an inhibitor of the Notch-related signaling system. In fact, higher Notch signaling activity can be observed in Numb-negative cancer cells, which lowers to basal levels following the expression of retroviral-encoded recombinant Numb (58). It was also observed that suppression of Numb is associated with an increase of Notch signaling activity both in normal breast cells and in Numb-positive breast cancer cells. Interestingly, a pharmacological inhibition of the Notch signaling pathway resulted in growth suppression of Numb-negative human breast tumor cells, whereas Numb-positive human cancer cells were not significantly affected by the treatment (58). The pharmacological agent used in this study was the proteosome inhibitor termed MG132 (58).

The targeting of the Hedgehog signaling pathway was investigated in the contexts of human prostate cancer (37) and mouse medulloblastoma (59). The Hedgehog signaling system has an essential role in developmental patterning and is required for the regeneration of the prostate epithelium (37). However, a constitutive activation of the Hedgehog signaling pathway transforms prostate progenitor cells, which, consequently, become malignant (37). In addition, it was observed that metastatic prostate cancer cells exhibit a higher Hedgehog signaling activity than other localized prostate cancer cells (37). The pathway activity can be triggered via Hedgehog ligands and depends upon the expression of the essential responsive element termed Smoothened (37). The latter is not expressed in benign epithelial cells of the prostate (37). On these grounds, the manipulation and the monitoring of Hedgehog signaling activity may result respectively in the development of novel therapeutics and diagnostics for cancer (37). Interestingly, the Hedgehog inhibitor cyclopamine, which is an alkaloid extracted from the corn lily *Veratrum californicum*, caused a complete and durable regression of human prostate tumors previously implanted in athymic mice (37).

The targeting of the Sonic Hedgehog signaling pathway was analyzed in a transgenic mouse model for medulloblastoma (59). This study utilized a small molecule inhibitor termed HhAntag, which blocked the functions of Smoothened in malignant cells (59). HhAntag is a benzimidazole derivative that showed an higher binding affinity for Smoothened than cyclopamine and, therefore, proved to be more efficient in inhibiting the Sonic Hedgehog signaling system in neuroblastoma cells (59). Furthermore, HhAntag has the capacity to penetrate the blood brain barrier and can be administered orally in animals (59).

In addition to the development of novel therapeutics, the cancer stem cell hypothesis may have important implications for the improvement of more effective preventive diagnosis for cancer. So far, tumor markers are associated with differentiated stages of cancer cells, such as the prostate-specific antigen (PSA) (60, 61) and CA125 (62), which are prognostic antigens for prostate and ovarian cancer, respectively. An exciting recent study focused on the proto-oncogene Bmi-1 (63, 64), which belongs to the polycomb group gene family (65). Bmi-1 plays a primary role in the self-renewal of adult hematopoietic stem cells (66) and neuronal stem cells (67). Intriguingly, a conserved Bmi-1-driven gene expression pattern was similarly engaged in normal adult stem cells and in eleven types of human malignancies (63, 64). The Bmi-1-driven gene expression pattern was termed the eleven-gene signature and was sadly associated with poor prognosis (63). These data derived from a total of 1,153 oncological patients and included both epithelial and non-epithelial tumors (63). Epithelial malignancies comprised cancers of the prostate, breast, ovaries, bladder and lung, whereas non-epithelial malignancies consisted of lymphoma, mantle cell lymphoma, acute myeloid leukemia, mesothelioma, medulloblastoma and malignant glioma (63).

Various techniques aiming at isolating and characterizing the phenotype of cancer stem cells are currently under development. The study of the biology of cancer stem cells is very important from a pathological standpoint, for the improvement of preventive cancer diagnosis and to design novel therapeutics. Several preliminary findings discussed in this review significantly outlined the importance of pursuing research programs focusing on the biology of cancer stem cells.

6. CONCLUSION

The field of stem cell research has had major repercussions in oncology, which, after the formulation of the cancer stem cell hypotheses, changed entirely its focus. Indeed, the study of the biology of cancer stem cells is currently shedding very useful insights into a better understanding of cancer pathogenesis and progression of the disease. These are essential requirements both to optimize preventive cancer diagnosis and to develop novel therapeutics for the treatment of cancer.

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