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TOPIC HIGHLIGHT

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Hallmarks in colorectal cancer: Angiogenesis and cancer stem-like cells

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Abstract

Carcinogenesis is a multistep process that requires the accumulation of various genetic and epigenetic aberrations to drive the progressive malignant transformation of normal human cells. Two major hallmarks of carcinogenesis that have been described are angiogenesis and the stem cell characteristic of limitless replicative potential. These properties have been targeted over the past decade in the development of therapeutic treatments for colorectal cancer (CRC), one of the most commonly diagnosed and lethal cancers worldwide. The treatment of solid tumor cancers such as CRC has been challenging due to the heterogeneity of the tumor itself and the chemoresistance of the malignant cells. Furthermore, the same microenvironment that maintains the pool of intestinal stem cells that contribute to the continuous renewal of the intestinal epithelia also provides the necessary conditions for proliferative growth of cancer stem-like cells. These cancer stem-like cells are responsible for the resistance to therapy and cancer recurrence, though they represent less than 2.5% of the tumor mass. The stromal environment surrounding the tumor cells, referred to as the tumor niche, also supports angiogenesis, which supplies the oxygen and nutrients needed for tumor development. Anti-angiogenic therapy, such as with bevacizumab, a monoclonal antibody against vascular-endothelial growth factor, significantly prolongs the survival of metastatic CRC patients. However, such treatments are not completely curative, and a large proportion of patient tumors retain chemoresistance or show recurrence. This article reviews the current knowledge regarding the molecular phenotype of CRC cancer cells, as well as discusses the mechanisms contributing to their maintenance. Future personalized therapeutic approaches that are based on the interaction of the carcinogenic hallmarks, namely angiogenic and proliferative attributes, could improve survival and decrease adverse effects induced by unnecessary chemotherapy.

Key words: Colon cancer; Stem cell; Cancer stem-like cell; Tumor-initiating cell; Microenvironment

Core tip: Recent progress in the therapeutic treatment of colorectal cancer has resulted from targeting the hallmark stem cell-like properties of tumor cells. The survival of colorectal cancer patients has been significantly prolonged with bevacizumab, which inhibits angiogenesis providing the proliferative conditions for tu-

mor cell growth. Personalized therapeutic approaches, centered on the angiogenic and proliferative properties of cancerous cells, could improve patient survival and decrease adverse effects induced by unnecessary chemotherapy.

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INTRODUCTION

Carcinogenesis is a multistep process reflecting a series of genetic and epigenetic alterations in normal human cells that drives their progressive transformation into highly malignant derivatives. The successful growth of metastatic cells depends on the interactions and the properties of the cancer cells and the potential target organs, proposed as the "seed and soil" hypothesis by Paget[1]. In 2000, Hanahan and Weinberg suggested that the malignant growth of nearly all types of tumors is a result of six essential alterations in cell physiology: self-sufficiency in growth factors, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and the ability to invade and metastasize[2]. Since then, two additional hallmarks of carcinogenesis have been described: the reprogramming of energy metabolism and the evasion of immune destruction, which have contributed to the reconceptualization of cancer cell biology. The notion of a tumor microenvironment[3] has led to profound changes in the study of cancer and in the therapeutic approach. Furthermore, the study of angiogenesis and stem cell-like limitless replicative potential of cancer cells in the tumor microenvironment has facilitated progress in cancer treatment, especially for colorectal cancer (CRC).

The formation of new vasculature, or angiogenesis, is actively involved in tumor development, progression, and metastasis. Although the initial step of tumor angiogenesis is not well understood, the recruitment of perivascular support cells is necessary for blood vessel formation[4]. Diverse tissue-specific tumor-associated stromal stem cell types contribute to the formation of the tumor niche, including carcinoma-associated fibroblasts (CAFs), tumor-associated macrophages, lymphocytes, pericyte cells, inflammatory cells, normal epithelial cells, and mesenchymal stem cells (MSCs). Recently, it was shown than MSCs migrate to tumors and can transition into CAFs[5,6]. These processes appear at the earliest stage of tumor development.

The tumor niche provides conditions favorable for cell proliferation and protection against conventional therapies[7], and contains cells that possess stem cell-like properties such as self-renewal and multipotentiality[8]. These cells have been termed tumor-initiating cells, or cancer stem-like cells (CSCs). Although CSCs represent only a small proportion of cell types within the tumor, they may be responsible for the resistance to cancer therapies and tumor recurrence in solid cancers such as glioblastoma[8] and CRC[9,10], which is composed of a heterogeneous population of dormant (or quiescent) and active cells[11]. CSCs are intrinsically resistant to apoptosis and express several members of the ATP-binding cassette (ABC) transporter family, which are overexpressed in the multidrug-resistant phenotype[12]. Microenvironment stimuli, such as hypoxia, also contribute to chemoresistance by inducing a stem cell-like phenotype in cancer cells[13]. The relationship between these cells and the angiogenic microenvironment is fundamental for understanding tumor progression and therapeutic resistance.

INTESTINAL STEM CELLS

Normal intestinal epithelial cells have a lifetime of around five days and are continuously renewed by stem cells (SCs) under microenvironmental influence[14]. The intestinal SCs are located at the crypt base and are involved in tissue homeostasis and repair. These cells undergo asymmetric division, giving rise to one identical daughter cell, and one cell with the potential to migrate to the top of the crypt and fully differentiate into an intestinal cell. Intestinal crypts contain two pools of SCs. At the lowest part of the crypt base is an active pool of SCs that express Lgr-5 (leucine-rich repeat containing G protein-coupled receptor 5). A second reserve pool of quiescent SCs resides at the +4 position, *i.e.*, the fourth cell above the lowermost cell of the intestinal crypt. These cells express Bmi-1 and TERT (telomerase reverse transcriptase) and have the ability to replace Lgr-5-expressing cells[15,16]. However, the identification of intestinal SCs remains an issue of debate. Many molecules have been proposed as putative markers (Table 1), such as the cell surface proteins Lgr-5, aldehyde dehydrogenase-1 (ALDH-1), and integrin-β1 (CD29), but none have been widely accepted as specific molecular markers.

CANCER STEM-LIKE CELLS

Any normal cell can accumulate mutations and become a cancer origin cell, such as a CSC. CSCs are multipotent cells that give rise to progenitors and differentiated cells causing tumor heterogeneity, and whose migration results in metastasis. The existence of CSCs was first hypothesized in 1994 by Lapidot *et al*[17] and later confirmed when Ricci-Vitiani *et al*[14] isolated CD133+ cells from colon cancer tumors and characterized them as CSCs. CSCs express markers common to stem and progenitor cells, and are similarly capable of unlimited growth *in vitro*. While only a small portion of cells within a tumor (< 2.5%) are endowed with tumor propagation[18], CSCs have the ability to reproduce the parental tumor *in vivo*.

Table 1 Markers used to identify normal colonic stem cells and colonic cancer stem-like cells

	Marker	Function
Normal stem cell	Integrin-β1 (CD29)	Cell surface receptor - cell adhesion molecule
	Hes-1	Transcriptional repressor - transactivated by Msi-1
	Msi-1	RNA binding protein - maintenance of undifferentiated state
	Bmi-1	Polycomb receptor - maintenance of chromatin silencing
	Lgr-5	Wnt target gene - potential of self renewal
	ALDH-1	Detoxifying enzyme
	DCAMKL-1	Kinase - radioresistance abilities
	TERT	Quiescent stem cells and radio resistant
	Ascl-2	Transcription factor - target of Wnt and Notch pathways
Cancer stem-like cell	CD133	Pentaspan transmembrane glycoprotein
	CD44	Hyaluronic acid receptor
	CD166	Cell adhesion molecule
	ALDH1	Enzyme
	4-Oct	POU-domain transcription factor
	SOX2	Transcription factor
	c-Myc	Transcription factor
	Integrin-β1 (CD29)	Cell surface receptor-cell adhesion molecule

Clonal evolution of proliferating CSCs may lead to a gain or loss of stem cell-like attributes through individual responses to microenvironmental stimuli, including epigenetic modifications and additional genetic aberrations[16]. The Wingless/Int (Wnt) signaling pathway plays a pivotal role in the regulation of SC self-renewal[19]. In normal cells, Wnt signals are transduced through the Frizzled/LRP5/6 complex to inhibit the phosphorylation-dependent degradation of β-catenin. In colon cancer cells, constant but heterogeneous mutations in adenomatous polyposis coli (APC) and β-catenin genes result in high Wnt signaling activity. Moreover, extrinsic signals given by neighboring or matrix cells, such as stromal myofibroblasts, can regulate Wnt activity and SC attributes of CSCs[20].

Four other major signaling pathways are also altered in CSCs. In normal colon tissue, TGF-β and Notch signaling pathways regulate cell proliferation, differentiation, migration, apoptosis, and SC maintenance and function. Altered TGF-β signaling in more advanced and metastatic CRCs results in an inhibition of its tumor suppressive activity. Notch-1 is abundantly expressed in the stem cell zone in normal colon tissue, and is upregulated in CRC[21]. Moreover, Notch signaling is 10 to 30 fold higher in CRC-CSCs compared to commonly used colon cancer cell lines[22]. This upregulation prevents CSC apoptosis through p27, a cell-kinase inhibitor, which maintains CSC renewal and represses cell lineage differentiation genes. Neighboring myofibroblasts produce bone morphogenetic protein antagonists Gremlin 1 and Gremlin 2 in addition to Wnt signaling ligands, which can also modulate Notch signaling[23]. The Hedgehog signaling pathway is one of the key regulators of animal embryogenesis, and is also involved in the proliferation, migration, and differentiation of cells. Hedgehog signaling has been implicated in tumor growth and CD133+ stem cells in CRC[20]. Recently, a role for neurotrophins was highlighted in CRC both *in vitro* and in tumors, where enhanced brain-derived neurotrophic factor signaling as a result of increased expression of tropomyosin-related kinase B (TrkB) receptors, was associated with advanced disease and a worse prognosis[24]. Moreover, some studies suggest that TrkB regulates epithelial-mesenchymal transition (EMT) in solid cancers[25], especially in CRC[26].

CSC IDENTIFICATION

Identification of CSCs is based on SC markers (Table 1), especially Lgr-5 and Bmi-1, the only markers rigorously evaluated *in vivo*[27,28]. CD133 and CD44 are two classical markers that have also been used, though they are not specific. Two transcription factors, Oct-4 and Sox2, are more promising CSC markers as they are involved in cell renewal. Levels of Oct-4 and Sox2 are elevated in CRC and correlate with increased CSC proliferation and poor prognosis[29,30]. Several methods have been developed to isolate CSCs based on the expression of these markers[16,31] using flow cytometry (fluorescence-activated cell sorting or FACS) or magnetic-activated cell sorting[32] (Table 2), though tumor heterogeneity as well as the low abundance and lack of differentiation has made the isolation of CSCs from patient tumors and *in vitro* cultures difficult. These methods rely on specific antigen recognition and thus are restricted by the availability of highly specific antibodies. In addition, labeling of cell-surface markers by antibodies could trigger signaling pathways and induce cell modification and differentiation. Therefore, the development of methods that do not rely on marker labeling is greatly needed. Tools based on intrinsic biophysical properties such as size or density may be of benefit. Counterflow centrifugal elutriation, which separates cells by weight, has been a valuable tool for obtaining homogeneous populations[33], though experiments to isolate CRC-CSCs have not yet been attempted. More recently, CSCs have been sorted from a panel of CRC cell lines using sedimentation field flow fractionation technology, in which sorting is based on cell size and density[34].

Table 2 Advantages and disadvantages of the cell sorting methods

Method	Advantages	Disadvantages
MACS	Fast, easy to make	Cell labeling indispensable
FACS	Fast	Cell labeling indispensable, flux cytometry indispensable
CCE	Cell labeling not necessary, cell weight based method	Time consuming, specific instrumentation indispensable
SdFFF	Cell labeling not necessary, cell size and density based method	Time consuming, specific instrumentation indispensable

MACS: Magnetic-activated cell sorting; FACS: Fluorescence-activated cell sorting; CCE: Counterflow centrifugal elutriation; SdFFF: Sedimentation flux force fractionation.

TUMOR NICHE AND MICROENVIRONMENT

The non-cancerous niche is a dynamic milieu, consisting of stem cells, neural cells, lymphocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells, and myofibroblasts surrounded by a stromal microenvironment. The niche adapts in response to environmental cues to ensure the optimal conditions for SC proliferation and differentiation, even in the absence of SCs[19]. Intestinal SCs can also be affected by the components of the crypt lumen, such as bacteria or epithelial cells[16]. One of the most extensively studied niche components is intestinal subepithelial myofibroblasts, which regulate intestinal SCs by secreting growth factors and cytokines.

CSCs can secure the niche microenvironment by displacing normal SCs and interact with it to generate vascular precursors[35]. The tumorigenic niche is composed of recruited myeloid cells, vascular and lymphovascular endothelial cells, macrophages, and transformed myofibroblasts surrounded by stromal tissue. Stromal fibroblasts secrete various cytokines and growth factors that act in an autocrine or paracrine fashion on tumor cells, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and hepatocyte growth factor, which is an enhancer of Wnt activity[36,37]. CAFs that are present in the tumorigenic niche secrete the cytokines CXCL1 and CXCL2, as well as IL-1β and IL-6 to enhance angiogenesis and tumor progression[38]. These cells are able to modulate the expression of oncogenic genes in cancer cells, such as Her2, EGFR, and Ras and thereby contribute to chemotherapeutic resistance[39]. CAFs are a major contributor to the tumor-prone microenvironment, and thus promote tumor growth. Targeting of these cells remains a challenge due to the presence of distinct CAF populations that do not express tumor-specific markers[40].

MSCs are non-hematopoietic precursor cells residing in the bone marrow that also contribute to the tumor microenvironment. MSCs have been shown to influence tumor development, progression, metastatic diffusion, and resistance to chemotherapy in many solid cancers, including colon cancer[41]. The interaction of MSCs and cancer occurs early in tumor formation *via* numerous pathways. MSCs in the colon express a high level of vascular endothelial growth factor (VEGF) when stimulated by interferon-gamma and TNF-α, thus leading to colon cancer growth[42]. They can recruit endothelial cells by secreting CXCL12[43], and their secretion of IL-6 can induce non-cancer stem cells to express CSC markers and cause tumor formation *in vivo*[44]. IL-6 induces the secretion of endothelin-1 (ET-1) from cancer cells and promotes tumor development by recruiting endothelial cells and activating signaling pathways that regulate protein synthesis. This was demonstrated in a study by Huang *et al*[45] showing that angiogenesis as a result of mixing non-tumorigenic MSCs and HT-29 cells, a colorectal cancer cell lineage, was blocked by IL-6 or ET-1 antibodies. Moreover, patients with CRC have significantly higher VEGF and IL-6 serum levels, which correlate with advanced stages and metastatic disease[46]. Additional studies have also implicated IL-6 in tumor development[47-50].

ANGIOGENESIS AND HYPOXIA

In normal adult tissues, angiogenesis is only transiently turned-on. Tumor angiogenesis generates neovascularization in response to the need for nutrients and oxygen and for the elimination of metabolic wastes and carbon dioxide. During tumor progression, an angiogenic switch is activated causing normally quiescent vasculature to continually sprout new vessels that sustain expanding neoplastic growth[3]. The angiogenic switch is governed by countervailing factors that either induce or oppose angiogenesis, such as VEGF and thrombospondin, respectively. VEGF signaling is complex with alternative splice variants that are regulated at multiple levels, and *VEGF* gene expression can be upregulated by hypoxia, through activation of the HIF1 transcription factor, and by integrin or oncogene signaling. HIF1 is known to induce self-destruction or autophagy of the tumor in order to preserve nutrients in hypoxic conditions[51]. VEGF is secreted through a K-ras/PI3K/Rho/ROCK/c-Myc axis in CRC[13]. VEGF ligands signal through tyrosine receptor kinases, two of which are implicated in angiogenesis, namely VEGFR1 and VEGFR2, and a third receptor, VEGFR3, which is implicated in lymphangiogenesis. VEGFRs are not only expressed in vascular endothelial cells, but also in other cell types, including macrophages and monocytes, suggesting they play a role in EMT[52]. Other signaling pathways that have been implicated in angiogenesis have crosstalk with VEGF signaling, such as the Ang/Tie or Notch signaling pathways[53]. Other factors, such as fibroblast growth factor, or platelet-derived growth factor, are involved in the maintenance of the

angiogenic process.

The blood vessels produced within the tumor are typically aberrant with abnormal endothelial proliferation and apoptosis[54]. Numerous cells originating from bone marrow play crucial roles in pathological angiogenesis and in the formation of primary tumor and metastatic sites, notably macrophages, neutrophils, mast cells, myeloid progenitors, and endothelial progenitor cells (EPC). EPCs account for 12% of the total number of endothelial cells in tumor vessels, and play a critical role in the metastatic angiogenic switch. Most of these cells can migrate into neoplastic lesions and become intercalated into the neovasculature as pericytes or endothelial cells[55].

CSCs AND MICROENVIRONMENT INTERACTIONS: IMPLICATIONS FOR PHYSICIANS

An understanding of angiogenic pathways has progressed the development of cancer therapeutics over the last decade, especially for treatment of CRC. In 2004, the first treatment with an anti-angiogenic compound, a monoclonal antibody against VEGF named bevacizumab, was recommended for use with first and second line adjuvant chemotherapy, FOLFOX (5-flourouracil, leucovorin, and oxaliplatin) or FOLFIRI (5-fluorourcil, leucovorin, and irinotecan). A meta-analysis conducted in 2009 including more than 3000 patients concluded that the addition of bevacizumab to chemotherapy for metastatic CRC prolonged both specific free survival and overall survival despite a higher incidence in grade Ⅲ/Ⅳ hypertension, arterial thromboembolic events, and gastrointestinal perforations[56]. However, a phase 3 randomized trial assessing the use of bevacizumab in combination with oxaliplatin-based therapy in adjuvant treatment of patients with resected stage Ⅲ or high-risk stage Ⅱ colon carcinoma (the AVANT study), suggested a detrimental effect of bevacizumab that involved more serious adverse effects without an improvement in disease-free survival[57]. Thus, anti-angiogenic therapy may only benefit CRC patients with liver metastasis, though further evaluation is needed. Other anti-angiogenic therapies, such as aflibercept, a VEGFA, VEGFB and placenta growth factor decoy receptor, or ramucirumab, a VEGFR1/2/3 and Tie2 tyrosine kinase inhibitor, have been validated by clinical trials[13]. Due to the increase in plasma VEGF levels and EPCs after partial hepatectomy in CRC metastatic patients, Pocard and Eveno[58] claim the following: (1) the primary cancer should be resected rapidly to minimize metastatic niche activation; (2) systemic chemotherapy associated with anti-angiogenic drugs should be administered after surgery; (3) liver metastases should be resected; and (4) immunomodulatory and anti-angiogenic treatments should be administered to minimize recurrence.

In practice, neither anti-angiogenic drugs nor adjuvant chemotherapy can completely eliminate recurrence or resistance events. It is now acknowledged that CSCs and EMT can induce chemoresistance through intrinsic and indirect mechanisms. Intrinsic mechanisms involve proficient DNA repair machinery, high expression of ABC transporters, and altered cell cycle kinetics. For example, the blockade of ABC transporters improves patient response to neoadjuvant radiotherapy[59]. Alterations in cell cycle leave some CSCs in a state of quiescence where they are protected from chemotherapeutic toxicity, thus enabling tumor regrowth[60,61]. In addition, the overexpression of IL-4 that occurs in CRC amplifies the expression of anti-apoptotic mediators, thus the *in vivo* efficacy of cytotoxic therapy is increased with IL-4 blockade[62]. Indirect contributions to chemoresistance come from the microenvironment[12]. The dynamic interactions between CSCs and the microenvironment result in a continuous remodeling of both compartments. These epithelio-mesenchymal interactions occur in the EMT, which in addition to promoting metastasis development, plays a role in chemoresistance. Furthermore, the chaotic and dysfunctional vasculature of the tumor, which inhibits supply of oxygen and nutrients, prohibits the accrual of optimal concentrations of chemotherapeutic agents within the tumor[12]. Therefore, targeting of both the intrinsic and indirect mechanisms with anti-angiogenic agents or inhibitors of EMT/hypoxia-associated effectors will more effectively deplete the CSC pool and contribute to increased chemotherapeutic response.

A main prognostic indicator for CRC is the identification of the predominant cell type. Traditionally, the basis for prognosis and care has come from the classification of CRC as outlined by the American Joint Committee on Cancer[63]. Surgery is curative for stages 1-3, adjuvant chemotherapy is indicated for high-risk stages 2 and 3 CRC, and anti-angiogenic drugs are recommended for metastatic patients. Unfortunately, it is still difficult to predict disease progression or treatment response. Studies of CRC have attempted to determine a signature capable of identifying the patient populations with high-risk for recurrence that need adjuvant therapy. Currently, patients are screened for mutations in the *KRAS* or *BRAF* genes that indicate resistance to therapy, though a large proportion of patients with wild-type KRAS are also chemoresistant[64].

Multiple molecular subtypes of CRC have been identified, and whereas the most differentiated CRC subtypes, named transit-amplified and goblet-like subtypes, have a good prognosis and do not need adjuvant therapy, the stem cell-like subtype has the poorest prognosis and requires adjuvant chemotherapy (FOLFIRI), even in cases of metastasis[65,66]. A new classification system has been proposed based on the cellular phenotype and therapeutic response. Sadanandam *et al*[67] combined gene expression analyses with differential responses to cetuximab to define six CRC subtypes in cultured cell lines and from patient tissues. The CRC subtypes were associated with cellular differentiation state and Wnt signaling activity from distinct anatomical regions of the colon crypts.

The heterogeneity of CRC indicates that a change to

therapeutic schemas is needed. Most importantly, therapeutic approaches should include multiple targets, aimed at disrupting the cooperative interaction between the tumor cell and its microenvironment. Advancement of personalized therapeutic approaches will help to improve patient survival, not only by increasing specific survival, but also by decreasing the adverse effects induced by unnecessary chemotherapy.

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