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Bacterial oncogenesis in the colon

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Abstract

The human colon plays host to a diverse and metabolically complex community of microorganisms. While the colonic microbiome has been suggested to contribute to the development of colorectal cancer (CRC), a definitive link has not been made. The role in which the colon microflora could contribute to the initiation and/or progression of CRC is explored in this review. Potential mechanisms of bacterial oncogenesis are presented, along with lines of evidence derived from animal models of microbially induced CRC. Particular focus is given to the oncogenic capabilities of enterotoxigenic *Bacteroides fragilis*. Recent progress in defining the microbiome of CRC in the human population is evaluated, and the future challenges of linking specific etiologic agents to CRC are emphasized.

Keywords

bacterial toxin; chronic inflammation; colonic microbiome; colorectal cancer; genotoxins; oncogenesis

Each year, approximately 1.2 million individuals are diagnosed with colon cancer worldwide [1]. As the second leading cancer affecting both men and women, colorectal

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cancer (CRC) claims the lives of over 600,000 individuals annually [1]. Greater than 90% of CRC cases are spontaneous, occurring in people with little or no family history of the disease. Once thought to be a cancer predominantly afflicting the western world, incidence rates of CRC are rapidly increasing in areas that have historically been considered low-risk, including South America, eastern Asia and eastern Europe [1]. This trend has been attributed to changes in dietary patterns, along with decreased physical activity, leading to a rise in obesity within these populations [2,3]. As a prominent public health threat, potential contributions to the development of CRC have been the focus of intense study. Colorectal carcinomas usually begin as benign tumors, called polyps or adenomas, which can develop anywhere along the colon from the epithelial cells lining the mucosa. Typically over a period of 10 or more years, some polyps become cancers. Importantly, however, colon cancer can be fully prevented by the early detection and removal of polyps. This progression from normal epithelium to adenoma to adenocarcinoma has been well characterized by Fearon and Vogelstein to involve the cumulative accumulation of genetic mutations [4]. The proposed classes of optimal target genes include tumor suppressors and oncogenes, along with mismatch repair genes. Common examples include *APC* (a tumor-supressor gene), *KRAS* (an oncogene), and *MLH1* and *MLH2* (mismatch repair genes) [4,5]. While there is general consensus about the stepwise transition to colorectal carcinoma, the initiating mechanism(s) remain unclear.

The notion that the endogenous enteric microbiome contributes to the etiopathogenesis of colon cancer has been proposed for decades. The human gastrointestinal (GI) tract is colonized by a vast and complex community of microorganisms totaling approximately 10^{13} bacteria composed of over 500 microbial species [6]. The commensal intestinal microbiota outnumbers human cells nine to one, and perhaps more impressively, their collective genes outnumber that of their human host 100 to one. The microbial community are immense. The colon is colonized soon after birth, facilitating the essential roles played by the colon microbiota in host physiology, including mucosal immune development, regulation of cell proliferation and modulation of gene expression in host epithelial cells [7,8]. Other beneficial functions of the metabolically complex microbiome include providing usable forms of nutrients as a byproduct of metabolism and protection against exogenous pathogens. In the healthy colon, the microbiota interactions with the host are at homeostasis; however, intrinsic or extrinsic factors can cause perturbations, leading to abnormalities in microbiome composition or function that have been associated with several diseases, including inflammatory bowel disease (IBD) and colon cancer [9,10].

The entirety of the healthy human colon is covered by a mucus layer that consists of an inner gel-like layer and a loose outer layer, both primarily composed of a secreted network of highly glycosylated MUC2 mucins. Among the family of mucin genes expressed in the human colon, the gene product of *MUCB* has also been detected in minor quantities at the base of the crypt [11]. In addition, MUC5AC and MUC6 have been associated with colorectal adenomas and ulcerative colitis [12]. The outer mucus layer serves as a semipermeable network providing a habitat for commensal bacteria to reside in, while the inner gel-like mucus layer acts as a physical barrier excluding bacteria from direct contact with the epithelium [13]. It is likely that bacteria transiently penetrate the inner mucus

barrier in a healthy state; however, they are thought to be cleared quickly through host immune responses [13]. The inner mucus layer ranges in width from 30 to 170 μm in the human colon, increasing in depth from the ascending to the descending colon [14]. Bacteria mainly colonize two major niches within the human colon: the lumen and the outer mucus layer. Characterization of these distinct microbial communities has been the focus of a series of recent studies [6,15–17]. The distinction between these communities is important, as the microbial milieu in these two ecological niches may contribute differently to the etiology of disease. It is well accepted that microbial dysbiosis (an imbalance of the microbiota) with bacterial invasion and persistence in the inner mucus layer (biofilm formation), contributes to the development or progression of IBD [9,18,19]. Massive bacterial biofilms within the empty mucus layer, constituting invasions of greater than 10^9 bacteria/ml, were identified in 94% of ulcerative colitis patients, 98% of Crohn's disease patients and 78% of self-limiting colitis patients, compared with just 11% in controls [19]. The phylum-level 16S profiles were observed to involve a shift in major populations, most notably an increase in Proteobacteria and a decrease in Firmicutes and Bacteroidetes [18]. IBD is associated with an increased risk for the development of GI malignancies. While the development of CRC in the setting of IBD involves many of the same genetic mutations as the stepwise transition to sporadic CRC, the timing and frequency of these mutations often differ [20,21]. Furthermore, chronic colitis-associated CRC tends to be macroscopically heterogeneous compared with sporadic CRC and arises from flat dysplastic tissue rather than distinct polyps [21]. This stresses the importance of characterizing the microbial–epithelial interactions in various CRC disease states, analyses that have been less detailed to date [17,22,23].

Mechanisms of bacteria-induced oncogenesis

A significant amount of effort has been employed to determine the mechanisms of microbially induced oncogenesis. Proposed mechanisms include the inhibition, alteration or exacerbation of normal host responses such as apoptosis, inflammation and cellular proliferation. Alternatively, bacteria may also promote cancer through production of secondary metabolites, such as reactive oxygen intermediates, or direct effects on cell transformation through the production of oncogenic toxins. An overview of the mechanisms of bacterial initiation or progression of oncogenesis is shown in Figure 1. It is also important to mention that it has been proposed that the microbiome may also serve a protective role against the development of CRC through its effects on host physiology [24].

Chronic inflammation

The association between inflammation and tumorigenesis has been appreciated since 1863, when Rudolph Virchow hypothesized that cancer developed from sites of chronic inflammation, termed 'the chronic irritation hypothesis' [25]. Today, the connection between inflammation and cancer is well established; however, the mechanisms and pathways are not fully characterized. Infection triggers inflammation as a means to effectively combat an invading pathogen. Polymorphonuclear phagocytes are typically normally the first cells recruited to the site of infection and serve as potent producers of proinflammatory cytokines and chemokines that amplify the response by recruiting more

immune cells [26,27]. These cells produce an abundance of reactive oxygen species that can damage lipids, proteins and DNA, leading to increased mutations in proliferating cells and ultimately alterations in cell turnover and death [26–29].

While bacterial infection was once thought of as an acute condition, it is clear that many bacteria are able to persist in the host and lead to chronic infections accompanied by inflammation. Study of the molecular mechanisms that link chronic infections to inflammation and cancer is an area of intense investigation. Persistent generation of microbially induced inflammation mediators such as TNF- α, IL-1 or even lipopolysaccharide on its own can lead to the induction of the NF-κB family of transcription factors, which have been shown to play a role in inflammation-driven carcino-genesis [28]. In an unstimulated cell, the family of NF-κB transcription factors are bound to the IκB inhibitory family of molecules, which effectively prevents translocation to the nucleus. Activation of this signaling pathway leads to the stimulation of the IKK complex, which phosphorylates the inhibitory IκB proteins, targeting them for ubiquitin-dependent degradation. This releases NF-κB proteins, allowing for translocation to the nucleus, in turn leading to the transcription of target genes [28]. Some of the genes targeted encode inflammatory cytokines, including IL-1β, IL-6 and VEGF, which leads to a positive feedback loop of continuing inflammation [26–28]. In addition, antiapoptotic genes, such as those of the *Bcl2* family, are upregulated by NF-κB, preventing routine cell turnover. Furthermore, expression of genes involved in cell cycle regulation is altered (e.g., cyclins are upregulated and cell cycle inhibitors are down-regulated). Ultimately, NF-κB plays a key role in inflammation-driven tumor development by generating an environment that promotes mutations and simultaneously prevents damaged cells from undergoing apoptosis, both key features of cancerous cells [26–29].

Reactive oxygen species and nitric oxide are also generated by inflamed epithelial cells under the stress of bacterial toxin exposure or chronic bacterial infection [29,30]. These molecules play important roles in the initiation and progression of carcinogenesis by directly altering DNA, leading to mutations, deletions and chromosomal instability; if left unrepaired, these can lead to carcinogenesis [26,31]. In addition to direct effects on DNA, reactive species canduction pathways. For example, reactive oxygen species can direct cell proliferation and inhibit apoptosis through activation of the transcription factors MAPK, AP-1 and NF-κB [31]. Persistent asymptomatic bacterial infection of the colon, in which the inner mucus layer is penetrated, is proposed as being capable of inducing chronic inflammation, resulting in a cascade of diverse and complex events that combine to generate a procarcinogenic microenvironment.

Oncogenic bacterial metabolites & toxins

In addition to the indirect bacterial infection, there are also direct bacterial mechanisms of oncogenesis. Through their metabolically complex processes, bacteria also produce reactive species, such as the derivatives of molecular oxygen, including superoxide, hydrogen peroxide and hydroxyl radicals [31,32]. These free radicals contribute to genomic instability by the mechanisms discussed above. Alternatively, several bacterial toxins have been identified that are predicted to be carcinogenic These toxins have the capacity to modify

host physiology, leading either to direct DNA damage, augmentation of cellular proliferation and/or disruption of cellular differentiation and apoptosis [32]. One thoroughly studied example is CagA of *Helicobacter pylori*, which is considered to be the most important risk factor that links *H. pylori* infection to the development of gastric cancer [33,34]. CagA binds the cellular tyrosine phosphatase, SHP2, leading to modulation of cell structure [28]. It has also been shown to target multiple host proteins that regulate inflammation, and several studies suggest that it has the ability to activate NF-κB and β-catenin signaling [35–38]. Recently, CagA was associated directly with a tumor-suppressor pathway when it was shown to usurp the tumor suppressor ASPP2 and modify its activity, thus promoting cell survival [39]. Strains of *H. pylori* expressing active VacA are associated with an increased risk of gastric cancer [40]. VacA has been shown to stimulate VEGF, increase cell proliferation and inhibit and induce apoptosis, as well as suppress the host immune response to favor long-term colonization, along with a heightened risk for transformation [41–43]. The *Pasteurella multocida* toxin is known to act as a highly potent mitogen and inhibitor of apoptosis [44]. *P. multocida* toxin is an AB toxin that activates G proteins through its deamidase function, influencing downstream signaling pathways, including MAPK cytoplasmic and JAK–STAT [45,46].

Another example of a bacterial toxin is CDT. This genotoxin is produced by several bacteria, including selected strains of *Escherichia coli*, *Actinobacillus actinomycetem-comitans*, *Campylobacter jejuni*, *Shigella* spp., *Salmonella* spp., *Helicobacter hepaticus* and *Helicobacter cinaedi*, as well as other entero-hepatic *Helicobacter* spp. [47]. CDT is composed of three subunits, one of which, CdtB, functions similarly to mammalian DNase by directly damaging host DNA [48]. Another CRC genotoxin family of interest can be found within the PKS genotoxic island of *E. coli*. Recent studies have shown that this island encodes a hybrid peptide–polyketide, colibactin, which is capable of directly inducing DNA double-strand breaks both *in vitro* and *in vivo* [49,50]. Furthermore, deletion of the genotoxic PKS island from an *E. coli* strain diminished its oncogenic potential [51]. In addition to the toxins mentioned above, the microbial community contains a repertoire of toxins, listed. in Table 1, proposed to have oncogenic abilities: CNF-1 and CIF in *E. coli*, and BFT discussed, in detail below [52–55]. Of this group, toxins produced by *E. coli*, *Salmonella* spp., *Shigella* spp. or *Bacteroides fragilis* are potential contributors to CRC pathogenesis within the microbiota. One consistent feature of these bacterial carcinogenic mechanisms is that, through either direct or indirect methods, they interfere with key eukaryotic processes.

APC^{Min} model of *B. fragilis*-induced CRC

One of the more compelling pieces of evidence displaying a direct link between an infectious bacterial agent in the induction of CRC lies with the murine models of enterotoxigenic *B. fragilis* (ETBF) infection. The genus *Bacteroides* is one of the most numerically prominent members of the intestinal microbial flora. One species in particular, *B. fragilis*, is a Gram-negative obligate anaerobe and common symbiote colonizing nearly all humans. However, *B. fragilis* is also an important opportunistic pathogen, as it is the most common anaerobe isolated from clinical infections despite comprising only a small portion (<1–2%) of the total micro-biota [56,57]. Long recognized for roles in intestinal

infections and more recently viewed as a molecular subtype, ETBF was revealed to induce colitis in wild-type C57BL/6 mice and promote oncogenic transformation in APC^{Min} mice (a murine intestinal cancer model) [54,58]. *B. fragilis* consists of two molecular subtypes, termed nontoxigenic *B. fragilis* (NTBF) and ETBF. NTBF is proposed to be a probiotic organism, serving a crucial role in immune development and providing the host with usable forms of dietary products [7]. By contrast, ETBF has been identified as a cause of inflammatory diarrheal disease in animals and humans, and has also been suggested to be associated with active IBD and CRC [55,59–61]. Interestingly, a recent study by Zitomersky *et al.* found that ETBF carriage is potentially quite common in the US population, as they detected ETBF in 40% (six out of 15) of healthy asymptomatic individuals between 31 and 66 years of age in Boston (MA, USA) [62].

To date, no ETBF strains have been fully sequenced. However, through identification and sequencing of a transposon-flanked pathogenicity island, ETBF was determined to encode a 20-kDa zinc-dependent metalloprotease termed BFT [63,64]. BFT is the only known virulence factor of ETBF, and all strains harbor one of three highly related *bft* isoforms (*bft-1*, *bft-2* or *bft-3*) present on the *B. fragilis* chromosome. All molecular isoforms are capable of exhibiting biological activity; however, the relationship between isoform and disease severity is not yet known. BFT binds to a currently unknown colonic epithelial cell receptor, triggering rapid cleavage of the tumor-suppressor protein E-cadherin, which, in turn, frees its associated β-catenin, allowing its nuclear localization [65]. The subsequent expression of the β-catenin/Wnt signaling pathway leads to an increase in colonic epithelial cell proliferation. It also stimulates additional signaling pathways through NF-κB. While the precise contribution of the plethora of colonic epithelial cell signaling triggered by BFT to ETBF pathogenesis remains unknown, one clear biologic outcome is the recruitment and activation of inflammatory cells, as well as epithelial cell secretion of pro-inflammatory cytokines and the production of reactive oxygen species [30,66,67]. A recent study by our group demonstrated that ETBF induces a rapid-onset acute symptomatic colitis, followed by chronic subclinical colonic inflammation and hyperplasia in specific pathogen-free wild-type C57BL/6 mice. Unique to this model is the ability of ETBF to persistently colonize the mice for an extended period of time after a single oral exposure to ETBF; in this study, mice infected for 16 months exhibited low-level colitis [58]. The acute ETBF murine colitis mimics the inflammatory diarrhea detected in humans with ETBF infection, whereas the long-term murine colonization is analogous to what is observed in ETBF colonization in the human population, suggesting that ETBF carriers may be susceptible to asymptomatic ETBF-induced colitis.

The pro-oncogenic cellular signaling induced by BFT in concert with the persistent chronic inflammation induced by ETBF in wild-type mice suggest that ETBF is an oncogenic bacterium. This was recently tested by our group using the APC^{Min} mouse strain, a well-established cancer model in which loss of a single copy of the *Apc* gene predisposes mice to the development of numerous tumors in the small intestine when the second allele spontaneously mutates [68]. However, importantly for this animal model of bacterial-induced carcinogenesis, adenomas are primarily observed in the small intestine and not in the colon [69]. APC is a multidomain tumor-suppressor protein that binds to and promotes

proteosomal degradation of β-catenin to regulate downstream Wnt signaling [69]. Loss or mutation of the *Apc* gene is the cause of the inherited disease familial adenomatous polyposis, and occurs in virtually all sporadic colon cancers [70]. By 4 months of age, sham APC^{Min} mice developed an average of one to three tumors in the colon. By contrast, APC^{Min} mice colonized with ETBF developed chronic asymptomatic colitis, with colon tumor foci detected as early as 1 week postinoculation. At 1 month of age, a marked increase in colon tumor formation (~12 tumors/mouse on average) occurs predominantly in the distal colon of ETBF-colonized mice. By contrast, APC^{Min} mice colonized with NTBF did not exhibit colon tumors in excess of the sham mice. ETBF induces rapid activation of Stat3 both in the colonic epithelial cells, which are the targets of transformation in the colon, and in a subset of mucosal immune cells. Stat3 activation is required for Th17 cell development and, consistent with this, ETBF induces a rapid mucosal Th17 inflammatory response within 1 week of colonization. Colon tumors induced by ETBF also have a marked increase in Stat3 activation. Furthermore, excess tumor formation is significantly inhibited by administration of IL-17-blocking antibody, indicating that IL-17 is necessary for tumorigenesis in this model [54]. These studies suggest that persistent long-term colonization with ETBF may induce chronic colonic inflammation, with the potential for oncogenic transformation [54,58]. Furthermore, while Th17 inflammatory responses typically help the host control bacterial and fungal infection, the ETBF murine model demonstrates that endogenous Th17 responses can yield oncogenesis in the colon, a result that is supported by additional murine and human data [71–75].

Other animal models of bacterial influences on CRC

Animal models of bacteria-driven oncogenesis have proven to be valuable tools in elucidating the link between microbes and CRC. Genetic knockout, germ-free and chemical mouse models have been developed and extensively used in studies connecting bacteria and CRC. The APC^{Min} model previously mentioned was the first mutant murine model for colon cancer and is an important tool, given the importance of inactivation of the *Apc* gene in the initiation of sporadic CRC. This initial APC^{Min} mutant, carrying a truncation at codon 850 of the *Apc* gene, was identified among a colony of mice following random ethylnitrosourea mutagenesis [76]. Utilizing gene-knockout technology, alternative *Apc* mutants have subsequently been constructed, including mouse strains with truncations at codon 716 and 1638 that also develop polyps [69,77]. There are several additional genetically engineered models of intestinal neoplasia, which are extensively covered in a review by Taketo and Edelmann, including single knockouts of *Muc2*, *IL10*, *Smad3* and *Gαi2*, and double knockouts of *APC* with *Smad4*, *TCRβ* with *p53*, *Gpx1* with *Gpx2* and *Tgfb1* with *Rag2* [78–86]. In the absence of intestinal microbiota, under germ-free conditions, *IL10*-knockout mice and *TCRβ* with *p53*, *Gpx1* with *Gpx2* and *Tgfb1* with *Rag2* double-knockout mice all showed decreased or completely inhibited tumor formation [84–90]. Together, these studies indicate a role for the intestinal microflora in the development of inflammation and neoplasia. Mixed results have been reported in germ-free APC^{Min} mice. While Dove *et al.* noted a twofold decreased tumor load in the medial small intestine, they did not see a significant overall decrease in tumors[88]. By contrast, a recent study by Li *et al.* found a significant decrease of tumor load in both the small intestine and the colon. Furthermore,

they identified two pathways triggered by microbiota, c-Jun/JNK and STAT3, which act to enhance tumor formation [89].

Mouse models of chemically induced colitis have also been used in studies to address bacterial involvement in colitis and tumorigenesis. The most commonly used agents are azoxymethane (AOM) and dextran sulfate sodium (DSS). A recent study by Uronis *et al.* found that *IL10*-knockout mice that were colonized with complex microbiota and exposed to AOM developed tumors; however, germ-free conditions abolished tumor formation [91]. It was further shown that conventional *IL10*/*MyD88* double-knockout mice showed no signs of tumor development upon treatment with AOM, suggesting that microbially induced tumorigenesis in this system was dependent on the TLR/MyD88 pathway [91]. Johansson *et al.* showed that bacteria penetrate the inner mucus layer before inflammation is observed in the DSS colitis model, suggesting that invasion of the protective inner mucus layer and subsequent bacterial contact with the epithelium triggers the host immune response and inflammation [92]. Further studies have shown that DSS-induced colitis can be ameliorated under gnotobiotic conditions, indicating that the presence of microflora facilitates DSS colitis [93]. A more recent study by Elinav *et al.* further supports the role of intestinal flora in DSS-induced inflammation [94]. Using mice deficient in the NLRP6 inflammasome, they showed that the resulting altered microbiota, characterized by increased levels of Bacteroidetes, led to an increased recruitment of inflammatory cells and worsened colitis upon DSS exposure when compared with wild-type mice. This report, among others, emphasizes that the microbiota composition is shaped not only by diet, but also by the host immune make-up, suggesting that human host polymorphisms modulating the inflammatory response may be important contributors to the influence of the microbiota on CRC pathogenesis [51,91,95–97]. Furthermore, several studies have shown that antibiotic treatment is capable of blocking colitis in the murine DSS colitis model [98–100]. Under germ-free conditions, DSS treatment alone, however, is able to induce slight inflammatory cell infiltration and edema, but without tumor induction [101].

While abundant data implicate the aggregate microbiome as a cofactor in colon tumor development, individual pathogens thought to promote colonic tumorigenesis have also been investigated using animal models. A study by Ellmerich and colleagues showed that *Streptococcus bovis*, long associated with colon cancer through epidemiological studies, is capable of markedly increasing the production of inflammatory cytokines and aberrant crypt foci in the colonic mucosa of rats through exposure to *S. bovis* cell wall antigens [102]. This study, however, lacked controls to demonstrate that the response was specific to *S. bovis* cell wall antigens. Furthermore, it is important to mention that the strain utilized in this study was later classified as *S. bovis* biotypeII/1 (*Streptococcus infantarius* subsp. *infantarius*), which shows a less convincing link to human CRC compared with bio-type I, a topic thoroughly covered in a recent review by Boleij and Tjalsma [103]. Another suspect, *Helicobacter hepaticus*, colonizes the liver and colon of several mouse strains and has been linked to hepatitis, chronic colitis and CRC, and is discussed in a recent review by Fox *et al.* [104]. It was recently shown that *H. hepaticus* triggers nitric oxide and TNF-α production, leading to inflammation and carcinogenesis in Rag2-deficient mice, implicating the innate immune response induced by *H. hepaticus* as carcinogenic [105]. A subsequent study

utilized transcriptional profiling of*H. hepaticus*-infected *Rag2*-knockout mice to reveal that colon and liver tissues exhibited different stress responses to infection. The colon was found to have a significant upregulation of genes involved in the generation of reactive species, while genes involved in DNA repair showed lower expression; this was directly contrasted with the liver, which showed upregulation of all major DNA repair pathways during infection [106]. These findings support the role of *H. hepaticus* inflammation-induced carcinogenesis, and also leads to interesting insights into the complexity of tissue-specific microbial pathophysiology. Similarly, the colon microbiota has been thought to play a role in the progression of certain diseases, such as HIV and HCV, both of which are conditions associated with an increased risk of cancer [107,108]. Other studies show that certain strains of *Enterococcus faecalis* produce extracellular superoxide and hydrogen peroxide, which induce aneuploidy and tetraploidy in colonic epithelial cells [109,110]. *E. faecalis* also encodes a metalloprotease, GelE, which contributes to the development of colitis, dysplasia and adenocarcinoma in monocolonized IL-10-deficient mice[87,111]. However, to date, a link between *E. faecalis* and human CRC has not been identified [112].

Another well-studied bacterial agent of interest is *Citrobacter rodentium*, which is known to induce self-limiting colitis, epithelial cell proliferation and tumorigenesis in the murine colon. *C. rodentium* is not a human pathogen, but is considered to be the mouse homolog of human attaching and effacing *E. coli* strains, which are yet another proposed procarcinogenic species. Early studies found that *C. rodentium* infection increases the carcinogenic effect of 1,2-dimethylhydrazine treatment in NIH Swiss mice [113]. Later, a study revealed that *C. rodentium* infection leads to cytokinetic alterations and is sufficient to promote colon tumor development in APC^{Min} mice [114]. Maddocks *et al.* reported that human attaching and effacing enteropathogenic *E. coli* strains downregu-late DNA mismatch repair genes and provided preliminary data identifying these bacteria in human CRC [115]. A recent publication by Arthur *et al.* showed that tightly adherent *E. coli* strains harboring the PKS genotoxic island were able to induce tumor formation in AOM-treated IL-10-deficient mice under germfree conditions [51]. The authors further showed that conventionally housed IL-10-deficient mice developed an altered microbiome in association with colitis in 100% of the mice. Importantly, they showed that inflammation, not the carcinogen AOM, modified the microbiota structure, with the emergence of potential procarcinogenic phyla. Furthermore, a specific microbial virulence factor (the PKS island), not inflammation alone, was required for microbially induced carcinogenesis in this model. This study stresses the interplay between specific carcinogenic species, the microbial community and the host. Consideration of these multifactorial influences is important when transitioning to studies concerning microbial involvement in human CRC.

Human studies

Despite a long quest, direct links between the bacterial microbiome and CRC in humans have not yet been established. Culture-based, observational or case–control studies largely focusing on fecal analyses from patients with CRC and healthy control patients have suggested that *Bacteroides*, *Streptococcus gallolyticus* subsp. *gallolyticus* (previously known as *S. bovis* biotypeI), *E. coli* and *Enterococcus* spp., among others, may be associated with the development of CRC. Particularly notable over time has been the association of *S.*

gallolyticus endocarditis and/or bacteremia with a high likelihood of having an underlying GI tract malignancy, most commonly CRC [116–122]. *Clostridium septicum* aortitis and/or bacteremia have also been suggested to be indicators of GI malignancy [123]. Culture-based human studies combined with recent experimental mechanistic studies have provided the greatest support for potential roles for ETBF, *S. gallolyticus*, enteroadherent *E. coli* and *E. coli* possessing the PKS island in human CRC [51,61,115,120].

Molecular approaches, in particular the advent of next-generation sequencing techniques, have facilitated studies to examine more comprehensively the microbial associations with CRC (Table 2). These approaches enhance culture-based methods because they allow the detection of 'noncultivable' microbes. Overall, the available data suggest that the tumor-associated microbiome differs from that detected on matched normal tissue in the same patient. Furthermore, the fecal microbiome of CRC patients appears to differ from that associated with their tumor and also from the fecal microbiome of healthy volunteers [124,125]. A wide range of bacteria have been reported to be enriched in tumor tissue samples, including *E. coli*, Proteobacteria (especially *Enterobacteriaceae*), *Bacteroides*-spp., *Prevotella* spp., *Streptococcus* spp., *Peptostreptococcus* spp., *Enterococcus* spp. and *Fusobacterium* spp. [22,23,126,127]. However, the differences detected between sample groups vary among studies, without clear patterns having yet been identified that might be useful, for example, to identify an individual at risk for or suffering from CRC. The methodologic differences, varying sample types analyzed, varying populations studied and limited patient data provided make differences among the studies difficult to interpret. Two recent studies, representing the largest set of CRC and matched normal tissue samples analyzed to date, identified a predominance of *Fusobacterium* spp. (*F. nucleatum* and other *Fusobacterium* spp.) to be associated with CRC as compared with adjacent normal tissue [23,126]. No healthy control populations were included in either study, and experimental models of *F. nucleatum* for testing its oncogenicity have not yet been reported. Most studies have focused on patients with CRC; however, to begin to implicate bacteria in the pathogenesis of CRC, it is important to determine bacterial associations with colonic adenomas, precursors of CRC. Similar studies considering the unique pathogenic associations for IBD (discussed earlier) would also be helpful. In the one molecular study evaluating adenomas available to date, the bacterial population distributions also differed between adenoma and control patients when rectal biopsies of normal tissue were compared using 16S rRNA sequence analysis [17]. It is clear that additional studies are needed not only to delineate the microbial populations associated with CRC compared with diverse control populations, but also to understand how the microbial populations may relate to disease outcome and contribute to the pathogenesis of CRC.

Conclusion & future perspective

Sporadic CRC is ultimately a genetic disease, where gene alterations and chromosomal instability are central to the stepwise progression towards neoplasia [4,5]. This complex process is undoubtedly the result of numerous influences ranging from age, gender, nutritional intake, physical activity and host genetic background to the diverse and variable intestinal micro-biome. The epidemiological and experimental evidence discussed here strongly suggest a role for several bacterial agents in CRC. However, traditional

bacteriological approaches are built on the assumption that an etiologic pathogen can be isolated, cultured and identified, and that pathogenesis can be explained through confirmation of disease. Throughout the 19th century and beyond, these concepts, grounded in Koch's postulates, have proven to be crucial in the identification of countless infectious pathogens, including the etiologic agent of gastric cancer, *H. pylori* [128]. Yet unlike the archetypal infectious disease consisting of a single causative agent, the colon plays host to a variety of commensal organisms, many of which have been implicated, both alone and in consort, to contribute to the genesis of colon cancer. The challenge for traditional epidemiological approaches to identify links between bacterial agents and CRC is further hampered by the long length of time between initiation and detectable carcinogenesis. Searching for the responsible agent(s) among the multiple constituents of the intestinal flora presents a challenging prospect, since it is possible that the critical inciting microbiotic agent or composition is no longer present at the time of disease discovery. As such, we are then potentially reliant on the detection of an immune signature of the microbe or microbiota to provide the epidemiologic link to CRC.

Two recent reviews draw attention to the potential for bacterial 'alpha'-bugs or drivers in the context of the aggregate flora to shape the microbial community in order to create a procarcinogenic environment [129,130]. This emphasizes the need for detailed knowledge about specific microbes, as well as alterations of whole microbial communities under diseased and healthy states, to better understand the etiology of CRC. The advent of next-generation sequencing technologies has facilitated these types of studies, which can take into account the community of a specimen, many of which were discussed here. However, limitations in the experimental evidence to date include small sampling numbers and absent or inadequate control populations for comparison. Furthermore, no information regarding host genetics has been analyzed. As revealed by the numerous mouse studies, commensal bacteria have pathogenic capabilities in the context of genetic abnormalities in the host.

While advances have been made in the early stages of characterizing what species are present on a tumor and its have been no attempts to determine the spatial organization of those microbes with respect to the host epithelium. The spatial arrangement of the bacterial community is likely to dictate both microbe–microbe interactions and microbe–host interactions. Proximity to the host epithelium facilitates the way in which microbes are recognized and responded to by the host innate and adaptive immune system [131]. A systematic study of the distribution of microbes along both the length of the colon, as well as a cross-sectional characterization of the lumen and mucus layer members, is essential to further elucidate the role of specific bacterial community members in the cancerous disease state.

As the field moves forward, several types of evidence will be needed to link the microbiota to human CRC [132]. Prospectively conducted studies, initiated at a time point before the onset of disease, and with relevant samples (ideally blood, tissue and stool) for analysis, would be ideal. Capturing information about the microbiome structure and composition in the early stages of disease initiation and throughout disease development would be invaluable. Ideally, the detection of microbiome dysbiosis or specific putative etiologic agents before disease development would help to address the 'cause or consequence'

conundrum. However, population-based microbiome studies are both cost-prohibitive and impractical for evaluating long-term (20–40 years in the case of colon cancer) disease development. Attention to designing control groups and using varied controls is also important in order to help determine whether a microbe or a microbiota composition exhibits a strong, consistent association with human CRC. We should seek to detect an immunologic response to the purported microbial etiologies of CRC. It was the combined criteria of either detection of *H. pylori* or an immune response to *H. pylori* that provided crucial data that defined *H. pylori* as the cause of most gastric cancers [128]. Murine models of colon oncogenesis will likely provide key insights into molecules and mediators with translational importance, enabling us to understand how the microbiota contribute to human CRC. Ultimately, elimination of the inciting microbe or restructuring of the microbiome, whether by diet, probiotics, antibiotics or vaccination, with subsequent prevention of CRC, is required for definitive declaration of disease association. While these criteria are stringent and create a necessarily high bar for investigators to reach, there has never been more interest in understanding the microbial inciters of human CRC. The emerging data are exciting and capture our imagination, making the future for discovery in this field bright.

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Executive summary

Background

- Colorectal cancer (CRC) is a public health issue both within the USA and globally, leading to the deaths of over 600,000 individuals annually.
- The majority of CRC is sporadic in nature. Accumulation of mutations in normal colonic epithelial cells characterizes the progression from adenoma to carcinoma.
- The microbiota have been implicated in contributing to the initiation or progression of CRC for years.
- In the human colon, bacteria are kept spatially segregated from the colonic epithelium by a mucus layer; perturbation of this mucus layer has been associated with states of pathology, including CRC.

Mechanisms of bacteria-induced oncogenesis

- Chronic bacterial infection associated with invasion of the inner mucus layer can lead to chronic inflammation through the persistent generation of inflammatory mediators, affecting cell turnover, apoptosis and increasing the likelihood of mutations.
- Reactive oxygen and nitrogen species may arise from several sources, including bacteria and epithelial and immune cells. Through direct DNA damage or modification of cellular signaling, these molecules can generate a procarcinogenic environment.
- Bacteria are capable of producing a variety of oncogenic toxins that can directly damage DNA, influence cellular signaling and/or induce mucosal inflammation in order to initiate or promote colon tumorigenesis.

APC^{Min} model of Bacteroides fragilis-induced CRC

- *Bacteroides fragilis* is a symbiotic organism with the capacity to become an opportunistic pathogen.
- Enterotoxigenic *B. fragilis* (ETBF) express the oncogenic BFT and has been associated with diarrheal disease accompanied by colitis in both humans and animals.
- ETBF induces acute colitis followed by chronic subclinical colitis in specific pathogen-free wild-type C57BL/6 mice, which is associated with long-term ETBF colonization.
- ETBF promotes tumor formation under specific pathogen-free conditions in the APC^{Min} model of murine colon cancer through, in part, a Stat3-mediated Th17 mucosal immune response.

Other animal models of bacterial influences on CRC

- Under germ-free conditions, several murine models of genetically engineered neoplasia show decreased colon tumor formation.
- Several mouse models of chemically induced colitis can be ameliorated with antibiotic treatment or through a gnotobiotic environment.
- Multiple pathogenic bacteria are capable of inducing tumorigenesis in genetically engineered mice.

Human studies

- Culture-based and observational studies have associated multiple bacterial species with CRC; however, the most convincing combined clinical and experimental evidence exists for ETBF, *Streptococcus gallolyticus* and *Escherichia coli* species, especially those harboring the PKS genotoxic island.
- Sequencing data suggest that the microbiome of CRC patients differs from that of healthy individuals, and that the tumor microbiome differs from that detected on flanking normal tissue from the same patient.

Future perspective

- Further experimental evidence is necessary to link members of the microbiome to CRC.
- Thorough characterization of the spatial arrangement of microbes along the length and cross-section of the colon in the disease and healthy state is needed.
- Future studies of the human population will benefit from increased sampling sizes, the inclusion of multiple and varied controls, consideration of host genetics and prospectively collected human samples, including colon tissues, to allow analysis before the onset of disease.

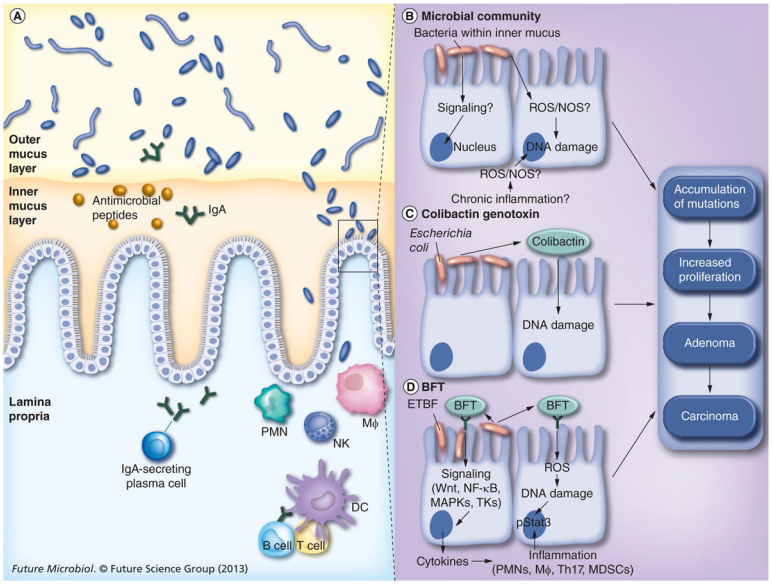

Figure 1. Overview of tissue- and cell-level mechanisms of bacterial oncogenesis

(A) In the healthy human colon, the inner mucus layer serves as a physical barrier separating the mucosal epithelium from luminal contents. The mucus layer is further protected through epithelial cell secretion of antimicrobial peptides and plasma cell secretion of IgA. This spatial segregation largely maintains the host–microbe homeostasis; nevertheless, bacterial invasion of the inner mucus layer does occur. **(B)** It is this perturbation that facilitates direct interactions between microbes and host cells, resulting in pathology. The precise mechanisms by which the bacterial community may induce oncogenesis when invading the inner mucus layer are, as yet, uncertain. **(C & D)** By contrast, for select bacteria for which preliminary epidemiologic data suggest an association with some human colorectal cancer, linkages between the mechanism of action of secreted toxins and colorectal cancer are shown. **(C)** Genotoxin colibactin secreted by *Escherichia coli* harboring the PKS island damages DNA. DNA damage by colibactin can be direct and/or through as yet unidentified colonic epithelial and/or other mechanisms. **(D)** Steps supported by experimental data regarding the action of BFT secreted by ETBF. See text for details.

DC: Dendritic cell; ETBF: Enterotoxigenic *Bacteroides fragilis*; Mϕ: Macrophage; MDSC: Myeloid-derived suppressor cell; NOS: Nitric oxide synthase; PMN: Polymorphonuclear cell; ROS: Reactive oxygen species.

Table 1

Oncogenic bacterial toxins and linkage to human colorectal cancer.

Toxin	Bacterial species	Potential oncogenic mechanisms	Human CRC studies†	Ref.
CagA	*Helicobacter pylori*	Binds cellular SHP2, leading to cytoskeletal rearrangements; dysregulates NF-κB and β-catenin signaling; hijacks the tumor suppressor ASPP2 of p53 to inhibit cell cycle progression	Meta-analysis of 16 case–control studies revealed that infection *with H. pylori* strains containing *cagA* increased the risk for gastric cancer 1.64-fold over *H. pylori* strains lacking *cagA*	[33–39]
VacA	*H. pylori*	Induces the formation of vacuolar compartments and increases proliferation through stimulation of VEGF; has been shown to suppress immune responses and inhibit apoptosis; has also been reported to induce apoptosis, an antitumor mechanism	Several epidemiological studies have reported that *H. pylori* strains that express active toxin have a higher association with gastric cancer than strains expressing inactive toxin (as measured *in vitro*)	[40–43]
PMT	*Pasteurella multocida*	Activates Rho GTPases, influencing cell signaling and proliferation	Not available	[44–46]
CDT	*Escherichia coli, Actinobacillus actinomycetemcomitans, Helicobacter* spp., *Shigella* spp., *Salmonella* spp., *Haemophilus* spp., *Campylobacter* spp.	A subunit of CDT called Cdtb (for binding subunit) acts as a DNase directly damaging host DNA	Not available	[47,48]
Colibactin	*E. coli*	Directly induces DNA double-strand breaks *in vivo* and *in vitro*	67 and 21% of CRC (n = 21) and control (n = 24) patients, respectively, were positive for PKS$^+$ *E. coli* (see text)	[49–51]
MAP	*Citrobacter rodentium*	Targets host mitochondria, causes epithelial barrier disruption and cytoskeletal rearrangement	Not relevant. *C. rodentium* is a murine pathogen	[47]
CNF	*E. coli, Yersinia pseudotuberculosis*	Modifies Rho signaling, inhibits cell cycle progression	Not available	[52]
CIF	*E. coli, Burkholderia pseudomallei, Y. pseudotuberculosis, Photorhabdus luminescens* and *Photorhabdus asymbiotica*	Inhibits apoptosis	Not available	[53]
BFT	*Bacteroides fragilis*	Cleaves E-cadherin, leads to Wnt/β-catenin nuclear signaling, leading to increased colon epithelial cell proliferation; direct DNA damage in colonic epithelial cells; induces oncogenic Stat3/ Th17 mucosal inflammation and cell signaling; induces spermine oxidase and NF-κB cell signaling	38 and 12% of CRC (n = 73) and control (n = 59) patients, respectively, were positive for ETBF	[54,55,58, 65–67]

† Except for *Helicobacter pylori*, where the associations of select virulence determinants to gastric cancer are provided.

CRC: Colorectal cancer; ETBF: Enterotoxigenic *Bacteroides fragilis*

Table 2

Molecular analyses of microbiota in human colorectal cancer.

Population studied	Method	Sites analyzed	Key findings	Comments	Ref.
Tubular-villous adenoma (29 patients/83 biopsies†), CRC (31 patients/83 biopsies‡), Screening colonoscopy (31 patients/97 biopsies), Colonoscopy for symptoms (34 patients/113 biopsies§); Germany	16S rDNA PCR (5′, 600-bp product) on colon biopsies with 40–100 cloned 16S sequences analyzed/ biopsied; gentamicin protection assay	Not stated	Number of 16S PCR-positive patients (number with *Eschericheria coli*): 1 (1) asymptomatic control, 10 (4) symptomatic controls, 27 (18) adenoma, 28 (24) carcinoma Gentamicin protection assay detected intracellular *E. coli* in biopsies of 81% of adenoma and CRC patients (n = 16) and 0% of controls (n = 25)	No antibiotics in prior 8 weeks; sites of biopsies and pathology of biopsies (i.e., tumor or normal mucosa) not stated; bowel preparation not stated	[127]
Adenoma patients (21), nonadenoma (control) patients (23); participants from DHS study; USA	16S rRNA PCR analyzed by T-RFLP; 16S rRNA clone libraries (=30 clones/ patient) for four adenoma and four control patients; 16S rRNA FISH	Colonoscopy-obtained biopsy samples, 10–12 cm from anal verge	T-RFLP data: bacterial community distribution differed between adenoma and control patients Clone data: adenoma patients had >Proteobacteria, <Bacteroidetes (phyla), >*Faecalibacterium*, >*Dorea*, <*Bacteroides* and <*Coprococcus* (genera) FISH: bacteria present in mucus layer in adenoma and controls without difference noted	No antibiotics in prior 8 weeks; only rectal biopsies analyzed (normal tissue); excluded patients with prior CRC or adenoma; all received polyethylene glycol bowel preparation	[17]
Fecal population: CRC patients (60) and normal colonoscopy patients (119) Mucosal population: normal colon (22) and CRC (22; 16 colon, 6 rectal); France	Replicate 16S rRNA (V3–V4) pyrosequencing of 6 controls and 6 CRC fecal samples (n = 24 samples); qPCR for select genera and species¶ on all stools and 44 mucosal DNA samples	Feces collected between 3 days and 2 weeks prior to colonoscopy; mucosal samples from surgery	PCA indicated the phylogenetic core of CRC and control stools differ qPCR revealed higher *Bacteroides*/ *Prevotella* in CRC versus control patients	No prior history of cancer or colitis; specific exposure to antibiotics not stated; fecal and mucosal results not discussed separately; site of mucosal tissue harvesting in colon not stated	[74]
6 CRC patients; The Netherlands	16S rRNA (V1–V3) pyrosequencing	Tumor and adjacent normal mucosa 5–10 cm from tumor in surgical resection specimens	On-and off-tumor bacterial populations differed significantly, although consistent patient-to-patient changes were not shown Overall, tumor tissue associated with Bacteroidetes and putative commensals, whereas Proteobacteria, especially *Enterobacteriaceae*, favored off-tumor tissue	No patient details provided; site of tissue acquisition in colon not stated; no healthy control population	[22]
CRC patients (46), healthy volunteers (56); China	16S rRNA (V3) pyrosequencing; PCR to detect *Bacteroides* species and butyryl-CoA transferase genes	Fecal samples collected before surgery for CRC patients and in association with routine clinic visits for controls	PCA differentiated CRC from control patients *Bacteroides* species, except *Bacteroides fragilis* and butyrate-producing bacteria, were enriched in control samples, whereas the family *Enterobacteriaceae* and genera *Streptococcus*, *Peptostreptococcus*, *Enterococcus* and *Fusobacterium*, among others, were enriched in CRC samples	Antibiotic exposure not defined for CRC group; no antibiotic exposure for 3 months in control group; other patient data not stated	[124]

Population studied	Method	Sites analyzed	Key findings	Comments	Ref.
CRC patients (46) including stool (21), rectal swab (32), tumor tissue with matched normal tissue (2–5 and 10–20 cm from tumor) (27); healthy volunteers (56) including stool (22) and rectal swab (34); China	16S rDNA (V1–V3) bacterial pyrosequencing	Feces and rectal swab prior to bowel cleansing; tissue samples from surgical resection	Microbiota of CRC and lumen differ By PCA analysis, tumor and matched normal tissues were similar; however, in additional analyses, genus-level[#], but not phylum-level, differences were detected in tumor versus matched normal tissues Both mucosally adherent bacteria (rectal swab comparison) and luminal microbial composition (stool) differed between CRC and healthy volunteers[††]	No antibiotics within 1 month of sampling; no patient data provided; 'within patient' versus 'between patient' analyses not clearly defined	[125]
11 CRC and matched normal tissue samples[‡‡] for metagenomic sequencing; 88 CRC and matched normal tissue samples for qPCR; Canada	Paired-end, PCR-amplified RNA libraries sequenced on Illumina® GAIIx platform; qPCR for *Fusobacterium nucleatum* rRNA	CRC and matched normal colon mucosa from tumor tissue repository of surgical specimens	Only *F. nucleatum* subsp. *nucleatum* was markedly disproportionate between CRC and matched control tissues (nine of 11 subjects =twofold higher for *F. nucleatum* Illumina reads in CRC tissue and ~70% of 88 patients evaluated by qPCR)	Only paper to date to evaluate transcriptionally active bacteria; colon sites of tissue samples not defined; no patient data provided; no healthy control population	[126]
Nine CRC and adjacent normal tissue samples for whole-genome sequencing; 95 CRC and adjacent normal tissue samples for PCR and 16S rRNA pyrosequencing; Spain; USA; Vietnam	Whole-genome Illumina sequencing; 16S rDNA (V3–V5) pyrosequencing; FISH using all bacteria and *Fusobacterium*-targeted probes	CRC and adjacent normal colon mucosa from surgical specimens	Overall *Fusobacterium* species enriched in CRC (range: <1 to >20% relative abundance in nine Illumina-sequenced samples; 16S rDNA sequencing, qPCR and FISH suggested increased *Fusobacterium* in CRC compared with normal colon tissues)	Colon sites of tissue samples not defined; no patient data provided; OTU with greatest similarity to *F. nucleatum* was the most dominant phylotype within CRC, but some tumors contained multiple *Fusobacterium* species; no healthy control population	[23]

[†] Nineteen with new adenoma and ten with colonoscopy within 1 year of polypectomy (six with recurrent adenoma).

[‡] Ten new diagnoses of CRC: 15 with colonoscopy within 1 year of partial colectomy (seven adenoma, five adenoma and CRC); three with colonoscopy 5–10 years after CRC resection.

[§] Normal colonoscopy.

[¶] *Clostridium/Leptum* group, *Clostridium/Coccoides* group, *Bacteroides/Prevotella* group, *Escherichia coli*, *Bifidobacterium* genus, *Lactobacillus/Leuconostoc/ Pediococcus* group and *Faecalibacterium prausnitzii* species.

[#] Eleven genera were reported as differing between tumor and matched normal tissues by unweighted UniFrac PCA.

[††] Sixteen and ten genera were reported as differing between rectal swab and stool, respectively, of CRC versus healthy volunteers by unweighted UniFrac PCA.

[‡‡] Distance of normal tissue from tumor not defined.

CRC: Colorectal cancer; OTU: Operational taxonomic unit; PCA: Principal component analysis; qPCR: Real-time PCR; T-RFLP: Terminal restriction fragment length polymorphism.