Colon Cancer Stem Cells: Promise of Targeted Therapy




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First developed for hematologic disorders, the concept of cancer stem cells (CSCs) was expanded to solid tumors, including colorectal cancer (CRC). The traditional model of colon carcinogenesis includes several steps that occur via mutational activation of oncogenes and inactivation of tumor suppressor genes. Intestinal epithelial cells exist for a shorter amount of time than that required to accumulate tumor-inducing genetic changes, so researchers have investigated the concept that CRC arises from the long-lived stem cells, rather than from the differentiated epithelial cells. Colon CSCs were originally identified through the expression of the CD133 glycoprotein using an antibody directed to its epitope AC133. It is not clear if CD133 is a marker of colon CSCs—other cell surface markers, such as epithelial-specific antigen, CD44, CD166, Musashi-1, CD29, CD24, leucine-rich repeat-containing G-protein–coupled receptor 5, and aldehyde dehydrogenase 1, have been proposed. In addition to initiating and sustaining tumor growth, CSCs are believed to mediate cancer relapse after chemotherapy. How can we identify and analyze colon CSCs and what agents are being designed to kill this chemotherapy-refractory population?

Keywords: Cancer Stem Cell; Colorectal Cancer; Metastasis.

According to the traditional model of carcinogenesis, a tumor can originate from any cell of the body following multiple mutations, conferring it unlimited proliferation potential. The resulting mutated progeny is thought to get additional mutations, forming a genetically varied tumor mass in which selection of resistant sublines occurs over time. In the last several years, evidence has suggested that the capacity of initiating a tumor could be rather a unique characteristic of cells with stemness properties. These so-called cancer stem cells (CSCs) have been isolated from a variety of tumor types, including colorectal cancer (CRC). Many markers and features of CSCs have been defined, but it is not clear how this information can be used in the clinic. CSCs are not always destroyed by chemotherapeutics, which target homogeneous populations of rapidly growing, differentiated tumor cells. CSCs therefore seem to have an important role in cancer recurrence. Reagents are being developed to target this refractory CSC population. To this aim, it is important to identify the regulatory mechanisms and signaling pathways involved in CSC self-renewal. These studies require testing the ability of reagents to kill CSCs and prevent the emergence of resistant clones following therapy in complex in vivo models.

Here, after reviewing the current knowledge on stem cell (SC) features, crypt biology, and CRC genetics, we present our current opinion on cancer biology, reporting the newly proposed and the re-evaluated old theories of tumorigenesis, ie, CSC and clonal evolution models, and suggest efficient strategies for the complete tumor eradication.

Abbreviations used in this paper: ALDH1, aldehyde dehydrogenase 1; *AML*, acute myeloid leukemia; *APC*, *adenomatous polyposis coli*; ASCs, adult stem cells; BMDCs, bone-marrow–derived cells; BMP, bone morphogenetic protein; CDC4, cell division cycle 4; CDX2, caudal type homeobox transcription factor 2; CHRDL1, chordin-like 1; CK20, cytokeratin 20; CRC, colorectal cancer; CSCs, cancer stem cells; DCAMKL-1, doublecortin and CaM kinase–like-1; *DCC*, *deleted in colorectal cancer*; ESA, epithelial specific antigen; FOLFIRI, 5-fluorouracil (5-FU), leucovorin, and irinotecan; FOLFOX, 5-FU, leucovorin, and oxaliplatin; GREM1, gremlin 1; GREM2, gremlin 2; IL-4, interleukin-4; ISEMFs, intestinal subepithelial myofibroblasts; Lgr5, leucine-rich repeat-containing G protein-coupled receptor 5; MMR, mismatch repair; Msi-1, Musashi-1; NOD/SCID, non obese diabetic/severe-combined immunodeficient; NOD/SCID *Il2grl*$^{-}$, NOD/SCID interleukin-2 gamma receptor knockout; PI3K, phosphatidyl inositol 3-kinase; SCs, stem cells; Shh, Sonic hedgehog.

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SC Definition, Classification, and Roles

SCs are undifferentiated cells that, through an asymmetric cell division, give rise to 2 different daughter cells. One daughter is identical to the mother and contains SC properties (self-renewal), whereas the other is a more specialized cell.[1] Based on their ability to differentiate, SCs are classified as either totipotent (cells able to give rise to a new individual on their own), pluripotent (cells able to give rise to almost all tissues of the body), or multipotent (cells able to generate more cell types on a certain location).[2] SCs' ability to differentiate depends on the developmental stage; loss of the full, unspecialized state (totipotency) occurs following blastocyst inner cell mass development—these cells, called embryonic SCs, are pluripotent. Adult SCs (ASCs) retain the undifferentiated state by self-renewal, but are committed to the specific lineages of the organ in which they reside. However, there is evidence that supports greater developmental potential of ASCs—that ASCs are committed but not restricted to a unique fate, as proven for bone-marrow–derived cells (BMDCs).[3]

ASCs are usually involved in tissue homeostasis but can also participate in tissue repair after injury.[4] Studies of neural SCs have shown that ASCs are usually quiescent and that cell division occurs infrequently under steady-state conditions in an asymmetric fashion to allow for maintenance of population size.[5] Nonetheless, following injury or disease, ASCs increase the proportion of symmetric divisions in order to replace and regenerate damaged tissue, even at the expense of a decrease in SCs number.[6] Studies in rapidly regenerating tissues, such as blood, intestine, and epidermis, have indicated that there is some heterogeneity in cycling kinetics among SCs.[7] Based on the number of cellular divisions accomplished throughout their whole lifespan, 2 groups with different proportions of SCs can be considered. One group, composed of the vast majority of SCs, frequently cycles and functions in normal homeostasis; the other, composed of a smaller pool of SCs, slow-cycles and functions as an SC reserve for time of crisis. The limited number of cellular divisions allows such dormant, master SCs to possess the highest long-term proliferation potential, at the same time preventing genome alterations. However, studies have shown that in the epidermis, actively cycling cells, which can undergo DNA replication errors, can still be used to recreate a niche following injury.[8] Such alterations in SCs' cell cycle properties could mediate cancer pathogenesis. CSCs might be derived from either self-renewing normal cells, as a consequence of anomalous differentiation, or from progenitor cells that can directly give rise to cancer cells or reprogram themselves, acquiring SC behavior before inducing cancer.[9] Moreover, SC expansion could occur through symmetric divisions in which SCs give rise to 2 identical SC progeny.[10] Genetic and epigenetic changes would then be required to fully transform this cell population.[11] Symmetric cell division could be induced by loss-of-function mutations of cell polarity and cell fate determinants, as observed in *Drosophila* neuroblasts.[12] Using an innovative assay with a fluorescent dye, Cicalese's group observed that breast CSCs can divide symmetrically.[13] They found that ErbB2-tumor mammospheres had 5-fold more SCs than those of normal breast, and associated such phenomena with increased numbers of self-renewing cell divisions. Increased numbers of self-renewing division do not result in increased numbers of SCs if they are accomplished through asymmetric mitotic divisions. Using time-lapse video microscopy and analysis of cell fate determinants such as Numb, Cicalese's group analyzed SCs divisions from normal and ErbB2-induced tumor mammary tissues, finding that symmetric and asymmetric divisions occur in both samples, but in different proportions. The normal cells divided mostly asymmetrically, whereas the tumor cells divided symmetrically. Other investigations led to the finding that normal breast SCs rapidly lose self-renewal potential in culture, whereas ErbB2-tumor SCs are nearly immortal, increasing approximately 5–fold with every passage. Interestingly, self-renewal and p53 loss promoted continuous expansion of mammary SCs. Figure 1 illustrates the different types of SC division in normal and tumor tissues.

Histology of the Colon

The colon, or large intestine, comprises the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anal canal. Four layers characterize the colon wall; from inside to outside these are the mucosa, submucosa, muscularis externa, and serosa. The most external mucosal surface is lined by an absorptive and secretory epithelium (simple columnar) that is folded to form a number of invaginations embedded in the connective tissue. These test-tube–shaped structures, called crypts of Lieberkühn, represent the functional unit of colon. Normal human colon consists of millions of crypts, each containing about 2000 cells.[14–17] Overall, 3 main epithelial cell lineages comprise a crypt: the columnar cells or colonocytes, the mucin-secreting cells or goblet cells, and the endocrine cells. Turnover of these cell lineages is a constant process, occurring every 2–7 days under normal circumstances and increasing following tissue damage.[18] This complex process is regulated by ASCs located within the crypt unit—the numbers and locations of these cells are topics of debate. The vast majority of the information comes from mouse studies of the small intestine, which differs from the colon because of the presence of finger-like projections called villi and a 4th cell type, the Paneth cells, with a central role in the host defense against microbes.[19] More than 30 years ago, Cheng and Leblond proposed the unitarian theory,[20] according to which all differentiated cell lineages within the gastrointestinal epithelium are clonal populations derived from a slowly cycling clonogenic SC. Next, stud-

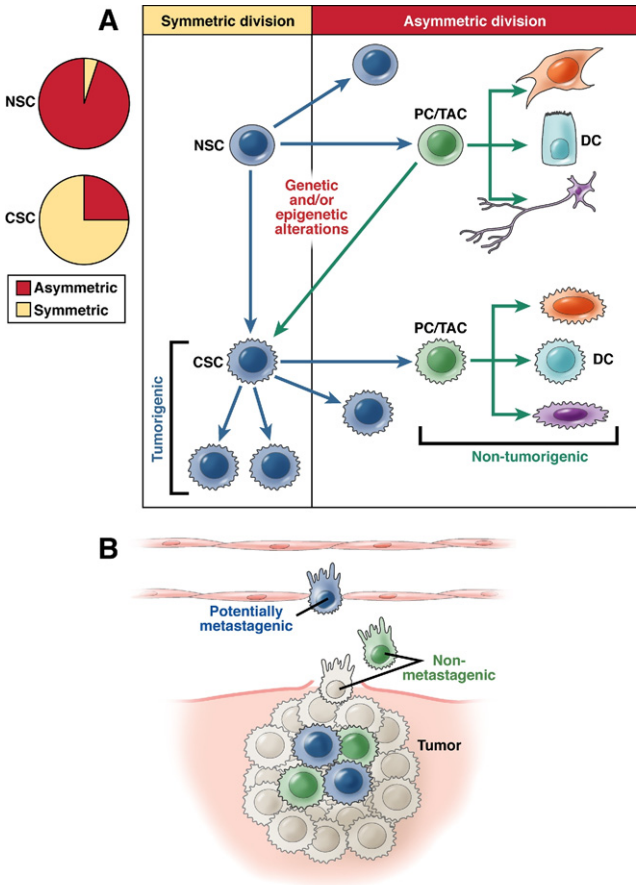


Figure 1. SCs division modalities. A normal stem cell (NSC) mainly divides symmetrically to give rise to another stem cell and a progenitor cell (PC) or a transit-amplifying cell (TAC) that in turn divide to produce differentiated cells (DC). Genetic and/or epigenetic alterations are needed to transform a NSC into a CSC. A CSC divides both asymmetrically and symmetrically giving rise to nontumorigenic/nonmetastatic PC/TAC and tumorigenic/potentially metastatic CSC.

ies located this cell in the midcrypt of the ascending colon and in the crypt base of the descending colon.[21] Other evidence indicated that each crypt contains >1 SC; in 1987 Potten et al proposed that the crypt contains a ring of about 16 functional SCs,[22] but the cells identified might also be long-lived Paneth cells. The unitarian theory is supported by the observation that after irradiation, only a single cell survives in each crypt and can regenerate it.[23] Accordingly, studies in a variety of model systems, such as mouse aggregation chimeras, have shown crypts to be derived from clonal populations.[24] In every case, $>10^{10}$ new cells are produced daily; these differentiate along a vertical axis within the gut.[25] SCs divide to produce transit cells that migrate up the crypt wall toward the luminal surface. Once at the top, they undergo apoptosis and are either shed to the lumen or engulfed by stromal cells.

A number of evidences have shown that right-sided colon cancers have a worse prognosis than left-sided colorectal cancers.[26] The reason for this may be due to a variety of factors, including embryologic, morphologic, physiologic, biochemical, environmental, and genetics differences, between the proximal and the distal colon.[27]

Molecular Markers of Normal Colon SCs

Bromodeoxyuridine labeling was initially used to identify the SC compartment of several tissues including colon,[28] based on the assumption that SCs divide infrequently and retain the DNA label for a longer time than the more rapidly dividing progenitor cells. This method of SC identification was replaced by the identification of stemness markers, usually on the cell surface, that allow SCs to be isolated by flow cytometry.

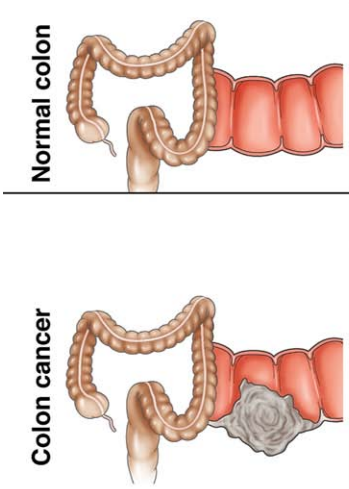
The RNA-binding protein Musashi-1 (Msi-1) was the first molecule identified as a putative human colon SC marker. Most information about its function came from studies in *Drosophila*, where it was found to be indispensable for asymmetric cell division of sensory organ precursor cells.[29] Similarly, mouse Msi-1 was proposed to be required for asymmetric distribution of intrinsic determinants in the developing mammalian nervous system.[30] Msi-1 expression was then reported in mouse small intestine and in human colon crypt SCs.[31,32] Nishimura et al[32] showed that most Msi-1$^+$ cells were located at the crypt base of human colon, between cell positions 1 and 10—a distribution that could match that of SCs.

Members of the Msi family could have distinct targets in different progenitor or SC populations. In mammals, Msi-1 is believed to maintain the undifferentiated state of SCs through the posttranscriptional control of downstream genes. Repression of translation of the mRNAs that encode the Notch inhibitor Numb and the cell cycle inhibitor p21WAF have been reported.[33,34]

Fujimoto et al reported that the integrin subunit β1 (CD29) was a candidate surface marker for the proliferative zone of the human colonic crypt, which includes SCs and progenitor cells.[35] They noticed that the cells located in the lower third of crypts expressed higher levels of CD29 than the cells in the remainder of the crypt. When crypt cells were isolated by flow cytometry based on CD29 levels, 2 cell populations that had different abilities to form colonies were identified.

More recently, Barker's group used lineage-tracking experiments to identify a unique marker of normal colon SCs, the Wnt target gene leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5).[36] Lgr5 is an orphan G-protein–coupled receptor of unknown function. In the intestine, it marks actively cycling cells, contradicting the concept that SCs are quiescent. However, Lgr5 did mark cells that were responsible for in vivo reconstitution of the complete small intestinal and colon epithelial lining. Moreover, a single Lgr5$^+$ cell from the intestine could regenerate a complete crypt-like structure in vitro (in Matrigel).[37] More recently, doublecortin and CaM kinase-like-1 (DCAMKL-1), a microtubule-associated kinase expressed in postmitotic

Table 1. List of Colon Stem Cell and Cancer Stem Cell Markers



Marker	Other name	Function	References
Msi-1		RNA-binding protein	29
CD29	Integrin b1	Cell adhesion molecule	35
Lgr5	GPR49	Unknown, Wnt target gene	36
DCAMKL-1		Kinase	38
CD133	Prominin 1	Self-renewal, Tumor angiogenesis	70, 71 72, 75
ESA	EpCAM, BerEp4	Cell adhesion molecule	79
CD44	CDW44	Cell adhesion molecule, Hyaluronic acid receptor	79, 81, 82
CD166	ALCAM	Cell adhesion molecule	79, 81
Msi-1		RNA-binding protein	80
CD29	Integrin b1	Cell adhesion molecule	81
CD24	HSA	Cell adhesion molecule	81
Lgr5	GPR49	Unknown, Wnt target gene	81
ALDH1	ALDC	Enzyme	87

neurons, has been proposed as a putative colonic SC marker.[38] DCAMKL-1 was found expressed in the same cells as Msi-1, but likely represented a subset of Msi-1–expressing cells. DCAMKL-1$^+$ cells were found apoptosis-resistant following radiation injury. Twenty-four hours after ionizing radiation exposure, only few stem/progenitor cells were in fact removed by apoptosis, and the potential descendants were able to divide and, at least transiently, express DCAMKL-1. Exposure to lethal doses of ionizing radiation highlighted that DCAMKL-1 expression is absent in the regenerative crypt when the proliferation is at its peak, but it is restored 7 days after irradiation. Importantly, DCAMKL-1 identified a population of quiescent cells, contrary to data obtained from Hans Clever's group, which identified a population of actively cycling SCs using the Lgr5 marker.

Table 1 provides a list of normal colon SC markers.

Intestinal SC Niche

Niches are the physical environments that maintain SCs in a variety of tissues, including human colon.[39] In the colon, they have been described as structures most likely formed by intestinal subepithelial myofibroblasts (ISEMFs) located at the base of the crypt. ISEMFs are activated and proliferate in response to various growth factors, including members of the platelet-derived growth factor family.[40] ISEMFs within the intestine are involved in organogenesis, protection from harmful agents, and repair after damage.[41] ISEMFs are also believed to regulate intestine SC self-renewal and differentiation by secreting hepatocyte growth factor, transforming growth factor-β, and keratinocyte growth factor, whose receptors are present on the epithelial cells.[42]

Extrinsic and intrinsic signals have been shown to regulate SC niches. Apart from ISEMFs, maintenance of the intestine SC niche is regulated by Eph/ephrin family of receptor tyrosine kinases. EphB/ephrinB signaling is required to establish cell compartments and to organize ordered migration of epithelial cells along the crypt axis.[43] Holmberg et al reported that Wnt proteins are present at the bottom of crypts and interact with receptors on epithelial cells, resulting in nuclear β-catenin-induced proliferation. β-catenin stimulates expression of the EphB receptors, which interact with ephrin ligands higher in the crypt to extend the domain of proliferation.[44]

Apart from Wnt, other signaling pathways, including those mediated by bone morphogenetic protein (BMP), Notch, and Sonic hedgehog (Shh), have been identified as key regulators of the SC niche.[42] This type of epithelial–mesenchymal signaling is likely to define a border between the proliferative SC niche and the differentiated epithelium by restricting Wnt-expressing cells to the crypt base.[45] Differential expression of BMP pathway components along the colon crypt axis has been reported. In the colon top, BMP1, BMP2, BMP5, BMP7, SMAD7, and BMP receptor 2 are highly expressed, whereas the basal crypt exhibits high expression of 3 BMP antagonists, gremlin 1 (GREM1), gremlin 2 (GREM2), and chordin-like 1. GREM1, GREM2, and chordin-like 1 likely originate from myofibroblasts and smooth muscle cells and contribute to create the colonic epithelial SC niche through modulation of Wnt activity.[42]

Niches regulate SC fate, ensuring the correct balance between SC self-renewal and differentiation. Contrary to immortal SCs, which always divide asymmetrically, SCs in niches sometimes expand by producing 2 daughters

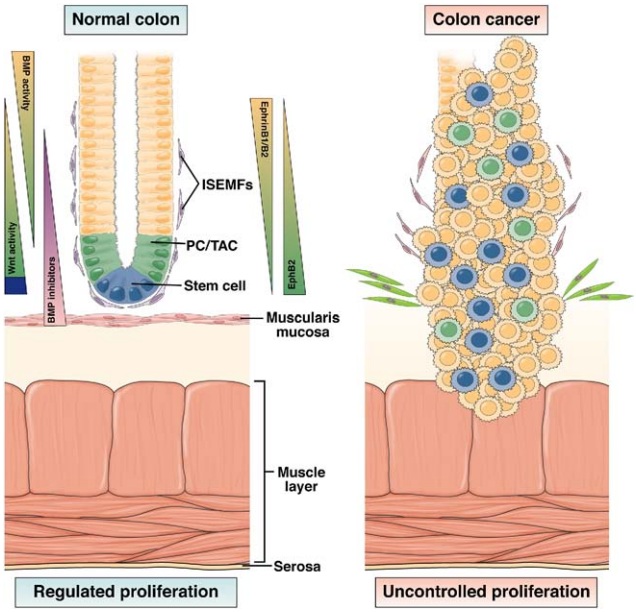


Figure 2. Model of the epithelial-mesenchymal signaling that defines SC niche in normal or cancer intestine. (*Left*) Intestinal subepithelial myofibroblasts (ISEMFs) surround the crypt base, a commonly proposed location for the intestinal SC niche and regulate epithelial SC function by paracrine secretion of growth factors and cytokines. High levels of Wnt signaling in the lower region of the crypt induce expression of EphB receptors, which in turn interact with EphrinB higher in the crypt. Bone morphogenetic protein (BMP) inhibitors, produced by muscolaris mucosa are expressed in a counter gradient, with the highest levels at the crypt bottom. As a consequence, BMP activity is highest in the upper region of the crypt and may permit Notch and Shh signaling to affect cellular differentiation of the intestinal lineages. (*Right*) The derangement of the above-mentioned pathways within SCs mediates the development of malignancy within the intestinal tract.

that remain within the niche or become extinct by producing 2 daughters that leave the niche and differentiate[39] (Figure 2).

Intestinal crypt-villus units could be self-organizing structures that can be generated from a single SC in the absence of a cellular niche. This was proven by Sato et al, who assessed a method for establishing long-term culture conditions whereby single SCs or single crypt structures isolated from intestinal crypts produced organoids that contained all the differentiated cell types and architecture of intestinal crypts present in adult mammals.[37] Further studies are required to determine how these findings relate to epithelial–mesenchymal interactions.

Colorectal Carcinoma

Maintenance of genomic integrity is ensured in colonic and other types of cells by a series of cell cycle checkpoints. These prevent transmission of damaged or incompletely replicated chromosomes by stalling the cell cycle until repairs are made or, if repairs cannot be made, by targeting the cell for destruction via programmed cell death. Factors involved in checkpoint signaling can be classified as sensors, mediators, transducers, or effectors.[46] The tumor suppressor p53 is an effector molecule that guards the genome by arresting cell cycle progression in G_1 or promoting apoptosis.[47] Apart from the G_1 checkpoint, other DNA damage checkpoints include the intra-S phase checkpoint and the G_2/M checkpoint.[48] Alterations in cell proliferation and apoptosis in colonic mucosa that result from deregulation of these intricate pathways increase risk for CRC. The multistep progression requires years and is accompanied by a number of genetic alterations. The pathologic transformation of normal colonic epithelium can lead to dysplastic epithelium, formation of adenomatous polyps, and, ultimately, invasive CRC. Each step in CRC tumor progression results from well-defined alterations in the genome. In one model, mutations in *adenomatous polyposis coli* gene (*APC*) lead to hyperproliferation and formation of an adenoma of class I; K-ras activation leads to adenoma of class II; loss of *deleted in colorectal cancer* (*DCC*) results in adenoma class III; and then invasive cancer results from *p53* mutations (Figure 3).[49] Not of these mutations are required for tumor progression, many more genes and steps can be involved and alternative pathways have been proposed for development of the inherited CRCs.[50] A better understanding on how many genes are mutated in a human

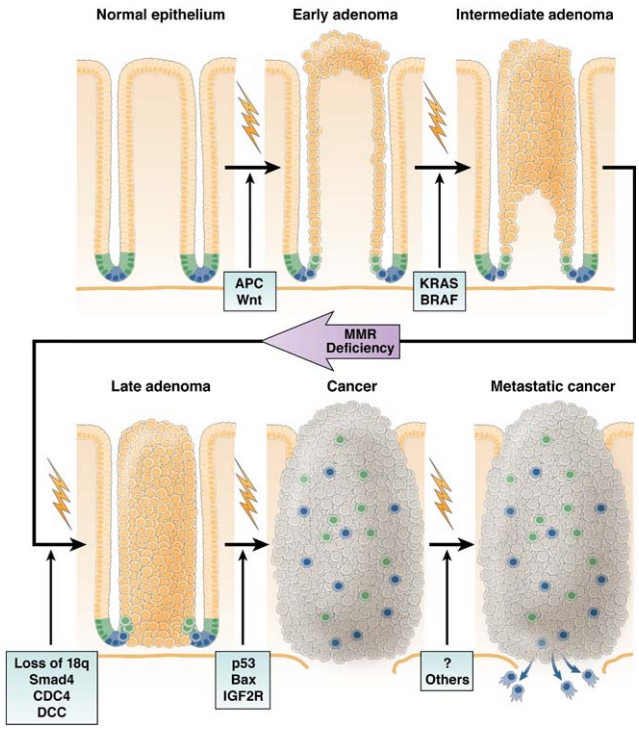


Figure 3. Schematic presentation of the development of colon carcinoma adapted from Vogelstein and Fearon. Mutations in *APC*, a strong negative regulator of the Wnt pathway, lead to hyperproliferation and formation of an early adenoma; *BRAF* and *KRAS* mutations occur at the stage of intermediate adenoma; loss of heterozygosity at 18q, comprising *Smad4, cell division cycle 4* (*CDC4*), and *DCC*, or mismatch repair (MMR) deficiency result in late adenoma; invasive cancer results from *p53, Bax, and/or insulin-like growth factor receptor 2* (*IGF2R*) mutations; other unknown factors are responsible for metastatic cancer.

tumor came from the study of Wood et al.[51] Through a genome sequence analysis on colon and breast cancers, this group has recently suggested a landscape composed by gene "mountains," represented by those that are frequently altered and have been the focus of cancer research for years (ie, *APC, K-ras, p53*), and gene "hills," mutated in relatively few cancers. These latter appear now to dominate the scene. Particularly, in a systematic search of 18,191 genes representing >90% of the protein-coding genes in the human genome, it was found that an average 77 genes are mutated in an individual colon cancer and 81 in breast cancer. Of these, about 15 are likely to be responsible for driving the initiation, progression, or maintenance of the tumor, and most of these genes may be different for each patient. Importantly, the type of mutations found in colon and breast cancers was quite different, being mutations converting 5′-CpG to 5′-TpG much more frequent in the first group than in the second, suggesting that epigenetic changes could have a driving role in colon carcinogenesis.

Constitutive activation of Wnt signaling represents one of the leading causes of CRC; alterations in the Shh signaling pathway that regulates normal colon cell proliferation are also involved in CRC pathogenesis. Douard et al reported increased transcription of *Shh* in neoplastic tissues from patients with CRC, compared with normal tissues.[52] Shh activation correlated with downstream activation of the transcription factors GLI1 and FOXM1, which induce proliferation. Dysregulation of the Notch pathway could also lead to CRC, because Notch activation expands the population of proliferating intestinal progenitors by inhibiting cell differentiation.[53] Finally, the BMP signaling pathway is involved in CRC. Up to 50% of individuals with juvenile polyposis, an inherited syndrome with a high risk for CRC, carry germline mutations in BMP pathway components.[54,55] Furthermore, the BMP pathway is inactivated in most sporadic CRCs.[56] The large-scale approach by Wood et al has actually revealed that the vast majority of pathways preferentially mutated in CRC, as well as in breast cancer, centers on phosphatidylinositol 3-kinase (PI3K) pathway, suggesting that drugs targeting this pathway could be efficacious in cancers with such mutations.[51]

CSC Markers

Cancer cells with stem-like features were first observed in acute myeloid leukemia (AML) and later found in other tumor types. In most cases, such cells have been identified through their expression of specific cell surface markers. The $CD34^+CD38^-$ and the $CD44^+CD24^-$ phenotypes were the first signatures associated with AML and breast tumors, respectively.[57,58] One of the next CSC markers identified was CD133, a pentaspan transmembrane glycoprotein also known in humans as Prominin 1. The $CD133^+$ population is enriched in cancer-initiating cells in many tissues, including retinoblastoma,[59,60] teratocarcinoma,[60] brain tumor,[61,62] kidney cancer,[63] prostate tumor,[64] hepatocellular,[65] and colon carcinomas.[66,67] Nonetheless, use of CD133 as a marker for identification and isolation of colon CSCs is a subject of debate; despite its use in isolating cell populations with cancer-initiating ability, studies have shown that CD133 is expressed by SCs and more differentiated progenitor cells.[68] CD133's function is unclear, although it is believed to have a role in asymmetric division and self-renewal. Bauer et al proposed that the polarized localization of CD133 indicates its role in regulating proliferation.[69] CD133 is concentrated in cell surface domains that correspond to the spindle pole region during metaphase. In telophase and cytokinesis, it is either equally or unequally distributed between the 2 nascent daughter cells. Studies have indicated a role for CD133 in tumor angiogenesis. $CD133^+$ glioma cells produce proangiogenic factors that can directly modify endothelial cell behavior.[70] Other data indicate that the $CD133^+$ cell population can itself give rise to endothelial cells that promote vascularization and tumor growth, like renal progenitor cells do.[71] Within the intestine, CD133 would mark SCs susceptible to neoplastic transformation. These cells would be in fact prone to aberrantly activate Wnt signaling and such event would disrupt normal tissue maintenance leading to their aberrant expansion, resulting ultimately in neoplastic transformation of the intestinal mucosa.[72]

Beyond colon and liver, other cell surface markers are used to identify gut CSCs. Pancreatic CSCs were initially characterized based on expression of CD44, CD24, and epithelial specific antigen,[73] yet recent publications indicate CD133 as a marker for tumorigenic CSCs in this tumor type.[74] CSCs from esophageal squamous cell carcinomas have been instead identified through aberrant Hedgehog pathway activation and ongoing data from the same group have been indicating that Bmi-1 signal activation could also be involved in emergence of aggressive esophagus cancer progenitor cells.[75] Finally, although data obtained from a mouse model of *Helicobacter*-induced gastric cancer have implicated BMDCs as a potential source,[76] a more recent work on a panel of human gastric cancer cell lines has proposed resident tissue $CD44^+$ cells as the candidate for CSCs.[77]

Identification of Colon Cancer–Initiating Cells

The existence of colon CSCs was first reported by the research groups of John Dick and Ruggero De Maria,[66,67] which independently described a small population of cancer cells capable of initiating tumor growth in immunodeficient mice. By implanting limiting dilutions of human colon cancer cell suspensions into preirradiated nonobese diabetic severe combined immunodeficient mice, O'Brien et al demonstrated that only a small subset of colon cancer cells ($1/5.7 \times 10^4$ total cells) initiated tumor growth.[66] Using flow cytometry, Ricci-

Vitiani et al detected a rare population of $CD133^+$/cytokeratin (CK) 20^- cells in colon tumor samples (2.5% ± 1.4% of total cells).[67] CK20 is considered a colonic epithelial terminal differentiation marker and therefore to be absent in the SC compartment. Based on immunohistochemical analyses, these cells were present in areas of high cell density.[67] The tumorigenic potential of colon $CD133^+$ cells was next analyzed by comparing the ability of $CD133^-$ and $CD133^+$ populations to engraft and give rise to subcutaneous tumors in severe combined immunodeficient mice. Low numbers of $CD133^+$, but not high numbers of $CD133^-$, engrafted and formed tumors; high numbers of unsorted cells gave rise to tumors but, despite the high number of $CD133^+$ among them, tumor formation took more time.[66,67]

Because $CD133^-$ cells were positive for epithelial specific antigen (ESA) and p53, when the tumors they were derived from were $p53^+$, O'Brien et al concluded that the $CD133^-$ cells were malignant cells likely generated from asymmetric division of $CD133^+$ cells. Importantly, although significantly enriched, not every $CD133^+$ cell could initiate tumor formation. Limiting dilution assays revealed that only 1 of 262 $CD133^+$ colon cancer cells could induce formation.[66]

$CD133^+$ cells could be isolated by plating of single cells from cancer tissues in serum-free medium in the presence of epidermal growth factor and basic fibroblast growth factor. In these in vitro cultures, $CD133^+$ cells formed sphere-like aggregates, proliferated at an exponential rate, and displayed long-term tumorigenic potential; increasing aggressiveness with number of in vivo passages.[67] Growth factor deprivation and the presence of serum in cultures induced differentiation along with loss of CD133 expression and gains of CK20 and caudal type homeobox transcription factor 2 expression.[67] Moreover, under differentiation conditions on Matrigel, only tumorigenic $CD133^+$ cells were able to generate colonies organized in crypt-like structures.[78] Importantly, $CD133^+$ cells were found resistant to apoptosis because they produce interleukin-4 (IL-4).[78]

Dalerba et al found that the $CD133^+$ cell population contained stem-like epithelial specific $antigen^{high}/CD44^+$cells and proposed that CD44 and CD166 were markers of colon CSCs.[79] We found that $CD133^+$ colon cancer cell spheroids grown in vitro also express Msi-1[80] and consist of heterogeneous populations of cells;[81] although all the cells express CD133, different subpopulations express CD166, CD44, CD29, CD24, or Lgr5 and have nuclear localization of β-catenin. Cells that express CD133 and CD24 have clonogenic potential and multilineage differentiation; $CD133^+/CD24^+$ cells differentiate into goblet-like, enterocyte-like, and neuroendocrine-like cells. Lineage is partly determined by activation of the PI3K pathway, because PI3K inhibition with LY294002 resulted in an enterocyte-like differentiation pattern. During differentiation, CD133 and CD24 were most rapidly down-regulated, followed by CD44. Surface levels of CD29 and CD166 underwent only limited changes upon differentiation. Du's group found that $CD44^+$ and $CD133^+$ cells did not colocalize in the same region of CRC tissues, and that a single $CD44^+$ cell could give rise to a sphere in vitro with SC features, and to a xenograft tumor in vivo with the properties of the original tumor, concluding that CD44 is a robust marker and is of functional importance for colon CSCs.[82] Choi et al demonstrated that levels of CD133 and CD24 correlated with invasiveness and differentiation of CRC cells, although Kaplan-Meier survival curves and log-rank tests showed no correlation between patient survival and these markers.[83] Horst et al reported that CD133 was a prognostic factor for CRC, but its functional role has not been defined; CD133 knockdown in colon cancer cell lines did not affect proliferation, migration, invasion, or colony formation.[84]

Additional discrepancies about CD133 expression and function have arisen from studies by Shmelkov et al, who stated that CD133 is not a specific marker of organ-specific stem and progenitor cells.[68] In reporter studies of the *CD133* promoter, the authors showed that the gene is ubiquitously expressed in differentiated colonic epithelium of adult mice and humans. When the extent of CD133 expression within the hierarchy of cells in primary colon cancers was investigated, CD133 was widely expressed in all primary colonic tumors examined. However, in such tumors, the majority of stromal and inflammatory cells were $CD133^-$. In analyzing samples of human CRCs that metastasized to liver, 40% were negative for CD133; Shmelkov et al concluded that $CD133^+$ cells are not necessary for metastasis. Conversely, $CD133^+$ and $CD133^-$ subpopulations isolated from the $CD133^+$ liver metastases were able to form tumors following subcutaneous injection into mice, even following 2nd, 3rd, and 4th rounds of transplantation. Interestingly, the $CD133^-$ population always initiated tumor growth earlier than the $CD133^+$ population and sustained a faster rate of tumor growth. Moreover, $CD133^+$ and $CD133^-$ tumor subpopulations were capable of forming colonospheres, in vitro at similar rates. $CD133^-$ fraction-derived clones were $CD133^-CD44^+CD24^-$, whereas $CD133^+$ fraction-derived clones were $CD133^+CD44^{low}CD24^+$. Thus, according to Shmelkov et al, the $CD133^-$ fraction of colon cancer is more enriched for colon cancer–initiating cells.

Yi et al reported that in CRC and glioblastoma, the absence (or low levels) of CD133 protein results from hypermethylation at a CpG island in the proximal promoter of CD133.[85] Jaksch et al found that in cultured cells, reactivity to an anti-CD133 antibody correlated with the cell cycle profile of colon CSCs,[86] because CD133 expression was highest in cells with 4N DNA content and lowest in cells with 2N DNA. Thus, the differential expression of CD133 could simply reflect stage of the cell cycle, rather than being a differentially expressed, stable SC lineage marker. Moreover, resistance

to chemotherapy or radiotherapy might be related to expression of molecules such as the antiapoptotic protein survivin, which increases in the G_2-M phase of the cell cycle. So, cells with surface CD133 might be resistant to death stimuli because of their cell cycle stage, rather than because they are apoptosis-resistant CSC.

The promoter region of human *CD133* contains 5 different promoter regions with multiple corresponding exons that can be alternatively spliced. No significant differences have been observed in promoter activity between CSCs and differentiated cancer cells or in protein expression pattern. Loss of CD133 during differentiation occurs at the posttranslational level, due to a conformational change of the protein on the cell surface that makes it undetectable by the antibodies used to analyze CD133 surface levels. Apart from the differentiation status of the cell, the level of CD133 measured can be influenced by the methods used to detect it; protocols that involve cell fixing or permeabilization could change the 3-dimensional structure of the CD133 protein. Cells isolated from the same tumor can have different protein expression profiles based on the detection procedure used.

Another potential colon CSC marker is aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme that oxidizes intracellular aldehydes and converts retinol to retinoic acid. Because of its function, ALDH1 could protect SCs against oxidative insult, allowing for longevity and also modulate SCs proliferation. Huang et al[87] described subsets of $CD44^+$ or $CD133^+$ cells that were positive for ALDH1 and located at the base of the normal crypt, During colon tumor progression to carcinoma, the number of cells positive for all 3 markers ($CD44^+$, CD133, and ALDH) increased and were distributed further up the crypt axis. Human cancer cells, isolated based on enzymatic activity of ALDH and injected into nonobese diabetic severe combined immunodeficient mice, formed tumors. Selection of $CD133^+$, $CD44^+$ cells with ALDH activity enriched somewhat the CSC population. Table 1 presents the markers that have been proposed to characterize CRC SCs.

Limitations of CSC Theory

The CSC theory has been proven in xenograft experiments. However, studies in animal models might underestimate the frequency of cells with tumorigenic potential. Quintana et al reported faster growth of human melanoma and a higher frequency of melanoma cancer-initiating cells in nonobese diabetic combined immunodeficient interleukin-2γ receptor knockout mice (NOD/SCID *Il2rgl*$^-$, which lack T, B, and natural killer cells.[88] Moreover, injection with or without Matrigel also strongly affected the frequency of cells with tumorigenic potential. Therefore, modifications in xenotransplantation assays can increase the detection of cancer-initiating cells. Expression analysis of 50 surface markers revealed that no marker could be used to distinguish tumorigenic from nontumorigenic cells.[88] Therefore, melanoma tumorigenic cells are phenotypically heterogeneous and are not organized in a hierarchical fashion, as the CSC model sustains. Instead, these melanoma cells appear to follow the clonal evolution model described by Nowell in 1976[89]: tumor progression results from acquired genetic variability within the original clone that allows sequential selection of more aggressive sublines. Each model supports the assumption that tumors originate from a single cell that has acquired multiple mutations and gained unlimited proliferative potential. However, the CSC hypothesis presumes that a normal stem or a progenitor cell is the target of malignant transformation and gives rise to a population of genetically identical cancer cells, of which only a small subset maintains the original SC properties and contribute to tumor progression. Actually, a CSC could originate from a differentiated cell through a mutation, conferring limitless replication potential. The clonal evolution model proposes that any normal cell can be transformed and that all of its daughter cells can acquire additional mutations, forming a mass of genetically varied cancer cells that promote tumor progression. As a consequence, the frequency of tumorigenic cells is small in the CSC model and high in the clonal evolution model.

The intrinsic differences between tumorigenic and nontumorigenic cells are thought to derive from epigenetic changes in the CSC model and a combination of epigenetic and genetic changes in the clonal evolution model.[90] Consequently, according to the clonal evolution model, a tumor can be composed of heterogeneous or homogeneous populations. This has implications for therapy—if most tumors arise through the clonal evolution model, all the cells should be targeted therapeutically. According to the CSC model, however, the few self-renewing CSCs that mediate tumor growth are difficult to kill and their persistence might explain tumor recurrence after therapy.

Therefore, to assess the efficacy of therapeutics, it is necessary to accurately distinguish tumorigenic from nontumorigenic cancer cells and to understand which progression model occurs in the tumor.

There might be a small fraction of CSCs that mediate CRC progression and recurrence. We propose that CRC begins as an SC disease but then progresses by clonal evolution of its CSCs. Recent evidence indicated that targeting APC mutations to the $Lgr5^+$ SCs in the small intestine of mice led to formation of large polyps, whereas targeting of the transient amplifying cells did not.[91] Most of the oncogenic mutations found in CRC cells are likely to accumulate during pretumor progression via sequential cycles of mutations in SCs followed by crypt niche dominance by the mutant SCs. Such mutations confer no visible changes during this phase.[92] Clonal evolution is likely to continue during tumor progression, because more-advanced neoplasms have more mutations, with re-

spect to premalignant lesions, such as adenomas.[49] Heterogeneity in CRC appears to be a clonal trait; colon spheroid cultures are heterogeneous, with respect to marker expression and Wnt signaling activity.[81] Although the majority of CRC cells are CK20$^-$, CD133 expression is heterogenous. Moreover, CD24, CD29, CD44, and CD166 are also expressed on a subpopulation in those spheroid cultures. Importantly, only a small number of cells have β-catenin nuclear localization, indicating varying degrees of Wnt signaling activity. Therefore, the CSC hypothesis is not likely a universal model that applies to all cancers or all patients with the same disease; care should be exercised when generalizing concepts derived from specific human malignancies or models.

Clinical Perspectives

CRC is the second leading cause of cancer-related death in the world.[93] Nearly all colon cancers begin as benign polyps that can slowly develop into malignant tumors. Colonoscopy can be used to screen for precancerous polyps so that they can be removed before malignant transformation. However, only about 39% of CRCs are found at an early stage; CRC is metastatic (CRM) at the time of diagnosis in >60% of cases. When metastases are found at distant sites, 5-year survival is <10%. The liver is the most common site of metastatic disease in patients with CRC.

Two different protocols are appropriate first-line treatments for patients with metastatic CRC to the liver: a combination of 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) and a combination of 5-fluorouracil, leucovorin, and irinotecan (FOLFIRI). FOLFOX and FOLFIRI have demonstrated good efficacy in phase III trials and are actually employed more frequently in younger than older patients with metastatic CRC, likely to improve resection rates.[94]

Neoadjuvant chemotherapy has been combined with antiangiogenic drugs, particularly with bevacizumab (Avastin; a recombinant monoclonal antibody that targets vascular endothelial growth factor) and cetuximab (Erbitux; an antibody that inhibits the epidermal growth factor receptor).[95,96] Although these types of combination therapies have increased disease-free survival and improved overall survival in patients with CRC, most patients with metastatic disease are not cured.

Because chemotherapeutics interfere with the ability of rapidly growing cells to divide, CSCs might be spared, leading to tumor recurrence and metastasis. Because CRC growth is believed to be mediated by CSCs, improving our understanding of CSC behavior could lead to targeted therapies for this cancer type. Unfortunately, the complex network of mechanisms that regulate SC renewal and carcinogenesis are not clear. It might be possible to modulate SC signaling as a therapeutic approach for CRC. Small-molecules that inhibit the Wnt pathway and γ-secretases that inhibit the Notch pathway have been recently identified as novel approaches to CRC therapy.[97] Advances in high-throughput technologies and bioinformatics will allow for development of additional reagents targeting SC signaling pathways. However, it is important to remember that CSC cultures and animal models do not reproduce, with high fidelity, what happens during human tumor initiation and progression.

Exposure of colon CSC-derived xenografts to oxaliplatin reduced tumor size, but significantly increased in the percentage of CD133$^+$ cells.[78] Enrichment of CD133$^+$ cells has been reported to occur also in pancreatic cancer following gemcitabine therapy.[74,98] Mueller et al[98] showed that neither inhibition of the Shh pathway with cyclopamine nor inhibition of mTOR signaling with rapamycin, but only the combination of inhibitors of these pathways could deplete the pancreatic CSCs pool. Interestingly, in an animal model for pancreatic cancer, combined therapy with cyclopamine, rapamycin, and gemcitabine was tolerated and resulted in tumor-free, long-time survival.

In CRCs, inhibiting the IL-4 signaling transduction pathway with an anti–IL-4 neutralizing antibody or an IL-4 receptor α antagonist sensitized CSCs to chemotherapeutics through down-regulation of antiapoptotic proteins, such as cFLIP, Bcl-xL, and PED.[78] Furthermore,

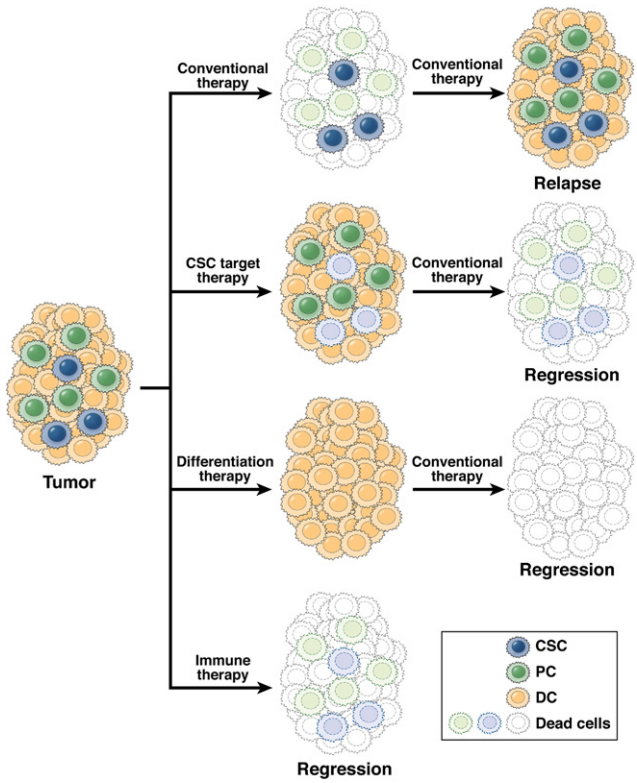


Figure 4. Strategies for CSC sensitization. CSCs are believed to be spared from most anticancer therapies, such as chemotherapy. Inhibitors of survival pathways, along with differentiation-inducing agents, immune cells, and cytotoxic chemotherapeutics, might be used to destroy CSCs and induce complete tumor regression.

incubation of colon CSCs with the bisphosphonate zoledronate induced an efficient γδ T-cell response. The potent major histocompatibility complex–unrestricted activity of these immune cells against different tumor cells in vitro has been documented, but this was the first report of using γδ T cell to target CSCs.[99] An immune therapy approach has been applied also to SCs from AML and human bladder.[100,101] In these cancers, the blockage of the immunoglobulin-like CD47 protein rendered the cancer-initiating population susceptible to innate and adaptive immune system clearance by restoring its phagocytosis by macrophages.

Other therapeutic options, such as the induction of CSC differentiation, are being developed. Salinomycin, a highly selective potassium ionophore, was recently described as the first compound that can selectively eradicate the tumor through induction of terminal epithelial differentiation of CSCs. Gupta et al revealed that salinomycin decreases the proportion of $CD44^{high}/CD24^{low}$ breast cancer cells, whereas paclitaxel has opposing effects. Importantly, cells exposed to salinomycin were less capable of inducing tumors following injection into mice; salinomycin also slowed the growth of the animals' tumors through unknown mechanisms.[102] Salinomycin is thought to inhibit potassium-positive channel-regulated migration and interfere with the epithelial-mesenchymal transition and metastasis.

BMP4 is able to activate a differentiation program and stimulate apoptosis in colon CSCs, reducing β-catenin activation through inhibition of PI3K/AKT pathway and up-modulation of Wnt-negative regulators. The anti-tumor activity of BMP4 is increased by oxaliplatin and 5-flouroucil; concomitant administration of these drugs induces complete, long-term regression of colon CSCs-derived xenograft tumors.

Inhibitors of survival pathways, along with immune cells, differentiation agents, and cytotoxic drugs, might be used in combination to treat patients with CRC and other cancers. Figure 4 shows a picture describing some strategies for sensitizing colon CSCs.

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Conflicts of interest

The authors disclose no conflicts.

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