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TOPIC HIGHLIGHT

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Cancer stem cells in colorectal cancer from pathogenesis to therapy: Controversies and perspectives

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Abstract

Colorectal cancer remains one of the most common and lethal malignancies worldwide despite the use of various therapeutic strategies. A better understanding of the mechanisms responsible for tumor initiation and progression is essential for the development of novel, more powerful therapies. The traditional, so-called "stochastic model" of tumor development, which assumes that each cancer cell is tumorigenic, has been deeply challenged during the past decade by the identification of cancer stem cells (CSCs), a biologically distinct subset of cells within the bulk of tumor mass. This discovery led to the development of the hierarchical model of tumorigenesis which assumes that only CSCs have the ability to initiate tumor growth, both at primary and metastatic sites. This model implies that the elimination of all CSCs is fundamental to eradicate tumors and that failure to do so might be responsible for the occurrence of relapses and/or metastases frequently observed in the clinical management of colorectal cancer patients. Identification and isolation of CSCs is essential for a better understanding of their role in the tumorigenetic process and for the development of CSC-specific therapies. Several methods have been used for this purpose and many efforts have been focused on the identification of specific CSC-surface markers. This review provides an overview of the proposed roles of CSC in human colorectal tumorigenesis focusing on the most important molecules identified as CSC-specific markers in colorectal cancer and on the potential strategies for the development of CSC-targeted therapy.

Key words: Colorectal cancer; Cancer stem cells; Tumorigenesis; Cancer therapy; Prognostic marker

Core tip: A better understanding of the mechanisms responsible for tumor initiation and progression is essential for the development of novel, more powerful therapies for colorectal cancer patients. In this paper, we review the basic concepts of both the traditional "stochastic", and of the more recent, "hierarchical" models of tumor development. We then introduce the so-called cancer stem cells (CSCs) and provides an overview of the proposed roles of CSCs in human colorectal tumorigenesis focusing on the most important molecules identified as CSC-specific marker in colorectal cancer and on the potential strategies for the development of CSC-targeted therapy.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in Western countries and, although it can cause symptoms at a very early stage and can be easily detected and treated by resection, it remains the second leading cause of cancer-related death in Europe and the third in the United States with a median survival time ranging from less than one to more than five years depending on the stage of disease at the diagnosis and the surgical techniques and/or chemotherapy used, especially for metastatic CRC[1]. Several studies have underlined the role of environmental and lifestyle factors in colorectal carcinogenesis showing an increase in CRC incidence in parallel with economic development and adoption of a western lifestyle in several countries.

CRC originates from epithelial cells lining the gastrointestinal tract which undergo sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal[2]. The intestinal tract consists of the small intestine (duodenum, jejunum and ileum) and the large intestine or colon, which comprises the cecum, ascending, transverse and descending colon, sigmoid colon, rectum and anal canal. The innermost layer of the colon wall (mucosa) is lined by an absorptive and secretory columnar epithelium which is folded into finger-like invaginations incorporated in the submucosa connective tissue to form the functional unit of the intestine, the crypts of Lieberkühn (Figure 1). Normal human colon consists of millions of crypts containing about 2000 cells and comprising the differentiated cell lineages (enterocytes, enteroendocrine cells and goblet cells). A fourth differentiated type, the Paneth-like cells, resides at the bottom of colon crypts and has been shown to synthesize and secrete a variety of antimicrobial factors[3]. Differentiated colon epithelial cells are subjected to a massive turnover throughout life, being replaced approximately every 5 d. The ability to maintain tissue homeostasis is provided by a subset of self-renewing undifferentiated, multipotent stem cells which generate transit-amplifying cells, committed progenitors[3]. These cells lie towards the bottom of the crypt in the proliferative zone and through an asymmetric division are responsible for generating all epithelial cell types along the crypt-villus axis. The number of long-lived stem cells per each crypt is commonly estimated to be between 4 and 6 cells even if the precise number and what controls their numbers remain uncertain (Figure 1). Two distinct populations of putative stem cells have been identified at the base of intestinal crypts. A population is marked by the expression of the G-protein receptor Lgr5, a Wnt gene target, and positioned just above the Paneth cells at the crypt base, while the other resides at +4 position from the bottom of the crypt and are marked by the expression of the polycomb group gene Bmi1 and the telomerase reverse transcriptase, Tert[4,5]. Both cell types have been demonstrated to fulfill the criteria for stem cells (pluripotency and self-renewal capacity)[4,5]. Several studies are trying to understand whether their stem cell characteristics are

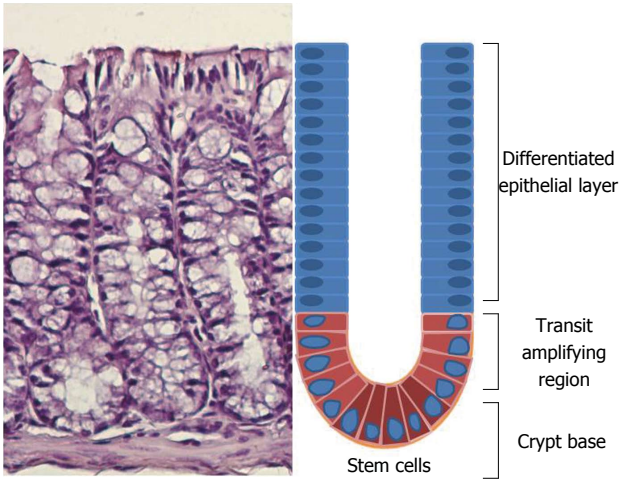

Figure 1 Schematic representation of an individual colon crypt showing the position of different cell types. Stem cells lie at the bottom of the crypt and through an asymmetric division are responsible for generating all epithelial cell types along the crypt-villus axis.

intrinsically determined or determined by the environmental niche. It is widely accepted, however, that stem cell niches are formed by cellular components and extracellular matrix which create a special microenvironment important for the maintenance of stem cells properties, protect stem cells from differentiating and apoptotic stimuli and regulate the balance between proliferation and differentiation through direct interaction and secretion of various cytokines and growth factors[6]. The stem cells self-renewal and differentiation are also influenced by components in the crypt lumen derived from bacteria or epithelial cells as well as by morphogenetic factors secreted by intestinal sub-epithelial myofibroblasts[7].

Mounting evidence suggests that stem cells might play an important role in the process of tumor development being able to acquire a tumorigenic potential and giving rise to the so-called cancer stem cells whose potential role as tumor initiating cells as well as targets of cancer therapies is discussed in this review.

Models of colorectal tumorigenesis

CRC has been an ideal model to study the malignant progression because different phases of the same malignancy often coexist within the same patient and have provided basic information concerning human tumorigenesis. Although most of the CRCs are sporadic, a small percentage arises in the setting of inherited syndromes, such as familial adenomatous polyposis (FAP), juvenile polyposis syndrome (JPS) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), which have been extremely useful for our understanding of human colorectal tumorigenesis. The study of these hereditary cancer syndromes, as well as of sporadic CRC, has led to a detailed knowledge of the sequence of genetic mutations underlying CRC development with the formulation of a model of multistep carcinogenesis which has been subsequently extended to the majority of human can-

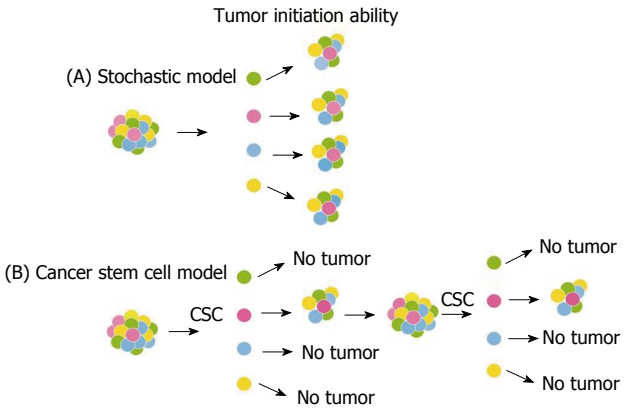

Figure 2 Models of tumor development. (A) Stochastic model: every cancer cell isolated from the bulk tumor is tumorigenic and thus has the ability to proliferate extensively and initiate tumor growth. (B) Cancer stem cell (CSC) model: only a rare subpopulation of undifferentiated cells has the unique biological properties necessary for tumor initiation, maintenance, and spreading.

cers[8]. It is now widely accepted that, regardless of the starting event, CRC is the end result of a variable concatenation of genetic alterations that lead a normal colonic epithelial cell to transform into a colon cancer cell. According to the model of colorectal tumorigenesis, initially proposed by Fearon *et al*[8] (also known as the adenoma-cancer sequence), CRC development occurs through a series of steps morphologically identifiable: initially there is localized proliferation of the colon epithelium with the formation of small adenomas which progressively grow with dysplasia and ultimately progress into invasive carcinomas. Most of the CRC is characterized by a dysfunctional regulation of the Wnt/β-catenin pathway, essential for the development of the normal colonic mucosa[9]. About 80% of patients with FAP have loss or mutation in the APC (adenomatous polyposis coli) gene which encodes a protein that participates to the formation of a complex that regulates the stability of β-catenin. In the absence of Wnt ligand this complex retains the β-catenin which is phosphorylated and degraded by the proteasome. When the APC molecule is mutated, the cytosolic β-catenin levels are stabilized and the protein can then accumulate in the nucleus where it serves as a coactivator for the Tcf family of transcription factors which activate the expression of specific target genes including some metalloproteinases, the fibronectin and oncogenes such as c-myc and cyclin D1[10]. The study of JPS, a condition that predisposes to hamartomatous gastrointestinal polyps formation, has revealed the important role of SMAD/BMP (bone morphogenetic protein) in intestinal architecture. JPS is due to germline mutations in the SMAD4 gene in 15%-20% of cases and to mutations in the gene encoding BMP receptor 1A in 25%-40% of cases. SMAD4 is an intracellular signal transducing transcription factor shared by the Transforming growth factor β, activin and BMP pathways. BMP family ligands are expressed by the villus mesenchyme, while epithelial cells display nuclear phosphorylated SMADs, implicating these cells as terminal recipients of the signal[11]. Wnt and BMP pathways interact to control intestinal stem cells self-renewal through the PTEN-Akt pathway that helps to control the nuclear localization of the Wnt pathway effector β-catenin[12]. Interaction between Wnt and Notch pathway, which maintains proliferative cells in normal scripts, is also deregulated in tumorigenesis[9]. Wnt signaling, Hedgehog, BMP, Notch and Platelet-derived growth factors are involved in the process of epithelial to mesenchymal transition and invasion[7].

According to the traditional model of carcinogenesis a tumor may arise from any cell of the body following a series of mutations conferring them an unlimited proliferation potential. The resulting mutated progeny is subject to additional mutations, due to genetic instability, and epigenetic changes, promoting the appearance of a genetically heterogeneous tumor mass.

This view has been initially integrated in the so-called "stochastic model" of tumor development which assumes that each cancer cell isolated from the bulk tumor is tumorigenic and thus has the ability to proliferate extensively and regenerate a tumor with the same characteristics of the original tumor when injected in immunodeficient mice (Figure 2).

The presence of cell types with various degrees of differentiation within human CRC[13] and of a stem cells overpopulation at the botton crypt during the process of adenoma development in patients with FAP, has suggested a hierarchical model of CRC development as opposed to the stochastic model. According to this model, only a small fraction of tumor cells would be able to support the neoplastic proliferation, the part that retains the characteristics of stem cells and that in itself has a unlimited proliferative potential. The tumors would be organized as a normal tissue with a rare subpopulation of undifferentiated cells having the unique biological properties necessary for tumor initiation, maintenance, and spreading[14] (Figure 2). These cancer cells displaying stemness features have been defined cancer stem cells (CSCs) and, similarly to normal stem cells, would be located in a niche with mesenchymal cells that would ensure their survival in a secure environment, regulating their proliferation through secretion of soluble factors[15]. According to this model, these slow proliferating CSCs display self-renewal, unlimited proliferative potential and multipotency and would be responsible for tumor initiation and development as well as local relapses and metastases. Moreover, CSCs would be highly resistant to traditional antineoplastic agents due to the expression of detoxifying enzymes, drug transporters and DNA repair mechanisms[15].

The origin of CSCs remains unclear and it is still object of a debate whether they derive from more mature cells that reacquire stem cell properties during tumor formation or are the direct progeny of mutated stem cells[15] (Figure 2). The discovery of stem cells in the majority of normal tissues, including colon crypts, supports the hypothesis that normal stem cells might represent a possible target for tumorigenic mutations and the origin of CSCs due to both their longevity and their ability

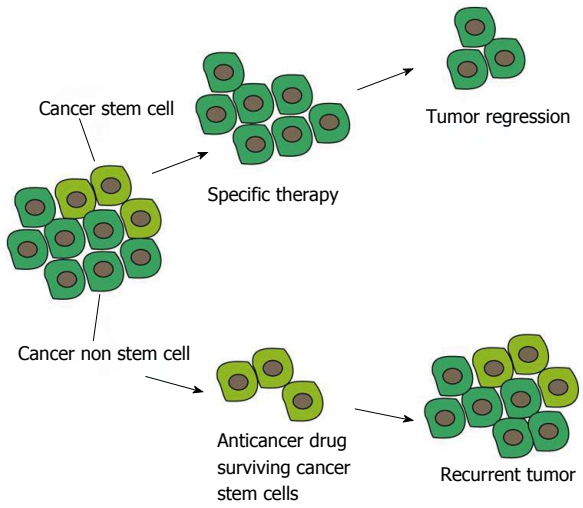

Figure 3 Advantages of a cancer stem cell-specific therapy compared to conventional anticancer therapies. The current anticancer drugs wipe out most of the bulk population but the surviving cancer stem cells (CSCs) can repopulate the tumor. Specific targeting of CSCs is essential for regression and complete eradication of the tumor.

to self-renewal. Furthermore, the fact that despite the emergence of new targeted agents and the use of various therapeutic combinations, none of the options currently available is curative for patients with CRC strengthens the model of CSCs and supports the hypothesis that most of the currently available therapies only target the bulk of the tumor mass (mainly constituted by proliferating cells) while sparing the rare, quiescent CSCs which would be able to re-initiate tumor growth thus giving origin to both recurrences and metastases (Figure 3). This hypothesis supports the need of a better characterization of CSCs with the aim to develop CSCs-specific therapies which might represent a great advantage in the fight against cancer.

COLORECTAL CANCER STEM CELLS MARKERS

Several methods have been proposed in the last years for the identification and isolation of CSCs but some of them require a great technical expertise, are extremely time-consuming or involve the use of animals. Thus, many efforts have been focused on the identification of specific CSCs-surface markers which would allow the identification, and likely the isolation, of CSCs using easier antibody-based techniques such as immunostaining, *Fluorescence-activated cell sorting* (FACS) analysis, cell sorting, immunomagnetic separation, *etc*. One of the first CSCs markers identified in human CRC was the CD133, a pentaspan transmembrane glycoprotein which was shown to specifically mark tumor-initiating cells within the bulk of human CRC[16,17]. In the same period Dalerba *et al*[18] found that $CD133^+$ cells population also express other specific stem cell antigens such as EpCAM, CD44 and CD166 which can help to identify CSCs while a subsequent study showed that CD133 colon cancer cells spheroids grown *in vitro* also expressed Msi-1[18]. Other potential markers of CRC stem cells have been more recently identified including CD29, CD24 and Lgr5[19-21] (Table 1).

CD133/Prominin-1

Human CD133, also known as Prominin-1, is a 120 kDa cholesterol-interacting pentaspan-transmembrane glycoprotein that belongs to the Prominin family. CD133 protein consists of an extracellular N-terminal domain, a cytoplasmic C-terminus that contains five tyrosine residues including a tyrosine phosphorylation consensus site, two small cysteine-rich cytoplasmic loops and two large extracellular loops containing four consensus sequences for N-linked glycosylation[22] (Figure 4).

CD133 was first recognized as a surface protein marker of a subset of hematopoietic stem cells and progenitor cells[22] and of bone marrow-derived circulating endothelial progenitors involved in postnatal angiogenesis, inflammation and tissue regeneration[23,24]. Subsequently, it was identified in several human normal tissues and on CSCs from a variety of solid tumors including brain, colon, liver, lung and prostate neoplasms[23,25].

Two studies first identified CD133 as a marker for stem cells in CRC. Ricci-Vitiani *et al*[16] showed the tumorigenic potential of $CD133^+$ human CRC cells and evidenced their ability to engraft and give rise to visible tumors in immunodeficient mice even after serial transplantations. Simultaneously, O' Brien *et al*[17] demonstrated an enrichment of more than 200-fold of cancer-initiating cells in the subsets of $CD133^+$ cells isolated from human CRC samples compared to unsorted cancer cell populations. Moreover, they showed that liver metastases are enriched with a population of $CD133^+$ cancer cells, a finding also confirmed by our group[26], and observed that tumor xenografts generated from $CD133^+$ cells reproduced the histological features of the original tumor[17].

CD133 is concentrated in plasma membrane protrusions, containing lipid rafts, and more recently several studies have suggested a link between the release of CD133 contained in the membrane vesicles and cellular differentiation, proving that CD133 might play a key role in maintaining stem cell properties[27,28]. However, the discussion on the effective value of CD133 and its usefulness as a CSC biomarker is still controversial because other studies have shown that the $CD133^-$ population of CRC cells is also able to initiate tumor growth in immunodeficient mice[29]. More recently, Feng *et al*[30] proposed another possibility to explain the central issue of the debate, showing that the sorted $CD133^+$ and $CD133^-$ SW620 colon cancer cells can undergo a conversion between the two cell subsets, this resulting in contradictory data. Moreover, Hsu *et al*[31], showed that the exposure to environmental stress, hypoxia and cell-adhesion free condition, promoted switching of $SW620CD133^-$ cells to $SW620CD133^+$ cells while exposure to ECM components promoted switching of $SW620CD133^+$ to $SW620CD133^-$ cells. The switching between the two

Table 1 Cell surface and intracellular molecules suggested as putative cancer stem cell markers in colorectal cancer and their most important features

Marker	Other name	Gene location[1]	Function	Ref.
CD133	Prominin-1, AC133	Chr 4 (p15.32)	Encoding of a pentaspan transmembrane glycoprotein which binds cholesterol in cholesterol-containing plasma membrane microdomains	[22]
CD44	PGP-1, HUTCH-1, GP90, EPICAN, CDW44, MIC4	Chr 11 (p13)	Cell adhesion molecule; involved in lymph node homing and lymphocyte activation	[55,56]
EpCAM	ESA, CD326, MK- 1, KSA, HEA125, BerEp4, 17-1A, GA733-2, KS1/4, EGP-2, EGP34, TROP-1	Chr 2 (p21)	Epithelial cell adhesion molecule	[70]
CD24	HSA	Chr 6 (q21)	Mucin-like cell adhesion molecule	[76]
CD29	B1 Integrin	Chr 10 (p11.2)	Receptor for extracellular matrix proteins; involved in regulation of cell migration, proliferation, survival, differentiation and death	[82]
Lgr5	GPR49	Chr 12 (q22-q23)	Receptor for R-spondin proteins; marker for adult stem cells	[89,90]
CD166	ALCAM	Chr 3 (q13.1)	Cell adhesion molecule	[96]

[1]From http://www.ncbi.nlm.nih.gov/gene.

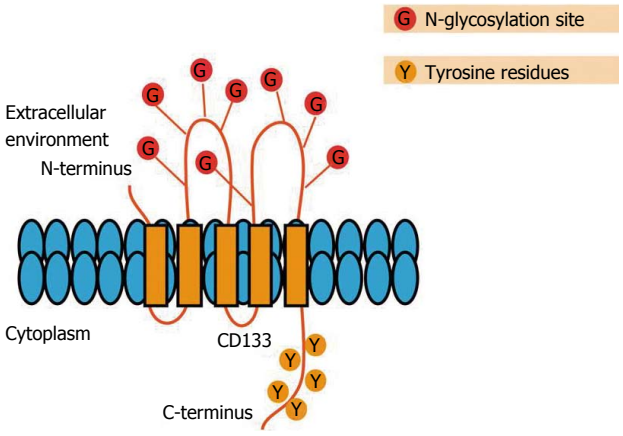

Figure 4 Schematic representation of the CD133 molecule. CD133 consists of an extracellular N-terminal domain, a cytoplasmic C-terminus containing five tyrosine residues, two small cysteine-rich cytoplasmic loops and two large extracellular loops, each containing four consensus sequences for N-linked glycosylation.

Table 2 Prognostic value of CD133

Marker	Prognostic value	Ref.
CD133	Worse outcome and higher risk of metastasis	[32]
	Independent prognostic marker for overall survival	[33]
	Association with CSC resistance to 5FU-based chemotherapy in CRC	[34]
	Association with resistance to conventional radiotherapy in CRC	[35]
	Prediction of distant recurrences after chemoradiotherapy in colon cancer patients	[35]
	High tumorigenicity of $CD133^+$ CRC cells compared to $CD133^-$ cells due to their interaction with CAFs by paracrine signaling axis of CXCR4-SDF1	[36]
	Risk factor for poor overall survival in stage Ⅱ and Ⅲ in colon cancer patients	[37]
	Relationship with K-Ras and B-Raf mutations in CRC patients	[38]

CSC: Cancer stem cell; CRC: Colorectal cancer.

subpopulations might be important for the adaptation to the microenvironment in tumor colonization (Figure 5). On this base, the current concept that CSCs unidirectionally differentiate into non stem cells could be challenged by the findings that non CSCs can convert in a stem-like state within the tumor depending on environmental stimuli[31].

Although the exact functional role of CD133 is still controversial, several studies have addressed its potential diagnostic and prognostic value as well as its intracellular signaling pathways. Several papers investigated the prognostic role of CD133 expression by immunohistochemistry and showed a high prognostic relevance for colon cancer progression and metastasis. Kojima *et al*[32] linked CD133 overexpression with a worse outcome and a higher risk of metastasis in CRC patients, a finding confirmed by Horst and others who showed that CD133 expression is an independent prognostic marker for overall survival[32,33]. Ong *et al*[34] demonstrated that high expression of CD133 is associated with resistance of CSC to 5-FU-based chemotherapy as well as with a significant worse survival. Moreover, $CD133^+$ cells have been shown to be more resistant to conventional radiation therapy, thus suggesting that post-chemoradiotherapy CD133 expression may predict the risk of distant recurrence and poor survival in radiotherapy-treated CRC patients[35].

Chao *et al*[36] proposed that $CD133^+$ CRC cells are more tumorigenic than $CD133^-$ cells due to their interaction with carcinoma-associated fibroblasts in tumor microenvironment by the paracrine signaling axis CXCR4-SDF-1 (Figure 6). This evidence was confirmed by Zhang *et al*[37] who showed that the co-expression of CXCR4 and CD133 on tumor cells was an independent risk factor for poor overall survival in stage Ⅱ and Ⅲ CRC patients. The prognostic role of CD133 in CRC patients was confirmed by Kemper *et al*[38] who showed a relationship between CD133 expression and the presence of mutations in K-Ras or B-Raf genes and suggested that CD133 might be regulated by the Ras-Raf-Mek-Erk pathway (Table 2).

The study of Mohammadi *et al*[39] was the first to evaluate the expression of CD133 in premalignant

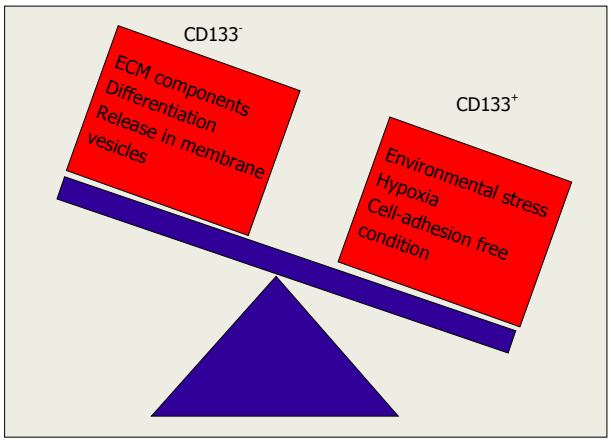

Figure 5 Signals regulating CD133 expression levels. The exposure to environmental stress, hypoxia and cell-adhesion-free condition promotes switching of CD133$^-$ to CD133$^+$ cells while exposure to ECM components promotes switching of CD133$^+$ to CD133$^-$ cells.

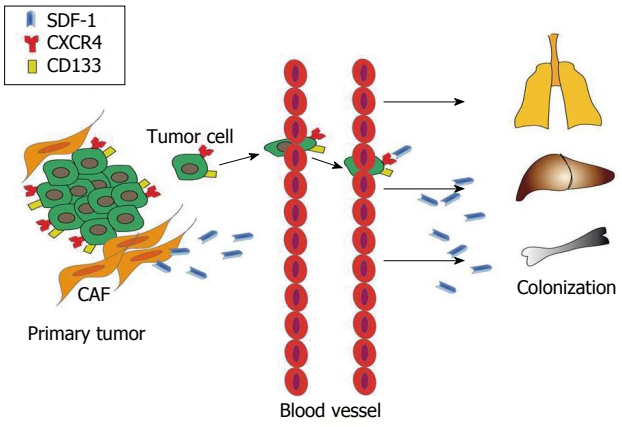

Figure 6 Possible role of the interaction between CXCR4/CD133 cancer cells and SDF-1 ligands. The SDF-1 ligand secreted by carcinoma-associated fibroblasts (CAF) in tumor microenvironment interacts with CXCR4/CD133 expressing cancer cells and could drive primary tumor cells towards metastatic sites.

Table 3 Prognostic value of CD44

Marker	Prognostic value	Ref.
CD44	Association of CD44 downregulation with a lower metastatic potential of CRC cells	[61]
	Association of CD44 downregulation with a higher metastatic and migratory potential of CRC cells	[62]
	Association with a reduced survival	[63]
	Correlation of CD44 loss with a higher tumor aggressiveness	[64]
	Relation with CRC cells proliferation but not with patients outcome	[65]
	Association with lymph node involvement and invasion depth	[66]
	Unfavorable prognostic factor for overall survival in advanced CRC	[66]
	Correlation of CD44 loss with advanced tumor stage, vascular invasion, lymph node involvement and infiltrating tumor border	[67]
	Association of CD44 loss in the lesion invasive front with adverse outcome of CRC patients	[67]

CRC: Colorectal cancer.

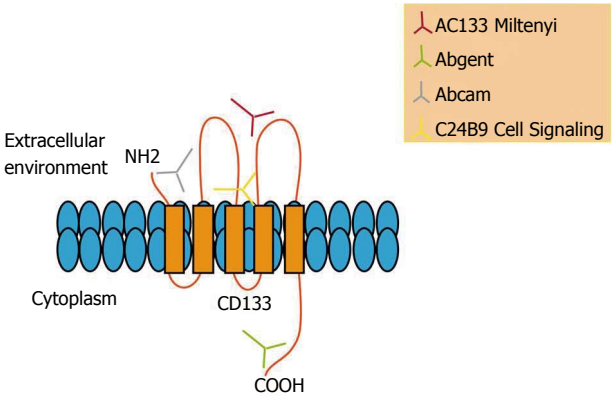

Figure 7 Epitopes recognized by different antibodies on CD133 molecule.

colorectal lesions such as non-dysplastic serrated polyps that comprise hyperplastic polyps (HP) and the non-dysplastic subset of sessile serrated adenoma-polyp-lesions (SSA/P/L) and its borderline variant. They showed that SSA/P/L and its borderline variant significantly express higher levels of CD133 than HP. They demonstrated that this premalignant colorectal lesion could be easily identified by determining the CD133 immunoprofile thus suggesting the usefulness of CD133 immunohistochemical evaluation in the diagnostic clinic routine[39]. Our group also reported that CD133 expression in human CRC is an independent risk factor associated with patient survival in multivariate analyses[40]. However, overall the data available in the literature do not allow a definitive and clear-cut assessment of the potential prognostic significance of CD133 expression which, as previously mentioned, is also the result of different antibodies, protocols and scoring criteria used for the evaluation of CD133 expression levels in clinical samples[41]. Therefore, some controversies could be a consequence of using different types of primary anti-CD133 antibodies to identify CD133$^+$ cells: most of the studies use the anti-human CD133/clone AC133 monoclonal antibody (Miltenyi) recognizing a glycosylated extracellular epitope of the CD133 molecule which can be downregulated independently from the corresponding mRNA and protein[28]. However, several other antibodies are available and are indistinctly used although they recognize different epitopes of the molecule and could give different results[41] (Figure 7).

The role of CD133 in colorectal tumorigenesis has been also investigate in mice. Zhu *et al*[42], demonstrated that in a murine model of colorectal tumorigenesis the endogenous activation of the Wnt signaling was associated with a marked expansion of CD133$^+$ cells which replaced normal mucosa architecture giving rise to neoplastic lesions. Our group analyzed by immunohistochemistry the expression of CD133 in a mouse model of colitis-related colon tumorigenesis induced by a combined treatment with azoxymethane and dextran sodium sulphate. In normal tissues rare scattered positive cells were detectable at the bottom of the crypts. The percentage of

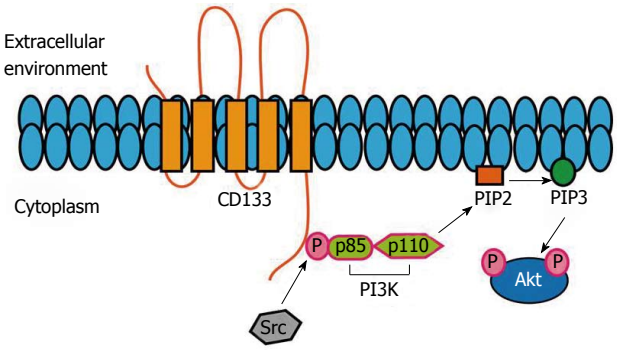

Figure 8 Potential molecular pathways associated with CD133. The phosphorylation of the tyrosine 828 is involved in the binding to p85 (PI3K regulatory subunit) and in the subsequent activation of PI3K/Akt pathway, which, finally, promotes the self-renewal and tumor formation of CSCs. CSCs: Cancer stem cells.

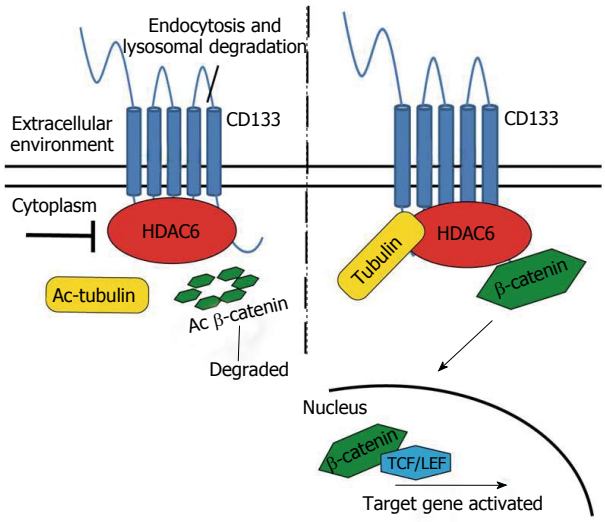

Figure 9 Schematic representation of HDAC6-mediated regulation of CD133 expression. HDAC6 physically binds to CD133 and stabilizes the β-catenin that in the nucleus promotes the activation of its target genes. Ac: Acetylated.

positive cells significantly increased in dysplastic lesions and appeared to progressively decrease in the passage from dysplasia to adenoma and then to cancer although remaining constantly higher than in adjacent normal tissues[43]. Overall these data, considered together with Mohammadi findings, suggest that upregulation of CD133 expression likely occurs at early stages and contributes to the entire process of colon tumorigenesis[43,44].

The identification of the potential molecular pathways involved in the enhanced tumorigenicity associated with CD133 expression is of great interest since it could be useful to identify and develop a targeted anticancer therapy against the CSC population. It has been reported that the CD133 glycoprotein is phosphorylated on the tyrosine-828 and tyrosine-852 residues within its C-terminal cytoplasmic tail, in a Src kinase-dependent manner. The tyrosine-828, upon phosphorylation could serve as a binding site for the SH2 domains of tyrosine kinases[44]. The phosphorylation of tyrosine-852 does not require the binding to the SH2 domains. In this regard, Wei *et al*[45] showed that, in the glioma CSCs, the phosphorylation of the tyrosine 828 is involved in the binding to p85 (PI3K regulatory subunit) and in the subsequent activation of PI3K/Akt pathway, which, finally, promotes the self-renewal and tumor formation of CSCs (Figure 8). Wang *et al*[46] reported that the inactivation of Akt and Erk pathways prevented the preferential survival of $CD133^+$ colon cancer cells isolated from primary CRC and decreased their tumorigenicity. Moreover, the down-regulation of Akt and Erk by short interfering RNAs attenuated the colony formation ability of $CD133^+$ cells[46]. More recently, it has been also showed that Silibinin, a chemo-preventive agent proved to be effective in several types of cancer, acts by inhibiting the PP2Ac/Akt/mTOR pathway which is associated with a reduction of CD133 expression in CRC spheroid cultures[47]. The Wnt signaling cascade plays various roles in stem cell maintenance, cell proliferation, differentiation and apoptosis and the deregulation of the Wnt pathway is associated with cancers. Corbo *et al*[48] reported a positive correlation among CD133 expression, Wnt pathway activation and increased SRp20 expression (splicing factor, a newly identified target gene of the Wnt/β-catenin pathway) in colon cancer cells. Furthermore, fibronectin, a major extracellular matrix glycoprotein, has been shown to be required for maintaining CD133 and CD44 positive subpopulations and tumorigenic capacity of CRC cells by activation of Wnt/β-catenin and its downstream integrin-Fak-Erk signaling pathways[49].

The regulation of CD133 expression is not fully understood but several evidences suggest the existence of multiple mechanisms involved in the regulation of CD133 expression and/or activity. As previously mentioned, CD133 is concentrated in plasma membrane protrusions and the release of CD133-containing membranous vesicles has been shown to contribute to the regulation of CD133 expression levels in several cell types[27]. Post translational modifications (*i.e.*, glycosylation) have been also suggested to play a role in the regulation of CD133 activity and its significance as a CSCs marker[28]. Indeed, it has been proposed that AC133, one of the most important epitopes of the molecule, rather than the entire molecule itself, might be important as CSCs marker.

Mak *et al*[50] proposed that CD133 expression is also regulated at a protein level by the deacetylase HDAC6, whose interaction with CD133 prevents its degradation by deacetylating α-tubulin and promotes the deacetylation of β-catenin and the activation of its signaling pathway. Moreover, they demonstrated that the inhibition of HDAC6 promotes CD133 trafficking into endosomes by increasing α-tubulin acetylation and is associated with β-catenin degradation (Figure 9).

The regulation of CD133 gene expression is also still poorly understood. Hypoxia and increased expression of hypoxia-inducible factors (HIFs) are associated with tumor progression and patient mortality in many solid tumors such as colorectal cancer, in which high expression levels of HIF-1α have been associated with poor

prognosis[51]. Ohnishi *et al*[52] suggested that the activity of one of the putative CD133 promoters (P5) is regulated by HIFs in human embryonic kidney and colon cancer cells. In particular, the CD133 promoter P5 appears to be activated by HIF-1α and HIF-2α through one of two E-twenty six (ETS) binding sites. This finding is consistent with the observation of Mao *et al*[53] that the $CD133^+$ populations in human CRC specimens express more HIF-1α than the $CD133^-$ cell population. Moreover, they engrafted human CRC specimens in BALB/c nu/nu mice and demonstrated that the majority of the $CD133^+$ population in tumor xenografts was localized in the hypoxic region. The same Authors also demonstrated that the percentage of $CD133^+$ cells increased following chemotherapy (5-fluorouracil, oxaliplatin or 5-fluorouracil plus oxaliplatin) thus indicating that $CD133^+$ cells were less sensitive to drugs than the $CD133^-$ counterparts and that the tumor hypoxic region could be associated with chemotherapeutic resistance of colon CSCs[53]. The possibility that potential epigenetic mechanisms might be also involved in the regulation of CD133 expression in CRC has been suggested by Yi *et al*[54] who described an abnormal DNA hypermethylation in a CpG island in the promoter region of the CD133 gene in colon cancer cells but further studies are required to definitively address this type of regulation for CD133 expression.

All these findings suggest a potential key role of CD133 in the initiation and progression of human CRC and support its value as a possible prognostic and diagnostic marker in CRC. The knowledge of the regulatory mechanisms upstream of CD133 and of the molecular mechanisms activated downstream could be useful in the development of targeted drugs specifically directed against CSCs, in an attempt to prevent recurrence, metastasis and chemotherapy resistance in CRC patients.

CD44

CD44 is member of a family of transmembrane proteins that include at least 20 variants resulting from a single gene by both alternative splicing and post-translation modifications[55]. The human CD44 gene includes 20 exons: exons 1-5 and exons 16-20 form a mRNA that code for a standard form of CD44 which is present in all tissues (CD44s); exons 6-15 are subject to alternative splicing that, in theory, may give life to more than 1000 variant isoforms of CD44 (CD44v)[56]. The standard isoform of human CD44 protein contains 363 amino acids and is formed by three regions: the extracellular (270 aa), the transmembrane (21 aa) and the C-terminal cytoplasmic (72 aa) domains. The presence of variable exons, mainly involving the extracellular domain, confers to CD44 a large variability of biological functions, that contributes to tumorigenicity when CD44 is expressed on tumor cells[56].

CD44 is a cell adhesion molecule that allows cell-cell and cell-ECM interactions through the binding to its principal ligand, hyaluronic acid (HA). It is also involved in lymph node homing and lymphocyte activation, myelopoiesis, lymphopoiesis, and angiogenesis[56]. CD44s, the smallest CD44 isoform that lacks variant exons, is abundantly expressed by both normal and cancers cells, whereas the CD44v isoforms that contain a variable number of exon insertions are mainly expressed by cancer cells[56].

CD44 is submitted to sequential proteolytic cleavages in the ectodomain and intramembranous domain, key events for the CD44 dependent cell-matrix interaction and signaling pathway. Cleavage of CD44 ectodomain is regulated by multiple stimuli such as extracellular Ca^{2+} influx, activation of protein kinase C or Ras and is mediated by membrane-associated matrix metalloproteinases. The release of the soluble ectodomain (soluble CD44) regulates cell attachment and migration and induces the intramembranous domain cleavage, mediated by the presenilin (PS)-dependent γ-secretase, that releases the intracellular domain of CD44 (CD44-ICD). CD44-ICD translocates to the nucleus, where it activates gene transcription, including CD44 itself, via binding to TPA-responsive elements[57] (Figure 10).

CD44 has been proposed as CSCs marker of several solid tumors, including breast, pancreas, head and neck, non-small cell lung, hepatocellular and colon cancers[18,56]. $CD44^+$ CRC cells display a greater ability to form colonies *in vitro* and a higher tumorigenicity *in vivo* compared to $CD44^-$ cells. Moreover, only $CD44^+$, but not $CD44^-$ CRC cells are able to retain the morphological and phenotypic characteristics of tumor lesions from which they were derived following serial transplantations[58]. The association of CD44 with CD54 (a member of the immunoglobulin super-family also called intercellular adhesion molecule-1) has been shown to specifically identify rectal CSC displaying the ability to self-renew *in vivo* and *in vitro*, form spheres and recapitulate tumor bulk[59].

CD44 expression is regulated by the Wnt signaling pathway *via* β-catenin. In fact, activation of β-catenin/Tcf-4 signaling in intestinal tumors is associated with CD44 overexpression and deletion of CD44 in APC Min/+mice inhibits the initiation of tumors[60]. CD44 appears to be essential for stemness maintenance of colorectal CSCs since it is involved in the activation of the tyrosine kinase receptor c-Met[58]; CD166, a mesenchymal stem cell marker (see below), has been suggested as a potential co-CSCs marker, together with CD44, in human CRC, since in xenograft $CD44^+/CD166^+$ cells have a higher tumorigenicity as compared to $CD44^+CD166^-$ cells. The surface phenotype $EpCAM^{high}/CD44^+/CD166^+$ has been proposed as an alternative to the CD133 positivity for the selection of colon CSCs[18] and $CD44^+$ CRC cells have been shown to display a higher proliferation, more robust formation of colonies, less spontaneous apoptosis and a higher resistance to drug-induced cell death compared to $CD44^-$ cells[47].

More controversial are the findings regarding the role of CD44 in tumor progression and in the development of metastases in CRC. Several studies showed that expression of CD44 on tumor cells is correlated with

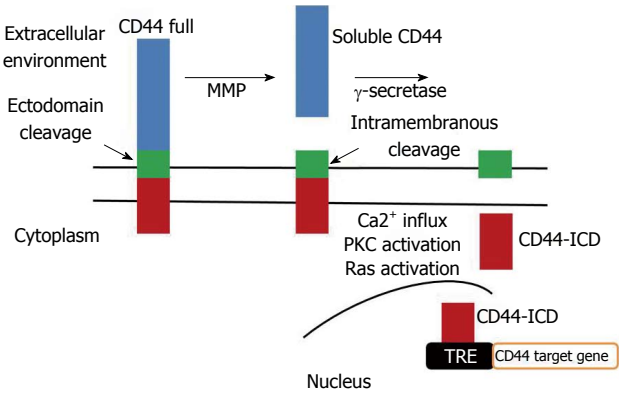

Figure 10 Schematic representation of CD44 sequential proteolytic processing. CD44 undergoes sequential proteolytic cleavages in the ectodomain and intramembranous domain. Cleavage of CD44 ectodomain generates soluble CD44 that regulates cell attachment and migration and induces the intramembranous domain cleavage, releasing the intracellular domain of CD44 (CD44-ICD). CD44-ICD translocates to the nucleus, where it activates gene transcription, including CD44 itself, via binding to TPA-responsive elements (TRE). MMP: matrix metalloproteinase.

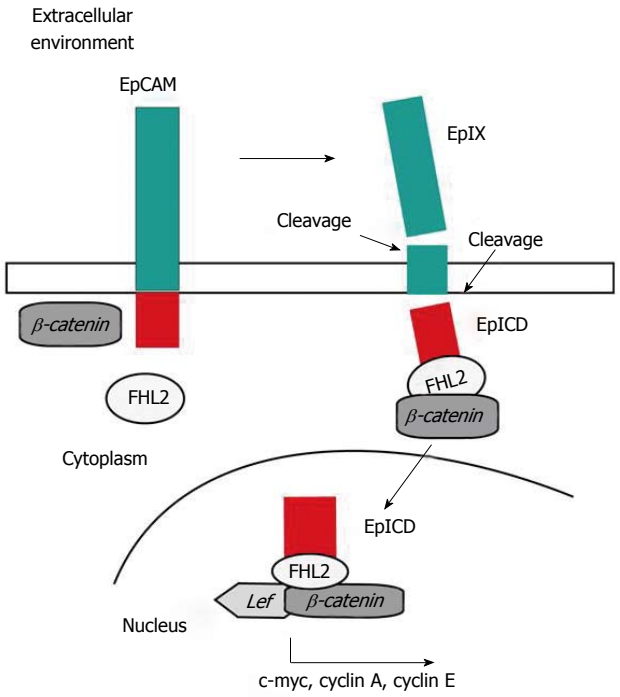

Figure 11 Schematic representation of epithelial cell adhesion molecule activation. Cleavage of full length epithelial cell adhesion molecule (EpCAM) generates EpEx (extracellular domain of EPCAM) and EpICD (EpCAM Intracellular Domain) fragments. EpICD binds the scaffold protein FHL2 and joins to the transcriptional regulators β-catenin and, within the nucleus, interacts with LEF, binds to DNA and induces gene activation.

tumor progression and metastasis while others have suggested an inverse correlation or no correlation at all[57,58].

Down-regulation of CD44 was initially related to a decrease in the metastatic potential of CRC cells[61], while more recently Dallas reported that down-regulation of CD44 leads to an increase of the metastatic and migratory potential of CRC cells[62]. It was observed that high-grade CRC have higher CD44 expression levels compared to low-grade tumors and this over-expression was associated with a reduced patients survival[63]. On the other hand, Ylagan *et al*[64] reported that the loss, rather than an increased expression, of CD44 is associated with an increased tumor aggressiveness while Fernández *et al*[65] demonstrated that CD44 expression levels were related to proliferation in CRC, but not with patients outcome. Subsequently, CD44 expression in human CRC was associated with the depth of invasion and lymph node involvement, and CD44s overexpression was suggested to be an independent unfavorable prognostic factor for overall survival in advanced CRC[66]. These findings were not confirmed by Lugli *et al*[67] who reported that the loss of CD44 is associated with more advanced tumor stage, the presence of vascular invasion, lymph node involvement and an infiltrating tumor border. Patients with tumors displaying a loss of CD44 or CD166 expression in the invasive front of the lesion had an adverse outcome compared with those expressing at least one of the two markers[67] (Table 3).

Further studies are warranted to further understand the suitability of CD44 molecule as a CSC marker in CRC and its role in human colorectal tumorigenesis.

EpCAM

Epithelial cell adhesion molecule (EpCAM), initially described in 1979 as a tumor associated antigen in human CRC[68], is a 30-40 kDa transmembrane glycoprotein showing frequent and high-level expression in a variety of human epithelial normal and cancer tissues, including colon[69]. It has been also detected on normal stem and progenitor cells and in cancer-initiating cells isolated from colon, breast, pancreas and prostate carcinomas[16,17,70]. Several evidences demonstrate that EpCAM is involved in cell adhesion, proliferation, differentiation and migration as well as in cancer and stem cells signaling[71,72].

The human EpCAM protein was independently identified by various research groups and, for this reason, several terms have been used to identify the molecule on the basis of the monoclonal antibody used to identify it[70]. However, it has been lately agreed the use of the term "EpCAM", without other specifications[70].

EpCAM displays a marked expression gradient from crypts to the apex of villi in normal colon tissue: adenoma development is associated with an increased EpCAM expression, and EpCAM overexpression is frequently observed in colorectal carcinoma[73]. Denzel *et al*[72] demonstrated that EpCAM is less accessible to antibodies in colon adenomas than in cancer because, in the last condition, EpCAM is activated by proteolysis in EpICD, the intracellular domain of EpCAM, and is intracellularly redistributed in dispersed patterns. They also showed that EpICD translocates into the cytoplasm together with the scaffold protein FHL2 and joins to the transcriptional regulator β-catenin to form a complex which, within the nucleus, interacts with Lef and binds to DNA inducing c-myc, cyclin A and cyclin E expression[72] (Figure 11). These findings were further confirmed by the ob-

servation that nuclear and cytoplasmic EpICD in solid epithelial cancers, such as colon, are increased, while the expression of membrane EpEx, the extracellular domain of EPCAM, is absent or reduced[74].

EpCAM was initially identified as a marker of human colorectal CSCs by Dalerba *et al*[18] who focused on two markers previously identified on human breast CSC: CD44 and EpCAM. Two main populations of epithelial cells were sorted from primary human CRCs by FACS: $EpCAM^{high}/CD44^{+}$ and $EpCAM^{low}/CD44^{-}$ and their tumorigenic properties were assessed. The results obtained demonstrated that the injection of 200 to 500 $EpCAM^{high}/CD44^{+}$ cells in NOD/SCID mice were sufficient to give rise to a tumor, whereas up to 104$EpCAM^{low}/CD44^{-}$ cells failed to form visible tumors. The xenograft tumors from $EpCAM^{high}/CD44^{+}$ reproduced the histopathology and phenotypic heterogeneity of the original tumors including the presence of variable percentages of both $EpCAM^{high}/CD44^{+}$ and $EpCAM^{low}/CD44^{-}$ cell populations[18]. They also verified that human $EpCAM^{high}/CD44^{+}$ cells from xenogenic colorectal tumors can be further stratified on the basis of the expression of the protein surface marker CD166, which could be used for the enrichment of colorectal CSCs[18]. Similar conclusions were also reached by Dylla *et al*[75] who suggested that one of the possible reasons of CRC resistance to chemotherapeutic agents might be at least in part attributed to the presence of $EpCAM^{+}/CD44^{+}$ CSC since residual tumors after chemotherapy are enriched of these cells.

All these findings about EpCAM signaling and its involvement in various cellular processes, provide a strong basis for further studies to better understand its potential clinical, prognostic and therapeutic value in CRC patients.

CD24

CD24 is a small, heavily glycosylated mucin-like adhesion molecule consisting of 27 amino acids with several potential O- or N-linked glycosylation sites, which lead to a molecular mass ranging between 38 and 70 kDa[76]. CD24 is attached to cell membranes by a phosphatidylinositol anchor and is expressed physiologically in the developing pancreas and brain and in pre-B lymphocytes, in regenerating muscle, in normal keratinocytes and in renal tubules[76]. It is physiologically localized in lipid rafts where it seems to be involved in the regulation of cell adhesion and signaling[76].

CD24 is expressed in various hematologic malignancies and solid tumors such as neuroblastoma, rhabdomyosarcoma, renal cell carcinoma, breast, ovarian, prostate, lung, colorectal and gastric cancer[76,77]. The observations that CD24 is one of the possible ligands of P-selectin and one of the adhesion receptors expressed by activated endothelial cells and platelets suggest that this molecule might play a role in the process of cancer metastasis[76].

Nestl *et al*[77] initially reported an increased expression of CD24 RNA in CRC: they showed that CD24 mRNA was weakly detectable in normal colonic mucosa but highly expressed in tumor cells, and to a lesser extent in the surrounding stroma. Later, Weichert *et al*[78] analyzed CD24 protein expression in colon cancer cell lines and human CRC and correlated it to clinic-pathological variables including patient survival. From this study emerged that the majority of CRCs showed both membranous and cytoplasmic CD24 staining, and that the membranous CD24 staining was associated with metastasis but was not significantly related to other clinic-pathological variables, while the cytoplasmic staining could be considered an independent prognostic marker related with a poor patient survival[78]. Conversely, Sagiv *et al*[19] failed to demonstrate any prognostic significance of CD24 expression level in CRC: in their study CD24 was similarly highly expressed in both adenomas and carcinomas. Moreover, unlike Weichert findings, they only reported a membranous staining. The same study also showed that CD24 is expressed early in the multistep process of CRC carcinogenesis, a finding consistent with its potential role as CSC marker.

Contradictory data have been reported in the literature concerning the prognostic value of CD24 whose expression levels have been reported to be not related with survival of CRC patients despite their significant relationship with conventional clinic-pathological factors such as tumor invasiveness and degree of differentiation[79]. Therefore, the real prognostic role of CD24 in CRC remains still unclear and controversial and it should be better elucidated by further studies.

Spheroid cultures of primary CRC have tumor-initiating capacity and are capable of inducing tumors upon xenotransplantation. These tumors resemble the original neoplasms both from a morphological point of view and the expression of specific markers[16]. Vermeulen *et al*[14] suggested that the co-expression of CD133 and CD24 could improve the identification of the clonogenic population within the spheroid cultures, and that both markers are downregulated during cell differentiation. CD24 was also used, in association with CD44, to identify and characterize CSCs from CRC cell lines by Yeung *et al*[80]. They demonstrated that the $CD44^{+}/CD24^{+}$ subpopulation of cells, isolated using FACS sorting, was the most clonogenic, giving rise to the highest proportion of megacolonies (complex structures resembling colonic crypts) compared to $CD44^{-}/CD24^{-}$ cells. $CD24^{+}$ subpopulation was also shown to exhibit cancer stem-like properties such as enhanced chemotherapy-resistance, self-renewal and tumorigenic capacity both *in vitro* and *in vivo*, compared to $CD24^{-}$ subpopulation isolated from CRC cell lines[80].

To our knowledge only few studies have investigated the underlying molecular mechanisms and the exact role played by this cell surface marker in CRC tumorigenesis. Thus, CD24 has been shown to activate Erk1/2 and p38 MAPKs and to increase the activity of Src and induce miR-21 expression, which in turn inhibits the expression of Pdcd4 and PTEN. On the other hand, the expression of CD24 and Src appears to be suppressed by miR34a through the downregulation of miR21[81].

Further studies are warranted to clarify the real activity of CD24 in CSCs and the key regulatory molecular

networks involved in its role in colorectal tumorigenesis.

CD29

CD29 (β1-integrin) is a member of the integrin family and consists of a large extracellular domain, a single transmembrane stretch and a short cytoplasmic domain. It acts as a receptor for extracellular matrix proteins and activates signaling molecules and pathways that regulate cell migration, proliferation, survival, differentiation and death[82].

In fact, CD29, by binding with fibronectin or Type I collagen, allows activation of Fak by Src leading to the activation of Erk that regulates cell proliferation. Erk, through phosphorylation of myosin light chain (MLC) by MLC kinase, also regulates cytoskeleton reorganization and cell motility. Moreover, CD29 regulates cell survival through the activation of Akt pathway[83].

CD29 has been initially described as an epidermal stem cell marker, and subsequently as a regulator of spermatogonial stem cells homing and of hematopoietic stem cells[22]. In normal human colon, CD29 is expressed at the bottom of the crypts, where it identifies a cell population that is capable of forming colonies in agar. For this reason, CD29 has been proposed as a stem/progenitor cell marker[27] and as a marker of colon CSCs. In fact, it has been shown that $CD133^+CD29^+$ colon CSCs are biologically characterized by self-renewal, proliferation and differentiation[14,20].

CD29, with E-cadherin, mediates cell-cell and cell-collagen interactions that are required for the maintenance of the differentiated phenotype of human CRC cells. Thus, CD29 downregulation may be responsible of the switch from differentiated to undifferentiated phenotype *in vivo*[84]. CD29 seems to be also implicated in the enhancement of the metastatic activity of CRC cells. In fact, Okazaki *et al*[85] showed that CD29 was significantly increased *in vivo* in metastases derived from human CRC cells. CD29 expression appears also to increase in the passage from adenoma to adenocarcinoma and with increasing tumor stage[86].

CD29 expression may be also associated with overall survival in CRC patients. In fact, loss of CD29 expression is associated with advanced stage and with poor prognosis and CD29 expression decreases in metastatic lesions[87], although other Authors have suggested that CD29, in combination with CD49b, might contribute to the acquisition of a metastatic potential in CRC cells. Finally, CD29 expression has been shown to identify the population of CRC cells that are more resistant to radio and chemo-therapy[88]. Further studies are needed to understand the specific role of CD29 as CSC marker as well as in the progression of CRC.

Lgr5

Lgr5, (Leucine-rich repeat-containing G protein-coupled receptor 5) also known as Gpr49, is an orphan G protein coupled receptor, characterized by a large leucine-rich extracellular domain and seven transmembrane domains. It is a receptor for R-spondin proteins which represent secreted agonists of the canonical Wnt/β-catenin signaling pathway[89,90].

Lgr5 is a member of the glycoprotein hormone receptor subfamily that includes the thyroid-stimulating, the follicle-stimulating and the luteinizing hormones receptors[21].

Lgr5 was first identified in human colon cancer cell lines harboring Wnt activating mutations as a Wnt target gene[4,91] and was then shown to be overexpressed in other human malignancies such as ovarian, hepatocellular, esophageal and basal cell carcinomas[92].

Since Lgr5 is one of Wnt target genes, it is not surprising that this protein is found expressed in different stem cells[5,93]. In the intestine Lgr5 is expressed in mature intestinal stem cells at the bottom crypt[4,5]; more specifically, Barker *et al*[4], using in situ hybridization demonstrated that Lgr5 is selectively expressed on few proliferating cells alternated with Paneth cells at the bottom of the crypts in the small intestine. These cells, known as crypt base columnar cells, are cycling cells and represent intestinal stem cells. These findings have suggested that Lgr5 could have an important role in colorectal carcinogenesis and that it could be an ideal marker of colorectal CSCs.

Several research groups have investigated whether Lgr5 could play a role in colorectal tumorigenesis and several studies suggested that there is a close correlation between Lgr5 expression and colon cancer progression[90,92].

In fact, Lgr5, which is normally localized to the basal intestinal crypt area, is expressed only in the peripheral region of adenomas and ubiquitously in established adenocarcinomas. It has been hypothesized that the accumulation of genome mutations occurring during the process of malignant transformation, might lead to loss of $Lgr5^+$ cells polarity that can thus migrate to the tumor-host interface (carcinoma *in situ*) and then in all the tumor (advanced cancer)[94]. The selective expression of Lgr5 in the peripheral region of adenomas supports the hypothesis that it might mark intestinal CSCs. In favor of this hypothesis is the work of Batlle *et al*[95] that reported that Lgr5 is selectively expressed on human colon CSCs. This finding has been further confirmed by Kemper[38] who demonstrated that Lgr5 identifies the CSC fraction in CRC and that it is expressed at high levels in spheroid cultures derived from primary CRC (that are known to be enriched for CSCs) and decreased following cellular differentiation[38].

Since Lgr5 is expressed at high levels in both colorectal adenomas and adenocarcinomas it likely plays an important role not only in the early but also in the late events of tumorigenesis, such as invasion and metastasis. Moreover, high Lgr5 expression has been shown to correlate with mesenchymal characteristics of tumors, such as high expression of vimentin and low expression of miR-200c, and with increased invasiveness and lymph node metastases[92,94].

Overall, the available evidence suggests that Lgr5 could play a key role in the development and progression

of CRC and might represent a useful marker to identify and/or target CSC in colon cancer.

CD166

Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, is a member of a subgroup of transmembrane glycoproteins in the immunoglobulin superfamily, characterized by the presence of five extracellular immunoglobulin-like domains (VVC2C2C2)[96].

CD166 is able to form low-affinity hemophilic interactions and much stronger heterophilic interactions with CD6 expressed on T lymphocytes, thymocytes and on a subset of B cells[97].

Beside hematopoietic cells, expression of CD166 has been reported in a wide variety of tissues and cells including selected epithelia, lymphoid and myeloid cells, fibroblasts, neurons, hepatocytes, pancreas acinar and islet cells[98]. CD166 is also present in a large number of tumors including breast, lung, colon and prostate cancer and melanoma[98].

In the small intestine and in the colon, CD166 was observed at high levels on the surface of cells within the stem cell niche at the base of the crypt, but little is known about its endogenous function. However, CD166 seems to be involved in the morphogenesis of tubular structures by cell-cell and cell-matrix interactions[99].

Expression of CD166 in colon cancer has been analyzed by several groups with conflicting results. Weichert *et al*[100] suggested that CD166 up-regulation is an early event in colon tumorigenesis because it was found in all adenomas of the colon. Moreover, they reported by immunohistochemistry both a cytoplasmic and membranous staining for CD166 in CRC and a correlation between high membranous CD166 expression and poor prognosis. On the contrary, Horst and collaborators did not find any correlation between CD166 expression and CRC patients outcome[101].

The study conducted by Lugli revealed an association between the loss of CD166 and an increase in tumor size, lymph node metastasis, tumor infiltration and a shorter overall survival[67].

These findings have been partially confirmed by a recent work showing that CD166 expression is a positive prognostic marker for overall survival in CRC patients. CD166 expression in well differentiated CRC suggests a role of the protein in the early stages of tumorigenesis and, since CD166 seems to be involved in cell-cell and cell-matrix adhesion, its loss may be associated with reduced cell adhesion and therefore with a higher metastatic potential of tumors[102].

It has been recently suggested that CD166 may contribute to the identification of colorectal CSCs[18] but its role in CRC tumorigenesis as well as a marker of CSC remains to be defined.

THERAPEUTICS RELEVANCE OF COLORECTAL CANCER STEM CELLS

Cancer stem cells are believed to be resistant to chemo- and radiation-therapy and are indicated to be the cause of cancer relapse and metastasis: conventional anticancer therapy wipes out the bulk populations but the surviving CSCs repopulate the tumor (Figure 3). Therefore, targeting both CSC and the bulk populations is essential for complete tumor eradication. Thus, the identification of colorectal CSC markers and their signaling pathway is crucial for the development of novel therapies which could specifically target these cells.

The potential therapeutic strategies aimed at selectively target CSCs, which are beginning to be experimentally validated, include the elimination of CSCs through agents which target specific markers of CSC (such as monoclonal antibodies) or interfere with CSC-specific pathways[103].

Todaro *et al*[104] demonstrated that $CD133^+$ colon CSC produce and use the cytokine IL-4 to protect themselves from apoptosis caused by conventional chemotherapy agents, 5-fluorouracil and oxaliplatin. In fact, the simultaneous treatment with antibodies to IL-4 greatly increased the antitumoral cytotoxic activity of the drugs.

It has been also reported that a 5-fluorouracil and oxaliplatin chemoresistant derivative of the HT29 human CRC cell line displayed an enrichment of $CD133^+$ and $CD44^+$ cells with an increased expression of the Type 1 insulin-like growth factor receptor (IGF-IR). Treatment with a monoclonal antibody to IGF-IR induced a significant inhibition of tumor growth, thus demonstrating an enhanced sensitivity of colon CSC to IGF-IR specific targeted therapy[105].

More recently, Bach *et al*[106] used measles viruses, oncolytically active against various types of human cancer, to generate CD133-specific measles viruses (MV) and to provide a new CSC-specific anticancer therapy. They were able to efficiently infect the primary colon spheres to test the oncolytic activity of CD133-MV on colon primary tumor cells. The infection caused a rapid loss of $CD133^+$ cells and, when implanted in NSG mice, the CD133-MV infected tumor spheres formed tumors smaller than uninfected tumor spheres. However, no effect in term of tumor volume was observed when the resected tumors were transplanted in secondary mice and the re-isolated tumors contained 70% of $CD133^+$ cells[106].

Given that CD133 is also expressed on normal stem cells, Bostad *et al*[107] have developed a site-specific strategy that allows to release the drug only in the tumor area. They developed an immunotoxin targeting CD133 by using the photochemical internalization (PCI) technology. The biotinylated anti-CD133 antibodies were mixed with streptavidin-saporin (sap) to form the model of anti-CD133-sap immunotoxin. Saporin, a plant toxin, is a potent ribosome inactivating protein and was used as the toxin component of the immunotoxin. The aim of this technology was to avoid the degradation of the drug by the lysosomes before the drug has interacted with its biological target, and the main advantage should be the accumulation of the photosensitizer preferably in the neoplastic tissue. This report demonstrated that the $CD133^{high}$ population of WiDr colon cancer cells is more resistant

to photodynamic therapy than the $CD133^{low}$ population but the PCI of a CD133-targeting toxin is able to sensitize and destroy these resistant cells. Thus, PCI-based anti-CSC strategy could be a specific method for a selective killing of $CD133^{+}$ CSCs while sparing normal stem cells[107]. Chen *et al*[108] tested the effects of CD133 monoclonal antibody (Miltenyi) on hepatocarcinoma cells. The CD133 monoclonal antibody treatment, under extracellular low glucose condition, inhibited the proliferation of hepatocarcinoma cells, suppressed spheroid and colony formation, attenuated xenograft tumors and improved the efficiency of chemotherapy. Moreover, Swaminhatan and others developed nanoparticles formulated using the biodegradable poly (D, Llactide- co-glycolide) polymer and surface functionalized with an anti-CD133 antibody (CD133NPs). The CD133NPs were loaded with paclitaxel and were able to reduce the fraction of tumor-initiating cells *in vitro* and tumor recurrence in the MDA-MB-231 xenograft tumor model[109].

EpCAM has been also suggested as a potential target for the development of a CSC-specific therapy for CRC. Several clinical trials have already evaluated the efficacy of a monoclonal antibody to EpCAM for a targeted treatment of CRC. Edrecolomab, a murine monoclonal anti-EpCAM antibody, was the first immunotherapeutic agent licensed for the use in a large-scale human anti-tumor immunotherapy trial. In 1994, Riethmüller *et al*[110] randomly assigned to adjuvant therapy with Edrecolomab a series of patients with a resected Dukes' C CRC: they showed an improved survival rate, and a reduction of mortality and disease recurrence[110,111]. These promising results were not further confirmed. In fact, Punt *et al*[111] showed that the addition of Edrecolomab to fluorouracil and folinic acid in the adjuvant treatment of resected stage Ⅲ CRC did not provide any further improvement in term of survival, and that the immunotherapy alone was associated with a significant shorter disease-free survival[111]. Similar findings have been reported by Fields *et al*[112] who adopted a combination of fluorouracil-based therapy and Edrecolomab for the treatment of stage Ⅲ colon cancer patients, getting poor results.

More recently, Waldron *et al*[113] have characterized a biospecific target toxin, which is composed by anti-EpCAM and anti-CD133 scFv (single-chain variable fragment), and have focused on three different types of carcinoma: head and neck, breast, and colon carcinoma. The toxin, called deimmunized CD133KDEL (dCD-133KDEL), was synthesized using an anti-CD133 scFv that recognized the loop two of the extracellular domain of CD133 and both the glycosylated and unglycosylated forms of CD133. The anti-CD133 scFv was fused with an anti-EpCAMscFv and with a truncated form of Pseudomonas exotossin A (PE38) and the construct called dEpCAMCD133KDEL and showed a strong inhibition of proliferation in CRC cell lines.

CD44 can represent a suitable therapeutic target for CRC, since it presents two distinct forms between normal and cancer cells. In fact, the different local environmental pressures are responsible for different splicing and post-translational modifications which give rise to different CD44 molecules that can be recognized by specific agents useful for both diagnosis and therapy[114].

CD44 knockdown was shown to inhibit tumor growth and metastasis *in vivo*[61], a finding confirmed by Du *et al*[58] who used lentiviral RNA interference to stably knock down CD44 or CD133 in CRC primary cells isolated from patients. These Authors reported that knockdown of CD44 reduced clonal formation, whereas CD133 knockdown had little effect compared to control.

The combination of curcumin and dasatinib has been also suggested as a therapeutic strategy for chemo-resistant CRC. In fact, the combination therapy with curcumin and dasatinib inhibited the growth of chemo-resistant HT29 and HCT-116 CRC cells, the formation of colonospheres and extracellular invasion. The expression of CSC markers CD133, CD44, CD166 and ALDH1 displayed a 25%-30% decrease in cells treated with curcumin and dasatinib thus suggesting that the combination may be used as a specific CSC targeted therapy to prevent recurrence of CRC[115].

Another study demostrated that difluorinated-curcumin in combination with 5-fluorouracil and oxaliplatin, the standard of CRC chemotherapy, was more potent than curcumin in reducing CD44 and CD166 expression in chemo-resistant CRC cells. This effect was associated with growth inhibition, induction of apoptosis and disintegration of colonospheres[116].

Misra *et al*[114] demonstrated that CD44v6 knockdown reduced the ability of CRC cells to signal through hyaluronan-CD44v6. They encapsulated plasmidic DNA coding CD44v6 shRNA into transferrin (Tf)coated nanoparticles which are recognized by Tf-receptor (TF-R) present at high level on tumor cells which then internalize the particles by receptor-mediated endocytosis. These nanoparticles were delivered within pre-neoplastic and neoplastic colon tissues in the Apc Min/mice model, causing inhibition of the CD44v6 expression. This inhibition was associated with a reduced adenoma number and growth through a hyaluronan/CD44v6/ErB2/Cox 2 interaction pathway[114].

Mesoporous silica nanoparticles (MSNs) have been proposed as nanocarriers for several anticancer treatments. Yu *et al*[117] have developed a targeted drug delivery system based on hyaluronic acid (HA) modified MSNs (HA-MSNs). HA-MSNs have a specific affinity to CD44 overexpressed on CRC cells and can enter cells *via* the HA receptor mediated endocytosis pathway. Doxorubicin (Dox), an anticancer drug, has been encapsulated into MSNs with or without HA. HCT-116 cells were treated with free Dox, Dox-HA-MSNs or Dox-MSNs. The cells treated with Dox-HA-MSNs presented a stronger inhibition of proliferation (51%) compared to the other two groups[117].

A new potential therapeutic approach targeting CSCs was suggested by Sagiv *et al*[118] who showed that the growth of human CRC cells lines, expressing the presumptive CSC marker CD24, was inhibited after treatment with three different anti-CD24 monoclonal

antibody which showed a synergistic effect with chemotherapeutic agents[19,118].

Downregulation of CD29 expression by antisense oligonucleotide in the HT-29 human CRC cell line reduced both tumor cells migration *in vitro* and hepatic metastasis *in vivo*[37]. Interestingly, barberine, a botanical alkaloid with cytotoxic effects on most type of cancer cells, can inhibit the migration of SW480 and HCT116 CRC cells through a decrease of CD29 expression level *via* AMP-activated protein kinase thus suggesting the possibility to use this drug to specifically target CD29-expressing CSCs[119].

Targeting of CSC can be also obtained by interfering with the pathways involved in stemness maintenance such as Wnt, Hedgehog and Notch. Several approaches for CRC therapy have focused on Wnt pathway inhibition. Wnt family proteins are secreted intercellular signaling molecules that act as ligands to activate a specific signal transduction pathway. Upon binding of Wnt to its receptor Frizzled (FZD), the protein Disheveled (Dvl/Dsh) is activated and inhibits the Glycogen synthase kinase 3 (GSK-3) activity. The latter binds to axin, APC and Casein Kinase 1 (CK1), and forms a complex that binds β-catenin and promotes its degradation. When Wnt signaling inhibits GSK-3, β-catenin dissociates from the complex and enters the nucleus, where it binds to the DNA binding protein Tcf/Lef, becoming a transcription factor[120]. Efforts have been mainly devoted to the identification of small molecules inhibiting this pathway.

Chen *et al*[121], in a screen of a synthetic chemical library, identified a new small molecule able to inhibit the Wnt signaling. This compound, called IWP (inhibitor of Wnt production), inhibits the activity of Porcupine, a membrane-bound acetyltransferase, essential for Wnt production[121]. Similarly, using report-based screening approaches, Huang *et al*[122] found a small molecule, XAV939, that inhibits Wnt through the tankyrase inhibition, an event leading to an increase in the stability of axin and subsequent β-catenin degradation[122].

Pyrvinium, another small molecule promoting the degradation of β-catenin was identified by Thorne *et al*[123]. This molecule, promotes β-catenin phosphorylation through casein kinase activation. It was shown that pyrvinium treatment of CRC cells bearing APC or β-catenin mutation inhibits both Wnt signaling and proliferation[123].

Several studies have focused on the identification of molecules capable of destroying the interaction between Tcf/Lef and β-catenin and therefore of inhibiting α-catenin-dependent transcription. Three of these compounds were shown to inhibit CRC cells growth both *in vitro* and *in vivo*[124].

Emami *et al*[125] developed a compound, ICG-001, which specifically inhibited a co-activator essential for Wnt pathway activation. Treatment of CRC cell lines bearing APC or β-catenin mutations with ICG-001 induced cell death in a dose-dependent fashion while not affecting normal epithelial cells. An analogue of this compound was recently approved for phase I clinical testing[126].

The Wnt pathway has been also successfully inhibited using a specific anti-Wnt monoclonal antibody, which inhibits the proliferation and induces apoptosis in CRC cells, even in those with downstream mutations[127]. Numerous groups have tried to inhibit the Wnt pathway by inhibiting the FZD receptor activity.

A member of FZD family, FZD7, results predominantly expressed in CRC cells and it is implicated in canonical Wnt signaling in cells with APC or CTNNB1 mutations. The use of specific siRNA to knockdown the expression of endogenous FZD7 has proved effective in reducing the metastatic potential of CRC cells[128]. Similar effects have been obtained using an antibody targeting FZD7[129]. Remarkable results have been also obtained using inhibitors of Delta-like ligand 4 (DLL4), an important component of the Notch pathway. Human CRC xenografts treated with an anti-DLL4 antibody in combination with irinotecan have showed a reduction of CSCs and of tumor growth whereas treatment with irinotecan alone increased the percentage of CSCs[130,131].

CONCLUSION

The CSC model of tumorigenesis postulates that tumors are not cellularly homogenous but display a hierarchical structure and contain a rare population of cells, the CSC, that display the same self-renewal and proliferative potentials as normal stem cells associated with the capacity to give rise to tumors (Figure 2). As previously described, mounting evidence suggests the existence of a CSC population in human CRC[16,17].

It has been hypothesized that CSC may derive from transformation of quiescent, normal long-term stem cells or could result from the de-differentiation of more mature cells[15-17]. In CRC, the first hypothesis is supported by the observation that normal and cancer stem cells share similar properties and surface markers (*i.e.*, CD133 and Lgr5). However, it cannot be excluded that CSC might derive from cells that, at some specific stages of differentiation, undergo malignant transformation acquiring new properties including stem-like features. This hypothesis might explain the different aggressiveness of tumors which might relate to the different differentiation degree of cells undergoing the transformation event(s) as evidenced by the different tumor grading[15] (Figure 12).

The ability to identify and isolate CSC is essential to fully characterize them and to understand the molecular mechanisms responsible for their establishment and their maintenance. As mentioned, several approaches have been used to identify and isolate CSC the most important being the antibody-based technologies targeting CSC-specific surface markers. However, different antibodies, techniques and protocols are used in different studies and these certainly contribute to the conflicting results present in the literature. Thus, it will be important to define standardized procedures and reagents to identify CSC in clinical samples. Moreover, several questions remain unresolved especially regarding the significance of CSC

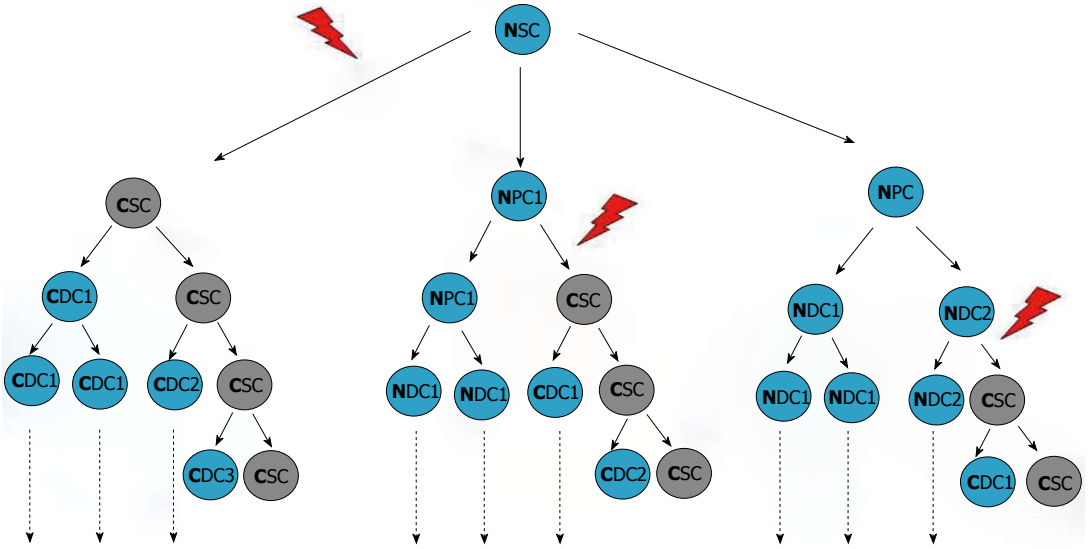

Figure 12 Origin of cancer stem cells and tumor heterogeneity. It has been hypothesized that CSCs may derive from transformation of quiescent, normal stem cells (left) or could result from the de-differentiation of more mature cells which might re-acquire the capability of self-renewal. Other mutations might occur following transformation in both cases. SC: Stem cell; PC: Progenitor cell; DC: Differentiated cell; **C**: Cancer; **N**: Normal; CSCs: Cancer stem cells.

markers and whether they play a direct role in essential CSC properties such as self-renewal and tumor-initiation ability or they are just markers of stem-like cells with no relevant physiological functions[44]. This is a very important issue in view of the possibility to develop specific anti-CSC therapies.

Indeed, the CSC model of tumorigenesis implies that targeting of CSC is essential for a complete eradication of the disease (Figure 3). This consideration fits well with the disappointing daily experience of oncologists facing occasional complete responses that do not translate into cure for patients. Indeed, the model hypothesizes that CSC must be completely eliminated in order to eradicate the disease and prevent recurrences/metastases. However, CSC have been reported to be relatively resistant to standard anticancer therapies, such as radiation and chemotherapy, which target rapidly proliferating cells[103].

Thus, initial responses to treatment could represent therapeutic effectiveness against the bulk cancer cells while sparing rare quiescent CSC which would then be responsible for tumor re-growth both at primary and metastatic sites. According to this model, a better understanding of the biology of CSC is essential to improve efficacy of anticancer therapies and several groups are pioneering the possibility of specifically targeting CSC through multiple approaches, as previously described[107,117,123]. A big issue will be the identification of substantial differences between normal and cancer stem cells and/or specific therapeutic strategies that would allow the development of drugs specifically targeting CSC while sparing normal counterparts. From this perspective, another important point to keep in mind is that standard response parameters might not be suitable to evaluate specific CSC-targeting therapies. Indeed, current evaluation criteria only take in consideration the effects of treatment on tumor bulk and thus might underestimate the effect of a therapy specifically targeting a rare population of cells within tumor mass. Thus, it will likely be important to re-examine the standard criteria to evaluate response therapy and other approaches, such as new CSC-specific imaging techniques, might be needed to this aim. This does not mean that conventional therapies will no longer have a place in the future anti-cancer protocols despite the fact that CSC may be resistant to them. Indeed, it seems realistic to anticipate that a useful approach to improve current treatment of solid tumors, including CRC, will be the combination of a specific anti-CSC treatment with traditional agents (*i.e.*, 5-fluorouracil and/or oxaliplatin) that can debulk the mass of cancer cells.

In conclusion, the CSC model of tumorigenesis has the potential to radically revolutionize the way how we look at malignant diseases as well as the clinical management of CRC patients. To this aim, it will be essential a definitive assessment of the roles that putative CSCs play in the development of human CRC and in specific aspects of malignancy. The ultimate proof of the relevance of CSCs in tumor development and in the clinical management of CRC cancer patients will be the demonstration that specific targeting of CSCs can improve patients outcomes, a goal strongly awaited by scientists, oncologists and, especially, patients.

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