Cancer Stem Cells and Differentiation Therapy: An Innovative Therapeutic Approach

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Abstract: Cancer stem cells are closely related to and may often originate with adult stem cells. Under normal circumstances, the regular turnover of cells in developed tissues is offset by the work of adult stem cells, which can divide to make more stem cells or progenitor (immature) cells. The progenitors then differentiate into the mature cells needed to maintain the organ or to respond to an injury, hormones, or other external signals. Cancer stem cells can produce more of their kind or progenitors that multiply and differentiate to become the malignant cells that make up the bulk of a cancer. Nowadays it is reported that, similarly to other solid tumors, colorectal cancer is sustained by a rare subset of cancer stem–like cells (CSCs) which survive conventional anticancer treatments, thanks to efficient mechanisms allowing escape from apoptosis, triggering tumor recurrence. To improve patient outcomes, conventional anticancer therapies have to be replaced with specific approaches targeting CSCs. In this review we provide strong support that BMP4 is an innovative therapeutic approach to prevent colon cancer growth increasing differentiation markers expression and apoptosis.

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1. INTRODUCTION

Stem cells are defined as undifferentiated cells that are capable of self-renewing and differentiating into a large number of diverse mature progeny. Amongst the various categories of stem cells, the embryonic stem (ES) cells are totipotent and able to differentiate into many cell types under appropriate conditions in vitro and contribute to all different tissues in vivo (1–3), making them a very promising foundation for stem cell-based therapeutics. Somatic stem cells from different organs, on the other hand, are pluripotent and responsible for tissue regeneration and repair. Adult stem cells have been identified in several organs, such as the hematopoietic system, brain, skin, mammary gland and lung, but it is not yet clear whether they are present in all other adult organs (4, 5). The best-studied somatic stem cells are hematopoietic stem cells (HSC). HSCs in mice and humans have been positively identified and successfully isolated by Weissman and colleagues (5, 6). HSCs are known to be responsible for the generation of all cell types in the blood, although their potential for giving rise to other tissues (or plasticity) is still controversial (4, 5). Dick et al. have recently revealed that, like the normal hematopoietic system, leukemia is organized as a hierarchy in which only a rare population retains a clonogenic capacity upon transplantation (7). Similarly, a solid tumor can be likened to an organ developed in an aberrant way, as it contains a heterogeneous mixture of cell types and abnormal tissue structures. More importantly, such an aberrant organ can be maintained and even formed at remote sites if no therapeutic intervention is performed. It is well established that tumor engraftment, although requiring a large number of cells, results in the formation of secondary tumors that recapitulate primary ones. The clonogenic and heterogenic nature of tumors suggests that a rare cell population in cancer, which acts like stem cells, is responsible for tumor growth and metastasis. These rare cells are named cancer stem cells (CSC) after normal stem cells, as both have similar abilities to self-renew and to give rise to heterogeneous differentiated cell types (8). Recent advances have begun to disclose the biologic identity and origin of CSC in several types of cancers and to elucidate the mechanisms underlying the transformation of normal cells into CSC.

1.1 Cancer Stem Cells (CSCs) Hypothesis

During the past years the process of tumorigenesis was explained by cancer biologists through the stochastic model, according to which all tumor cells share common genetic and epigenetic mutations, reflective of their clonal origin (9). In addition to the genomic instability, intrinsic factors (levels of transcription factors, signaling pathways) and extrinsic ones (host factors, microenvironment, immune response) influence tumor cells behavior leading to significant heterogeneity in terms of features, surface markers expression, proliferation kinetics and tumor

initiation capacity (10). More recently, the hierarchy model has been proposed, according to which cancer consists of a heterogeneous population characterized by various stages of differentiation. Accumulating evidence has posited that tumor mass is characterized by the presence of a small population of cells, necessary and sufficient to initiate and sustain indefinitely tumor growth and subsequent progression. These "tumor-initiating cells" are also called cancer stem cells (CSCs) since they share the hallmarks of normal stem cells (e.g., unlimited self-renewal, quiescence, multipotentiality and expression of drug and apoptosis resistance genes), expand the stem cell compartment undergoing symmetric division and differentiate into the multiple lineage via asymmetric division (11) (Figure 1).

Dick and colleagues demonstrated that only a small minority of acute myeloid leukemia (AML) cells were able to produce leukemia in NOD/SCID murine model (11). From the initial studies in hematological malignancies, CSCs have been identified in a variety of solid tumors including breast, prostate, brain colon, pancreas, ovary, lung, and, recently in thyroid, as assayed either by their in vitro clonogenity or by their ability to initiate new tumor growth after xenotransplantation into immunocompromised mice which recapitulate the phenotypic heterogeneity of the primary tumor (12-19). The emergence of CSCs and subsequent cancer development may arise from deregulation of the processes that regulate self-renewal, cell fate and differentiation of normal stem or progenitor cells (20), but moreover CSCs may originate from mutations in differentiated cells favoring timeless proliferative potential (21). Several signaling pathways such as Wnt, Notch and Sonic Hedgehog (Shh) have been found to regulate the self-renewal of normal stem cells in a variety of cancers.The importance of a self-renewal pathway in maintaining Leukemia Stem cells (LSCs) has been first underlined by Jamieson group. Their results showed that an aberrant activation of Wnt pathway is implicated in human blast crisis LSCs propagation. They also identified an increased activation of Wnt signaling in breast CSCs growth. Shh signaling pathway is also known to play a critical role in maintaining human LSCs, breast, glioblastoma and colon CSCs. Finally, Notch pathway has been shown to be activated in colon CSCs subset but also in breast and glioblastoma CSCs (22).

1-2 Colorectal Cancer Stem Cells

Normal colonic stem cells (NCSCs) are localized at the base of the crypts surrounded by intestinal subepithelial myofibroblasts (ISEMFs). Defined by properties of self-renewal and multilineage differentiation, they ensure a high rate of tissue renewal: by asymmetric division NCSCs generate another SC and a progenitor cell also known as a transit-amplifying cell (TAC) which, in turn, generates more mature cells of colonic epithelium. It has been suggested that ISEMFs play a critical role in the regulation of a correct balance between SCs self-renewal and differentiation, by paracrine secretion of growth factors and cytokines (21, 23).

In addition to ISEMFs, maintenance of colonic epithelial SCs niche is modulated by high Wnt activity in the lower region of the crypt which induces the expression of EphB receptors and the subsequent interaction with ephrin ligands located in the higher position of the crypt (21, 24). Another signaling pathway identified as a key regulator of the SCs niche is that mediated by Bone Morphogenetic Proteins (BMPs). As a consequence of the high expression of BMP antagonists in the colon bottom, the BMP activity is higher in the upper region of the crypt inducing differentiation of colonic epithelial cells (21, 23). In 1990, Fearon and Volgestein suggested a genetic model for colorectal tumorigenesis in which gene mutations occurred with a specific time defining a particular stage of tumor development (25). In patients with familial adenomatous polyposis, mutations in the Adenomatous Polyposis Coli (APC) gene are reported as the initiating gatekeeper regulating positively Wnt machinery and causing hyperproliferation and early adenoma formation (26).

The stage of intermediate adenoma is promoted by B-RAF and K-RAS mutations. Late adenoma results from loss of heterozygosis involving the chromosome 18q, mutations in Small Mother against DPP homolog 4 (Smad4), Cell Division Cycle 4 (CDC4) and Delected in Colorectal Cancer (DCC) or alternatively mismatch repair deficiency. P53, Bax and insulin-like growth factor receptor2 mutations are responsible for invasive cancer; lastly, unknown factors lead to metastatic cancer (21, 27). Even in cancers caused by alterations in genomic integrity, neoplastic change might initiate through subsequent mutations in morphogenetic pathways regulating normal proliferation of intestinal epithelium, such as Akt/ PKB, Wnt, Shh, Notch and BMPs (26).

These multiple genetic mutations, restricted to TACs, would be acquired by their progeny resulting in increased proliferative potential, independence of extrinsic growth control signals and autonomous control over all metabolic activities that feed tumor progression (28). Although it has long been assumed that neoplastic formation derives from alterations within adult colonic stem cells, the existence of colorectal cancer stem cells (CR-CSCs) has been demonstrated through the finding that colon CD133+ cells are able to grow exponentially in vitro as undifferentiated tumor spheres, when cultivated in serum-free medium, and initiate tumor growth in

mouse models, thus reproducing the same morphological and antigenic pattern of the original human tumor (29-31).

Many studies have provided proof that, within the CD133+ subpopulation, there exists a minority of cells possessing tumor-initiating ability. Dalerba et al. (15) suggest cell surface glycoprotein CD44 and Epithelial Cell Adhesion Molecule (EpCAM) as specific markers of CR-CSCs: in the context of CD133+ tumor population, they have identified a subset of stem-like CD44+/EpCAMhigh cells able to generate tumor xenografts upon serial transplantation into NOD/SCID mice. A further isolation of colon cancer cells using the mesenchymal stem cell marker CD166 enhanced the success of tumor xenograft. A recent study performed by Huang et al. (32) showed that enzymatic activity of ALDH1 can be used as a potential CR-CSCs marker being expressed by cells positive for CD44+ or CD133+ located at the base of normal crypts. It has been reported by the same group that during tumor progression the selection of CD44+, CD133+ cells with ALDH activity increases in number and reaches the crypt axis.

2. Clinical Implications of CSCs

The discovery of CSCs in a variety of tumors has changed the view of carcinogenesis and therapeutic strategies. According to the stochastic model, the tumor chemoresistance is due to preexisting clones with mutations that confer drug-resistance. The CSCs model postulates that CSCs evade death signals induced by current therapeutic drugs through a variety of strategies including upregulation of multidrug-efflux pumps able to exclude exogenous substances, alterations in DNA-repair mechanisms, altered cell cycle checkpoint controls and impaired apoptosis machinery. In addition, CSCs survive to current treatments, evaluated for the ability to kill only more differentiated and highly proliferating cells, because CSCs are proliferatively quiescent, less differentiated and overcome apoptosis resistance evading the control mechanisms. A combination of 5-FU, oxaliplatin and leucovorin (referred to as FOLFOX) and a combination of 5-FU, oxaliplatin and irinotecan (referred to as FOLFIRI) are the current therapy for colon cancer patients. Actually, the therapeutic approach for CRC includes anti-VEGF or EGFR monoclonal antibodies which improve positive outcomes in patients suffering from metastatic colon cancer and severe hepatic dysfunction (21). However, none of these anticancer therapies is curative in most patients with metastatic disease due to failure to eradicate the CSCs compartment. The development of targeted therapies for this cancer type would therefore require a better knowledge of the different aspects of stem cell biology in the context of CRC such as complex network of mechanisms that regulate tumor development and resistance to chemotherapy. It is therefore evident that a therapeutic approach to selectively target CSC pool bypassing their chemoresistance could be more effective to eradicate bulk tumor. Thus, the purpose of new therapeutic regiments is to eliminate the self-renewal compartment of tumor mass by:

- Targeting stem cell properties inducing the inactivation of survival pathways in CSCs.
- Forcing CSCs to differentiate [1] (Figure 2).

3. BMPs: An Example of Differentiation Therapy

Considerin the role of Bone Morphogenetic proteins (BMPs) in development and differentiation stages, these molecules have been studied over the past decade in tumorigenesis and metastasis formation.

BMPs belong to a subgroup of the transforming growth factor-beta (TGF-β) super-family; so far 20 BMPs have been discovered (33). According to current models, BMPs bind two distinct serine/threonine kinase receptors; different combination of type I and type II receptors determine the specificity for the ligands. Upon ligand binding, the type II receptor trans-phosphorylates type I receptor in its GS domain; initiating the signal transduction by phosphorylating Smad1/5/8 proteins (RSmads). Then RSmads form a complex with Smad4 (CoSmad) and translocate into the nucleus, where this complex could bind directly to gene regulatory elements or interact with other transcription factors regulating target gene expression (34). In addition to the Smad pathway, BMPs activate an alternative pathway, which includes p38 and ERK MAP kinases (35). Moreover, BMPs activation is tightly regulatregulated by the presence or the absence of antagonists, such as Gremlin, Chordin and Noggin (36).

Originally these proteins have been studied and characterized for their chondrogenic and osteogenic abilities, as they are able to induce ectopically bone formation in rodents (37). Afterwards, BMPs were analyzed for their role in cell growth, differentiation and apoptosis.

It is well established that several BMPs have a function in multiple developmental processes. Studies in Drosophila melanogaster and Xenopus laevis established that BMPs are required for correct dorsal-ventral axis formation and mesoderm induction in embryos (38, 39). Since these data prove that BMPs pathway is essential for the development of embryos invertebrates, further studies were carried out on murine models to strengthen the hypothesis that BMPs are important during vertebrate embryogenesis. Many knockout mice were generated for BMPs, BMPs receptors and molecules involved in the signaling pathway. Most of these models (BMP2, BMP4, BMPR

I and II, Smad 4 and 5 KO mice) are lethal, as mutant embryos, exhibiting multiple gastrulation defects, among which include lack of mesoderm formation and incorrect left-right axis asymmetry, morphogenesis and organs positioning (38-43). A different phenotype was observed in BMP7 null mice: these mice present postnatal lethal mutations with various developmental skeleton-kidney and eye defects. Given that BMPs have a role in embryonic development; it was supposed that these proteins may play a role during SCs differentiation. Pera et al. (44) demonstrate that human embryonic SCs treatments with Noggin impair their spontaneous differentiation, suggesting that in these cells BMPs pathway activation induces differentiation.

In the colon crypts ISEMF cells contribute to stem cells niche maintenance balancing different and opposite signals that promote self-renewal (Wnt and Notch pathways) and differentiation (BMPs pathway). The understanding of these mechanisms is important because it is hypothesized that the existence of a CSCs niche may have a role in maintaining and increasing the CSCs pool (45). In CRCs, an abnormal activation of Wnt signaling pathway leads to nuclear β-catenin accumulation and subsequent abnormal CSCs proliferation; moreover, BMPs signaling inhibition promotes nuclear β-catenin activity through PTEN inactivation and activation of PI3K-Akt pathway (46). A subsequent microarray study identifies a list of genes ifferentially expressed in colon bottom crypts and in the tops: the first group includes genes involved in Wnt and Notch pathways, but also BMPs inhibitors, such as Gremlin 1, Gremlin2 and Chordin-like 1; the second genes involved in BMPs and apoptosis pathway and cell cycle inhibitors (23). These data suggest that in the colon bottom crypts a balance between Wnt/Notch and BMPs pathways is necessary in order to maintain and regulate CSCs niches.

More evidence for their putative role in CRC was provided by genetic studies and transgenic mice models. Germline mutations in genes encoding SMAD4, BMPRIA and BMP4 are found in up to 50% of individuals with juvenile polyposis, an autosomal dominant syndrome with a high risk for CRC (47-49).

Furthermore, Noggin transgenic mice phenocopy the intestinal histopathology of patients with this syndrome (50); subsequently it was described that mice with an inducible mutation of BMPRIa develop intestinal polyps (46). This body of data argues that BMPs signaling disruption leads to precancerous lesions (51).

CRC develops as a result of increasing proliferation and apoptosis deregulation and TGF-β signaling inactivation have a key role in this pathology (52). It has been reported that SMAD4 is frequently deleted in CRC and that BMPs pathway is inactivated in the majority of colorectal tumors (53,54). Indeed, BMP2, BMP3, BMP4 and BMP7 inhibit proliferation and induce apoptosis and differentiation in colon cancer cells that do not have Smad4 mutation and loss of PTEN [51, 55-57].

Considering BMPs' role in regulating SCs differentiation and inducing apoptosis and differentiation, it is possible to suppose that CSCs treatment with these molecules could induce differentiation and following chemotherapies sensitization. Some preliminary studies have been performed on both CSCs of glioblastoma and CRCs. Preliminary studies in glioblastoma demonstrate that BMP2, BMP4 and BMP7 treatment inhibits sphere forming and induces differentiation of CD133+ cells; moreover CD133+ cells pre-treatment with these cytokines attenuates tumor formation in mice (58-60). Recently, the same results are obtained in CR-CSCs. The treatment of CD133+ CR-CSCs with BMP4 induces in vitro differentiation and reduces their tumorigenic potential; moreover in vivo the combined treatment with BMP4 and conventional chemotherapeutics reduces the tumor size (61). These data open the possibility to use BMPs or analogue-drug to induce the differentiation of CSCs and to make them more sensitive to conventional chemotherapy.

4. PROSPECTIVE

Despite recent progress in CSC research, our knowledge of these rare populations is still limited and many questions remain to be answered. Certain types of cancer are known to be multi-stage diseases, which generally progress into more malignant forms with the sequential accumulation of genetic and molecular alterations. For instance, hematological malignancies,such as CML, are often found to have two distinct phases: chronic phase and blast crisis (or leukemia). Similarly, some epithelial tumors, e.g. colon tumors, are thought to progress through at least five stages: pretumor patches/fields, hyperplasia, carcinoma in situ, invasive carcinoma and metastasis. One of the central questions in the CSC research is: how to link CSC to cancer progression in these tumors? Given sequential requirements of genetic and molecular alterations and distinct pathologic abnormalities associated with different stages of cancer progression, one may postulate that there could be multiple CSC populations, either intrinsically linked or generated independently, responsible for different stages of cancer progression.

To advance CSC research, we need to first understand the normal stem cells and critical pathways controlling stem cell properties. For this, identification of cell surface molecules for prospective stem cell isolation and biologically relevant stem cell assays are essential. In addition, technical improvement will expedite the studies of these rare and heterogeneous

population(s). We should investigate the molecular mechanisms for the CSC formation and maintenance, especially their self-renewal regulation, which holds the key for the development of effective therapeutic strategies against CSC. Although stem cell niches have been shown to play an instructive and pivotal role in the regulation of stem cells, their implication in the CSC formation remains to be elucidated. Ultimately, with further improvements in our understanding of CSC, we will be able to develop better diagnostic and therapeutic methodologies, with which to classify, treat, and cure cancer.

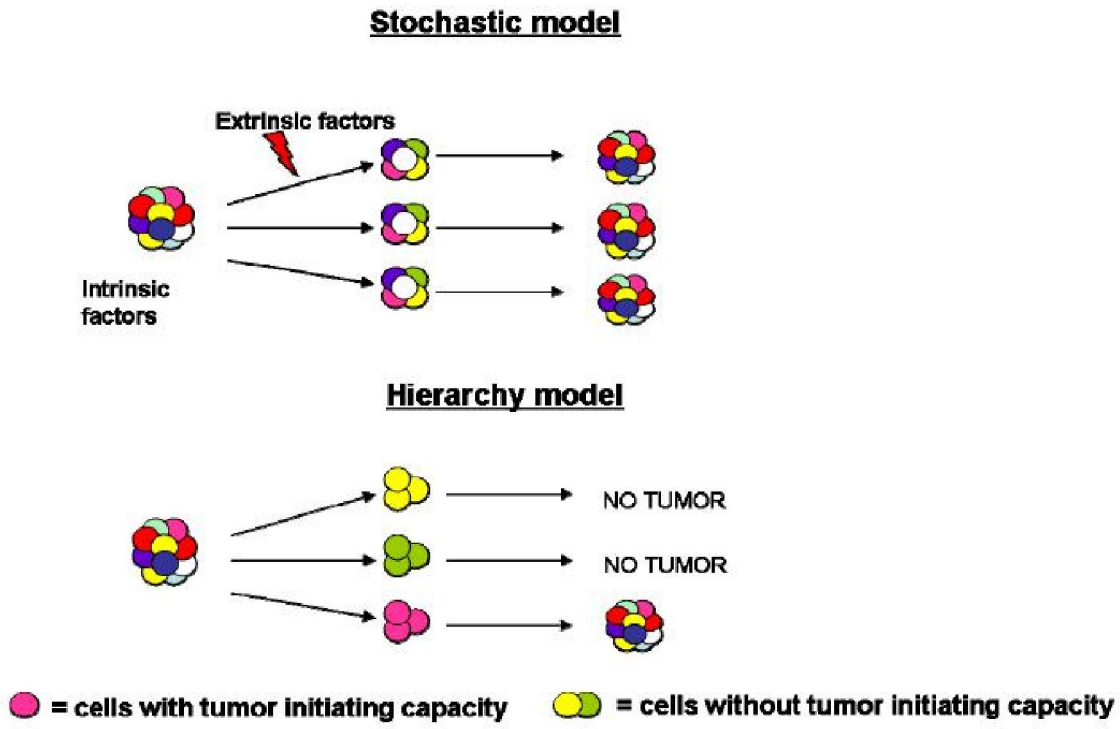

Figure 1. Models of tumor heterogeneity. Tumor heterogeneity has been explained by two theories: according to the stochastic model, tumor cells are influenced by intrinsic and extrinsic factors; by contrast, in the hierarchy model, tumor cells have different functional abilities and only a subset can initiate tumor growth

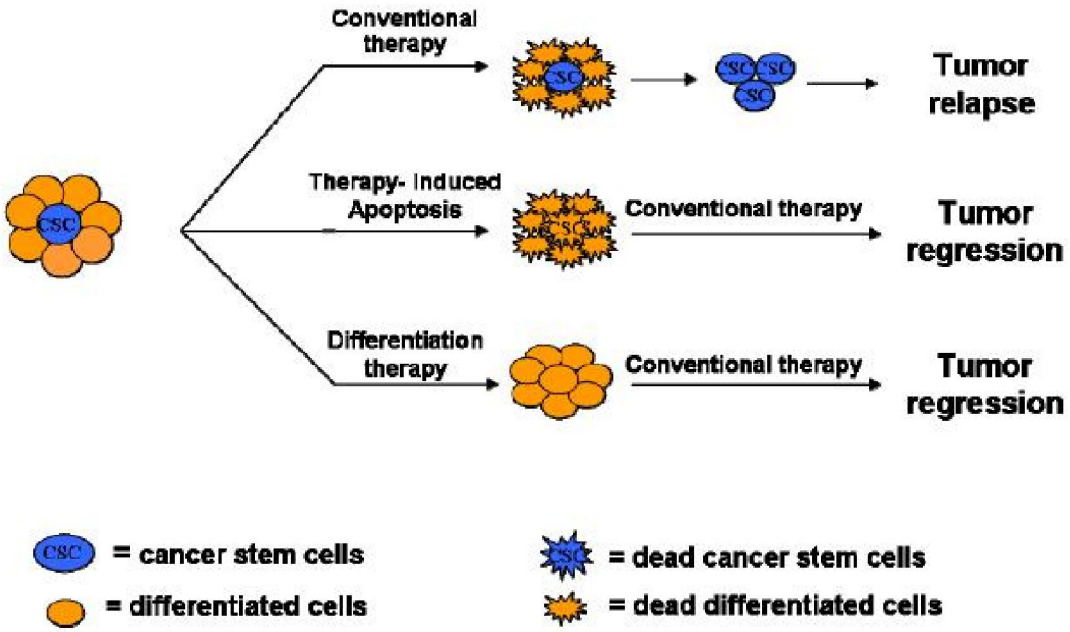

Figure 2. Therapeutic Strategies for CSCs sensitization.

5. CONCLUSIONS

CSCs are believed to play a critical role in tumor initiation and recurrence. Current chemotherapeutic regiments target the most actively cycling cells, which represent the tumor bulk, sparing the CSC compartment. Thus, novel and more efficient stem cell-based therapies, able to kill this chemotherapy-refractory population, are needed to improve patients' survival. In this scope, the identification of agents that can inhibit the CSCs survival machinery forcing apoptosis or induce their differentiation represents the first step to achieve in the near future, providing important advances for cancer treatment.

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