Review

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Personalized Medicine

Stem cells as therapeutic targets in colorectal cancer

Alicja Zalewski[1], Adam E Snook[2] & Scott A Waldman*,[1]

[1]Department of Surgery, Thomas Jefferson University Hospital, 1020 Locust St, JAH368, Philadelphia, PA 19107, USA

[2]Department of Pharmacology & Experimental Therapeutics, Thomas Jefferson University, 1020 Locust St, JAH368, Philadelphia, PA 19107, USA

*Author for correspondence: scott.waldman@jefferson.edu

Colorectal cancer continues to represent a significant burden on public health as the second highest cause of cancer mortality, when men and women are combined, in the US. About 50% of patients either present with late-stage metastatic disease, or develop metastatic recurrences, and ultimately die. In turn, this mortality largely reflects cancer stem cells, tumor-initiating cells that are responsible for cancer progression, drug resistance, recurrence and metastasis. This review summarizes the unique properties of colorectal cancer stem cells, and the emerging strategies by which they can be selectively targeted as a therapeutic approach to eradicating this disease.

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Burden of colorectal cancer in the USA

Despite improvements in screening practices over the last 20 years in Americans aged 45 years and older, colorectal cancer remains a substantial public health burden. It remains as the third most common cause of cancer mortality in both men and women in the USA and ranks second when men and women are combined [1]. In 2020, the number of incident cases is projected to be 147,950, and the number of deaths to be 53,200 [1]. Typically, colorectal cancer is treated with a combination of surgical resection with chemotherapy and radiation. The standard chemotherapy is FOLFOX, a combination of folinic acid, fluorouracil and oxaliplatin, or FOLFIRI, a combination replacing oxaliplatin for irinotecan [2]. Despite these therapies, the most important predictor of survival is the stage at diagnosis. For patients diagnosed with Stage 1 or localized disease, 5-year survival rates are 90%, but decline to 14% in patients with Stage 4 metastatic disease [1]. Therefore, it is important to develop treatment strategies that could improve the survival of patients with late-stage disease, or prevent metastases altogether.

Cancer stem cells

The mutations and epigenetic modifications that result in cancer ultimately confer several properties that are described as hallmarks of cancer cells. These include sustained proliferation, invasion, metastasis, replicative immortality, angiogenesis and the ability to evade growth suppression and apoptosis [3,4]. Ultimately, a single mutated cell produces a heterogeneous population of different cancer cell states, and recruits and reprograms the supporting cells of the tumor microenvironment (TME), to comprise a solid tumor [3–7]. Tumor heterogeneity accounts for several subpopulations of cells with differential expression of surface markers as well as different properties. The majority of tumor cells lack self-renewal capacity, and because of their rapidly-dividing nature, they can easily be targeted by conventional chemoradiation therapy. However, there exists a small subpopulation of cells that possess a unique capacity for self-renewal, differentiation and tumor initiation. These are termed cancer stem cells (CSCs), and comprise 0.01–10% of cells within the tumor [7]. CSCs play an important role in cancer progression, recurrence, metastasis and drug resistance [3–7].

CSCs were first described in acute myeloid leukemia by Lapidot *et al.* in 1994 with a landmark discovery that not all leukemic cells propagate leukemia when transplanted into immunodeficient mice [3,8]. The authors

demonstrated that it is a specific subpopulation of leukemic cells bearing the surface marker CD34 and lacking CD38 (CD34+CD38- cells) that most efficiently propagate leukemia in severe combined immunodeficient mice. They defined these as CSCs and further defined their functional properties of self-renewal, propagating the tumor over an extended period and multipotency, recapitulating the different cell lineages found in the primary tumor [3,8].

CSCs also were identified in solid tumors, first reported by Al-Hajj in breast cancer in 2003 [9,10]. Subsequently, CSCs were discovered in lung cancer [11], colon cancer [12], prostate cancer [13], ovarian cancer [14], brain cancer [15], pancreatic cancer [16] and melanoma [17]. As CSCs were discovered in these solid tumors, two models were developed to explain the heterogeneity within tumors. The first, the cancer stem cell model, proposes that all the fundamental hallmarks of cancer, such as initiation, progression, metastasis and recurrence, rely on a rare population of stem cells [9,18]. Therefore, there exists a hierarchical organization of all the cells comprising the bulk of the tumor, with the CSCs at the top with their unique self-renewing and pluripotent properties, giving rise to the heterogeneous population through asymmetric division [18]. The second, the clonal evolution model, assumes all tumor cells contribute to tumor maintenance with differing capacities and that intercellular variation is primarily attributed to subclonal differences that result from genetic or epigenetic changes during cancer development [18].

The evidence for the existence of CSCs in both leukemia and solid tumors strongly reinforces the theory of the cancer stem cell model [9–17,19]. These authors successfully demonstrated the hierarchical nature of tumors by purifying and assaying distinct cell types and subsequently xenografting these populations into immunodeficient mice. As such, xenografting is central to the CSC model, since tumor initiation is one of the defining features of CSCs [19]. The others include: self-renewal, meaning the CSC population can be serially transplanted through multiple generations to form tumors; expression of distinctive surface markers that can be used to sort CSCs; and finally multipotency, meaning that tumors arising from CSCs contain the mixed tumorigenic and nontumorigenic cells of the original tumor.

Importance of targeting CSCs

Standard cancer treatment in the form of chemotherapy and radiation was designed to kill the majority of cancer cells and induce a dramatic regression of a large tumor by targeting all rapidly-dividing cells without selectivity, thereby also killing normal, healthy dividing cells associated with systemic side effects including bone marrow suppression, alopecia and diarrhea and malabsorption. Cancer stem cells have a slower rate of division as well as a greater ability to correct DNA defects, making them more resilient to standard treatments, which can promote the propagation of resistant clones of cancer cells [20]. In this way, CSCs persist and cause tumor recurrence, cancer progression and metastases. Therefore, it is increasingly important to develop CSC-specific therapies to kill this unique population of cells, thereby exhausting the tumor's self-renewing potential. Otherwise, cancer cannot be eradicated. Moreover, specific targeting of CSCs will allow for administration of highly-potent cytotoxic agents with limited systemic toxicity [5]. The lack of current therapies effectively targeting CSCs highlights the profound clinical need for advances in this important chemotherapeutic field [6].

Identifying colorectal cancer stem cells

In the colon, stem cells reside at the base of the crypts of Lieberkuhn, amounting to less than 20 stem cells per crypt. During normal regeneration of the intestinal epithelium, the stem cells replicate and as the new cells differentiate, they migrate up the crypt to ultimately replace the apoptotic cells at the top [12,20–23]. Disruption of this delicate homeostasis can lead to malignant transformation.

Colorectal cancer stem cells (CCSCs) can be identified by their expression of specific cell surface markers. As described by O'Brien *et al.* in 2007, purification experiments established that CCSCs expressed the cell surface marker CD133 [12]. These cells rapidly formed tumors after injection into immunodeficient mice, whereas the CD133- cells that comprised the majority of the tumor were unable to initiate tumor growth [12]. Moreover, $CD133^+$ cells maintain themselves and differentiate and re-establish tumor heterogeneity upon serial transplantation [12]. $CD133^+$ CSCs make up approximately 2.5% of colorectal tumor cells [24,25].

In addition to CD133, other CCSC markers have been identified, including CD166, CD44, Lgr5, ALDH1, EphB receptors and others [23,25–28]. A promising study by Huang *et al.* in 2009 discovered populations of cells located at the base of the normal crypt that expressed CD44, CD133 and ALDH1 [22,29]. As colonic epithelium progressed to carcinoma, the number of cells that expressed all three markers increased and were distributed further up the crypt. Isolation of human colorectal cancer cells based solely on enzymatic activity of ALDH and injection into immunodeficient mice formed tumors and further perpetuated the tumorigenic paradigm essential to

CCSCs [22,29]. In that context, the function of ALDH as a detoxifying enzyme explains the insensitivity of CCSCs to toxic insults that underlie their canonical chemotherapeutic resistance.

Beyond sorting CCSCs based on expression of specific cell surface markers, they can be identified functionally based on their colony-forming ability [30]. After tumor tissues are dissociated into single cells, a single CCSC can form a cell colony, or sphere, under specific conditions, including low-density cell culture in serum-free media containing epidermal growth factor and basic fibroblast growth factor [30,31]. Moreover, drug resistance can be used to identify CCSCs. Indeed, since CCSCs primarily reside in G0 in cell cycle arrest, they are static and nondividing, evading toxicities of chemotherapeutic drugs [30,32]. Once CCSCs are identified and isolated, their unique properties can be studied to develop targeted therapies.

Targeting signal pathways unique to CCSCs

Signal pathways implicated in self-renewal and increased CCSC survival include Notch, Hedgehog, Wnt/β-catenin and JAK/STAT [6,30,33–35]. Considerable progress has been made in early clinical trials for Notch and Hedgehog pathway inhibitors, while targeting the Wnt pathway has proven to be challenging [30]. More recently, advancements in genomic analyses have identified other pathways implicated in CCSC survival.

Notch

The Notch signaling pathway is initiated by recognition of a transmembrane ligand protein by the transmembrane Notch receptor on a neighboring cell [30]. Notch is known to function as both an oncogene and a tumor suppressor in different types of cancer. In colorectal cancer, Notch acts as an oncogene, playing an important role in CCSC survival, self-renewal, differentiation and metastasis [30]. Conversely, when Notch is inhibited, chemoresistance and self-renewal capabilities of CCSCs are reduced [36]. One study by Zhang *et al.* demonstrated that a circadian clock protein PER3 promotes CCSC apoptosis and is significantly downregulated in colorectal cancer compared with normal adjacent tissue [36]. The PER3 and Notch pathway are inversely linked, and overexpression of PER3 leads to decreased expression of one type of Notch receptor (Notch1) and one of its ligands (Jagged1) [36]. In this way, PER3 acts as a tumor suppressor, with potential as a target for eliminating CCSCs by inhibition of Notch signaling.

Currently, there are different classes of Notch signaling inhibitors being investigated for treating colorectal cancer, including γ-secretase inhibitors and antibodies against the Notch receptor or its ligands [6,30]. γ-secretase inhibitors prevent the final proteolytic cleavage of the intracellular Notch receptor domain required for transmembrane signaling [6,33]. A Phase II clinical trial in 37 patients in 2012 evaluated responses to R04929097, a selective γ-secretase inhibitor, as a single agent for patients with metastatic colorectal cancer [6,37]. No radiographic responses were observed, and time to progression was short, suggesting minimal efficacy [37]. However, further studies are warranted to evaluate the therapeutic effect of γ-secretase inhibitors in combination with other standard therapies, rather than as a monotherapy. Indeed, in one study combining a γ-secretase inhibitor with platinum compounds enhanced cell death in a large subset of colorectal cancer cell lines [38]. However, to date, no clinical studies have translated these observations.

An antibody against one Notch ligand in particular, DLL4, has been the most studied as an antitumor target. DLL4 plays a role in tumor angiogenesis, and anti-DLL4 antibodies disrupt the formation of capillary networks necessary for tumor growth [6]. Demcizumab is a humanized monoclonal antibody targeting DLL4 that was well tolerated in a Phase I clinical trial and demonstrated disease stabilization and decreases in tumor size [4,30]. In human xenograft models of colon and other cancers, demcizumab markedly reduced tumor growth, regrowth and the number of cells expressing CCSC markers [39]. In a Phase I trial, 55 patients with previously treated solid tumors experienced disease stabilization and reductions in tumor size with demcizumab, although prolonged administration of the drug was associated with increased risk of congestive heart failure [39]. Enoticumab, another humanized anti-DLL4 antibody, inhibits growth in a dose-dependent fashion in tumor xenograft mouse models and demonstrated clinical activity in several tumor types [40]. Indeed, enoticumab prolonged stable disease to >6 months in a colorectal adenocarcinoma patient with familial adenomatous polyposis syndrome [40].

Hedgehog

The hedgehog pathway involves the hedgehog ligand and several transmembrane proteins including the receptor Patched (PTCH) and Smoothened (SMO) that ultimately lead to activation of GLI transcription factors. It is required for proper cell differentiation and leads to an increase in angiogenic factors and anti-apoptotic genes and a decrease in apoptotic genes. In several types of cancer, hedgehog signaling promotes self-renewal and metastasis of CSCs [30]. One study demonstrated that the tumor suppressor RUNX3 inhibits metastasis and stemness of CCSCs

by promoting ubiquitination and degradation of GLI, and through this suppression of the hedgehog pathway, agents that induce RUNX3 may be useful in targeting CCSCs [41].

The most commonly studied mechanism to silence the hedgehog signaling pathway is with SMO antagonists. Of these, vismodegib was tested in a randomized Phase II clinical trial in combination with standard therapy, including FOLFOX or FOLFIRI with bevacizumab, in patients with previously untreated metastatic colorectal cancer [42]. Unfortunately, there was no survival benefit in patients receiving vismodegib, in addition to standard therapy. However, the cohort administered vismodegib received a treatment intensity that was lower for all regimen components suggesting that combined toxicity may have contributed to lack of efficacy [42].

Wnt/β-catenin

The Wnt/β-catenin pathway is complex and includes more than 19 ligands and 15 receptors [30]. It is activated at the base of intestinal crypts and is important in regulating normal stem cell renewal, clonogenicity, and maintenance, and when dysregulated, plays a significant role in tumorigenesis [6]. In colorectal cancer, APC or β-catenin mutations are frequently seen, which upregulate Wnt signaling, leading to tumor initiation and progression [43]. Aberrant upregulation of Wnt signaling is observed in both nonhypermutated microsatellite stable (MSS) and hypermutated microsatellite instability (MSI) colorectal cancers. In the majority of MSS cancers, loss of the tumor suppressor APC leads to adenoma formation, which is an early event in the multistep model of colorectal transformation [44]. In MSI cancers, frequent mutations are observed in multiple oncogenes and tumor suppressor genes in the Wnt pathway, including APC, β-catenin and AXIN2 [44]. Induction of Wnt signaling activates dormant CCSCs, promoting their cell cycle progression [30]. Moreover, activation of β-catenin has been associated with migration and metastasis of CCSCs [45].

The classes of Wnt pathway inhibitors encompass generic targets, including NSAIDs, COX2 inhibitors and vitamins, as well as inhibitors of the Wnt-receptor complex, β-catenin destruction complex and nuclear/transcription factor complex [44]. Several studies suggest that NSAIDs and COX2 inhibitors, like celecoxib, inhibit β-catenin transcription and prevent polyp formation in patients with familial adenomatous polyposis (FAP) [44]. Indeed, inhibiting COX2-dependent prostaglandin E2 production has chemopreventive effects in colorectal cancer [44]. In addition, vitamin A decreases β-catenin protein levels [44]. Inhibitors of porcupine, a membrane-bound O-acyltransferase responsible for processing Wnt ligand secretion, specifically targets the Wnt pathway without suppressing Notch or hedgehog-mediated signaling [44]. The porcupine inhibitor LGK974 caused 63% tumor regression in mice with no adverse effect in normal Wnt-dependent tissues [46]. Also, antibodies targeting individual components of the Wnt pathway, including Wnt-1 and Wnt-2, suppress tumor growth *in vivo* and drive apoptosis in colorectal and other cancer cells [44]. OMP18R5, OMP-54F28 and OMP131R10 are in Phase I trials as combined therapies in pancreatic cancer, hepatocellular carcinoma, metastatic colorectal cancer and other non-gastrointestinal (GI) cancers [6]. These have shown promise in xenograft models [44]. Further, small molecule inhibitors targeting tankyrases (polyADP-ribosylating enzymes) antagonize Wnt effects in colon cancer cells. The tankyrase inhibitors JW55, JW67 and JW64 suppress tumor growth in APC mutant mice, although they also caused significant intestinal toxicity [47]. A small molecule, ICG-001, which inhibits the transcription of β-catenin, eliminated drug-resistant tumor cells by inducing apoptosis [44]. The promising tumor-suppressive effect and lack of toxicity highlight the therapeutic potential of ICG-001 for cancer treatment.

There are many challenges associated with developing therapies targeting Wnt signaling. Systemic toxicity remains a key issue, since Wnt signaling is crucial in normal tissue regeneration and homeostasis. In addition, there is significant integration and crosstalk between Wnt and other pathways such as Notch, so it is important to develop therapies with high specificity. Since there are so many components to the pathway and a diverse mutational spectrum, it is important to identify agents that treat cancer patients carrying specific mutations. For example, hypermutated MSI colon cancers harboring inactivating APC mutations are unlikely to respond to porcupine inhibitors because of the dominant downstream mutations of APC [44,46]. Tankyrase inhibitors specifically target APC-mutated tumors, which constitute greater than 80% of colorectal cancers, however, these have intestinal toxicity as a drawback. As a result, despite several Phase I and II trials investigating Wnt inhibitors, there are no approved drugs available in the clinical setting thus far [44].

JAK/STAT

The JAK/STAT signaling pathway incorporates the tyrosine kinase-related receptor, JAK and the transcription factor STAT. Several cytokines and growth factors transmit signals through this pathway, including interleukins,

EGF, interferon and others [30]. It is important in cell proliferation and differentiation. Park *et al.* identified JAK/STAT as a crucial resistance mechanism for the persistent growth of CCSCs after radiotherapy [35]. JAK2 was preferentially overexpressed in CCSCs and contributed to radioresistance by limiting apoptosis and enhancing clonogenic potential [35]. The activation of STAT3 and increased transcription of cyclin D2 led to persistent CCSC growth by maintaining an intact cell cycle and proliferation with low levels of DNA damage accumulation [35]. A Phase II single arm clinical trial investigated the effect of pacritinib, a potent JAK inhibitor which was previously effective in hematologic malignancies with a favorable safety profile, on metastatic refractory colorectal cancer [48]. Unfortunately, the trial was discontinued for futility and lack of treatment benefit [48]. A separate study utilized a STAT3 inhibitor, napabucasin, which was effective in a Phase II trial as a combination therapy with FOLFIRI [49]. Subsequently, a Phase III trial revealed no difference in overall survival among patients with metastatic colorectal cancer who received napabucasin [50].

Next-generation sequencing analyses

Recently, genomic analysis techniques have allowed for identification of many other pathways implicated in CCSC propagation and survival. Patient-derived organoids (PDO) propagated from a population of CCSCs expressing the marker Lgr5 were analyzed by next-generation sequencing and found to have amplification of oncogenic drivers, such as ERBB2 [51]. In this way, Vlachogiannis *et al.* profiled 151 cancer-related genes in both PDOs and their parental biopsies, and subsequently screened 55 drugs currently being used in Phase I–III clinical trials or in clinical practice [51]. For example, by specifically propagating CCSCs as PDOs, those that had an ERBB2 amplification responded well to lapatinib, a dual ERBB2 and EGFR inhibitor [51]. Personalized chemotherapy can thus be developed by sequencing each individual patient's tumor. However, chemosensitivity to specific regimens based on the specific mutations can still be difficult to predict. As demonstrated by Maekawa *et al.*, patient-derived spheroid xenografts (PDSX) from isolated CCSCs can be used to compare chemosensitivities [52]. It is most exciting that PDSX models provided more predictable tumor growth with less variance than conventional PDX models, demonstrating that identification and propagation of CCSC populations provide a powerful tool in developing personalized chemotherapeutics [52].

Noncoding RNAs as targets

Noncoding RNAs include RNA molecules which are not translated into proteins. There are many types including ribosomal RNA and transfer RNA, but the two types that have increasingly been shown to play a role in tumorigenesis and can serve as potential targets include miRNAs and long noncoding RNAs (lncRNAs).

miRNAs

miRNAs are small noncoding RNAs about 20 base pairs in length with significant regulatory roles through the process of binding to the 3′UTR of target genes, leading to degradation of mRNA and inhibition of translation [53]. MicroRNAs play a role in CCSC self-renewal, differentiation and tumorigenesis. For example, miR-21 is one of the most notable carcinogenic miRNAs and is termed an oncomiR, targeting multiple tumor suppressor genes associated with proliferation, apoptosis and invasion [53,54]. Specifically in colorectal cancer, miR-21 induces stemness by downregulating TGF-β receptor 2 and stimulating invasion and metastasis by suppressing PDCD4 [55]. In addition, miR-21 induces resistance in HT-29 colon cancer cells to fluorouracil *in vitro* [56], and it is associated with significantly shorter disease-free and overall survival in patients with Stage II colorectal cancer [57]. Other miRNAs contributing to CCSC therapeutic resistance include miR-140, which confers resistance to methotrexate and 5-FU in $CD133^{high}$ $CD44^{high}$ colon cancer cells [58] and miR-215, which enhances chemoresistance of these cells to methotrexate and raltitrexed, an antimetabolite chemotherapeutic [54]. On the other hand, a tumor suppressive miRNA identified in colorectal cancer, miR-34a, is downregulated in colorectal cancer cell lines and clinical specimens and likely suppresses metastasis by regulating Notch signaling [59].

Many miRNAs are being investigated as diagnostic markers for colorectal cancer screening. For example, Wang *et al.* have shown that miR-29a is upregulated in tumors and blood of colorectal cancer patients and can be detected in patients with liver metastases, so it can potentially be used as a diagnostic biomarker [60]. Beyond diagnosis, therapeutic targeting of miRNAs may be achieved using inhibitors called anti-miRNA oligonucleotides (AMOs) which block the binding of miRNAs to the 3′UTR [61]. Further, entities called sponges bind to the miRNAs themselves inhibiting their activities [53]. In a study by Tao *et al.*, transfection of a miR-21 specific AMO into HCT116 human colon carcinoma cells decreased the expression of miR-21 and impaired proliferation and clone

formation [61]. Downregulation of miR-21 by the AMO also reduced the expression of vascular endothelial growth factors, which is necessary for the metastatic capacity of CCSCs [61]. In this way, miRNAs can be useful targets in eliminating CCSCs, although further studies must be conducted before they can be advanced into clinical trials.

Long noncoding RNAs

As with miRNAs, lncRNAs also play a role in tumorigenesis and drug resistance. LncRNAs are typically >200 nucleotides and regulate a variety of processes, such as proliferation, differentiation, apoptosis, invasion and metastasis, all of which are important features of CCSCs [62]. One example is the growth-arrest specific transcript 5 (GAS5) lncRNA, which is upregulated during growth arrest induced by the absence of growth factors or serum starvation. Under normal conditions, it can bind to the glucocorticoid receptor, and acts as a tumor suppressor by inhibiting proliferation and promoting apoptosis [63]. GAS5 is downregulated in several cancer types, including colorectal cancer, and this reduced expression is inversely associated with tumor size, stage and lymph node metastasis, and lower overall survival [64,65]. Furthermore, by overexpressing GAS5, cell proliferation and migration were inhibited *in vivo* and apoptosis was promoted [64]. Another example is HOTAIR, a lncRNA which, when overexpressed, promotes CCSC migration and invasion and when downregulated suppresses the epithelial-to-mesenchymal transition [66]. In addition to their role in cell proliferation and apoptosis, lncRNAs also contribute to chemotherapeutic drug resistance. In colorectal cancer, dysregulation of several lncRNAs, including UCA-1, have been implicated in the acquisition of 5-FU resistance [62].

As with miRNAs, lncRNAs have been investigated as potential clinical biomarkers. For example, CCAT1 is upregulated not only in colorectal cancer, but also in inflammatory bowel disease and polyps [67]. It can be detected in the blood and stool samples of colorectal cancer patients. Beyond their clinical utility as biomarkers, lncRNAs also could serve as therapeutic targets to eliminate CCSCs, although the most effective strategy remains to be defined. For instance, the plasmid BC-819 was created utilizing the promoter of the lncRNA H19, which is over-expressed in embryonic and malignant tissues but minimally in adult tissues [68]. Intratumoral or intra-arterial treatment with BC-819 produced a significant suppression of subcutaneous or metastatic colorectal tumor growth *in vivo* [68]. A different technique targeting oncogenic lncRNAs includes silencing by siRNAs. Indeed, siRNAs have been developed against HOTAIR, CCAT2 and other lncRNAs, and they decrease tumor proliferation and invasion [66]. More recently, Pichler *et al.* administered nanoparticles containing siRNA against FLANC, a lncRNA upregulated in colorectal cancer (CRC) cells, *in vivo* [69]. These nanoparticles significantly decreased the number of metastases, with low tissue toxicity, by upregulating phosphorylated STAT3, a component of the JAK/STAT pathway as discussed above [69].

Immunological targets

Immunological targeting of CCSCs has been well-studied and offers promising advantages over other therapies. By harnessing the immune system, systemic cytotoxicity can be reduced, since immune cells exert their effects in an antigen-specific manner [6]. Additionally, a memory response could be created to prevent cancer recurrence. It has been challenging, however, to demonstrate that the sole target of these therapies are CCSCs.

MSI, neoantigens & immune checkpoint inhibitors

There are different molecular phenotypes of colorectal cancers, including chromosomal instability, CpG-island methylator phenotype and MSI. In turn, these molecular subtypes, in part, predict the efficacy of immunotherapy [70]. MSI constitutes a small subset of about 15% of colorectal tumors, typically reflecting defects in DNA mismatch repair. In contrast to the majority of tumors which are MSS, MSI tumors have a higher tumor mutational burden and neoantigen load. In turn, this is more favorable for targeting with immunotherapy, and the subsequent antitumor immune response has greater efficacy [70].

Immune checkpoints, including CTLA-4 and PD-1/PD-L1, are important inhibitors of innate and adaptive immune responses and contribute to self-tolerance [7]. In that context, by stimulating immune checkpoints, cancer cells can escape immune destruction. Checkpoint inhibitors have been developed, but their success among different cancer types has been mixed, dependent on the TME [7]. Tumors in which the TME is considered 'inflamed' with high levels of T-cell infiltration and neoantigen expression are more sensitive to immune checkpoint inhibitors. Nivolumab and pembrolizumab which block PD-1, have been approved for patients with MSI refractory or metastatic colorectal cancer as a result of a recent clinical trial [70]. In a Phase II study, patients with MSI tumors had higher objective response and progression-free survival rates with pembrolizumab compared with patients

with MSS tumors [70,71]. This study also identified a much higher number of potential neoantigens in patients with MSI tumors (mean of 578 vs 21) [71]. Similarly, nivolumab in combination with ipilimumab, an antibody against CTLA-4, produced better progression-free and overall survival than nivolumab alone in a Phase II study in patients with MSI tumors [70]. Despite these promising results, immune checkpoint inhibitors have, so far, only been effective in treating MSI and a small subset of MSS tumors, leaving nearly 85% of colorectal cancer cases unaddressed.

Tumor associated antigens (TAAs) can be recognized by T cells when exposed on the surface of tumor cells. TAAs include over-expressed self-antigens and differentiation antigens which are also expressed in normal tissues, and oncofetal and cancer testis antigens not present on most normal adult cells [7]. Targeting tumor-specific neoantigens derived from unique mutations in cancer cells is ideal with respect to creating therapeutic efficacy with minimum potential for adverse reactions in normal tissues. In that context, tumor mutational burden correlates with the quantity of neoantigens in many cancers, including colorectal tumors [70]. Moreover, there were more neoantigens identified in MSI tumors than MSS tumors [70]. Neoantigens are being used as targets in cancer vaccination. In that context, identifying tumor-specific neoantigens can aid in the development of personalized vaccines. Indeed, neoantigen vaccines effectively inhibit tumor growth and elicit an effective antitumor T cell response in murine colon carcinoma models [72]. There are several clinical trials investigating neoantigen vaccines in colorectal cancer [70]. These include a personalized synthetic neoantigen vaccine in combination with an adjuvant (NCT02992977), an mRNA-based individualized vaccine targeting the patient's tumor-associated peptides (NCT03289962), and a neoantigen-loaded dendritic cell vaccine (NCT01885702).

Adoptive cell therapy

Adoptive cell therapy (ACT) has been pivotal in treating hematologic malignancies, and has shown promise in treating solid cancers. ACT describes the process by which T lymphocytes are isolated from cancer patients, engineered and/or expanded *ex vivo*, then reinfused back into patients. This includes use of unmodified tumor-infiltrating lymphocytes as well as peripheral blood T cells engineered to express T-cell receptors (TCRs) or chimeric antigen receptors (CARs). Similarly, CAR-NK cells have been developed to exploit the innate, rather than adaptive, immune system. Both CAR-T cells and BiTEs (described below) require development of high affinity antibodies targeting a TAA. CAR-T cells express an artificial receptor composed of a targeting domain derived from an antibody connected to intracellular signaling domains [6]. CAR-T cells targeting CD133, EGFR and EpCAM, for example, have been developed and deemed safe for clinical use [7], though many other CAR-T cell approaches have produced significant toxicity and death in patients [73]. A Phase I trial demonstrated the efficacy of CAR-T cells against CD133 in treating patients with late-stage metastatic malignancies, including colorectal, pancreatic and hepatocellular carcinoma [74]. A CAR-T cell targeting EpCAM was studied in the setting of peritoneal carcinomatosis, and delayed tumor growth with an excellent safety profile [75]. Carcinoembryonic antigen (CEA), a glycoprotein involved in cell adhesion, is used as a serum biomarker in colorectal cancer patients and also is being used as a target for CAR-T cells. A Phase I study reported that CEA-specific CAR-T cells resulted in stable disease in patients with CRC and CEA+ metastases [70,76]. This therapy was well tolerated, and 2 of 10 patients experienced tumor shrinkage. NKG2D also has been used to target CAR-T cells. NKG2D is involved in NK cell-mediated lysis and T-cell signaling. NKG2D CAR-T cells are effective against CRC cell lines, and a Phase I trial of CYAD-101 (one type of NKG2D CAR-T cell) is currently being conducted in patients receiving FOLFOX for unresectable metastatic CRC [75,77]. Another CAR-T cell that has successfully treated colorectal cancer metastases in mice is directed toward guanylyl cyclase C (GUCY2C), an intestinal transmembrane protein involved in fluid homeostasis [78]. GUCY2C CAR-T cells killed human colorectal cancer cells expressing GUCY2C and provided durable survival in a human xenograft model in mice, without intestinal toxicity [78,79]. These results suggest that the GUCY2C CAR-T cells can potentially be used in patients with GUCY2C-expressing gastrointestinal malignancies [78]. Like conventional T cells, CAR-T cells may produce a memory response against recurrent tumors, which may be enhanced by appropriate starting population selection and manipulation of CAR-T signaling [80]. On the other hand, a drawback to ACT is off-target toxicity when normal tissues share the same antigen.

While CAR-T cells employ *ex vivo* genetic modification to redirect T cells to target tumors, Bispecific T-cell Engagers (BiTEs) are soluble molecules administered to patients to redirect endogenous T cells to target tumors. BiTEs have two linked single-chain variable fragments from different antibodies, one targeting a cell surface molecule on T cells and the other an antigen on the surface of cancer cells, independent of MHC molecules [80].

Solitomab is a BiTE with specificity for CD3 and EpCAM, which has been tested in the treatment of solid tumors [80], has been effective in eradicating CCSCs in immunodeficient mouse models [21].

Selective CCSC antigenic targets

Directing monoclonal antibodies against cell surface markers found on CCSCs, including CD133, CD166, CD44, ALDH1, Lgr5 and EpCAM, can potentially lead to tumor shrinkage and reduce metastases [21]. An example is catumaxomab, an antibody targeting EpCAM, which was first approved in 2009 in Europe for treating malignant ascites [21]. It causes T-cell mediated lysis by acting as a BiTE, binding to EpCAM on CCSCs and CD3 on T cells. However, it is important to consider that CCSC surface phenotypes can vary in different patients and even in the same patient upon relapse [30]. Thus, developing specific antibodies with global utility can be challenging. Beyond isolated antibodies, antibody-drug conjugates have been created to deliver cytotoxic agents to targeted cells [21]. These require conjugate internalization by CCSCs and lysosomal processing to activate the cytotoxic payload. There are antibody-drug conjugates targeting Lgr5 that displayed antitumor efficacy and safety in mice [81]. CCSC targeted antibody-drug conjugates have not yet been translated into the clinic.

Targeting the dysplastic TME

The TME plays a critical role in the support and modulation of CCSCs, allowing them to maintain their stemness and multipotency [7]. This dynamic niche is composed of fibroblasts, endothelial, stromal, mesenchymal and immune cells [7]. It is the cross-talk between these cells and CCSCs that is crucial in dictating their plasticity, and also inducing angiogenesis and promoting tumor invasion and metastasis [30]. These interactions occur through adhesion molecules and paracrine factors. One chemokine receptor, CXCR4, has been described in many cancers [30]. A drug targeting CXCR4, plerixafor (AMD3100), induced remission in relapsed or refractory acute myeloid leukemia (AML) patients [30]. Another commonly studied target is VEGF, which contributes to angiogenesis and tumor growth and is blocked by the monoclonal antibody bevacizumab, or Avastin [9]. Bevacizumab decreases the number of glioblastoma stem cells in mice [9]. In the context of colorectal cancer, a pivotal Phase III study demonstrated improvement in response rate, progression-free survival and overall survival with the addition of bevacizumab to FOLFIRI [80]. As a result, bevacizumab is now incorporated into the standardized therapy for metastatic disease [82]. In addition, hypoxia is a potent driver for maintaining CCSC stemness and inhibiting apoptosis. Hypoxia-inducible factors are transcription factors that are upregulated in CCSCs, allowing them to survive and adapt to hypoxic conditions [30]. Hypoxia-inducible factors also induce the epithelial-to-mesenchymal transition, promoting CCSC invasiveness and resistance to chemoradiation [30]. These factors represent future therapeutic targets to potentially eliminate CCSCs.

Targeting the unique metabolism of CCSCs

A unique phenomenon discovered in cancer cell metabolism is the Warburg effect, which describes the tendency for cancer cells to undergo aerobic fermentation, meaning they preferentially undergo glycolysis rather than oxidative phosphorylation, even in the presence of oxygen [83]. This is especially true for CCSCs, in which the Warburg effect creates an environment that favors CCSC survival and the reprogramming of non-CCSCs into CSCs [84]. This suggests that even killing of CCSCs may not suffice as a therapeutic approach, since they can be regenerated from non-CCSCs [83]. Interestingly, the first-line drug for Type II diabetes, metformin, improves the efficacy of other anticancer therapies and is used as a complementary agent to conventional chemotherapy [83,85]. By activating adenosine monophosphate-activated kinase (AMPK) most notably, as well as a host of other metabolic enzymes, metformin blocks protein synthesis and cell growth and ultimately halts the cell cycle [85,86]. Its effects have been more pronounced in cancers associated with hyperinsulinemia, which includes colorectal cancer [83]. It lowers glucose and insulin levels within the cancer cell niche, which reduces cancer progression [83]. Besides affecting CCSC metabolism, metformin also triggers apoptosis and autophagy by p53 and p21 and prevents angiogenesis [85,86]. Further, in the human colorectal cancer cell lines SW620, SW480 and HCT116, metformin enhanced the effects of 5-FU specifically on $CD133^+$ CCSCs and prevented cell proliferation [83]. Moreover, metformin reduced tumor burden and prevented relapse more effectively than doxorubicin alone in xenograft models in mice [86]. Taken together, metformin, alters the metabolism of CCSCs and is an attractive complement to conventional chemotherapeutics to improve their efficacy [83].

Epigenetic targets

There is evidence of CCSC regulation by epigenetic mechanisms like histone modification and DNA methylation [3]. For example, Ezh2 is a histone methyltransferase enzyme that leads to transcriptional repression of E-cadherin, with a role in cancer initiation and progression [3,87]. Indeed, several studies have associated loss of E-cadherin with invasiveness and advanced tumor stage in several cancers, including colorectal cancer [87]. Furthermore, inhibition of Ezh2 sensitizes tumors to chemotherapy, suggesting that Ezh2 inhibitors could be promising therapeutics [3]. Because of the reversible nature of epigenetic mechanisms, there is a substantial therapeutic potential for targeting enzymes responsible for modification of DNA and histones [86].

Another example includes SIRT1 (the silent mating type information regulation 2 homolog 1), a histone deacetylase protein that is over-expressed in cancer cells resistant to 5-FU [83]. SIRT1 expression correlated with invasion, lymph node metastasis and TNM stage in colorectal cancer patients, indicating poorer prognosis [88]. By inhibiting SIRT1 with miR-34a, human colorectal cancer DLD-1 cells were more sensitive to 5-FU [83]. SIRT1 is closely associated with stemness features of CCSCs, reflected by its co-expression with the surface marker CD133 and its role in maintaining CCSC tumorigenicity and ability to form colonospheres in culture [89]. These findings suggest that SIRT1 plays an integral role in maintaining CCSC stemness and should be considered as a target for colorectal cancer therapy.

Future perspective

As highlighted, CCSCs are a unique population of cells within the colorectal tumor bulk with specific properties that, if left unchecked, result in increased tumorigenicity, metastases and recurrence. Therefore, it is essential to continue developing and optimizing therapies that selectively target CCSCs in order to improve patient survival. While a multitude of strategies have been discovered and developed, their translation and achievement of significant improvement in overall survival continues to be challenging. A promising method of testing specific therapies to develop personalized chemotherapy for each patient is by cultivating CCSC organoids. Miyoshi *et al.* have successfully propagated over 100 patient-derived CCSC spheroid lines, however, the techniques still need to be optimized in order to achieve higher efficiency to be able to apply this practically [90]. Additionally, safety is a concern since many therapies target stem cells generally, and not just CCSCs. Discovering each patient's unique tumor-associated antigens and developing personalized therapies against them likely will be optimized in the future with the expansion of more advanced technologies and bioinformatics methods, especially for cancers with high mutational burdens like MSI tumors. Additionally, rather than relying on one class of CCSC-targeting therapy in combination with conventional chemoradiation, a multifaceted approach may be necessary to include therapeutics directed at different mechanisms to effectively target CCSCs and achieve lower recurrence rates and improve survival in patients with colorectal cancer.

Executive summary

- As the second most common cause of cancer mortality in the USA, colorectal cancer remains a substantial public health burden.
- Many patients present with metastatic or recurrent disease, having a worse prognosis than patients who present with localized disease which is amenable to operative resection.
- Cancer stem cells are a small subpopulation (0.01–10%) of tumor cells that are responsible for chemoresistance, metastasis and recurrence, and they exhibit the unique properties of self-renewal, multipotent differentiation, and marked tumorigenicity.
- Colorectal cancer stem cells (CCSCs) constitute roughly 2.5% of the total tumor cell population and can be most commonly identified by the cell surface markers CD133, CD166, CD44, ALDH1, Lgr5, EphB2.
- Signal pathways implicated in self-renewal and increased CCSC survival include Notch, Hedgehog, Wnt/β-catenin and JAK/STAT.
- Both miRNA and lncRNAs play a role in tumorigenesis and drug resistance, although agents targeting these pathways require further investigation prior to translation into the clinical setting.
- Immunotherapies offer promising results in preventing cancer progression and treating metastases. These include immune checkpoint inhibitors, adoptive cell therapies and antibody-based agents against specific CCSC surface markers.
- Targeting multiple components of the TME, CCSC metabolism, as well as epigenetic regulators, is an emerging strategy to eradicate CCSCs.
- In the future, patients likely will benefit from a combined approach that incorporates multiple CCSC-targeted therapies to enhance efficacy while limiting toxicities from conventional chemoradiation therapies.

Author contributions

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