Published in final edited form as:

Curr Colorectal Cancer Rep. 2011 June ; 7(2): 128–135. doi:10.1007/s11888-011-0093-2.

Colorectal Cancer Stem Cells: Biology and Therapeutic Implications

Brian J. Wilson

Transplantation Research Center, Children's Hospital Boston and Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, 300 Longwood Avenue, Enders 814, Boston, MA 02115, USA
bwilson2@bics.bwh.harvard.edu

Tobias Schatton

Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital and Transplantation Research Center, Children's Hospital Boston, Harvard Medical School, 77 Avenue Louis Pasteur, HIM 660, Boston, MA 02115, USA tschatton@partners.org

Markus H. Frank

Transplantation Research Center, Children's Hospital Boston and Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, 300 Longwood Avenue, Enders 816, Boston, MA 02115, USA
markus.frank@childrens.harvard.edu

Natasha Y. Frank

Department of Medicine, Boston VA Healthcare System, Transplantation Research Center, Children's Hospital Boston and Division of Genetics, Brigham and Women's Hospital, Harvard Medical School, 300 Longwood Avenue, Enders 814, Boston, MA 02115, USA

Abstract

The hypothesis that cancer is driven by a subpopulation of tumor-initiating or cancer stem cells (CSC), defined by their selective ability for extensive self-renewal and capacity to give rise to nontumorigenic cancer cell progeny through differentiation, has been validated experimentally in diverse human malignancies. Translational relevance of the CSC hypothesis is underlined by emerging novel strategies designed to target all subpopulations within a given tumor in order to effect cancer eradication and improve patient outcomes. Colorectal cancer stem cells (CRSCs) have been identified and successfully isolated by several research groups based on distinct cell-surface marker characteristics. Identification of CRSC populations has led to a wave of discoveries describing novel self-renewal and drug resistance mechanisms in colorectal cancer that represent novel future therapeutic targets. In this review, we will discuss emerging CRSC-specific pathways and the therapeutic promise of targeting this cancer population in colorectal cancer patients.

Keywords

Colorectal cancer; Stem cells; CD133; CD44; ALDH1; ESA; CD166; Therapy; Chemoresistance; Radioresistance; Targeting; ABCB5; 5-FU; Oxaliplatin; Wnt; APC; Lgr5; SMAD4; BMP4; Notch; Il-4; Aurora-A kinase

Introduction

Despite the wealth of novel approaches to cancer therapy, colorectal cancer (CRC) remains the leading cause of cancer-related deaths for men and women in the United States [1]. While the majority of CRC patients present with surgically resectable tumors, many of them eventually succumb to metastatic disease originating from residual microscopic malignancy not evident at the time of surgery [2]. Adjuvant therapies, including radiotherapy and chemo-therapy, are designed to target residual tumor cells; however, their current success is limited by the existence of therapy-resistant cancer cell populations [3, 4]. This highlights the urgent need for development of novel therapeutic strategies to target alternative tumor pathways with the ultimate goal of successful cancer eradication.

CRC displays a well-defined multistage progression in terms of both pathogenesis and molecular events [5], which has been associated, at the DNA level, with cumulative alterations in oncogenes, tumor suppressor genes, and repair/stability genes. At the molecular level several signal transduction pathways have been identified as key regulators of the malignant transformation process, and studies of familial CRC syndromes have led to discovery of distinct critical genetic alterations that also have been subsequently implicated in sporadic carcinogenesis. Functional importance of Wnt signaling in colorectal carcinogenesis was highlighted by the discovery of dysfunctional mutations in the adenomatous polyposis coli (APC) gene as the cause of the hereditary cancer predisposition syndrome, familial adenomatous polyposis (FAP) [6, 7]. In addition to FAP-related malignancies, APC mutations have been also found in 80% of sporadic CRCs, where, in addition to APC, mutations in other Wnt signaling cascade genes, including β -catenin, have been described [8]. Similarly, genetic alterations of the BMP4 signaling pathway, such as mutations in the *BMPRIA* and *SMAD4* genes, have been first identified in the hereditary cancer predisposition syndrome, juvenile polyposis (JP) [9, 10] and later, *SMAD4* somatic mutations were found in 30% of sporadic CRCs [11]. Studies of hereditary nonpolyposis CRC (HNPCC) led to the discovery of mutated mismatch repair genes, including *MLH1*, *MSH2*, *PMS1*, *PMS2*, and *MSH6*, which were subsequently also detected in many sporadic cancers [12]. This genetic heterogeneity is also reflected in the phenotypic heterogeneity of CRC, which is characterized by differences in tumor localization, aggressiveness, and therapeutic response.

At the cellular level, human cancers can consist of phenotypically heterogeneous cell populations [13]. In addition, tumor cells can exhibit functional heterogeneity, differing, for example, in growth characteristics in proliferation assays in vitro [14] or differing in their potencies to initiate and sustain tumorigenic growth in vivo [15]. These differences can only in part be explained by ongoing genetic mutations. This conclusion has led to the development of the CSC model of cancer initiation and growth [16]. The CSC theory suggests that only a CSC subpopulation of cells within a tumor can proliferate extensively and drive tumorigenic growth [3, 16], and give rise to morphologically and functionally diverse descendant cell populations, including non-tumorigenic bulk components (Fig. 1). Thus, the term “cancer stem cell” [3, 17, 18, 19•, 20•, 21•, 22], characterizes cancer subpopulations that contain a cell component exhibiting increased in vivo tumorigenicity, a perpetual capacity for prolonged in vivo self-renewal, and an ability to generate phenotypically diverse populations of non-tumorigenic cancer cells resembling the original patient tumor in serial xenotransplantation experiments.

There is now increasing evidence in support of the CSC hypothesis [3]: CSC capable of self-renewal and differentiation, and responsible for experimental tumor growth, have been identified in human hematological malignancies [23, 24] and several solid tumors. These include cancers of the breast [17], brain [18], human malignant melanoma [22, 25], and

colon cancer [19••, 20••, 21••]. Furthermore, CSC have been found to be associated with tumor progression in human patients [3, 22]. These findings and the possibility that CSC-dependent cancer growth and metastasis might contribute to the failure of existing chemotherapies to eradicate malignant tumors [3, 16, 22], indicate that targeting of CSC represents a promising strategy to eradicate cancers currently resistant to systemic therapy [3].

Defining a Colorectal Cancer Stem Cell

There is now accumulating evidence for the existence of colorectal cancer stem cells (CRSCs) in human colorectal cancer [19••, 20••, 21••, 26] and for a potential contribution of CRSCs to clinical tumor progression, chemoresistance, and therapeutic failure. Highly tumorigenic and self-renewing CRSC population in human colon cancers have been successfully enriched in two separate studies using the CD133 surface marker [19••, 20••]. Performing limiting dilution xenotransplantation experiments with CD133-sorted cancer subpopulations in immunodeficient mice, both groups independently demonstrated that the capacity to initiate tumor growth was confined to the CD133⁺ colon cancer cell subset. The percent positivity of tumor cells identified by the CD133 marker ranged from 2% to 25% [19••] and 1% to 6% [20••], and as few as 1×10^2 [19••] and 1.5×10^3 CD133⁺ human cancer cells [20••] were found capable of inducing tumor formation while 1×10^5 CD133⁻ cancer cells failed to do so, respectively. However, in one study, 1 of 9 mice injected with 2.5×10^5 CD133⁻ tumor cells developed a tumor [19••], indicating that cancer bulk components might possess a limited capacity to proliferate. Alternatively, the observed tumorigenic growth could be explained by CD133⁺ contaminants in this cell inoculum. CRSC within the CD133-expressing population were able to self-renew as well as differentiate into tumor bulk populations and to initiate tumor growth and re-establish the original patient tumor heterogeneity in serially transplanted secondary, tertiary [19••], and quaternary mice [20••]. In addition, Ricci-Vitiani et al. [20••] showed that CD133⁺ colon cancer cells, unlike CD133⁻ cells, could be maintained in vitro as colon tumor spheres for more than 1 year, without losing their in vivo tumorigenic potential. A follow-up study by Shmelkov et al. [27], however, questioned the utility of CD133 as a colorectal cancer stem cell marker by demonstrating that CD133⁻ sorted colorectal cancer cells could form primary tumors and metastases and be serially passaged in vivo. In contrast, in support of the role of CD133 as a candidate CRSC marker, Li et al. [28] have shown that CD133⁺ single cell-derived clonal populations isolated from the SW480 human colorectal cancer cell line could generate tumors more efficiently than CD133⁻ cells, and that CD133^{high} populations displayed a much more aggressive phenotype than CD133^{-/low} cells, in terms of metastasis, implicating CD133 as a marker of increased tumorigenicity. In addition, only CD133⁺ single cell clinical tumor-derived cultures were shown to possess multilineage differentiation potential and were capable of tumor initiation in vivo [29].

Another subset of tumorigenic human colon cancer cells was identified by Dalerba et al. [21••] based on an epithelial cell adhesion molecule (EpCAM, also known as ESA)^{-high}CD44⁺Lineage⁻ phenotype. The frequency of tumor cells expressing this marker combination ranged from 0.03% to 38% of total viable tumor cells. In xenotransplantation experiments to NOD/SCID mice as few as 2×10^2 ESA^{high}CD44⁺Lineage⁻ cells were capable of forming xenografts that mirrored the morphologic and phenotypic heterogeneity of the respective patient tumors, while 1×10^5 ESA^{low}CD44⁻Lineage⁻ cells consistently failed to initiate tumor development. However, inoculation of 2×10^5 ESA^{low}CD44⁻Lineage⁻ colon cancer cells resulted in tumorigenic growth in one recipient mouse, and serial xenotransplantation experiments with sorted tumor cell populations for assessment of long-term self-renewal were not performed in this study [21••]. In contrast to the findings identifying CD133 as a marker of colon cancer-initiating cells [19, 20••], Dalerba et al.

[21••] described tumors scoring homogeneously negative for CD133 that were capable of forming tumors in NOD/SCID recipients. Some colon cancers, however, displayed high levels of CD133-positivity with only a minority of this cell population co-expressing the CD44 antigen. Interestingly, characterization of the co-surface marker repertoire of $\text{ESA}^{\text{high}}\text{CD44}^+\text{Lineage}^-$ cells identified the mesenchymal stem cell marker CD166, which was previously also found to be associated with clinical colon cancer progression [30] and stem-like cancer cells in human malignant melanoma [31], as a candidate marker for the enrichment of CRSC. Results from initial screening experiments indicated that tumorigenic cells might be restricted to the $\text{ESA}^{\text{high}}\text{CD44}^+\text{CD166}^+\text{Lineage}^-$ colon cancer population [21••]. Moreover, the possibility that there was a subset of colorectal cancer cells that co-expressed both CD133 and CD44 was independently confirmed in two established human colorectal cancer cell lines, HT-29 and Caco2, and the $\text{CD133}^+/\text{CD44}^+$ double-expressing cells were demonstrated to be more tumorigenic in vivo in a xenograft model relative to either marker alone [32]. In a later study the detoxifying enzyme, aldehyde dehydrogenase 1 (ALDH1), which confers chemoresistance to alkylating agents via oxidation of intracellular aldehydes and identifies stem cell populations in human breast cancer [33], was found overexpressed in both hyperplastic colonic crypts from FAP patients and, to a greater degree, in colonic adenomas [26]. ALDH1 activity-sorted cells (ALDH1^+) were shown to efficiently generate xenograft tumors, and could be serially passaged in vivo, while ALDH1^- cells were unable to do so. ALDH1^+ cells also represented a subfraction of CD133^+ or CD44^+ cells, implying that ALDH1 may serve as another putative CRSC marker.

Recent results, using genetic lineage tracking in murine cancer models, indicate that CRSCs may originate from physiological tissue stem cells. For example, Barker et al. utilized the novel intestinal stem cell marker, Lgr5 [34], to demonstrate that deletion of the Wnt signaling regulator APC in Lgr5^+ cells but not in Lgr5^- transient amplifying cells leads to the development of intestinal adenomas within 3 to 5 weeks [35]. In a separate study, Zhu et al. [36] showed that activation of endogenous Wnt signaling in CD133^+ cells via mutant β -catenin resulted in disruption of crypt architecture and in a disproportionate expansion of CD133^+ cells in the stem cell niche at the bases of intestinal crypts. Thus, several potential markers or marker combinations exist for the enrichment of CRSCs.

The functional relevance of the cell-surface markers used to identify cancer stem cells is one of the most important questions to be addressed, particularly when considering the design of future therapies. Determining cause or consequence in CRSCs for the CD133 or CD44 markers, Du and colleagues [37] used an RNA interference approach and showed that knockdown of CD133 had no effect on in vitro clonogenicity or in vivo xenograft tumor formation, demonstrating CD133 as a passive marker of CRSCs. However, in the same assays, knockdown of CD44 resulted in limited colony formation and dramatically reduced tumor formation in xenografts, strongly implying a functional role for CD44 in CRC tumorigenesis. These data found independent support in a separate genetic model that used crossing of the murine $\text{Cd44}^{-/-}$ knockout mice to $\text{Apc}^{\text{Min}/+}$ mice, which resulted in a significant reduction in the development of intestinal tract adenomas [38]. The CD44 transmembrane glycoprotein is a cell adhesion molecule linking extracellular matrix signaling from hyaluronate to the cell [39]. CD44 expression can also be controlled by Wnt signaling, the implication being that targeting of this pathway in colorectal cancer may also target the CRSCs [40, 41, 42••].

CRSC Therapeutic Resistance

Conventional cancer therapeutics, including radiation and chemotherapy, mainly rely on the premise that cancer cells are fast-cycling, and target certain phases of cell-division, such as

the S-phase thymidylate synthase inhibitor 5-fluorouracil (5-FU) or the DNA-adduct forming agent oxaliplatin. However, there is accumulating evidence that CRSCs exhibit chemoresistant behavior, which could be related to their slower-cycling growth characteristics. A large clinical study of 501 cases of human colorectal cancers showed that CD133-overexpressing tumors were more resistant to 5-FU-based chemotherapy and that CD133 expression was associated with poor prognosis [43]. Similar increase in CD133 cell frequency was observed in oxaliplatin-treated SW620 and LoVo human colorectal cancer cells [44]. A recent study by Dallas et al. [45••] showed that treatment of human HT-29 colorectal cancer cells with high doses of 5-FU or oxaliplatin resulted in enrichment of CD133⁺ and CD44⁺ CRSCs, which also exhibited decreased in vitro proliferation rate. The resistant HT-29 cells were observed to over-express insulin-like growth factor-I receptor (IGF-1R), and treatment with the IGF-1R inhibitor AVE-1642 resulted in significant inhibition of tumor growth in an in vivo xenograft model. Todaro et al. [46••] reported specific expression of interleukin-4 (IL-4) in CD133⁺ CRSC populations where it modulated death receptor-induced and chemotherapy-induced apoptosis. Pretreatment of CD133⁺ CRSCs with an anti-IL-4 neutralizing antibody significantly sensitized the cells to 5-FU-based or oxaliplatin-based therapy in vivo [46••]. Another mechanism of CD133⁺ CRSC chemoresistance to 5-FU and oxaliplatin was described by Cammareri et al. [47••], who showed that specific gene silencing of a spindle checkpoint regulator, Aurora-A kinase, resulted in decreased anti-apoptotic protein expression and sensitized CRSCs to chemotherapy-induced death. MicroRNAs (miRNAs) are known regulators of cellular proliferation and may also contribute to CRSC therapeutic resistance by modulating CRSC-specific signaling pathways. For example, miRNA-140 has been shown to confer a methotrexate and 5-FU resistant phenotype in CD133^{hi}CD44^{hi} colon cells by inhibiting cell proliferation through targeting histone deacetylase, HDAC4 [48••], whereas miRNA-215 enhanced the chemoresistance of CD133^{hi}CD44^{hi} colon cells to methotrexate and Tomudex by inhibiting cell proliferation and inducing G2-arrest through the suppression of a cell cycle-regulated nuclear and centrosome protein DTL [49••]. Taken together, these studies demonstrate preferential survival of CRSCs following chemotherapy, and highlight the importance of specific simultaneous targeting of these aggressive cancer subpopulations or their resistance mechanisms to maximize the success of conventional cancer therapies [3].

The effectiveness of chemotherapy in the treatment of metastatic or disseminated cancer is often impaired by either intrinsic or acquired tumor resistance to multiple therapeutic drugs with different mechanisms of action, a phenomenon termed multidrug resistance (MDR) [50]. Decreased intracellular drug accumulation is among the best-studied mechanisms of cancer MDR, and has been shown to result in part from tumor cell expression of the ATP-dependent drug efflux transporter P-glycoprotein (P-gp, MDR1, ABCB1) [51]. In clinical human cancers, ABCB1 has been found highly expressed in CRC, in kidney, adrenocortical, and hepatocellular tumors and in acute myelogenous leukemia (AML) [50]. However, a specific relationship between ABC transporters and CRSCs has not been established to date. We have recently cloned and characterized ABCB5, a novel human MDR family member, which was found expressed in human skin [52] and human malignant melanoma [22, 31, 52]. In addition, analysis of ABCB5 mRNA expression across diverse physiological and malignant human tissues revealed the molecule to be expressed, among other tissues, in physiological colon tissue and CRC [31]. Remarkably, we found ABCB5 also to be specifically expressed on CD133⁺ tumor stem cell phenotype-expressing subpopulations among heterogeneous malignant melanoma cultures and within clinical human malignant melanomas of both primary and metastatic origin [31]. Analysis of ABCB5 gene expression across a panel of human cancer cell lines used by the National Cancer Institute for drug screening (NCI-60) by our laboratory and others demonstrated that ABCB5 correlates significantly with cancer resistance to multiple chemotherapeutic agents [31, 53]. Our studies demonstrated that ABCB5 mediates chemoresistance in CD133⁺ stem cell

phenotype-expressing melanoma cells via its function as a drug efflux transporter [31], as shown for doxorubicin, a chemotherapeutic agent to which clinical melanomas are otherwise refractory. Subsequent work by others has confirmed the role of ABCB5 mediated doxorubicin resistance in human melanoma and extended this finding to hepatocellular carcinoma [54, 55]. In addition, Huang et al. [53] validated the role of ABCB5 in melanoma resistance to camptothecin and to 5-FU, a drug commonly employed in colon cancer therapy. Because ABCB5 represents a chemoresistance mechanism [31, 53–56], our findings have provided evidence for a novel, potentially critical link between CSC, cancer progression, and chemoresistance in a solid malignancy [3], raising the possibility that ABCB5⁺ CSC may be responsible both for the progression and chemotherapeutic refractoriness of advanced malignant disease, and that CSC-targeted approaches might therefore represent novel and translationally relevant therapeutic strategies to disseminated cancer growth [3]. These findings are highly relevant to additional CSC-driven cancers where ABCB5 is expressed [31, 55, 57], and warrant investigation whether ABCB5 expressed in colon cancer [31] might represent an additional chemoresistance mechanism through which tumorigenic, chemoresistant CRSC subpopulations could potentially be targeted to increase therapeutic efficacy in this malignancy.

Several studies suggest that cancer stem cells are also more resistant to radiation therapy. For example, increased radioresistance of CD133-expressing human glioblastoma stem cells was attributed to their ability to preferentially activate the DNA damage checkpoint in response to radiation and repair radiation-induced DNA damage more effectively than CD133-negative tumor cells [58]. While the link between CRSCs and radioresistance is more uncertain at present, some studies have shown preferential survival of CD133⁺ CRSCs after adjuvant chemoradiotherapy of rectal cancers [59], which correlated with significant increase in CD133 expression observed in a radiation dose-dependent manner in vitro [60].

CRSC Targeting Therapies

Recent research advances have led to the development of novel targeted therapies for advanced colon cancer (Table 1). Such therapies aim to disrupt cellular pathways essential for tumor growth, survival, and metastasis, and to reduce the toxicity associated with less specific cytotoxic therapies [61]. Currently there are two established targets for such therapies: epidermal growth factor receptor (EGFR), which belongs to the ErbB family of tyrosine kinase receptors and is abnormally activated in many tumors [62], and vascular endothelial growth factor (VEGF), which is known to promote formation of new vessels by inducing growth and differentiation of endothelial cells and to be expressed by 40% to 60% of colon cancers, where it activates processes involved in tumor progression and metastasis [63, 64]. Several clinical trials have demonstrated that introduction of targeted therapies with monoclonal antibodies against EGFR (cetuximab) and VEGF (bevacizumab) in addition to 5-FU resulted in more than 20 months increase in the median survival for patients with advanced disease [61]. These data indicate that targeting specific cell populations within tumors that remain viable after chemotherapy might eventually lead to successful tumor eradication and curative forms of therapy.

Additional future potential CRSC targets have been identified based on the recently discovered CRSC-characteristic molecular pathways which confer CRSC chemoresistance and radioresistance (described in the previous section) as well as CRSC-specific mechanisms of self-renewal and tumorigenicity. The Wnt/ β -catenin pathway has been implicated in the maintenance of the intestinal crypt stem cell phenotype, and Wnt signaling dysregulation through either loss of APC function or oncogenic β -catenin mutations has been shown to cause the majority of sporadic cancer cases [65]. Disruption of Tcf/ β -catenin complexes by selected small-molecule antagonists has been shown to antagonize cellular

effects of β -catenin and to result in inhibition of cellular proliferation of both HCT116 (mutant β -catenin, wild-type APC) and HT-29 (wild-type β -catenin, mutant APC) colon cancer cells [66•]. Similarly, the Notch signaling pathway has been reported to be overexpressed in CRSCs, where it was found to play a role in CRSC viability, tumorigenicity, and self-renewal through repression of cell cycle kinase inhibitor p27 and transcription factor ATOH1 [67]. Targeting Notch in the Apc^{min} colorectal cancer model through pharmacological Notch signaling inactivation by γ -secretase inhibitor resulted in conversion of Apc^{min} proliferative cells in intestinal adenomas into postmitotic goblet cells and subsequent tumor growth arrest [68•]. BMP4 plays a critical role in physiological crypt development and disruption of BMP4 signaling by either overexpression of the BMP4 antagonist, Noggin, in mice or by mutations in BMP4 pathway genes, ie, *SMAD4* and *BMPRIA*, in humans results in aberrant crypt development and leads to the cancer predisposition syndrome, juvenile polyposis [69]. Immunohistochemical analyses of 72 cases of sporadic colorectal cancers also revealed abrogated BMP2 and SMAD4 protein expression, highlighting the potential contribution of disrupted BMP4 signaling to colorectal cancer tumorigenesis [70]. In this regard, Lombardo et al. [71•] showed that treatment of CD133⁺ CRSCs with exogenous BMP4 induces their differentiation and sensitizes tumor xenografts to oxaliplatin and 5-FU treatment in vivo. Carcinogenesis is characterized by alterations in cell morphology and adhesion characteristics driven by dysregulation of actin homeostasis and production of anchorage-independent cells with retracted cytoplasm that lack actin cables [72]. An actin-binding peptide, thymosin β 4 (T β 4), has been reported overexpressed in CD133⁺ CRSCs [20•]. Targeting T β 4 impaired CRSC in vitro growth and migration, and resulted in reduced tumor size of CD133⁺ CRSC-derived xenografts in vivo through modulation of ILK/Akt signaling cascade [73•].

Together, these data illustrate the therapeutic utility of the cancer stem cell concept, which, by enabling specific examination of more aggressive cancer-initiating and cancer-propagating subpopulations, provides the tools for discovery of novel mechanisms of cancer therapeutic resistance that might otherwise remain concealed if only unsegregated tumor cells were studied.

Future Perspectives

Surprisingly, despite the observed slower cellular proliferation rate of CRSCs, little is as of yet known with regard to the metabolism of this cancer cell population, leaving an exciting avenue unstudied in the dawn of the emerging field of metabolomics. The Warburg effect, the premise of which is that cancer cells restrict use of fatty-acid oxidation in favor of glycolysis as an ATP energy source, can also be harnessed to create novel broad-spectrum anticancer agents [74, 75]. A recent study by Akao et al. provided initial evidence of metabolic changes in therapy-resistant cell populations by demonstrating significant overexpression of a metabolic “master-regulator” Sirt1 [76] in DLD-1 5-FU-resistant cells [77]. Future large-scale screens of isolated CRSC populations employing techniques such as RNA-interference libraries, drug-screening libraries, mass-spectrometry, differentiation therapies, microarrays, and ChIP-on-chip analyses will allow further characterization of distinct CRSC molecular pathways and tailor therapies toward their efficient eradication.

Conclusions

Recently, large strides have been made with regard to identification and targeting of CRSCs, since their initially reported existence in 2007. There are currently many new studies with powerful conclusions that remain to be validated independently, and novel emerging molecular signatures of CRSCs are currently being intensively explored. New therapies and strategies for patient-oriented treatment, targeting both the CRSC and bulk tumor

populations side-by-side, should result in a dramatically enhanced patient survival once efficacy can be unequivocally proven.

Acknowledgments

This work was supported by funds provided by the NIH/NINDS K08 NS051349 to N.Y. Frank and the NIH/NCI IR01CA113796 to M.H. Frank.

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Fig. 1.

Targeting colorectal cancer stem cells (CRSCs) versus conventional therapies. Top: Conventional therapies, including chemotherapies and radiotherapies, target the bulk population of rapidly proliferating colorectal cancer cells, resulting in removal of a large proportion of tumor mass. However, therapy-resistant CRSCs remain (cell indicated in *yellow*) and can differentiate into non-CRSC cancer cells and repopulate the tumor over time, resulting in a more aggressive phenotype than the initial pretreated malignancy. Bottom: Targeting of the CRSC subpopulation is hypothesized to remove the cells responsible for tumorigenesis resulting in tumor regression, ultimate remission, and cure of the colorectal cancer

Table 1

Novel colorectal cancer stem cell targeting approaches

Gene/pathway	Targeting mechanism	Status	Reference
VEGF	Bevacizumab, humanized anti-VEGF mAb	FDA approved	[61]
EGFR	Cetuximab, an mAb against the EGFR extracellular binding domain	FDA approved	[61]
Insulin-like growth factor-I receptor (IGF-IR)	IGF-1R inhibitor, AVE-1642	Experimental	[45••]
Interleukin-4 (IL-4)	Anti-IL-4 neutralizing antibody	Experimental	[46••]
Aurora-A kinase	Specific gene silencing	Experimental	[47••]
Tcf/ β -catenin complex	Small molecule inhibition	Experimental	[66••]
Notch pathway	γ -secretase inhibitor	Experimental	[68••]
BMP4 pathway	Recombinant BMP4	Experimental	[71••]
Thymosin β 4	Specific gene silencing	Experimental	[73••]