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How the interplay among the tumor microenvironment and the gut microbiota influence the stemness of colorectal cancer cells

Colon cancer stemness: influence of TME and microbiota

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Abstract

Colorectal cancer (CRC) remains the third most prevalent cancer disease and involves a multi-step process in which intestinal cells acquire malignant characteristics. It is well established that the appearance of distal metastasis in CRC patients is the cause of a poor prognosis and treatment failure. Nevertheless, in the last decades, CRC aggressiveness and progression **have** been attributed to a specific cell population called colorectal cancer stem cells (CCSC) with features like tumor initiation capacity, self-renewal capacity, and acquired multidrug resistance. Emerging data highlight the concept of this cell subtype as a plastic entity that has a dynamic status and can be originated from different types of cells **through** genetic and epigenetic changes. These alterations are modulated by complex and dynamic crosstalk with environmental factors by paracrine signaling. It is known that in the tumor niche, different cell types, structures, and biomolecules coexist and interact with cancer cells favoring cancer growth and development. Together, these components constitute the tumor microenvironment (TME). Most recently, researchers have also deepened the influence of the complex variety of microorganisms that inhabit the intestinal mucosa, collectively known as gut microbiota, on CRC. Both TME and microorganisms participate in inflammatory processes that can drive the initiation and evolution of CRC. Since in the

last decade, crucial advances have been made with respect to the synergistic interaction among the TME and gut microorganisms that condition the identity of CCSC, the data exposed in this review could provide valuable insights into the biology of CRC and the development of new targeted therapies.

Key Words: Colorectal cancer; Colorectal cancer stem cells; Tumor microenvironment factors; Tumor stroma; Gut microbiota; Cancer progression

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Core Tip: Colorectal cancer (CRC) represents one of the most prevalent tumors worldwide. The tumor microenvironment (TME) through its proinflammatory role, among others, actively participates in CRC progression and the disturbance of gut microbiota (dysbiosis) can influence on this inflammatory process. Colorectal cancer stem cells (CCSC) are a tumor cell subpopulation that drives CRC initiation, progression and treatment failure. The features and behavior of CCSC are modulated by several factors including TME and gut microbiota. Here, we will give an overview of the synergistic interaction among TME and intestinal microorganisms that condition CRC environment and shape CCSC characteristics allowing CRC evolution.

INTRODUCTION

Colorectal cancer (CRC) is a multi-step process where intestinal cells acquire malignant phenotypic characteristics that allow them to proliferate, migrate, invade and establish in new tissues [1]. In the last decades, screening strategies and treatments have been improved, decreasing the proportion of CRC patients by as much as 65–88% [2]. However, this disease remains the third most prevalent type of cancer, having an incidence of 10% and ranking second in mortality (9.4% among all cancer deaths)

according to global cancer statistics [3]. The leading cause of patient deaths and relapses is the appearance of new CRC subtypes and the acquired resistance to currently used therapies [4]. Moreover, a great number of CRC are diagnosed by distal metastases and these patients have a poor survival rate due to a lack of response to therapy [2]. One of the causes that affect the treatment of this type of tumor by inducing resistance and the appearance of recurrences, is the presence of a small subpopulation of cells called colorectal cancer stem cells (CCSC). This small number of cells have mutations in specific oncogenes that allow them to develop the ability to induce tumor initiation, self-renew, differentiate, dedifferentiate, and acquire multidrug resistance [1] [5]. The origin of this cell subpopulation is still controversial. They may originate from colorectal normal cells, colorectal normal stem cells, or colorectal cancer cells by genetic alterations or by the influence of environmental factors that induce epigenetic changes [5].

It is known that in the tumor niche, different cell types, structures, and biomolecules coexist and interact with cancer cells favoring the growth and development of the tumor. Together these components constitute the tumor microenvironment (TME). In the last decades, several investigations have demonstrated that tumor surrounding ambience through its proinflammatory role, among others, actively participates in the development, progression and chemoresistance of CRC [1] [4].

Researchers have deepened the study of the influence of the complex variety of microorganisms that inhabit the intestinal mucosa, collectively known as the gut microbiota, on this inflammatory microenvironment. Besides contributing to innate and adaptive immune function, it has been observed that the imbalance in the species present in the intestinal microbiota and the consequent variation in microbial products can promote the development of CRC and compromise the efficacy of its therapy [6].

Since all the factors mentioned are involved in the CRC progression and therapy resistance and considering the great influence of CCSC in several events of this disease, this review aims to analyze the available literature that is focused on the interaction of

TME and the intestinal microbiota that favors the development and maintenance of CCSC properties.

COLON CANCER STEM CELLS: FEATURES AND BEHAVIOR

CRC is a heterogeneous pathology that has a variable clinical course and prognosis [7]. The etiology of this disease combines genetic alterations in colorectal epithelial cells with unhealthy lifestyles, such as smoking, alcohol consumption and poor nutritional habits [8]. In addition, it has been seen that sex, age, family history of CRC and the persistence of inflammatory processes or infectious agents in the intestinal tract, can be also considered as risk factors [5], [9]–[12]. In all these cases, the synergy among genetic mutations, epigenetic alterations and the influence of the TME and gut microorganisms promote the acquisition of molecular and phenotypic features that allow tumor progression [5], [6], [11], [13], [14]. Therefore, within the tumor niche, cells present great heterogeneity but are still strictly organized. In the last 20 years, the focus has been on the study of cancer stem cells (CSC) derived from colorectal tissue (CCSC), a subpopulation of cells that have a substantial tumorigenic capacity and maintain intestinal tumor growth [15]. CSC are responsible for resistance to multiple drugs maintaining a state of undifferentiation and slow cell division and also favoring the efficiency of desoxyribonucleic acid (DNA) damage repair mechanism [16]. Besides, they have similar features to normal stem cells, such as self-renewal, multipotency, cell cycle arrest, quiescence, and reversibility from their resting state [17], [18]. As shown in **Figure 1**, the ability of CSC to maintain their population response to symmetric/asymmetric division, resulted in the first situation in two identical daughter stem cells and in the second situation in two distinct cells with or without CSC properties [19], [20]. In addition to the division theory, CSC undergo a bidirectional conversion process between stem and non-stem phenotype [20]. Although initially a hierarchical model has been established, in which CSC are the initiators of a monoclonal developmental hierarchy, emerging data highlight the concept of phenotypic plasticity of CSC. This new theory is

supported by a dynamic state of interconversion between CSC and non-CSC that can be driven by the TME [21]-[23]. As the reader can see in **Figure 1**, during this phenomenon, cells can easily exchange their status within the tumor transforming from CSC to intermediate phenotypes to stemless states and vice versa [15], [18], [22], [24]. Therefore, based on the data provided by the literature and shown in **Figure 1**, it can be concluded that any cell type is capable of initiating and promoting cancer development [24]. This model contributes with new concepts to the classical theory of the origin/behavior of CSC that highlight the importance of taking into account the study of phenotypic plasticity and the reversible state of this type of cells and that support the criterion that cancer cells with or without stem characteristics must be eradicated for successful therapy.

CCSC constitute about 2% of the cell population in the tumor nest and this percentage can be higher with tumor progression, particularly after chemotherapy or radiotherapy treatments [17], [18], [25]. Since an increase in the proportion of this cell subtype is an indicator of poor prognosis, in the last decades the identification and targeting of CCSC have become one of the key topics of study [26]. The recognition of CCSC is possible by the detection of typical phenotypic characteristics such as the expression of surface markers, membrane transporters and enzymes. Some of them are Prominin-1/cluster of differentiation 133 (CD133), a transmembrane glycoprotein that is associated with metastasis, invasiveness and chemoresistance in CRC [18]; cluster of differentiation 44 (CD44) a receptor of hyaluronic acid in extracellular matrix related to the epithelial to mesenchymal transition (EMT) program and poor survival in CRC patients [5], [27]; cluster of differentiation 166 (CD166) and cluster of differentiation (CD24), both adhesion molecules whose expressions are associated with the aforementioned markers, and that contribute to stratify low, intermediate, and high-risk CRC cases [5], [28]; leucine rich repeat containing G- protein coupled receptor 5 (LGR5) a key CCSC biomarker that decreases in advances stages of CRC [20], [29] and aldehyde dehydrogenase (ALDH), an intracellular enzyme found in high concentrations in most of CSC participating in self-renewal, differentiation and self-protection [20], [30], [31]. In addition, the study of the ATP-

binding cassette (ABC) transporter superfamily through Hoechst 33352 dye efflux is also employed to detect CCSC [15], [32]. In experimental models, the identification and characterization of CCSC can also be performed by fluorescence-activated cell sorting, selection by cell culture properties, *in vivo* transplantation of cells derived from spheroids or organoids, and lineage tracing techniques with labeled CCSC [22]. The above mentioned markers are hallmarks of CCSC and are involved in CRC pharmacotherapy and pathophysiology [33], [34], but can also be present in enterocytes and cells of other tissues [20]. Hence, to increase the detection sensitivity and specificity, it is essential to combine the analysis of different biomarkers with CCSC isolation techniques.

Another substantial aspect to consider in the study of CSC is their association with other cellular processes such as EMT, autophagy and the response to cellular stress [15]. In particular, EMT is a physiological process that is also involved in tumor progression. The activation of this program ⁷ reduces intercellular adhesion and causes epithelial cells to acquire mesenchymal properties that increase invasiveness and migration of tumor cells [35]. Several studies have reported a link between EMT and the acquisition of CCSC characteristics in both, *in vitro* and *in vivo* assays [35]–[38]. These investigations show that transcription factors and signaling pathways that are altered in the EMT program are also deregulated in CSC, generating this subpopulation to exhibit phenotypes like EMT [39]. However, recent evidence indicates that EMT may not be necessary to acquire CSC properties. Then, although these processes can go along with each other, they can also happen through independent paths [15]. One of the tumor events that is known to be related to EMT and CSC is the high metabolic demand of TME and the existence of a tortuous vasculature that promotes a hypoxic environment. This phenomenon induces the release of factors such as hypoxia-inducible factor 1 α (HIF-1 α) that promotes not only EMT, but also autophagy associated with CSC. In CRC it was demonstrated that blocking this factor with the consequent inhibition of autophagy reduces cell proliferation and the acquisition of stem-like characteristics [40].

Another cause that has been reported that promotes a stem-like phenotype on several types of tumor cells is the cellular imbalance derived from oxidative stress [15]. In breast and lung cancer cell lines, studies demonstrate that oxidative stress upregulates the CSC marker SRY-box transcription factor 2 (Sox2) activity, and stem-like properties [41], [42]. However, in CRC cells it was shown that the reduction of intracellular reactive oxygen species inhibits the formation of colorectal cancer stem-like cells [43]. Since this type of cellular stress is considered potentially cytotoxic, more studies are necessary in order to know the mechanisms by which it has a positive effect on the development of CCSC [15].

Furthermore, it is important to note that like all processes and phenomena related to tumorigenesis and progression, CCSC and their features are modulated by the aberrant activation of various signaling pathways. Wnt, NOTCH, Hedgehog, and Transforming Growth Factor- β (TGF β) are important cascades that are usually misregulated in CCSC and play a central role in the therapy resistance of these cells [5], [44].

Thus, understanding CCSC features and all the events and factors associated with cell plasticity constitute a fundamental tool for the development of new target therapeutic strategies.

INFLUENCE OF THE TME ON CCSC FEATURES

It has been reported that multiple links exist between inflammatory processes and stemness in CRC [2]. In this context, the role of the tumor stroma is crucial. The TME in CRC is a physical shelter for CSC [5] composed by biomolecules from the extracellular matrix, an aberrant vasculature and multiple stromal and immune cell types. These cells include ¹mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), endothelial cells (ECs), pericytes, and tumor infiltrating immune cells (TIICs) which comprehend: macrophages, neutrophils, natural killer cells, Treg cells and cytotoxic T lymphocytes [2], [4]. The interaction between CRC cells and the different types of cellular and non-cellular elements of TME involves complex and dynamic crosstalk by paracrine signaling [22]. Therefore, self-renewal, differentiation and properties of

colorectal cancer cells and CCSC are modified by factors released by the surrounding stroma [1]. These factors are cytokines, growth factors and small nucleic acids, which have different mechanisms of action. Next, we will discuss those derived from TME that modulate CCSC properties and that are summarized in **Table 1**.

Cytokines have been shown to play a key role in CRC stemness. It was reported that TME-derived factors with a pro-inflammatory action such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) foster EMT phenotype and stem cell proliferation in human colon cancer cells [45], [46]. Besides, it is known that CAFs, one of the most studied cells in the TME, produce interleukin-6 (IL-6), which promotes the expression of CCSC markers such as ALDH1 and LGR5 [1], [47].

The acquisition of a stem-like phenotype is also influenced by the expression and secretion of growth factors [48], [49]. It was demonstrated that the epidermal growth factor (EGF) and the insulin-like growth factor (IGF) regulate and promote CCSC growth [50]. Moreover, Muñoz Galván and colleagues have proved that the treatment of CRC derived cells with hepatocyte growth factor (HGF) and/or macrophage migration inhibitory factor (MIF) increases the number and size of colonospheras and significantly enhances the expression of putative markers like CD133 [49].

Proangiogenic factors like vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) are also implicated in promoting growth and metastasis, both processes directly related to stemness [50]. Furthermore, it was demonstrated that clusters of ECs improve the survival of CCSC and promote their spread [51].

As it is known, all these TME factors modulate the activation of different signaling pathways, altering gene expression and thus modifying the molecular and phenotypic profile of tumor cells [5], [44], [50]. Wnt signaling is a key stem cell pathway involved in the maintenance of the CCSC and the TME [13], [52]. One decade ago, Vermeulen and colleagues observed that high activity of the Wnt signaling was associated with CCSC features. Furthermore, this activity was mainly observed near fibroblasts in the tumor niche. Vermeulen then demonstrated that HGF derived from CAFs activates Wnt signaling and the clonogenicity from CCSC [53]. This research had a great impact on the

study of CSC and recently, Essex and collaborators replicated these studies and obtained similar results. They found that TME regulates the activation of the Wnt signaling pathway, increases CCSC characteristics and enhances tumor-initiating potential [54]. Regarding this, it is known that several Wnt ligands are secreted mostly by CAFs [53]–[56]. Moreover, other TME factors participate in the activation of the Wnt/ β -catenin pathway (**Table 1**)

Some ligands from other signaling pathways are also related to stem cell phenotype. TGF- β is a growth factor that belongs to a superfamily of molecules including inhibins and bone morphogenetic proteins (BMP) [13]. It has the ability to promote or suppress tumor development depending on the interactions that take place in the TME [57]. As a pro-tumor factor, TGF- β regulates immune responses and participates in many neoplastic events such as proliferation, EMT and stemness [13]. TGF- β signaling pathway mutations and CCSC are linked [58] and in accordance with this, Zhou and colleagues found an association between TGF- β signaling and the expression of LGR5 biomarker in CRC [29]. Even more, Shoujun and colleagues have recently demonstrated that the expression of genes related to CCSC features like the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) alters TGF- β signaling and promotes CRC [59]. Some other members from the TGF- β family, like bone morphogenetic protein 4 and bone morphogenetic protein 2 (BMP4 and BMP2, respectively), have the capacity to induce CCSC differentiation and increase the response to standard chemotherapy [60] [16], [61], [62]. Besides, the modulation of the BMP4 pathway by hormones like triiodothyronine (T3) was reported in CCSC, decreasing its tumorigenic potential [63], [64] [44]. This result suggests that CCSC features are modulated not only by local molecules from the TME but also by endocrine factors [44].

Notch signaling is also associated with the expression of CSC features in CRC cells [16], [65]. In fact, it was reported that delta like canonical Notch ligand 4 (DLL4) and jagged1 (JAG1), both Notch ligands, are overexpressed in this type of tumor providing essential signals for CCSC maintenance [44], [66]. Moreover, since Hedgehog (HH) signaling is implicated in CRC development [20], in the last years several investigations were

conducted on the association of this pathway and CCSC properties. Regan and colleagues, have shown that the activation of the non-canonical Hedgehog pathway is required for CCSC survival and depends on sonic hedgehog protein (SHH) ligand [67]. Recently, it has been also observed that the modulation of HH-related proteins expressions by non-coding ribonucleic acids (ncRNAs) impacts on CCSC self-renewal capacity and drug resistance [68]. In line with this, Skoda and collaborators showed that treatment with hedgehog pathway inhibitors such as vismodegib and sonidegib weakens the ability of CCSC [69]. Since no significant differences have been found in clinical trials [70], more studies are needed to determine the effects of the inhibition of this pathway in CRC patients.

Besides the aberrant activation of several signaling pathways, hypoxia is known as a hallmark of CCSC and TME interaction [5]. This is a condition in the tumor niche whose main cause is the poor vasculature associated with the tumor and the upregulation of HIF-1 α , a factor released mainly by EC [40], [71], [72]. This condition activates Wnt/ β -catenin pathway inducing self-renewal and maintenance of CCSC [50], [73]. Also, HIF-1 α promotes cancer cell proliferation and CCSC survival [40].

Furthermore, short ncRNAs like microRNAs (miRs) and long ncRNAs (lncRNAs) are secreted not only by tumor cells but also by stromal cells in the TME [4]. In the last decades, the study of ncRNAs has gained importance in CRC. In the framework of factors and signaling pathways related to CCSC biology, these small nucleic acids have a key role [74]. miRs related to stemness in CRC are exposed in **Table 1**.

As previously mentioned, the interaction between CRC cells and their TME also involves non-cellular elements. Colonic stromal cells mediate the remodeling of extra-cellular matrix favoring the healing or progress of the disease [75]. Recently, it has been demonstrated, by lineage tracing, that components of extra-cellular matrix regulate dormancy in CCSC [76]. Tenascin, fibronectin, collagen type I, secreted protein acidic and rich in cysteine (SPARC), galectin and some other components of the tumor matrix are associated with stemness and CCSC activities [1].

Finally, another important concept to consider in the tumor nest is that CCSC also release various factors and cytokines that enable them to communicate with stromal cell, maintain their properties and evade the immune system, such as interleukin 4 (IL-4) and the cluster of differentiation 200 (CD200) [5], [44].

The aforementioned data (and shown in **Table 1**) suggest that TME instructs the development, properties, plasticity, maintenance and dissemination of CCSC. In the last decade, the remarkable influence of the stroma on CRC development prompted the postulation of a novel classification of this disease based on its impact on tumor gene expression [5]. This CRC staging contains four consensus molecular subtypes (CMS) plus an unclassified group are summarized in **Table 2** [1], [5], [77]-[79]. As the reader can see in this Table, the influence of TME determines low or high degree of immune and inflammatory response depending on the CMS, highlighting the importance of factors from TME in the distinctive characteristics of each CRC subtype. Taking into account that the mentioned inflammatory/ immune process (that is relevant for CRC classification) can be influenced by the intestinal microorganisms, next we will discuss the interactions of this microbiota with tumor cells and their microenvironment that modulate the behavior and characteristics of CCSC, since it is the focus of this review.

ESTABLISHED DYNAMICS BETWEEN THE GUT MICROBIOTA, THE TME AND CCSC

As we mentioned in this work, the inflammatory microenvironment contributes to promoting CRC initiation and progression. However, the role of the cell types involved in this process, including intestinal microorganisms, has not been completely understood yet.

The human microbiome, a concept that is mentioned throughout this section, represents microorganisms with their genetic elements and the interactions that arose with the environment in which they are found [94]. Advances in the characterization of this human microbiome have led to the consideration that the role of the microbiota in

metabolic functions and maintenance of homeostasis is more important than previously believed. Currently, the human is considered as a holobiont organism inhabited by millions of microorganisms including bacteria, archaea and fungi ^[95]. The gut microbiota is a complex ecosystem that contains more than 500 bacteria species involved in physiological processes like immune regulation and maintenance of human health ^[6] and its composition relies fundamentally on diet and lifestyle ^[74]

In physiological conditions, stromal and immune cells from the gut mucosa interact with this ecosystem to maintain the intestinal equilibrium ^[96]. Cells from the immune system recognize antigens from foreign cells and generate memory and effector cells, which control or avoid the generation of diseases ^[96].

It has been observed that sustained shifts in this ecosystem, known as intestinal dysbiosis, have unfavorable repercussions on health ^[74], ^[97]. In this sense, the presence of harmful microorganisms (“drivers”) could induce changes in the intestinal mucosa and favor the colonization by opportunistic bacteria (“passengers”)^[98]. This model is known as driver-passenger ^[98] and could involve changes in immune system allowing the advance of the damage in the intestinal epithelium tissue^[99], ^[100]. This imbalance of the local microbiota promote the restructuration of the intestinal environment and alter the immune status of the host contributing to the appearance of malignant cells and a favorable niche for tumor development, invasion and metastasis^[99], ^[101], ^[102]. The mechanisms of these microorganisms that influence directly on the immune system are different and involve the synthesis of immunomodulatory compounds and metabolites, like short-chain fatty acids (SCFA), polyamines and other fermentation products^[103], ^[104]. Moreover, it is known that the intratumoral composition of microorganisms affects T-cell-mediated cytotoxicity and anti-tumor immune surveillance^[105]. The unfavorable changes in the intestinal microbiota can promote a pro-inflammatory environment and impair anti-cancer immunity^[105]. In this context, cells from TME secrete factors like interferon- γ , TGF- β , IL-6, IL-8, CXCL1 and TNF- α , and favoring the differentiation of T helper 17 Lymphocytes (Th17) to develop an adaptive immune response that contributes to immune-prone carcinogenesis and CRC development ^[79], ^[101], ^[106]. In this

regard, increasing evidence suggests that gut microorganisms condition CRC patients response to immunotherapy, because they alter the expression of elements such as anti-programmed cell death protein 1 (PD-1) and its ligand (PD-L1) and anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4)^{[105], [107]}. PD-1/PD-L1 has been highly studied in the last years on the tumor-microbiome-immune axis^[105]; in fact, several investigations provide evidence that PD-L1 is overexpressed on different tumor cells and stromal immune cells, allowing tumors to evade attacks *via* T-cell infiltration ^{[105], [108], [109]}.

The increased expression of PD-L1 in CRC cells both *in vitro* and *in vivo* is a mechanism involved in the influence of certain pathogenic bacteria associated with an immunosuppressive tumor microenvironment^[110]. In contrast, bacteria associated with a healthy microbiota improves the efficacy of anti-PD-L1 therapy by enhancing the accumulation of cytotoxic T cells in the tumor microenvironment ^[111]. This suggests that TME reprogramming through manipulation of the microbiota can modulate the response to immunotherapies in CRC^[112]. Concerning all this information, CRC could be considered as a bacterial-induced disease and disturbance in microbiota could be a potentially useful as diagnostic biomarker, indicator of risk and predictor of response to therapies for this type of cancer ^{[74], [102]}.

On the other hand, CRC modifies the local metabolic environment ^[113]. In this context, it is important to mention that metabolites and factors derived from CRC cells and TME cells such as spermidine, L-valine, L-lysine or stearic acid confer an advantage for the growth and development of certain bacterial species, conditioning changes in the intestinal microbiota^[113]. Although different factors produces changes in gut microbiota, recently it has been seen that the shift in the metabolome of tumor cells and TME cells is a key aspect in this event^{[100], [113], [114]}. Thus, TME can be the consequence or the cause of intestinal dysbiosis.

The gut microorganisms cited below in this section are described in the available literature due to their role in CCSC development and maintenance. They are also summarized in **Table 3**.

Regarding CSC properties, some pathogenic bacteria such as *Helicobacter pylori* and *Porphyromonas gingivalis* can promote the expression of markers associated with stemness such as CD44 and CD133 in gastrointestinal tumors [115], [116]. This association between the presence of certain bacteria genera in the gut and the expression of CSC markers has led to the study of the effects of microorganisms shifts and bacterial metabolites on CRC. Several models of tumorigenesis induced by bacteria have been proposed, suggesting how the interactions of host-microorganism promote the development and progression of this type of cancer [115]. In fact, it is known that the metabolites from the intestinal microbiota have the potential to act as tumorigenic factors. However, others can act as anti-tumorigenic factors since many of these microbiota-derived products are capable of inhibiting CRC progression [117]. Kim and colleagues have demonstrated that ursodeoxycholic acid, a secondary bile acid produced by *Clostridium* species, including *Clostridium absonum* and *Clostridium baratii*, regulates the oxidative stress suppressing CCSC growth and CRC cells proliferation [43]. Moreover, it has been observed that niacin, a product of the metabolism of some intestinal bacteria, such as *Lactobacillus acidophilus*, has different effects on CCSC. Depending on the dose, this vitamin can promote proliferation or death in this cell subtype [118]. Additionally, bowel microorganisms produce short-chain fatty acids (SCFAs) such as butyrate, propionate and acetate [106]. It has been reported that these SCFAs favor beneficial bacteria proliferation and stimulate regulatory T cells to reduce inflammatory mediators, regulating immune response [119]. Butyrate participates in epithelial integrity maintenance and has antitumor effects. Several investigations show that in CRC, this product inhibits events associated with CSC such as invasion and proliferation [6]. Interestingly, butyrate inhibits cell proliferation to a greater extent in CRC derived cells than in non-cancerous cells [106]. Although butyrate was reported as an anti-tumor and chemopreventive agent [106], [120], other studies have shown that it has variable outcomes on CCSC [106]. So, more investigations are necessary to determine the mechanistic action of this type of fatty acid. Experiments with other SCFAs like acetate and propionate with similar results demonstrated that these acids have opposing effects

[6], [121]. Besides that, a large number of microbial products such as deoxycholic acid (DCA), lithocholic acid (LCA) chenodeoxycholic acid (CDCA), taurochenodeoxycholic acid (TCDCA) and others, are associated with the promotion of gastrointestinal tumors including CRC [6], [122]. Recent studies have found that in CRC patients the microbial composition of the colonic crypt is different from that of the intestinal lumen. In the environment of the crypt of the colorectal tumor, groups such as *Proteobacteria* and anaerobes, such as *Acinetobacter*, *Stenotrophomonas* and *Delftia* were found [123]. Therefore specific microorganisms could have a role in the maintenance of CCSC, located in the crypt, through the production of specific metabolites [124]. However, more studies are needed in this field since the molecular mechanisms underlying the effects of intestinal microbial products on CCSC have not yet been fully elucidated.

Currently, the study of mechanisms involved in the communication between the microbiota, the tumor cells and their microenvironment has gained impact on CRC. One reported mechanism for this interaction is through pattern recognition receptors (PRRs) located on intestinal epithelial cells that have the ability to detect distinctive microbial macromolecular ligands called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) and peptidoglycans [125]. Congruently, a recent work documented an altered function of CSC in a CRC murine model due to intruding bacteria like *Escherichia*, *Shigella*, and *Citrobacter*. This effect results in the activation of a toll-like receptor (TLR), a class of PRR, and the consequent upregulation of stem cell-associated genes such as *Cd44v6* and *Lgr5* [124], [126]. In line with this, the microorganisms are capable of activating several signaling pathways in tumor cells and/or TME cells and induce the secretion of factors associated with CCSC features. In this context, it has been observed in a murine model that microorganisms such as *Enterococcus faecalis* cause colitis after infection and induce expression of TGF- β , thereby activating the Smad signaling pathway [127]. A recent study has demonstrated an inverse correlation between the expression of molecules associated with TGF- β signaling pathway and stem cells- related genes in CRC. Moreover, the authors of this work have compared feces from mice with defects in TGF- β signaling with feces from wild-

type (WT) mice, and have shown that the first ones had increased bacterial species associated with the development and progression of CRC, such as *Clostridium septicum*, and diminished amounts of favorable microorganisms including *Bacteroides vulgatus* and *Parabacteroides distasonis* [59]. Similar results were obtained by Wang and collaborators who showed that the amounts of beneficial species (*Bacterioides dorei*, *Lachnoclostridium* sp., and *Mordavella* sp.) are recovered in WT mice but not in those with mutated TGF- β signaling after chemotherapy treatment [128]. These investigations demonstrate the close relationship between the microbiota, the production and release of TGF- β and CCSC in the tumor.

Concerning other signaling pathways, Wang and colleagues have shown that *Enterococcus faecalis* are capable of polarizing macrophages by activating Wnt/ β -catenin signaling and pluripotent transcription factors associated with the dedifferentiation, reprogramming and development of CCSC such as cellular myelocytomatosis oncogene (c-Myc), Kruppel-like factor 4 (Klf4), octamer-binding transcription factor 4 (Oct4), and SRY-Box Transcription Factor 2 (Sox2) [129]. These events respond to the microbiota-induced bystander effect (MIBE) theory based on the fact that macrophages induce genetic mutations and chromosomal instability in intestinal cells [130].

Other signaling pathways associated with pro-inflammatory and growth factors can be activated in response to bacterial products. For instance, the unbalance in the amount of the gut bacteria *Escherichia coli*, correlates with CRC progression by producing the genotoxin colibactin [79]. This toxin accelerates tumor progression and involves the production of growth factors related to CCSC, such as the HGF and the consequent activation of its signaling pathway [79], [131]. Also, the enterotoxin produced by *Bacteroides fragilis* (ETBF) promotes immune TME cells activation with the secretion of IL-17 which favors CCSC properties [132]. Furthermore, as we have previously mentioned, gut microorganisms shape the immune environment promoting tumor evolution and CCSC features. For example, *Fusobacterium nucleatum* stimulates IL-8 secretion by TME cells and the inhibition of T and NK cell functions [79]. This bacteria has been deeply studied, since clinical analysis of specimens from CRC patients showed that the levels of *F.*

nucleatum are significantly higher in neoplastic tissues than in adjacent normal tissues, and correlate with tumor invasion and metastasis [133]. These results support the role of *F. nucleatum* in the regulation of CCSC plasticity and EMT [115]. Also, it is known that *F. nucleatum* and other microorganisms like Epstein-Barr virus are capable of incorporating human ncRNAs favoring microbial growth [74]. In this regard, Tarallo and colleagues found a human and microbial ncRNA signature in CRC in which many miRs associated with CSC features, are overexpressed including miR-21 and miR-200 [74], [134]. A recent study conducted by Wang showed that *Citrobacter rodentium* infection induces the inhibition of miR-34a, which protects the inflammatory CCSC niche [135]. These investigations suggest a close relationship between the intestinal microbiota and the regulation of ncRNAs involved in CCSC properties.

Finally, not only the shift in the amount of microorganisms is responsible for stemness and CRC progression, the interaction and collaboration between several types of bacteria in biofilm communities also participate in bowel inflammation and CRC. It was demonstrated that biofilms correlate with an increase in IL-6 secretion by TME cells playing a key role in proliferation, cell transformation and stemness [79].

The data in this section demonstrate a close interrelationship between the gut microbiota, the TME, and CCSC. This information highlights the relevance of further investigating the intestinal microbiota switch in patients with CRC and the associated mechanisms that lead to TME changes and promote stemness.

THERAPEUTIC TARGETING OF TME AND THE GUT MICROBIOTA: A KEY TOOL TO MODULATE STEMNESS IN CRC

Standard chemotherapeutic approaches for CRC are based on attacking the replicative mechanisms of tumor cells to induce tumor regression. However, considering CSC properties, this subpopulation usually results unharmed by the treatment because they present a low division rate as well as a great capacity to correct DNA defects [136]. This entails therapy resistance of CSC and the subsequent treatment failure and disease progression. It is interesting to note that in CRC, CSC represent around 2.5% of

neoplastic cells but due to their phenotypic plasticity they constitute a dynamic population [137], [138]. This fact, together with the lack of response to therapies, highlights the need of new clinical strategies targeting CCSC [139].

As we explain throughout this review, the influence of the TME and the intestinal microorganisms on CSC properties makes these factors a promising tool in therapy. Many therapeutic agents are capable of inhibiting those events associated with the maintenance of CCSC. For instance, Apatinib, nabucasin (BBI-608), Bigelovin, Wogonin and Metformin are drugs whose mechanisms are associated with the inhibition of EMT or angiogenesis in CRC [1]. Moreover, it has been demonstrated that therapeutic agents such as Genistein cause the inhibition of CSC characteristics by glioma-associated oncogene1 (Gli1) signaling pathway [140]. Targeting the activation of those signaling pathways associated with CCSC can also be considered as a mechanism to reduce stemness in CRC tumors and thus improve the response to the therapy. LGK974, Foxo-5, PRI-724 [141] and DKN-01 [140] are agents that act targeting the Wnt/ β -catenin pathway. However, the clinical application of most of these drugs is still under study.

The tumor protective niche also could be modified to eradicate CCSC and overcome chemoresistance. As we have mentioned in previous sections, in the TME, immune cells modulate cancer development and progression. For that reason, in the last decade the treatment of patients with immune checkpoint inhibitors such as CTLA-4 and PD-1/PD-L1 has been studied. Even though employing these drugs leads to various systemic and organic complications, immunotherapy may be promising in sorting these obstacles and could ameliorate the response of CRC patients to the treatment [1], [142]. In fact, coadjuvant therapy with FOLFOX (a combination of leucovorin, fluorouracil and oxaliplatin which are first-line chemotherapeutic drugs) [143], and PD-1/PD-L1 inhibitors had an objective response rate of 50% in clinical trials [144]. In addition, in a phase II trial in CRC metastatic patients, immune checkpoints inhibitors like nivolumab and nivolumab-ipilimumab show improvement in patients survival rate [144]. Moreover, monoclonal antibodies against CAFs and antifibrotic drugs were also tested in clinical

studies [5]. Another type of antitumor therapy was accomplished through the production of a cell-based vaccine with specific antigens of CCSC [5].

In addition, plenty of compounds were designed in the last decade to target CCSC signaling pathways [5]. These strategies include the inhibition of HH signaling components, NOTCH pathway inhibitors, anti-angiogenic agents and Wnt ligand blockers. All these drugs are undergoing clinical trials [143]. Despite being an encouraging strategy, it still has limitations like the inhibition of signaling pathways involved in physiologic processes.

In the last years, the particularities exhibited by extracellular vesicles (EVs) have led researchers to consider them as a therapeutic delivery strategy of great value in CRC and other types of tumors. Within the different types of EVs are the exosomes, which are secreted by a variety of cells. These vesicles carry out the molecular content of donor cells and enable cellular communication over short and long distances. These EVs are loaded with coding nucleic acids, ncARNs and bioactive proteins which determine their functions. Exosomes can target a specific tissue and internalize in a cell type by the recognition of surface ligands/receptors [145]. In this regard, Han and collaborators investigated the delivery of human cord blood-derived MSC exosomes loaded with miRs as CRC targeted therapy. The results showed an inhibition of tumor growth *in vitro* and *in vivo*, as well as a selective increase of these ncRNAs in CRC cells [146]. The relation between miRs and CCSC was mentioned in previous sections so their delivery may be strong weapons to confront drug resistance and CCSC maintenance [5]. Circular RNAs (circRNAs) are ncRNAs that exhibit cell-type and tissue-specific signatures. There has recently been considerable attention on these ncRNAs as they modulate miRs expression [143]. In CRC, recent studies have focused on their study as biomarkers. However, they have not been applied in patients' therapy yet [147], [148]. Moreover, the importance that these small molecules could have in colorectal cancer is unknown [143].

Foods containing biologically active ingredients are termed functional foods or nutraceuticals [149], [150]. In the past years, the influence of diet on CRC development and evolution was demonstrated. A diet with natural products like phytochemicals and

nutritional herbs has shown protective effects in overcoming CRC associated dysbiosis [151], [152]. Diets enriched in dairy are a major source of products that are known to have a protective effect on CRC development such as, calcium, vitamin D and folate [152]. Sulforaphane, a sulfur-rich compound found in cruciferous vegetables like broccoli, has been documented to diminish CSC markers and improve the chemotherapeutic efficacy of drugs commonly used in CRC treatment such as cisplatin, doxorubicin and fluorouracil [151]. It has been observed that dietary polyphenols like quercetin have similar effects [151], [153]. Other polyphenols or flavonoids are known to target ABCG-2 transporters and miRs strictly associated with CCSC [153]. Curcumin is one of several substances present in turmeric plants. It has been demonstrated that this bioactive agent inhibits the activation of several signaling pathways related to CSC characteristics. The treatment with this natural product on a CSC model diminished the expression of CD44 and CD133 markers [151]. Moreover some other natural products have been observed that interfere with intrinsic CSC pathways, like epigallocatechin-3-gallate (EGCG), resveratrol and genistein [154].

Diet can also manipulate the gut microbiota. Indeed, this is achieved by the administration of probiotics in the diet. As probiotics and their active metabolites can exert immunomodulatory and anti-tumorigenic effects [149], the study of them and their metabolites has gained ground in recent decades. Probiotics are live microorganisms, normally lactic acid bacteria, ¹¹ recognized as safe by the United States Food and Drug Administration (FDA) [149]. Defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [155], they can improve health by administration along vegetable fibers and other prebiotics stimulating beneficial bacterial growth in the intestine [156].

Probiotics administration can be done by different routes, commonly through functional foods, but also by commercial supplements or vaccines [149], [152]. It is known that probiotic oral vaccines promote mucosal immunity that prevents enteric infections and could complement the standard therapy in the patient [157]. Microorganisms administration including probiotics and synbiotics (pharmaceutical preparation that

contains probiotics and prebiotics that implies a synergy between both) are a potential resource for prophylaxis and therapy in CRC [152], [158]. In addition, the luminal cocktail of microorganisms in the bowel can be modified not only by dietary approaches but also with the use of antibiotics or fecal microbiota transplantation (FMT) [159], [160]. In particular, FMT has gained considerable interest in recent years as a strategy to treat different gastrointestinal disorders [161]–[163]. It consists of introducing a healthy microbial population from a disease-free host into a diseased host that has a dysbiotic community to restore microbial homeostasis [164]. Although there are limited data on the use of FMT in the treatment of CRC, several studies are under development to answer relevant questions such as if CRC can be detected, treated or prevented with this method. Rosshart and collaborators observed that mice treated with this method improved their resistance against colorectal tumorigenesis induced by azoximetane [165]. Besides, it has been seen that FMT in Balb-c mice prevents intestinal damage, and chemotherapy-induced toxicity [166]. Interestingly, the fecal microbiota from CRC patients has been shown to cause tumors in healthy and germen-free *Apc^{min/+}* mice through the activation of the Wnt signaling pathway. In these mice, the intestinal barrier is also altered and the presence of pro-inflammatory cytokines is increased [167]. These data reveal that the composition of the microbiota may play a determinant role in TME conditions during tumorigenesis. Nevertheless, the subjacent mechanisms of all these treatments or how they ameliorate the side effects of chemotherapy it is not clear yet.

In summary, we need a favorable and efficient clearance of tumor cells, all tumorigenic cells including CCSC and a restructuring of the TME for the complete eradication of CRC. Based on everything described in this review, a specific combination of techniques and therapies for each tumor and patient would be necessary to achieve this goal.

FUTURE PERSPECTIVE

According to the information stated in the previous sections, in CRC occurs an alliance between the TME, intestinal microorganisms and CCSC that favors tumor progression.

In this scenario it is emerging a new query regarding the direct effects of CCSC on gut microbiota. Perhaps the appearance of CCSC by spontaneous mutations favors (through paracrine signals and the release of specific factors) a dysbiotic and pro-inflammatory environment but in this regard new investigations are necessary to evaluate the regulation of CCSC on CRC microbiota. So, there is great potential in the study of the interrelationship between these three components in the tumor niche, mostly for the development of new therapies aimed at the eradication of CCSC and non-stem cells, the restructuring of the TME and the growth induction of microorganisms that are beneficial to the intestinal mucosa.

Many of the therapies currently in use or under clinical evaluation are associated with systemic toxicity since they do not act on a well-defined target ^[151]. Therefore, the combination of radiotherapy and chemotherapy has still remained the strategy of choice in CRC ^[159] and not much attention is paid to nutritional accompaniment. Since the gut microbiota seems to be a pivotal factor in inflammatory disease and CRC development, overcoming therapy resistance could also improve with changes in diet. For this purpose, is crucial the development of foods containing compounds with anti-CCSC activity such as flavonoids but with better bioaccessibility and bioavailability ^[168]. Moreover, bacteriotherapy is a great opportunity to customize CRC treatment and the following tools that we will mention could be useful in this type of therapy. The modification of patient microbiome tending to resolve dysbiosis through the administration of beneficial bacteria could significantly improve conventional treatment ^[107]. Even more considering that some microbial species exhibit tumor targeting specificity, this strategy could ameliorate cytotoxicity in non-tumor cells. Regarding bacterial products, given their low molecular weight and hydrophobicity, they can easily enter tumor tissues and exert their action ^[169]. These facts result in the use of microorganisms with potential preventive or palliative action in CRC currently receiving special attention. In fact, many of them are studied to be used for the delivery of drugs to the tumor site and to produce anti-cancer vaccines ^[159], ^[169]. However, the information about the toxins, metabolism of microbial-derived agents and

complications from bacteriotherapy is still limited [169]. Thus, placing emphasis on clinical research that allows the use of these new therapies, overcoming the obstacles related to it, will be essential in the coming years.

In addition, as we discussed in the previous section, it is also necessary to focus on the restructuring of the TME in favor of improving conventional CRC treatment. Restructuring the extracellular matrix, modulating the immune response with vaccines, antibodies, or inhibitory drugs, employing drugs that induce changes in the secretion profile of TME cells, switching macrophages polarization and inhibiting CAFs and processes like fibrosis and inflammation are some of the potentially effective techniques under investigation. [1], [5], [130], [142].

The development of vaccines containing CSC-specific antigens is also under investigation [5]. However, since many of the antigens present in this cell subtype are also found in differentiated cells or normal stem cells, this is a challenge to overcome for successful therapy.

So, the combination of conventional therapies with new targeted inhibitors (e.g. inhibitors of signaling pathways or molecules derived from TME) plus an appropriate diet that favors beneficial colonic microbiota, as well as the use of targeting methods such as charged nanoparticles or specific bacterial species, could constitute a reliable alternative to fight with CRC chemoresistance and relapses. The use of different *in vitro* and *in vivo* preclinical models of CCSC such as colonospheras, organoids and xenografts, is essential to achieve this goal and bring it to clinical research.

In the near future, the challenge will be the development of selective and combined therapies to promote: 1) CSC eradication; 2) due to phenotypic plasticity, eradication of cancer cells without CSC features is also necessary; and 3) reduction of the damage to cells outside the tumor bulk.

In any case, it is clear that the standardization of treatment protocols is not always effective for this disease. It is advisable to resort to a combined and personalized therapy that considers the needs and responses of each patient.

CONCLUSION

Figure 2 shows the interplay between the TME and the gut microbiota that influences in the properties/behavior of CCSC. Besides, the reader can appreciate that CCSC influence on cells from TME favoring CRC progression but probably also on gut microbiota. The present knowledge described in this review provides data to guide future research that will need to be addressed the complexity of components of the CRC-associated microenvironment and microbiota. Not as an isolated neoplasm but as a dynamic pathology whose actors are capable, regrettably, of contributing to evasion mechanisms of the current therapeutic strategies. Although the incidence and mortality from CRC have decreased in recent years, a large number of patients still suffer from relapses due to resistance to treatment. The development of metastases and chemoresistance is undoubtedly one of the greatest challenges in CRC therapy. As we have seen in this work, the properties of the CCSC make this cell subtype have the main responsibility for the recurrences. The shift in the tumor niche and the intestinal microbiota favors the acquisition of CSC characteristics, promoting a worse prognosis of CRC. Although much is currently known about the interrelationship between components of the TME, the microorganisms present in the intestinal mucosa and CCSC, there is still much to be discovered in this field.

1

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