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Review

The Intestinal Microbiota in Colorectal Cancer

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Experimental evidence from the past years highlights a key role for the intestinal microbiota in inflammatory and malignant gastrointestinal diseases. Diet exhibits a strong impact on microbial composition and provides risk for developing colorectal carcinoma (CRC). Large metagenomic studies in human CRC associated microbiome signatures with the colorectal adenoma-carcinoma sequence, suggesting a fundamental role of the intestinal microbiota in the evolution of gastrointestinal malignancy. Basic science established a critical function for the intestinal microbiota in promoting tumorigenesis. Further studies are needed to decipher the mechanisms of tumor promotion and microbial co-evolution in CRC, which may be exploited therapeutically in the future.

Introduction

The impact of the intestinal microbiota on health and disease is increasingly emerging. While the microbiota evolved as a "neglected organ" in the early 2000s, our perception in 2018 has vastly changed in that the microbiota reflects a biological ecosystem that intensely communicates with the host (Charbonneau et al., 2016; Lynch and Pedersen, 2016; Marchesi et al., 2016; Sonnenburg and Backhed, 2016). A hype in microbiota research in the last decade allowed to delineate the composition and some functions of the intestinal microbiota, which was empowered by the introduction of modern molecular techniques. Current estimates suggest that the gastrointestinal tract contains as much bacteria as cells composing the human body (Sender et al., 2016). Although we can adequately depict the enormous diversity of the human microbiota, the role of most bacterial species in health and disease remains largely unknown. Key examples for a deranged interplay between the host and the microbiota may be derived from complex diseases including inflammatory bowel disease (IBD) and the tumor (micro-) environment in colorectal carcinoma (CRC). Despite enormous efforts and substantial progress in understanding the composition of the human intestinal microbiota, many functional aspects remain unresolved. This may be partly due to the complexity of the human intestinal microbiota, with a plethora of unpredictable host-microbe, microbe-microbe, and environmental interactions.

CRC, one of the most common malignancies in the western world, frequently causes death and is emerging worldwide. It is expected that CRC burden will substantially increase in the next two decades consequent to adoption of a western lifestyle (Arnold et al., 2017). It is well established that consumption of foods and nutrients affect the risk for developing CRC (Song et al., 2015). Dietary habits have substantially changed in the last decades especially in the western world and might affect colorectal carcinogenesis at various steps. Diet may exhibit effects on the host's immune response and elicit inflammation. In addition, dietary behavior tremendously influences the composition of the intestinal microbiota, which in turn impacts on the susceptibility to intestinal diseases (Shanahan et al., 2017) (Figure 1). Exposure to antibiotics early in life is associated with an increased risk for colorectal adenoma at the age of 60 (Cao et al., 2017), which suggests that a dysbiotic microbiota is acquired and sustained over a longer period of time (Gensollen et al., 2016). The colon (i.e., large intestine) and ileocecal valve exhibit the highest bacterial density along the gastrointestinal tract, which might point toward an important role for the microbiota in CRC. Indeed, studies in CRC patients and experimental evidence in animals linked the intestinal microbiota with CRC, which led to the identification of specific bacterial species that promote tumorigenesis (Drewes et al., 2016; Louis et al., 2014; Sears and Garrett, 2014). This review summarizes clinical information on the microbiota in CRC and aims to bridge to preclinical (i.e., experimental) evidence that the microbiota is a driving force in the development of intestinal tumors.

Specific Bacterial Strains Associated with CRC

CRC is frequently associated with dramatic alterations in the microbial composition of the tumor and adjacent mucosa, commonly termed as dysbiosis (Feng et al., 2015; Liang et al., 2017; Nakatsu et al., 2015; Tsoi et al., 2017; Yazici et al., 2017; Yu et al., 2017a). Dysbiosis is partly characterized by the expansion of bacterial taxa; however, dominant species in CRC evolution remain poorly defined. Experimental evidence for an important role of *Fusobacterium nucleatum* (*Fn*), *Escherichia coli*, and *Bacteroides fragilis* is emerging (Figure 2). In contrast, the role of depleted bacterial species in intestinal tumorigenesis is less well studied due to a lack of appropriate techniques. However, it may be speculated that the absence of certain (e.g., protective or beneficial) bacterial strains could be as important as tumor-associated bacterial overgrowth. Furthermore, we would like to highlight that the intestinal microbiota also comprises viruses and fungi; however, their role in CRC is not covered in this review as clinical evidence is largely lacking (Collins et al., 2011).

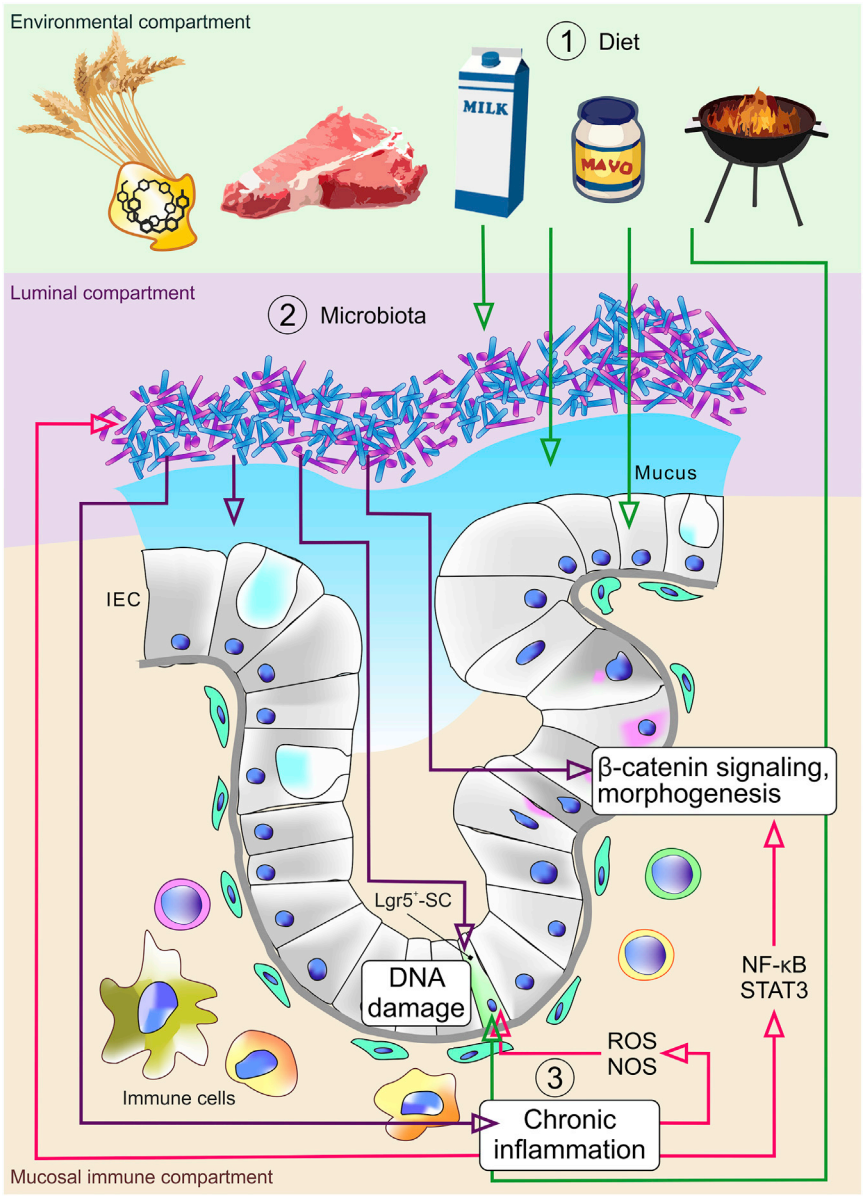

Figure 1. Multidimensional Framework of CRC Evolution

Malignant degeneration of intestinal epithelial cells (IECs) and progression to CRC involves a complex interplay from various layers of extrinsic and intrinsic factors. Together these influences result in oncogenic mutations in Lgr5$^+$ intestinal stem cells (SCs), in altered β-catenin/Wnt signaling, and in pro-inflammatory programs that drive CRC. (1) Extrinsic, predominantly nutritional factors can directly damage host DNA, modulate the composition and metabolic activity of the gut microbiota, interfere with gut barrier functions, affect IEC metabolism, and influence immune functions. (2) The microbiota influences intestinal tumorigenesis through several mechanisms. Several CRC-associated species such as *Fusobacterium nucleatum*, colibactin-producing *Escherichia coli*, and enterotoxigenic *Bacteroides fragilis* have been implicated in DNA damage and tumor progression. Microbial metabolism of complex carbohydrates, bile acids, and luminal iron, including heme iron, are important for barrier function and immune homeostasis as are bacterial signals integrated by surface and intracellular pattern recognition receptors such as Toll-like receptors, NOD-like receptors, and inflammasomes expressed by various cells types. (3) Chronic inflammation represents an important intrinsic factor that promotes carcinogenesis by inducing DNA damage, and reactive oxygen and nitrogen species, by modulation of IEC polarization and the tumor microenvironment, by activating transcriptional programs such as nuclear factor κB and STAT3 in IECs, and by hampering anti-tumoral immunity.

Fusobacterium nucleatum

The Gram-negative, anaerobic commensal *Fn* was associated with human CRC in two initial studies by genomic analysis (Castellarin et al., 2012; Kostic et al., 2012). *Fusobacterium* sequences were shown to be enriched in a small number of CRC patients and were also visualized within tumors using fluorescence *in situ* hybridization (Kostic et al., 2012). Castellarin et al. (2012) linked CRC with *Fn* expansion as demonstrated by qPCR analysis of 99 patients, which correlated with lymph node metastasis. Ahn et al. (2013) performed one of the first 16S rRNA-based analyses in CRC and demonstrated a decreased bacterial diversity in feces, depletion of Gram-positive fiber-fermenting *Clostridia*, and an increase in oral commensals *Fn* and *Porphyromonas*. These data appeared particularly interesting in light of the pro-inflammatory role of *Fn* in the intestine and its association with human IBD (Ohkusa et al., 2003).

Furthermore, *Fn* expansion is associated with periodontal disease which is linked to an increased CRC risk (Momen-Heravi et al., 2017). One study not only revealed increased presence of *Fn* both in high-grade dysplasia and established CRC but also a correlation with patient outcome (Flanagan et al., 2014). Enrichment of *Fn* in cancer tissue was also associated with shorter survival in another study and may therefore act as potential prognostic marker (Mima et al., 2016b). *Fn* expression was related to microsatellite instability (MSI) independent from CpG island methylator phenotype (CIMP) and BRAF mutation status (Mima et al., 2016b). *Fn* presence also correlated with CIMP-high lesions, and *Fn* was increasingly detected in malignant (CRC 56%) compared with benign lesions (hyperplastic polyp 24% and sessile serrated adenomas 35%) (Ito et al., 2015). As *Fn* abundance increases from rectum (2.5%) to cecum (11%), this bacterial strain may be particularly relevant in specific colonic locations (e.g., right-sided tumors) (Mima et al., 2016a). A large patient cohort from Japan demonstrated *Fn* colonization in 8.6% of CRC subjects and confirmed the association with MSI (Nosho et al., 2016). In another clinical study, *Fn* was detected in CRC tissue in 76 (13%) of 598 cases, and was inversely associated with the density of CD3$^+$ T cells supporting the idea that disease mechanisms involve regulation of immune responses (Mima et al., 2015). *Fn* abundance was not only associated with promotion of CRC

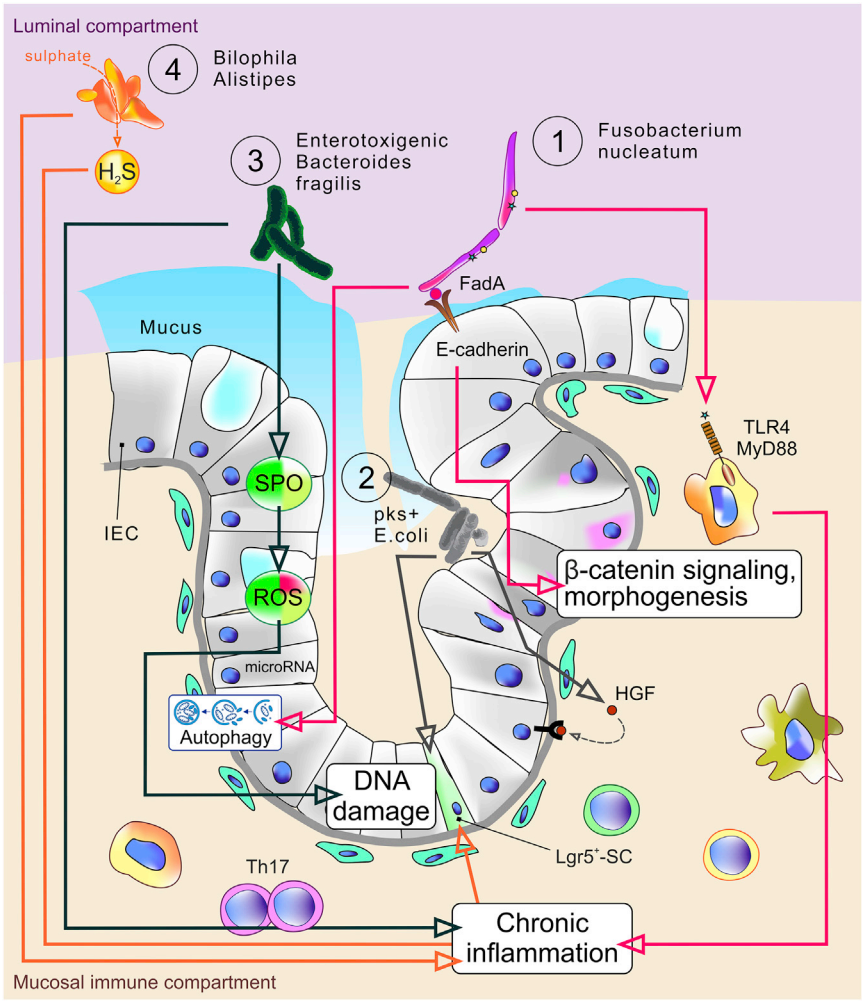

Figure 2. Microbial Mechanisms Influencing Cancer Development and Progression

The tumor-associated luminal environment represents a niche characterized by an impaired barrier function and a cluster of commensal bacteria that have been implicated in tumor initiation and progression. (1) *Fusobacterium nucleatum*'s (*Fn*) FadA antigen binds E-cadherin on IECs to activate β-catenin. This leads to uncontrolled cell growth, acquisition of a stem cell-like phenotype, loss of cell polarization, and possibly MSI instability. By mechanisms including TLR4 and MyD88, *Fn* has pro-inflammatory effects on the tumor microenvironment. Furthermore, *Fn* has been shown to modulate autophagy in IECs by activating regulatory microRNAs. (2) *E. coli* strains harboring the polyketide synthases (pks) island encoding the genotoxin colibactin are frequently observed in human colorectal tumors. Besides the genotoxic activity of colibactin, cell growth is sustained by cellular senescence associated with the expression of hepatocyte growth factor (HGF). (3) Enterotoxigenic *Bacteroides fragilis* (ETBF) causes inflammation and tumors in animal models. ETBF induces spermine oxidase (SPO) generating reactive oxygen species (ROS), thereby inducing DNA damage. ETBF is associated with Th17 responses. (4) Certain sulfate-reducing bacteria such as *Bilophila wadsworthia* or *Alistipes* spp. produce hydrogen sulfide (H_2S) capable of inducing genotoxic insults. Both strains promote inflammation in susceptible animals.

but furthermore conferred chemoresistance and recurrence in CRC patients by interfering with TLR4 and MyD88 signaling (Yu et al., 2017b). *Fn* was shown to target specific microRNAs resulting in activation of the autophagy pathway, thereby altering the chemotherapeutic response (Yu et al., 2017b). Remarkably, the CRC-associated microbiota including *Fn* can be detected in metastases (Bullman et al., 2017). Mouse xenografts of human primary CRC retained viable *Fn*, and antibiotic treatment reduced *Fn* load, tumor cell proliferation, and growth (Bullman et al., 2017). As such, clinical evidence is accumulating that *Fn* might promote colonic neoplasia.

This notion is also supported by experimental evidence. *Fn* is able to inhibit natural killer (NK) cell killing of various tumors, and this effect is mediated by the human (but not mouse) T cell immunoglobulin and ITIM domain (TIGIT) (Gur et al., 2015). The inhibitory receptor TIGIT is present both on human NK cells and T cells, and this inhibitory effect has been demonstrated to be dependent on the Fap2 protein of *Fn.* These data indicate that *Fn*-derived factors are able to regulate tumor-immune evasion. Furthermore, infection of CRC cells with *Fn* increases their proliferation rate, invasive activity, and potential to induce xenograft tumors in mice (Yang et al., 2017). *Fn* has been shown to modulate the tumor-immune microenvironment and the E-cadherin/β-catenin signaling pathway. Rubinstein and colleagues demonstrated that *Fn*, through FadA adhesion, binds to E-cadherin thereby activating the β-catenin signaling pathway resulting in induction of oncogenic and inflammatory responses (Rubinstein et al., 2013). Importantly, FadA gene expression in human CRC tissue is extraordinarily high compared with healthy controls, and inhibition of this pathway protected against pro-oncogenic activity. *Fn* was able to increase tumor multiplicity in the adenomatous polyposis coli/multiple intestinal neoplasia ($Apc^{Min/+}$) model of intestinal tumorigenesis (Kostic et al., 2013). Increased tumor load was paralleled by increased tumor infiltration of myeloid cells and pro-inflammatory cytokine expression. Notably, *Fn* did not deteriorate colitis, enteritis, or inflammation-associated intestinal carcinogenesis, demonstrating that *Fn* particularly controls tumorigenesis in this model. These studies support the idea that *Fn* plays a role in human CRC. The mechanisms deserve further attention but may involve immune evasion and/or the promotion of an inflammatory tumor microenvironment.

Escherichia coli

While *Fn* is among the most prevalent bacterial strains in CRC tissues and its relevance in CRC is supported by both clinical and pre-clinical studies, the evidence for *E. coli* is mainly based on preclinical investigations. *E. coli* are gut commensals, although certain strains have acquired the ability to promote intestinal inflammation and to produce toxins such as colibactin with oncogenic potential (Denizot et al., 2015). A western diet affects microbial composition and enhances susceptibility toward

the pathogenic potential of adherent invasive *E. coli* (Agus et al., 2016). Mucosa-associated *E. coli* are significantly more prevalent in CRC tissue and correlate with tumor stage and prognosis (Bonnet et al., 2014). Interestingly, pathogenic colibactin-expressing *E. coli* strains were more prevalent in advanced disease, and colonization of a colon cancer-associated *E. coli* strain into $Apc^{Min/+}$ mice resulted in a marked increase in number of polyps, suggesting that certain *E. coli* strains might indeed promote tumorigenesis. Studies using bioluminescent inflammation probes and fluorescence optical imaging also showed a correlation of *E. coli* with pro-inflammatory infiltrates which may propagate tumor growth (Veziant et al., 2016). This idea is strongly supported by earlier experiments in mono-colonized $Il10^{-/-}$ mice, which demonstrated that host inflammation is essential for *E. coli* cancer-promoting activities (Arthur et al., 2012, 2014). Disease phenotype was linked to changes in the *E. coli* gene catalog such as tumor-promoting *polyketide synthase* (*pks*) islands encoding colibactin (Arthur et al., 2014). Cultured mammalian epithelial cells exposed to *pks*-positive *E. coli* exhibited a transient DNA damage response with impaired DNA repair and an increase in gene mutation frequency (Cuevas-Ramos et al., 2010). Although exact pathomechanisms remain unclear, data derived from a xenograft and inflammation-associated tumor model provide evidence that *E. coli* that express the *pks* virulence island encoding genotoxins (such as colibactin) enhance tumor growth (Cougnoux et al., 2014). This effect was partly mediated by colibactin-induced cellular senescence and may involve the production of hepatocyte growth factor, which is highly expressed in human CRC. Interestingly, a small-molecule inhibitor of ClbP, an enzyme involved in colibactin synthesis, controls colibactin production and tumor formation *in vivo* (Cougnoux et al., 2016). A role for *E. coli* in CRC has also been supported by recent metagenomic studies in large CRC patient populations (Feng et al., 2015). However, the contribution of *E. coli* and *Fn* may vary in different experimental settings. For example, in one study, *Fn* clinical CRC isolates with FadA and Fap2 adhesins failed to induce inflammation and cancer, while colibactin-producing *E. coli* promoted tumorigenesis in $Apc^{Min/+}$;$Il10^{-/-}$ mice (Tomkovich et al., 2017). The varying degree of *Fn* colonization and the intestinal tumor location might have also affected the observed clinical phenotype in these experiments.

Bacteroides fragilis

Similar to *E. coli,* largely experimental evidence supports a role for *Bacteroides fragilis* (*Bf*) in intestinal tumorigenesis. *Bf* composes about 1%–2% of the commensal microbiota in most humans. *Bf*-derived toxin (BFT) causes inflammatory diarrhea and inflammation-related tumorigenesis (Sears et al., 2014; Wu et al., 2009). A study by Wu et al. (2009) revealed that enterotoxigenic *Bf* (ETBF), which secrete the toxin BFT, induce colitis and colonic tumors in $Apc^{Min/+}$ mice. This phenotype was driven by inflammatory Th17 cells as neutralization of both interleukin-17 (IL-17) and IL-23 reduced inflammation and tumor formation. *Bf* toxin gene expression has been detected more frequently in CRC subjects compared with controls, which also correlated with tumor prognosis to some extent (Boleij et al., 2015). ETBF colonization can be completely cleared by cefoxitin treatment, which was paralleled by reduction in murine colon tumorigenesis and IL-17A expression (DeStefano Shields et al., 2016; Housseau et al., 2016). BFT upregulates spermine oxidase, a polyamine catabolic enzyme, thereby generating reactive oxygen species and DNA damage, which propagates inflammation and tumorigenesis (Goodwin et al., 2011). Some reports observed that CRC tumor tissue was indeed associated with the presence of all three aforementioned strains, i.e., *Fn*, enteropathogenic *E. coli*, and ETBF (Viljoen et al., 2015).

CRC-Associated Bacteria: Results from Metagenomic Studies

Recently performed metagenomic studies have further supported the notion that CRC is associated with a certain gut microbiome signature. A metagenome-wide association study on stools from patients with advanced adenomas and CRC demonstrated that certain *Bacteroides* spp. (e.g., *B. dorei, B. vulgatus*, *B. massilensis*, and *E. coli*) correlated with systemic inflammation and tumor stage (Feng et al., 2015). In accordance with other studies, also *Parvimonas, Bilophila wadsworthia*, *Fn*, and *Alistipes* spp. were overrepresented in CRC patients; the latter may be of particular interest in inflammation-associated tumorigenesis as discussed later (Figure 2). When studying different ethnical cohorts, novel strains such as *Parvimonas micra* and *Solobacterium moreii* were associated with CRC (Yu et al., 2017a), and a set of 20 microbial genes significantly differentiated a CRC microbiome from healthy controls. qPCR abundance of two genes (butyryl-coenzyme A dehydrogenase from *Fn*, and RNA polymerase subunit β, *rpoβ*, from *P. micra*) clearly separated CRC microbiomes from those of healthy controls (Yu et al., 2017a). Detection of certain bacterial strains, i.e., the combination of four bacteria *Fn, Bacteroides clarus, Roseburia intestinalis*, and *Clostridium hathewayi* demonstrated a sensitivity >90% and a specificity >80% for CRC (Liang et al., 2017). All these studies suggest that certain bacterial strains and/or bacteria-derived factors could be used in the future as new screening tools for CRC (Shah et al., 2017).

Another important study investigated mucosal microbial communities in the human gut in different CRC stages (Nakatsu et al., 2015). In a metagenomic analysis these authors observed an association of CRC with certain metacommunities dominated by *Fn, Bf*, and *E. coli*, and new players such as *Gemella* or *Peptostreptococcus.* Notably, the finding that members of the oral microbiome are primarily associated with CRC is paralleled by previous studies and may have pathophysiological implications. Of note, the proportion of discordant metacommunities between tumor and tumor-adjacent mucosa were similar in adenoma and CRC samples (~40%) in this study. Certain metacommunities showed striking overlaps between cancer tissue and adjacent non-transformed mucosa, suggesting that, especially in the context of prevalent cancer, a substantial degree of dysbiosis may have already preceded in the colonic environment. As such, assessment of fecal and not necessarily mucosal microbiomes might be appropriate in CRC, which is supported by one study showing that colonic lavage reflected the microbial composition of a mucosal biopsy adequately (Watt et al., 2016).

Intestinal Microbiota and CRC: Potential Disease Mechanisms

Pattern Recognition Receptor Signaling, Inflammasomes, and Cytokines

There is growing evidence that the host immune system critically controls intestinal carcinogensis. The microbiota communicates

with the immune system through various mechanisms including Toll-like receptor (TLR) signaling (Luddy et al., 2014) and inflammasome sensing (Zmora et al., 2017). Importantly, TLRs and NOD-like receptors (NLRs) have been implicated in the progression of colonic tumorigenesis. For example, Nod1 recognizes bacterial antigens, which triggers an immune response, and Nod1 deficiency resulted in increased development of tumors in $Apc^{Min/+}$ mice and in an inflammation-related tumor model (Chen et al., 2008). The phenotype was accompanied by a disrupted intestinal barrier and was ameliorated by antibiotics. A similar phenotype can be achieved in case of Nod2 deletion, characterized by a highly pro-inflammatory milieu (Couturier-Maillard et al., 2013). In this study, disease was transferred to wild-type (WT) mice by fecal transplantation and improved by antibiotic therapy. These data and the study by Rutkowski et al. (2015) advocated a central role for the resident microbiota in TLR-controlled tumorigenesis. In line with this, germ-free mice developed fewer tumors in an inflammatory tumor model compared with conventional housing (Uronis et al., 2009). Mechanistically, TLR signaling and the microbiota may license activation of epithelial nuclear factor of activated T cells, which promoted intestinal tumorigenesis (Peuker et al., 2016). In accordance with a critical role for TLR signaling in intestinal tumorigenesis, IRAK-M, a negative regulator of TLR signaling commonly expressed in human CRC, forced colorectal tumor formation by suppressing antimicrobial defenses (Kesselring et al., 2016). Mutations in the innate immune sensor absent in melanoma 2 (AIM2) are frequently observed in patients with CRC. *Aim2*-deficient mice are highly susceptible to colonic carcinogenesis, which was mainly attributed to uncontrolled proliferation of intestinal stem cells and aberrant Wnt signaling (Man et al., 2015). This phenotype was paralleled by a dysbiotic microbiota and could be ameliorated by a healthy mouse microbiota, highlighting that pattern recognition receptor defects promoted microbiota-mediated tumorigenesis.

Mice deficient in an inflammasome protein, the NOD-like receptor family pyrin domain containing 6, exhibited enhanced tumor load, and this phenotype could be transferred to WT mice (Hu et al., 2013). Tumorigenesis in this model was microbiota driven involving chemokine (C-C motif) ligand 5 (CCL5), and IL-6 overexpression. Inflammation and IL-6 in particular have been linked to tumorigenesis as excellently reviewed recently (Lasry et al., 2016; West et al., 2015). These data demonstrate that microbial sensing controls inflammation and tumorigenesis and that disruption of microbial sensing instigated microbiota-mediated disease processes.

Inflammation, Microbiota, and CRC

It is estimated that 20%–30% of CRC cases are associated with inflammation. Inflammatory mechanisms are well-established drivers of tumorigenesis, which is also reflected by a substantial CRC risk in patients with IBD (Brennan and Garrett, 2016; Choi et al., 2017; Kang and Martin, 2017; Lasry et al., 2016; Mantovani et al., 2008) (Figure 1). The microbiota harbors the potential to shape an inflammatory microenvironment and, *vice versa*, inflammation might affect microbial composition. Carcinogenesis in the intestine is driven by the presence of microbes, inflammation, and modulation of intestinal immunity (Tlaskalova-Hogenova et al., 2014). Colon polyposis in $Apc^{Min/+}$ mice is accompanied by accumulation of microbes in polyps triggering local inflammatory responses. This phenotype could be reverted by antibiotic therapy and IL-10 specifically secreted by regulatory T cells (Dennis et al., 2013). Defects in alarmin/IL-33 expression renders mice highly susceptible to an IL-1α-dependent colitis and colitis-associated cancer that was promoted by a dysbiotic microbiota (Malik et al., 2016).

We recently demonstrated an important role for the siderophore-binding protein lipocalin 2 (*Lcn2*) in intestinal inflammation. $Lcn2^{-/-}Il10^{-/-}$ mice not only exhibited a severe colitis, but also spontaneously developed right-sided colonic tumors. Disease was abolished by antibiotic therapy, depended on IL-6, and could be transferred to WT mice (Moschen et al., 2016). Importantly, we demonstrated that certain bacterial strains, such as *Alistipes* spp., were crucially involved in inflammation-related carcinogenesis, as *Alistipes* gavage induced tumorigenesis in $Il10^{-/-}$ mice. This is of particular note, as several human metagenomic studies revealed an association between *Alistipes* and CRC (Feng et al., 2015). *Alistipes finegoldii* can be increasingly found in an "adenoma-carcinoma sequence" mouse CRC model induced by 1,2-dimethylhydrazine (Sun et al., 2017).

Several other microbiota-regulated pathways ranging from oxidative stress to autophagy and fatty acid metabolism have been linked to intestinal tumorigenesis. *Peptostreptococcus anaerobius*, an abundant bacterium in human CRC, increases colonic dysplasia in mice by promotion of TLR-mediated reactive oxygen species (Tsoi et al., 2017). Conditional inactivation of Atg7, a protein required for the autophagic process, in intestinal epithelial cells ameliorated tumorigenesis in $Apc^{Min/+}$ mice and enhanced anti-tumor responses. Intestinal inhibition of Atg7 evoked dysbiosis, which was critically involved in anti-cancer responses (Levy et al., 2015). *Atg7* deficiency resulted in p53-mediated cell-cycle arrest in tumor cells but not in normal tissue. Furthermore, microbiota-derived toxins may play a role (see section on *Bf* above) or bacterial-derived metabolites such as lipoteichoic acid (Khazaie et al., 2012). The notion that human CRC is indeed promoted by a dysbiotic microbiota stems from recent experiments by Wong et al. (2017). The authors transferred a human CRC microbiota (or a healthy microbiota as a control) into conventionally housed or germ-free WT mice that had been exposed to azoxymethane to induce tumor formation. In both models, the microbiota from CRC patients promoted the formation of polyps, dysplasia, and proliferation, indicating that the human CRC-associated microbiota modulates tumorigenesis in mice.

Role of Short-Chain Fatty Acids: "The Butyrate Paradox"

In recent years, bacterial metabolites, including the short-chain fatty acid (SCFA) butyrate, emerged as regulators of immune responses (Figure 3). SCFAs are produced when dietary fiber is fermented into the colon and represent a major energy source for colonocytes. Several bacteria have been identified as potential butyrate producers, among them *Fn* (Vital et al., 2014). Removal of carbohydrates from the diet promotes the growth of mucus-utilizing bacteria and alters the microbiota, which resulted in susceptibility to infectious colitis (Desai et al., 2016). Although most studies suggest that butyrate suppresses both inflammation and carcinogenesis in the colon, some studies revealed opposite results (the butyrate paradox). The tumor load emerging in $Apc^{Min/+}$;$Msh2^{-/-}$ mice was not only reduced by antibiotic treatment, but also by a carbohydrate-deficient diet

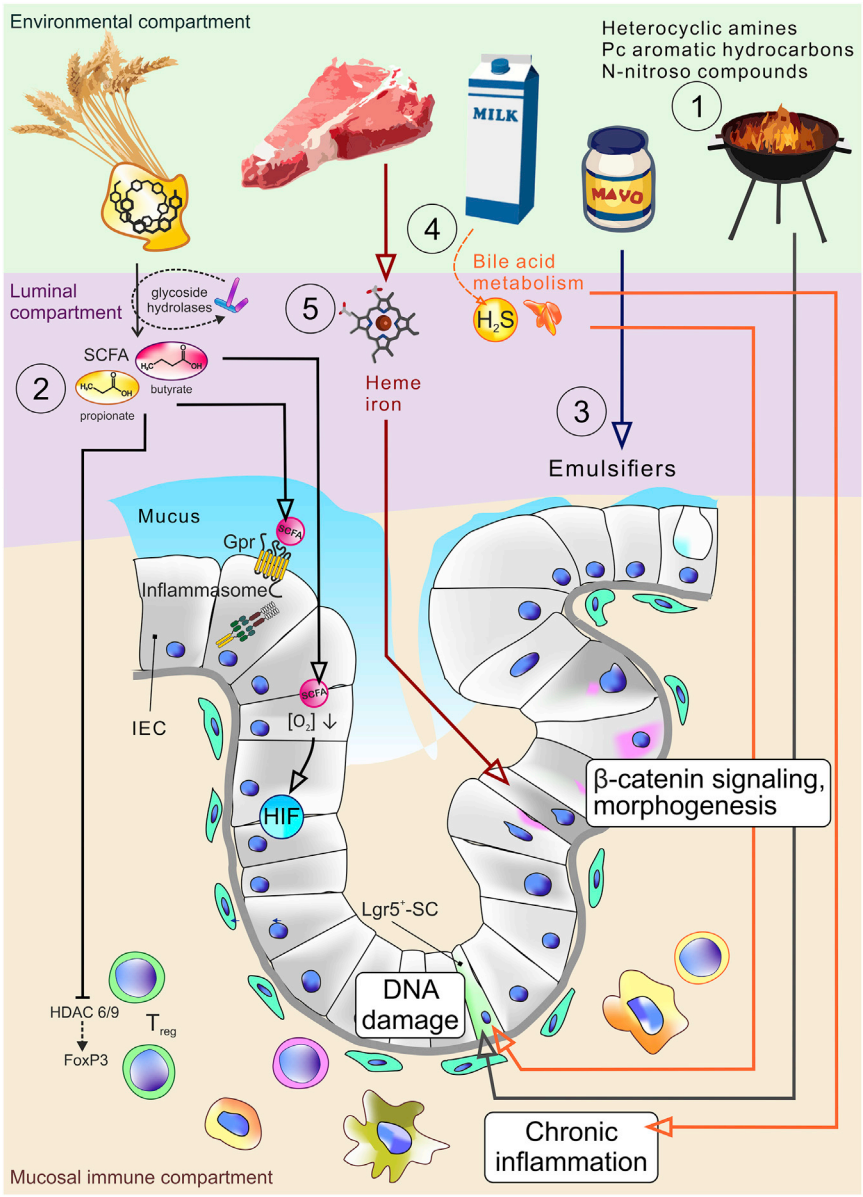

Figure 3. Nutritional Impacts on Intestinal Tumorigenesis

(1) Certain nutrient carcinogens including heterocyclic amines, polycyclic aromatic hydrocarbons, and N-nitroso compounds exert direct genotoxic effects. Intestinal homeostasis and an intact gut barrier function ensure spatial segregation and exclusion of luminal threats. Nutrition may be a key modulator of gut microbial composition and barrier function. A diet deprived in microbiota-accessible fiber promotes mucus-degrading species and deprivation of short-chain fatty acids (SCFAs). (2) SCFAs strengthen barrier functions through mechanisms such as G-protein-coupled receptor (Gpr)-mediated sensitization of the IEC inflammasomes and reducing IEC oxygen concentrations and induction of hypoxia-induced factor (HIF). Furthermore, SCFAs exert anti-inflammatory and tolerogenic effects on immune cells. (3) Gut barrier function may be further deteriorated by certain food additives including dietary emulsifiers. (4) Diets enriched in red meat and animal fat promote the overgrowth of pro-inflammatory and pro-tumorigenic species by altering bile acid metabolism. (5) Heme iron further exerts direct cytotoxic and hyperproliferative effects.

(Belcheva et al., 2014). Mechanistically, butyrate promoted hyperproliferation of colonic intestinal epithelial cells, which may account for the tumor phenotype. In line with a potential anti-carcinogenic effect for butyrate it could be shown that butyrate and its receptor Gpr109a suppress colonic inflammation and carcinogenesis (Singh et al., 2014). Native Americans with a low CRC risk exhibit lower total SCFA stool levels compared with African/Caucasian Americans having a considerably higher CRC risk (O'Keefe, 2016). The butyrate paradox remains, and probably several factors including local concentrations of this metabolite might determine its biological functions.

Biofilm: Pro-carcinogenic and Relevant in CRC?

Another possibility how the microbiota affects tumorigenesis may be the formation of biofilms (Dejea et al., 2014; Johnson et al., 2015). Dejea et al. (2014) observed that bacterial biofilms might be associated with CRC. In this small study, a bacterial biofilm was typical for right-sided CRC (observed in 13/15 subjects), whereas it was less commonly found in left-sided cancer (2 out of 15 CRCs). Interestingly, in this study a biofilm was also observed on normal mucosa, linked to a more pronounced inflammatory phenotype (increased epithelial IL-6 and STAT3 expression) without a consistent association with a tumor-typical microbial signature. The relevance of such bacterial biofilms is also supported by a recent study in patients with familial adenomatous polyposis (FAP) demonstrating patchy bacterial biofilms composed primarily of *E. coli* and *Bf* (Dejea et al., 2018). Interestingly, in this study colonic mucosal gene expression for colibactin and *Bf* toxin were highly increased in FAP patients versus healthy controls. Bacterial biofilms might be linked to certain metabolomic phenotypes as biofilm-positive cancers were associated with an upregulation of polyamine metabolites such as N1- or N12-diacetylspermine, which was reduced to normal levels after antibiotic therapy (Johnson et al., 2015). Bacterial biofilms might constitute an oncogenic risk factor for CRC reflecting a complex composition of bacterial and host metabolites (Dejea and Sears, 2016). A recent high-resolution bacterial 16S rRNA gene profile meta-analysis, including biofilm assessment, showed that CRC tissues are indeed enriched for biofilm especially in right-sided CRC, which was associated with *Bf* and certain oral pathogens such as *Fn, P. micra*, and *Peptostroptococcus stomatis* (Drewes et al., 2017). The oral microbiota in CRC seems distinctive and predictive, partly via oral biofilm-forming bacteria, which may allow for an alternative CRC screen in the future (Flemer et al., 2017).

Diet, Microbiota, and CRC

Diet can rapidly alter the intestinal microbiota potentially contributing to disease susceptibility (David et al., 2014; Desai et al., 2016). An expansion of sulfidogenic bacteria such as *Bilophila wadsworthia* was found in African American CRC subjects (Yazici et al., 2017). Hydrogen sulfide, which is mainly produced by autochthonous sulfidogenic bacteria, is genotoxic, and triggers inflammation and hyperproliferation. Abundance of these bacteria was linked to certain dietary habits such as consumption of fat and red meat intake (Yazici et al., 2017). In an experimental approach, diet-induced blooming of *B. wadsworthia* promoted inflammation in genetically susceptible mice (Devkota et al., 2012). How *B. wadsworthia* mediated inflammation and whether this also impacted on tumorigenesis is currently unclear.

Interestingly, Sonnenburg and colleagues demonstrated that changes in dietary habits even impact on the gut microbiota over generations (Sonnenburg et al., 2016; Sonnenburg and Backhed, 2016). A western diet (high fat and simple carbohydrates, deprived of microbiota-accessible complex carbohydrates and fiber) used in mice over generations resulted in an unrecoverable loss of diversity. Such data are of potential clinical relevance considering recent epidemiological trends in CRC in North America with an increased risk in younger people (Siegel et al., 2017). Several other typical components of a western diet such as emulsifiers lead to gut microbiota alterations and favor inflammatory and malignant intestinal diseases in mice (Chassaing et al., 2015; Viennois et al., 2017) (Figure 3). These data are increasingly relevant beyond CRC, as a western diet contributes to disease processes outside the intestine (Sonnenburg and Backhed, 2016). In this regard, one study in humans deserves attention: African Americans fed a high-fiber, low-fat African-style diet and vice versa rural Africans fed a high-fat, low-fiber western-style diet exhibited remarkable alterations on the colonic microbiota and its metabolome within 2 weeks, which also included major changes in inflammatory and proliferative markers (O'Keefe et al., 2015).

This and many other studies highlight that the microbiota could indeed reflect a "missing link" in the close interaction between dietary factors and CRC. The intestinal microbiota exhibit a high diversity in cultures that eat less-processed high-fibre diets (De Filippo et al., 2010; Schnorr et al., 2014). In contrast, westernization of dietary habits throughout the world and its impact on the gut microbiota might substantially contribute to current epidemiology of global CRC prevalence (Arnold et al., 2017). Data from the Nurses' Health Study and the Health Professionals Follow-up Study investigating 1,019 incident colon and rectal cancer cases demonstrated that diets rich in whole grain and dietary fiber are associated with lower *Fn* positivity, also supporting a connection between diet, microbiota, and CRC (Mehta et al., 2017). One possibility how westernized dietary habits may affect CRC risk has been experimentally revealed: heme, the pigment of red meat, is able to induce cytotoxicity of colonic contents and causes epithelial damage and hyperproliferation. Mice on a heme diet develop hyperproliferation associated with differential expression of oncogenes and tumor suppressors, which could be reversed by a 2-week antibiotic therapy (Ijssennagger et al., 2015) (Figure 3). The intestinal microbiota has recently also emerged as a key player in cancer therapy (Geller et al., 2017; Gopalakrishnan et al., 2018; Routy et al., 2018). As the role of the microbiota in the modulation of treatment efficacy is beyond the scope of this review, we kindly refer to an excellent recent review (Roy and Trinchieri, 2017).

Future Therapeutic Approaches Targeting the Microbiota

It appears plausible that microbial interventions in CRC may be beneficial. Several approaches are currently being considered targeting the gut microbiota including pre-, pro-, and antibiotics although dietary strategies may be equally effective (O'Toole et al., 2017). However, studies that would convincingly address this idea (even at the preclinical level) remain scarce. Probiotics such as VSL#3 affected microbial composition but failed to reduce colitis-associated CRC (Arthur et al., 2013). However, *Actinobacteria* (e.g., *Streptomyces*), *Proteobacteria* (e.g., *Acinetobacter*), and *Firmicutes* (e.g., *Brevibacterium* or *Bacillus*) might exert anti-carcinogenic effects (Zhou et al., 2017). Dietary components might be suitable to modulate the intestinal microbiota probably by promoting a large microbial diversity. As dietary factors are critically involved in evolution of CRC (O'Keefe, 2016), dietary approaches might reflect the most reasonable and cost-effective approach. Berberine, a plant-derived dietary component, was able to rescue tumor formation induced by *Fn* associated with reduced mucosal inflammation (Yu et al., 2015). Resistant starch, which has anti-inflammatory and anti-cancer properties, reduced tumor loads in a rat model of inflammation-associated CRC, probably through modulation of the microbiota (Hu et al., 2016). As such, dietary components and products of the intestinal microbiota are involved in CRC pathogenesis, but therapeutic interference at this stage is still in its infancy.

Conclusions

Epidemiologic studies have identified several lifestyle factors that affect the risk for developing CRC. Interestingly, many of these features, such as diversified diet, limited use of processed foods, consumption of adequate dietary fiber, exercise, and body weight all converge in an altered composition of the intestinal microbiota (Kyrgiou et al., 2017). Despite enormous progress and appealing observations, which address the role of the microbiota in the pathogenesis of intestinal tumorigenesis, major issues deserve our attention. Do we mainly describe an association of limited biological relevance between mechanistic insights in experimental approaches and observations in human CRC? This issue may be solved by translational studies that link mechanistic insights with human CRC that are eagerly awaited.

Furthermore, the regional specificity and metabolic capacity of the intestinal microbiota might be highly relevant but is poorly investigated. We still have a limited understanding of microbiome profiles at different sites (e.g., right- or left-sided CRC), which may define molecular subtypes of CRC (Guinney et al., 2015). Easily accessible fecal material probably reflects a suitable surrogate for the colonic microbiota; however, compelling evidence in CRC is missing. Studies from IBD patients demonstrated good correlations to colonic inflammatory phenotypes in contrast to IBD involvement of the small intestine (Gevers et al., 2014; Halfvarson et al., 2017; Pascal et al., 2017). Indeed, the concept of non-invasive determination of microbiome-based

biomarkers in feces for the diagnosis of CRC is appealing but not established. With more standardized and sophisticated clinical protocols (Falony et al., 2016; Vandeputte et al., 2016), a complete microbiome reference should become available. Especially with the help of larger aligned international sample panels we might expect definite microbial signatures in CRC.

Reduced microbial diversity in the intestine is characteristic for many intestinal and extra-intestinal disorders including IBD, obesity, type 2 diabetes, asthma, and chronic liver diseases (Tilg and Kaser, 2011). It is likely that a combination of alterations, rather than increased or decreased abundance of a particular strain in the intestinal microbiota promotes tumorigenesis. A high diversity might reflect a key feature of a healthy gut enabling a species-rich ecosystem to deal with environmental challenges that promote disease processes. How microbial diversity protects against tumorigenesis and which species (or their interplay) are indeed relevant in human CRC remains a conundrum. Furthermore, decreased abundance of specific strains within the commensal microbiota may have its impact on CRC development, but is challenging to explore experimentally. Remarkably, Crohn's disease patients exhibit similar microbial alterations (e.g., increased abundance of *E. coli* and *Fn*) as seen in CRC (Pascal et al., 2017) suggesting that not only inflammation but also a shared dysbiosis may contribute to IBD-associated CRC. In the future, dietary interventions with pre- or probiotics or a shift of dietary habits could successfully reduce the prevalence of CRC or may allow to guide treatment. Despite (or rather because of) many uncertainties in this emerging field, a new world of intestinal microbes is currently being discovered and many aspects support their fundamental role in malignant and non-malignant disease processes. For CRC, we propose that a better understanding of the host microbe mutualism and disturbances caused by environmental cues may shape novel therapeutic approaches.

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