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REVIEW

The gastrointestinal microbiota in colorectal cancer cell migration and invasion

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Abstract

Colorectal carcinoma is the third most common cancer in developed countries and the second leading cause of cancer-related mortality. Interest in the influence of the intestinal microbiota on CRC emerged rapidly in the past few years, and the close presence of microbiota to the tumour mass creates a unique microenvironment in CRC. The gastrointestinal microbiota secrete factors that can contribute to CRC metastasis by influencing, for example, epithelial-to-mesenchymal transition. Although the role of EMT in metastasis is well-studied, mechanisms by which gastrointestinal microbiota contribute to the progression of CRC remain poorly understood. In this review, we will explore bacterial factors that contribute to the migration and invasion of colorectal carcinoma and the mechanisms involved. Bacteria involved in the induction of metastasis in primary CRC include *Fusobacterium nucleatum*, *Enterococcus faecalis*, enterotoxigenic *Bacteroides fragilis*, *Escherichia coli* and *Salmonella enterica*. Examples of prominent bacterial factors secreted by these bacteria include Fusobacterium adhesin A and Bacteroides fragilis Toxin. Most of these factors induce EMT-like properties in carcinoma cells and, as such, contribute to disease progression by affecting cell-cell adhesion, breakdown of the extracellular matrix and reorganisation of the cytoskeleton. It is of utmost importance to elucidate how bacterial factors promote CRC recurrence and metastasis to increase patient survival. So far, mainly animal models have been used to demonstrate this interplay between the host and microbiota. More human-based models are needed to study the mechanisms that promote migration and invasion and mimic the progression and recurrence of CRC.

Keywords Colorectal cancer · Microbiota · Metastasis · Epithelial-mesenchymal transition

Abbreviations

APC	Adenomatous polyposis coli
BFT	*Bacteroides fragilis* toxin
CNF1	Cytotoxic necrotizing factor 1
CRC	Colorectal cancer
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth factor
GAP	GTP-ase activating protein
GelE	Gelatinase E
GPCR	G-protein coupled receptor
JAM	Junctional adhesion molecule
LEE	Locus of enterocyte effacement
MMP	Matrix metalloproteinase
TGF	Transforming growth factor

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Introduction

Colorectal carcinoma (CRC) is the third most diagnosed cancer in developed countries and the second leading cause of cancer-related mortality [1]. CRC comprises many different phenotypes. For example, CRCs from diverse molecular backgrounds vary in growth patterns, histomorphological characteristics and protein expression [2]. One of the best-studied molecular backgrounds of CRC formation is a mutation in the tumour suppression gene Adenomatous

Polyposis Coli (*APC*). This mutation in *APC* activates the Wnt/wingless signalling pathway, promoting proliferation and proto-oncogene expression [3]. Besides different molecular phenotypes, there is also a difference in tumour location in the colon [4]. The tumours in the proximal and distal colon show differences in histology and patterns of metastasis. Tumours in the proximal colon generally show flat histologies and commonly spread to the peritoneum. Tumours in the descending colon demonstrate polypoid-like morphologies and tend to spread to the lungs or liver [5]. Approximately 20% of patients with CRC already have metastases at diagnosis [1], and the most common sites of CRC metastases are the lung, liver, and peritoneum [6].

For hematogenic metastasis to be successful, five general steps are required. These steps consist of the detachment of tumour cells from the primary tumour site (1), intravasation (2), survival within the circulation (3), extravasation (4) and colonisation at the secondary site (5) [7]. CRC cells can undergo epithelial-mesenchymal transition (EMT), a vital process in the migration and invasion stage of the metastatic cascade. EMT refers to a cell re-programming enabling epithelial cells to lose their adherence to neighbouring cells and the extracellular matrix (ECM). Simultaneously, the cells acquire mesenchymal properties essential for migration and invasion [8]. A typical change in EMT is the loss of E-cadherin accompanied by dysregulation of the Wnt signalling pathway [9].

Many factors contributing to or initiating this transition have been extensively studied, such as a high-fat diet, smoking, and alcohol use [10–12]. The role of the intestinal microbiota on CRC has sparked interest in recent years. Several studies have shown an altered composition of the gastrointestinal microbiota in CRC [13], and the proximity of microbiota to the tumour region provides a unique microenvironment. The microbiota that are part of the tumour microenvironment in CRC contribute to the disease progression and recurrence [7].

Although metastasis in CRC is well-studied [14], mechanisms by which gastrointestinal microbiota contribute to the initiation, invasion, migration and metastasis of CRC remain poorly understood. Suggestions are made that the gastrointestinal microbiota are of influence on all aspects of cancer development. Stakelum et al. previously reviewed the role of the gastrointestinal microbiota in the other stages of the metastasis cascade [7]. Current evidence suggests that the microbiota impacts multiple processes in this cascade. For example, *F. nucleatum* was suggested to contribute to local invasion, but can also stimulate the secretion of the cytokine CXCL1 [15]. This cytokine participates in premetastatic niche formation in the liver [16]. The gastrointestinal microbes could also secrete factors that can contribute to local invasion through the induction of mesenchymal properties. Therefore, the primary goal of this review is to explore how bacterial factors can contribute to the migration and invasion of CRC. Additionally, this review aims to generate a deeper understanding of the potential mechanisms involved.

Mechanisms of CRC invasion and migration

During EMT, loss of the epithelial phenotype at the invasive front results in increased immune system evasiveness [17]. This change enables tumour cells to migrate through the basal membrane and extracellular matrix, and migrate into the bloodstream and lymph nodes [17, 18]. Characteristics frequently found in tumour cells at the invasive tumour front include the loss of junctional proteins, induction of EMT-related pathways, activation of Matrix Metalloproteinases (MMPs) and membrane ruffling [19]. More recently, autophagy gained interest as a regulator of metastasis initiation [20]. Below, these concepts will be discussed in the context of CRC invasion and migration.

Disruption of junctional proteins

Differentiated epithelial cells exhibit apicobasal polarity [19], determined by tight junctions and adherence junctions (Fig. 1). A steady adherence junction requires the binding of E-cadherin to the actin cytoskeleton via the cytoplasmic domain of β-catenin. E-cadherin expression inversely correlates with the malignancy of the tumour [21, 22]. In progressing tumours, loss of E-cadherin or its original localisation was consistently found. Adherence junctions can link a cell to either an adjacent epithelial cell or to the ECM. The transmembrane composition of adherence junctions depends on the linkage to epithelial cells or ECM. When the adherence junction links two epithelial cells, adherence junctions consist of cadherins. When the adherence junction links epithelial cells to the ECM, adherence junctions consist of integrins. On the inside of the cell, the integrin or cadherin is connected to the contractile protein actin. The actin forms belt-like structures on the cytoplasmic surface of the cell membrane, supporting the epithelial barrier [23]. As metastasis initiation progresses, the E-cadherin-β-catenin complex disconnects and the β-catenin is translocated to the nucleus [24]. In the nucleus, β-catenin functions as an inducer for transcription factors of the Wnt pathway [9]. These transcription factors cause a diversity of cellular effects regarding cellular adhesion, morphology of the tissue and tumour progression [23]. Wnt target genes include E-cadherin repressors *ZEB1* and *SNAI1*. Other Wnt target genes are *MT1-MMP-9* and *LAMC2* [14]. All these upregulated genes are associated with EMT and invasiveness [25]. Nuclear β-catenin expression and the loss of membranous E-cadherin showed separately to be prognostic factors for cancer prognosis [26].

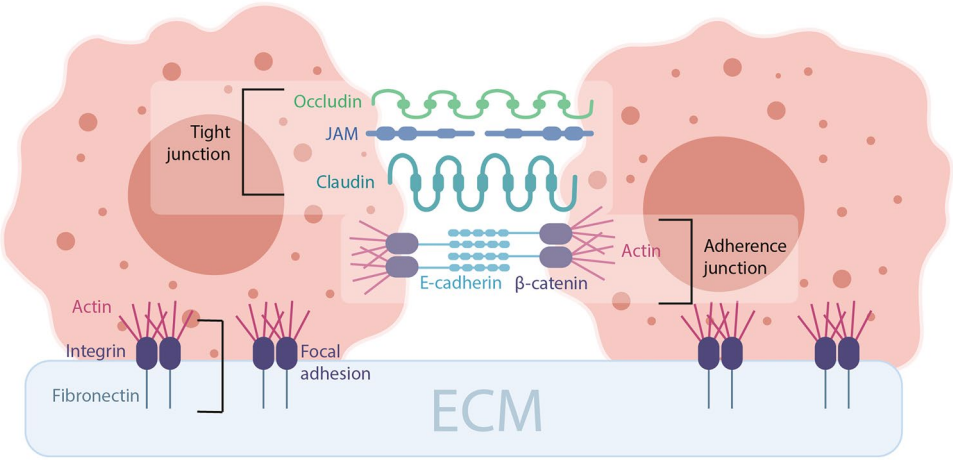

Fig. 1 Prominent cell-cell and cell-ECM connections in epithelial tumour cells. Cell-cell contact is maintained via desmosomes (not depicted), gap junctions (not depicted), tight junctions and adherent junctions. Cell-ECM contact is maintained via hemidesmosomes (not depicted) and focal adhesions. Tight junctions consist of occludin, a junctional adhesion molecule (JAM) and claudin. These molecules form a complex that is dividing the cell into apical and basal compartments. In adherence junctions, β-catenin and E-cadherin form a complex at the membrane that binds to the actin cytoskeleton, maintaining cell-cell adhesion. Focal adhesions consist of integrins, connecting proteins and is attached to the actin cytoskeleton on the inside of the cell

A stable epithelial barrier also requires tight cell-cell and cell-ECM connections. Tight junctions link adjacent epithelial cells to the basolateral membrane (Fig. 1). The function of tight junctions is to divide the epithelial cells into apical (body) and basal (blood) compartments. Tight junctions contribute to the barrier function of epithelial cells by controlling the diffusion of ions and other small molecules through the intercellular space [27]. Essential components of tight junctions in EMT are occludin and claudin [28]. Occludin is a 65 kDa protein that forms tight associations comparable to claudins [29]. Occludin is repressed during EMT of cancer cells by transcription factor SNAI1 [28], which is involved in the Nf-κB pathway [30]. The downregulation or disruption of occludin and claudin promoted cancer cell migration in SW620 cell lines [29, 31, 32].

MMP activation

After the loss of contact with the primary tumour, the cells need to break through the ECM to spread [33]. During this phase, tumour cells secrete enzymes to degrade the ECM. One type of enzyme involved in ECM degradation is MMPs. MMPs can be divided into six categories, according to their substrates. Examples of categories are interstitial collagenases, gelatinases and matrilysins [34]. A substantial enzyme in CRC spread is MMP-9 [34–36], also known as gelatinase B or 92 kDa type IV collagenase [37]. Pro-MMP-9 is cleaved into its active form by a subfamily of MMPs attached to the plasma membrane in the tumour microenvironment [38, 39]. There are two critical steps in the process by which MMPs enable cancer cells to degrade the ECM. First, MMPs work by degrading ECM macromolecules such as collagens, laminins and proteoglycans to remove any physical obstacles to invasion [40]. Second, MMPs break down the basement membrane of the ECM by cleavage of type IV collagen and laminin [41, 42]. Next to this direct approach, MMPs serve numerous other roles in the invasion process, such as enhancing vascular permeability [43].

Membrane ruffling

A weakened epithelial barrier also contributes to the migration and invasion of cancer cells by reorganisation of the cytoskeleton. Reorganising the cytoskeleton, thereby impeding barrier integrity, is the first step in the detachment of metastatic tumour cells [44]. One way of cytoskeleton reorganisation during metastasis is the occurrence of a ruffled membrane. A ruffled membrane often appears on the leading side of a motile (metastatic) cell, and an increase in ruffling is associated with the active movement of cells [45, 46]. Membrane ruffling is a complex and rapid process in which the protrusion of the cell membrane margins fluctuate abnormally. Ruffles on an adherent cell's periphery and leading edge, as well as ruffling on the dorsal surface, are the two most common forms of ruffling. Several cytokines have been shown to cause membrane ruffling. Examples of these include transforming growth factor (TGF) and fibroblast growth factor (FGF) [45]. Overall, membrane ruffling has been linked to metastatic status in tumour cells [47]. Additionally, membrane ruffling signifies tumour cell motility and metastatic potential in in vitro [48, 49] and animal

[50] studies. A ruffled membrane is also a characteristic of autophagosome induction [51].

Autophagy

Autophagy is known as the degradation of intracellular components within autophagosomes and plays an ambiguous role in metastasis [20, 52]. Autophagy in metastasis is most likely influenced by the cancer stage and tissue type [53]. It promotes genome stability and limits necrosis and inflammation in the developing stage [54–56]. On the other hand, autophagy is an essential process in all steps of metastasis [57]. For example, in highly metastatic tumour cells, autophagy induces motility and invasion by promoting focal adhesion turnover [58]. However, the exact mechanism by which autophagy contributes to EMT remains unclear.

Healthy human gut microbiota

The colon is the portion of the gastrointestinal tract that is most densely colonised by microbiota. It is estimated that the colon contains around 70 % of the entire human microbiota [59]. Spatial differences in bacterial composition were found in mice and divided into crypt, faecal and interlaced regions [60]. With a 12 times higher prevalence than carcinomas in the small intestine, the colon is also the most likely segment to develop malignancies [13]. After birth, the bacterial composition depends on, for example, the type of child delivery and the type of milk feeding. During this period, the hosts' composition is made, and external factors such as antibiotic use during childhood can severely influence it [61]. Later in life, the microbiota is, amongst other factors, shaped by diet and gut epithelial metabolism. Diet and gut epithelial metabolism cause both beneficial and unfavourable health effects. Members of the *Bifidobacterium* genus are among the first bacteria to colonise the human gastrointestinal tract and are considered beneficial to the host's health. For example, *Bifidobacterium longum* produces acetate, which causes upregulation of the barrier function in the host's gut epithelium [62]. Another example is the involvement of *Bacteroides* in carbohydrate metabolism. *Bacteroides* strains, such as *Bacteroides fragilis*, can metabolise complex carbohydrates and amino acids in the intestinal environment [63]. The gut epithelium itself also metabolises dietary fibres and, in this way, shapes the colonic microbiota [64]. As such, dietary fibre is metabolised into, amongst others, butyrate. This process maintains the epithelium in a metabolic state, characterised by high oxygen consumption. This high oxygen consumption results in epithelial hypoxia, which ensures that the microbiota in the colon consists mainly of obligate anaerobic bacteria. These anaerobic bacteria aid in the digestion of nutrients that the host enzymes cannot process. A shift in dietary fibre causes a shift in the microbial composition to facultative anaerobic bacteria, a hallmark of dysbiosis in the colon. Examples of such facultative anaerobic species are *Enterococcus faecalis* and *Escherichia coli*, which can act as a driver of CRC initiation [65]. During the initiation and progression of CRC, the composition shifts and bacteria from the *Fusobacterium* and *Bacteroides* genera merely colonise the gastrointestinal tract [66, 67]. To better understand the relationship between an altered composition of gastrointestinal microbiota and CRC initiation and progression, researchers proposed the driver-passenger model, as discussed below [68].

The driver-passenger model

An initial model proposed that colorectal cancers arise from chronic immune responses that synergize with microbial products to drive carcinogenesis [69]. This model was called the alpha-bug model. Recent developments in high-throughput sequencing technologies have enabled researchers to analyse the gut microbial structures of healthy and diseased body sites, contradicting this model. Several experimental data sets support a possible role for gut microbiota both in CRC initiation and progression [70–72]. From these data sets, the driver-passenger model was derived. The driver-passenger model deviates from the alpha-bug model in the sense that disease progression causes changes in the microenvironment of the growing tumour, thus creating a division between bacterial drivers and passengers [68].

Bacterial drivers of CRC initiation were defined as an abundancy of bacteria with pro-carcinogenic characteristics during cancer initiation [68]. An example of bacteria that show these characteristics is the production of DNA-modifying compounds by *E. faecalis* and *E. coli*. *E. faecalis* produces an extracellular superoxide, which is converted into hydrogen peroxide by the cellular metabolism of the gut epithelium, resulting in oxidative DNA damage [73]. Furthermore, some *E. coli* strains produce a genotoxin called colibactin, which can induce single-strand DNA breaks. The DNA damage inflicted increases the mutation rate of affected cells [68]. As a result of the changing microenvironment of the growing tumour, gut commensals with either tumour-promoting or tumour-suppressing capabilities (bacterial passengers) gradually replace the bacterial drivers (alpha bugs and their helpers).

Bacterial passengers in CRC are bacteria that, in a healthy gastrointestinal tract, are poor colonisers. However, these bacterial strains have a competitive advantage in the tumour microenvironment that eventually outcompetes the bacterial drivers. Considerable changes in the microenvironment during colon carcinogenesis include an altered epithelial barrier function and rupture of the epithelium [74–76]. As some microbial species are more adapted to this new environment, the bacterial passenger species likely have a

competitive advantage and are likely to contribute to disease progression [68]. Examples of bacterial passengers in CRC are *Fusobacterium nucleatum* and enterotoxigenic *Bacteroides fragilis* species. Although bacterial passengers mainly colonize the tumour microenvironment, bacterial drivers and passengers can contribute to CRC metastasis via various mechanisms. These mechanisms mainly work through the previously mentioned induction of the invasion and migration driving EMT [77].

Bacterial factors contributing to CRC invasion and migration

As mentioned, bacterial factors can contribute to these processes influencing CRC invasion and migration. Bacteria that produce these factors are *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Escherichia coli* and *Salmonella enterica*. Their secreted factors and possible contributions to CRC invasion and migration are described below.

Fusobacterium nucleatum

Fusobacterium nucleatum is a gram-negative, anaerobic bacterium commonly found in saliva and biofilms in the oral cavity. *F. nucleatum* is an invasive bacterium that contributes to the emergence of several periodontal diseases and diseases of the gastrointestinal tract [78]. However, *F. nucleatum* is also prevalent in CRC patients [71, 79], and patients with high levels of *F. nucleatum* have a worse prognosis and develop metastases more often [80, 81]. A recent hypothesis concerning the gastrointestinal abundance of *F. nucleatum* is that the species originates from the oral cavity and traverses via the gastrointestinal tract to the colon as a bacterial passenger in CRC [82]. *F. nucleatum* attaches to other bacteria and cells via fimbriae and non-fimbrial adhesins. Via these proteins, *F. nucleatum* colonises and contributes to colonise the gastrointestinal tract [83]. Mechanisms by which *F. nucleatum* promotes migration and invasion in CRC are based on the excretion of protein factors. The most prominent protein factors include Fusobacterium adhesin A (FadA), Keratin7 (KRT7) and Caspase activation and recruitment domain 3 (CARD3).

Fusobacterium adhesin A

The factor Fusobacterium adhesin A (FadA) contributes to migration and invasion by dissociation of adherence junctions. FadA is a fimbrial adhesin protein. Oligomerisation of pre-FadA and mature FadA form an aggregate with a high molecular weight, which can attach to and allow the bacterium to invade host cells signalling (Fig. 2) [84]. Solubility assays showed that pre-FadA is anchored in the inner membrane of the bacteria, and mature FadA is secreted outside of the bacteria, serving as an anchor. During the invasion of the host cells, the aggregate intrudes the outer membrane of the epithelial cell. FadA contributes to tumour cell dissociation by binding to E-cadherin and subsequent activation of β-catenin. Additionally, co-incubation of HCT-116 CRC cells with *F. nucleatum* increased IL-8 and CXCL1, which are correlated with increased metastatic potential [15].

Additionally, FadA modulates β-catenin signalling in cancerous cells via Annexin A1, a protein specifically expressed in proliferating colorectal cancer cells [85]. Annexin A1 is involved in the activation of oncogene Cyclin D1 (*CCND1*). Cyclin D1 plays an essential role in CRC progression [86, 87].

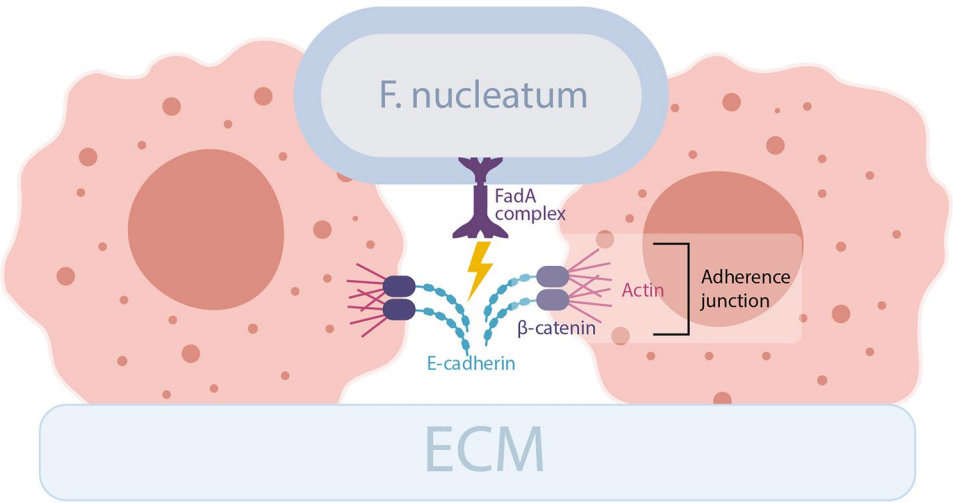

Fig. 2 Schematic overview of the proposed effect of the FadA complex on adherence junctions in epithelial tumour cells. FadA binds E-cadherin in adherence junctions, resulting in altered binding between the E-cadherin molecules. Consequently, there is a reduction of cell-cell contact between the tumour cells. Note: bacterium and epithelial cells not drawn to actual scale

Bacterial peptidoglycan

Bacterial peptidoglycan from *F. nucleatum* could contribute to metastasis initiation through activation of autophagy signalling. The detection of bacterial peptidoglycan downstream induces Caspase activation and recruitment domain 3 (CARD3) in HCT-116 cells. CARD3 is a protein encoded by the Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) gene. RIPK2 is a mediator of inflammatory responses after bacterial infections, during which it is activated by NOD-like receptors [88, 89]. This inflammatory response mainly depends on the recruitment of adaptor protein CARD3. The presence of bacterial peptidoglycan in early endosomes activates the NOD1-NOD2-RIPK2 complex. The complex signals through NF-κB and MAP kinase (MAPK) for activating immune cells and promoting pro-inflammatory cytokines. Although the evidence is still preliminary, CRC patient tissue colonised by *F. nucleatum* showed an upregulation in CARD3 [90]. This upregulation may promote CRC metastasis by activating autophagy signalling [91].

Enterococcus faecalis

Enterococcus faecalis is the most prevalent bacterial species found in the GI tract with a standard diet [92, 93]. *E. faecalis* is a gram-positive, commensal bacterium that belongs to the lactic acid-producing bacteria. The role of *E. faecalis* in CRC is controversial. Although part of the literature indicates a harmful function, strains of *E. faecalis* are also considered to have probiotic abilities with great applicability in food products. This controversial role of *E. faecalis* in CRC was reviewed previously elsewhere [92]. Nonetheless, *E. faecalis* strains are abundant in CRC tissue [94]. These bacteria grow facultative anaerobic and are resistant to extreme environmental challenges. Besides the GI tract, *E. faecalis* can be found in the human oral cavity [95]. In the context of metastasis, *E. faecalis* was shown to release gelatinase E (GelE), which can activate the collagen-degrading matrix metalloproteinase 9 (MMP-9). Below, we will describe GelE in more detail.

GelE

GelE can disrupt adherence junctions by degradation of E-cadherin. Steck et al. showed that *E. faecalis* secretes GelE, which directly disrupts the intestinal barrier and causes inflammation [96]. In transwell cultures, GelE derived from *E. faecalis* triggered a reduced epithelial barrier function and E-cadherin expression when combined with pro-inflammatory cytokines. GelE cleaves murine recombinant E-cadherin, which suggests that loss of E-cadherin can be a direct consequence of exposure to bacterial GelE. Indeed, in a colitis susceptible mouse model, GelE induced the degradation of E-cadherin, causing loss of the epithelial barrier [96]. In contrast, this was not demonstrated after GelE exposure in a wild-type mouse model, suggesting the involvement of pro-inflammatory cytokines. Furthermore, GelE regulates enteric epithelial permeability via protease-activated receptor 2 (PAR2) [97]. As such, activation of PAR2 leads to disruption of tight junctions [98], whereas E-cadherin degradation leads to the disruption of adherence junctions (Fig. 3a). GelE from *E. faecalis* was also shown to activate MMP-9 in colonic tissue by cleaving pro-MMP-9 into its active form and activate human plasminogen, causing supraphysiological degradation of collagen [99, 100] (Fig. 3b). Further studies by this group showed that this GelE producing *E. faecalis* promotes colonic cancer after colonic surgery in mice, a model for cancer recurrence. They also show an increase in colon cancer micrometastases in the liver with the presence of this *E. faecalis* strain in the colon [101].

Enterotoxigenic *B. fragilis*

To date, more than 20 different species of *Bacteroides* have been identified. *B. fragilis* is an obligate anaerobe bacterium found in the entire length of the gastrointestinal tract [102]. *B. fragilis* can be divided in two classes: non-toxigenic *B. fragilis* and enterotoxigenic *B. fragilis* [103]. In stool samples from CRC patients, the frequency of enterotoxigenic *Bacteroides fragilis* was found to be increased [104]. CRC patients also showed a high rate of enterotoxigenic *B. fragilis* infection [104], and detection of high levels of *B. fragilis* DNA fragments in the blood was associated with metastatic disease [105]. These bacteria maintain a healthy gastrointestinal microflora in humans by preventing and alleviating gastrointestinal inflammation [106, 107].

Bacteroides fragilis toxin

B. fragilis Toxin (BFT) also disrupts adherence junctions. Enterotoxigenic *B. fragilis* produces BFT. The toxin is a 20 kDa zinc-containing metalloprotease, also known as fragilysin [108]. In the CRC cell-line HT-29, BFT altered cell-cell attachment when placed on the basolateral membrane of epithelial cells [109]. More specifically, BFT cleaved E-cadherin, the extracellular domain of adherence junctions (Fig. 4), which was shown to be essential to decrease cell-cell attachment [110]. Cleaving E-cadherin in CRC cells can contribute to disease progression by weakening adherence junctions [111].

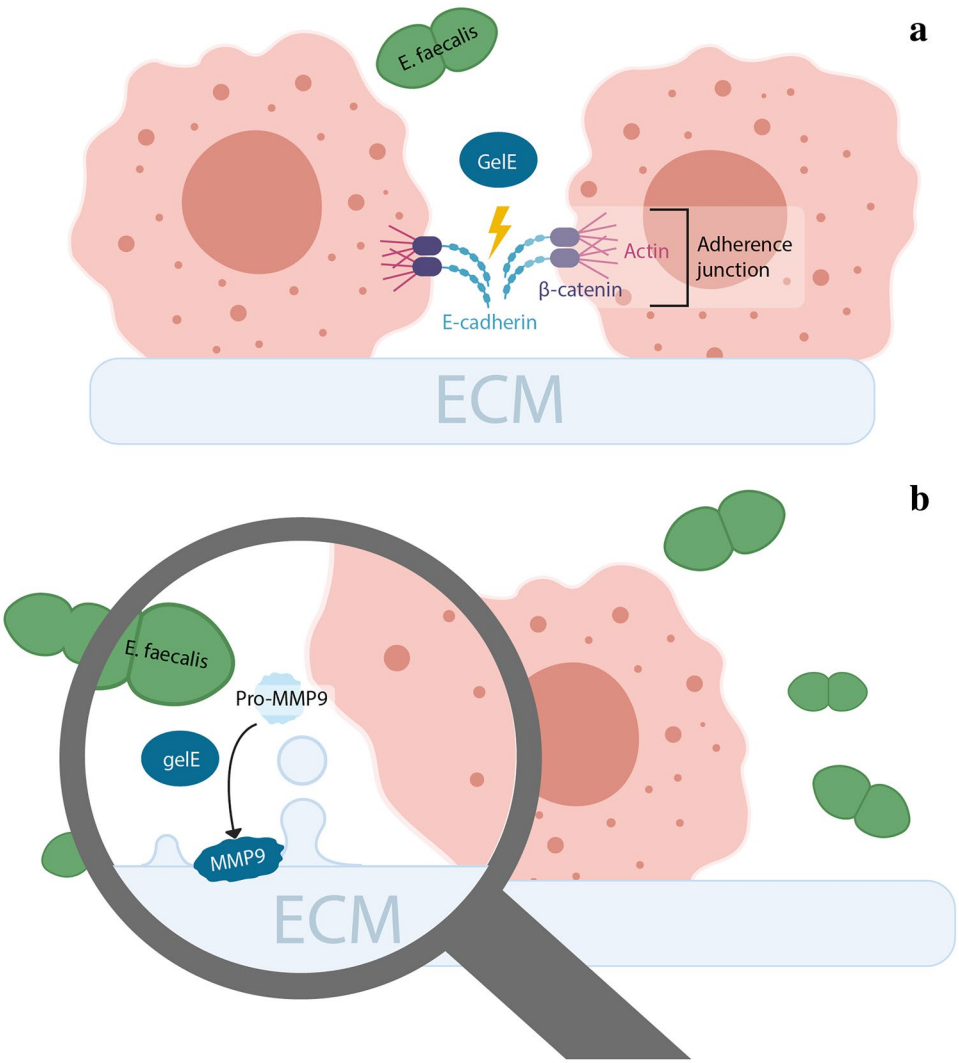

Fig. 3 Schematic overview of the proposed effect of GelE on adherence junctions and MMP-9 activation. **a** GelE degrades E-cadherin in adherence junctions, resulting in a reduction of cell-cell contact between the tumour cells. **b** Mechanisms of CRC invasion and migration. GelE cleaves Pro-MMP-9 into the active form MMP-9. As a result, MMP-9 degrades the ECM resulting in a reduction of cell-ECM contact. Note: bacterium and epithelial cells not drawn to actual scale

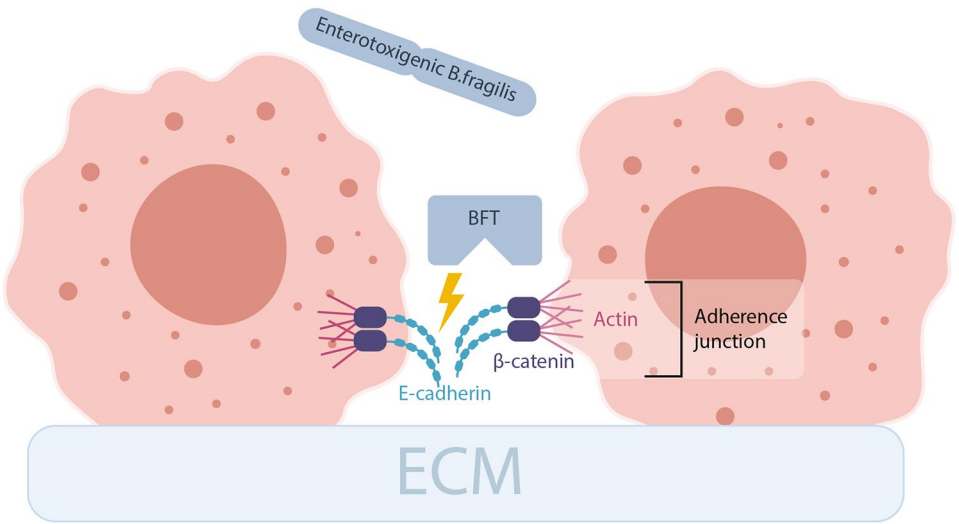

Fig. 4 Schematic overview of the proposed effect of *Bacteroides fragilis* toxin (BFT) on adherence junctions. BFT cleaves E-cadherin in adherence junctions, resulting in a reduction of cell-cell contact between the tumour cells. Note: bacterium and epithelial cells not drawn to actual scale

Escherichia coli

In the intestinal flora, *Escherichia coli* strains are aerotolerant anaerobic Gram-negative bacteria. *E. coli*, as a commensal, coexists peacefully with its mammalian host, promoting healthy intestinal homeostasis and causing disease only rarely [112]. Some virulent *E. coli* strains, on the other hand, may colonise the human gastrointestinal tract and cause disease. Pathogenic *E. coli* have been found in colon tissue from patients with adenocarcinomas more often than in healthy colonic tissue [113, 114], and bacterial *E. coli* DNA fragments in the blood proved an indicator for metastasis [105]. These are different from commensal strains because they contain pathogenicity islands in their genomes, coding for proteins that play a role in the dispersing virulence factors [115]. More specifically, *E. coli* contributes to CRC cell invasion and migration via Cytotoxic Necrotizing Factor 1 (CNF1) and effector protein EspF, which we will discuss below.

CNF1

CNF1 is a Rho GTPase-activating toxin that induces molecular changes and membrane ruffling in cancerous epithelial cells [116]. Examples of these changes include the activation of Nf-κB, COX2 expression, the release of pro-inflammatory cytokines, and, more importantly, enhanced cellular motility. As the transformation of a healthy epithelial cell to a carcinoma cell coincides with the same pathways, it is conceivable to hypothesize that CNF1-producing *E. coli* colonisation can influence cancer development. CNF1 contributes to disease progression by activating Rho GTPases, which are involved in the configuration of the actin cytoskeleton [117]. Overactivation of the actin cytoskeleton causes ruffling of the cell (Fig. 5a) [118].

EspF

EspF disrupts the epithelial barrier through the disassembly of tight junction proteins (Fig. 5b) via dephosphorylation and dissociation of occludin, a crucial part of the molecular structure of tight junctions. The effector protein EspF is a protein encoded in the locus of enterocyte effacement (LEE), a pathogenicity island. EspF is shown to be critical for decreased transepithelial resistance, a parameter for epithelial barrier function [119].

Salmonella enterica

Salmonellae are Gram-negative, facultative anaerobic pathogens that can infect a diversity of organisms [120, 121]. *Salmonella enterica* is a rod-shaped bacterium with an actin-like bacterial cytoskeleton that supports this rod [122, 123]. Several *S. enterica* serovars can cause severe human infections, leading to acute gastroenteritis. Although no correlation between *S. enterica* and CRC progression has been shown yet, colonisation of the tumour microenvironment by *S. enterica* can contribute to the progression of CRC via the secretion of enteric bacterial protein AvrA, which we will discuss below.

AvrA

The *AvrA* gene in *S. enterica* encodes a multifunctional pathogenic protein that is injected into host cells and activates the β-catenin signalling pathway. AvrA modulates inflammation, epithelial apoptosis, and proliferation by enhancing the ubiquitination and acetylation of target proteins in eukaryotic cell pathways [124, 125]. AvrA is transferred into host epithelial cells through a bacterial needle-like apparatus, defined as the type three secretion system [126] (Fig. 6). This system creates a translocation pore when it encounters the target cell. Through this pore, the effector proteins are injected. Once in the host cell, AvrA activates the β-catenin signalling pathway [127] and suppresses the degradation of β-catenin [128, 129]. Notably, the AvrA protein was found in samples from human CRC tissue [130], and as such, may affect disease progression and metastasis.

Conclusion and future perspectives

Here, we provided an overview of potential mechanisms by which gastrointestinal microbiota may promote CRC invasion and migration. The close presence of microbiota in the tumour area creates a unique microenvironment in CRC. Prominent bacteria contributing to metastasis of CRC include *F. nucleatum*, *E. faecalis*, *B. fragilis, E. coli*, and *S. enterica*. Most of these bacteria produce virulence factors that contribute to EMT, and via this transition, to disease progression. The direct mechanism has not been fully elucidated yet, though indirect effects have been reported for some virulence factors. Other mechanisms by which gastrointestinal bacteria contribute to invasion and migration are eliminating cell-cell adhesion, ECM degradation, membrane ruffling, and altering pro-metastatic cell-signalling pathways.

To increase patient survival, it is crucial to elucidate the mechanism by which bacterial factors promote CRC metastasis. Although we are only starting to uncover the complex interactions between CRC, the host and the gastrointestinal microbiota, the information available to date has implications for clinic intervention. Mechanistic insights can lead to the establishment of clinically relevant biomarkers indicative of the risk of metastasis. These biomarkers could be obtained by screening for, for example, specific bacterial

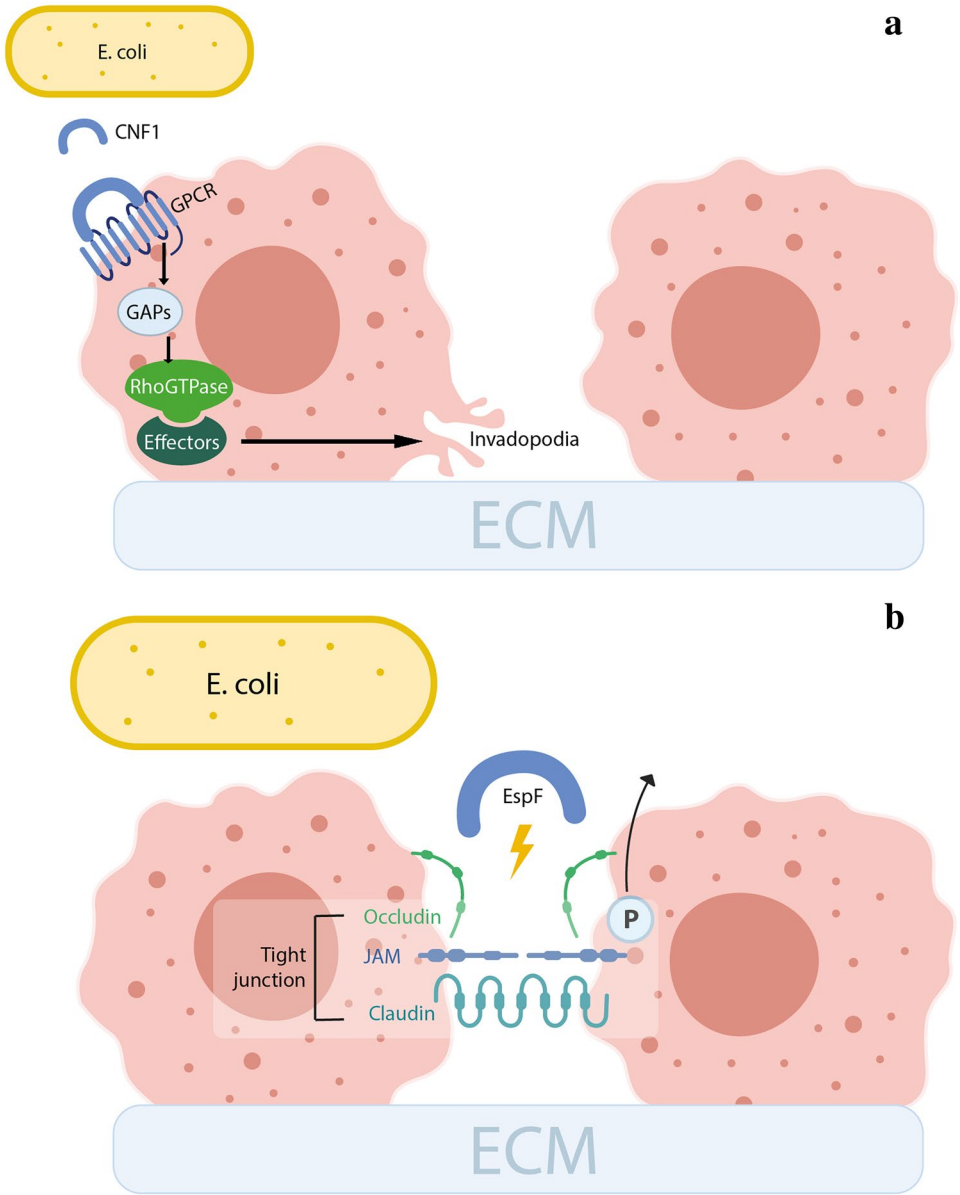

Fig. 5 Schematic overview of the proposed effect of *E. coli* on tight junctions and membrane ruffling. **a** CNF1 binds to a G-protein coupled receptor (GPCR) on the cell membrane. The GPCR activates GTPase-activating proteins (GAPs), leading to Rho GTPase activation. Rho GTPase, in turn, activates effector proteins that cause the cytoskeleton to reorganise the membrane in a ruffled shape (occurrence of invadopodia). Reorganisation of the cytoskeleton is regarded as the first step in the detachment of metastatic tumour cells. **b** EspF disassembles occludin via its dephosphorylation in tight junctions, resulting in a reduced cell-cell contact. Note: bacterium and epithelial cells not drawn to actual scale

strains, the metabolites or the response of the tumour cells. The biomarkers can help optimize treatment plans on a personal level, increasing the chance of patient survival [131]. Recent hypotheses state that each tumour comprises a unique on-tumour microbiota consisting of specific bacterial species and metabolic profiles [132].

Since the microbiota is closely associated with diet, mechanistic insights on how bacteria can promote metastasis can also lead to specialised dietary advice for CRC patients at risk for metastasis. The dietary advice could be based on the composition of the patient's microbiome or bacterial toxins in the blood, indicating which bacterial toxins are present in the colon. This strategy is already proposed in inflammatory bowel disease [133, 134] and used as a preventive measure for CRC [135]. For the latter, research suggests avoiding the intake of nutrients that stimulate dysbiosis and intestinal inflammation, like, red meat and high-fat products [136]. On the other hand, diet can enhance colonisation resistance to prevent the colonisation of pathogenic bacteria or an overabundance of CRC promoting bacteria [137]. Counteracting the colonisation of pathogenic bacteria is a strategy also used in the prevention of CRC. Especially high-fibre products like flaxseed, soy and oat promote the proliferation of phylae that guarantee a favourable modulation of the immune system and protection against pathogens [135]. Although the microbial composition of the tumour microenvironment in metastasizing tumours may differ from that of a developing tumour [68], the same principles might apply in terms of dietary advice. Nonetheless, research into the establishment of a 'metastatic microbiome' and preventative

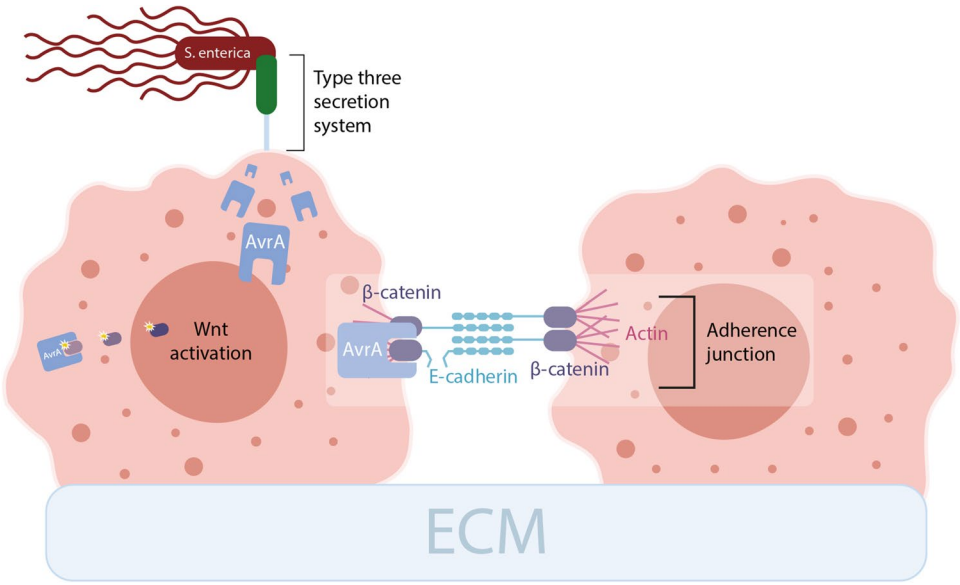

Fig. 6 Schematic overview of the proposed effect of AvrA on intracellular β-catenin and β-catenin in adherence junctions. AvrA disassembles the β-catenin-E-cadherin complex, resulting in reduced cell-cell contact. AvrA also promotes intracellular β-catenin migration to the nucleus. In the nucleus, β-catenin activates the transcription factor Wnt. Wnt upregulates genes associated with EMT and cancer cell invasiveness. Note: bacterium and epithelial cells not drawn to actual scale

dietary advice is still scarce. Other possible strategies to prevent the colonisation of pathogenic bacteria include faecal transplantation, probiotics and antibiotics, as will be discussed below.

Firstly, faecal microbiota transplantation (FMT) has been recently considered for the management of colorectal cancer. The main goal of FMT is to diminish inflammatory, proliferative and procarcinogenic pathways. Nonetheless, FMT has not yet been thoroughly investigated in CRC, especially not in a metastatic context, FMT could aid in reducing metastatic disease by the replacement of the pathogenic bacteria, but warrants further investigation.

Secondly, pathogenic bacteria would preferably be replaced by probiotics with the ability to strengthen, for example, epithelial barrier integrity [138]. This barrier would subsequently prevent metastasis [139]. However, when introducing probiotics there are also some limitations. For example, there is a risk of triggering fatal systemic inflammatory response syndromes and inadvertent transfer of pathogenic organisms [140]. Recent research with *E. coli* showed that in an environment with low microbial diversity, probiotic bacteria can accumulate genetic mutations that can be potentially harmful to the host [141]. Nevertheless, probiotics are well-recognized for the treatment of several diseases, including CRC when administered in the right dose [142, 143].

Thirdly, antibiotic treatment in the management of CRC metastasis showed promising effects. For example, oral administration of metronidazole to mice bearing patient-derived xenografts infected with *F. nucleatum* resulted in a decrease in tumour volume and tumour cell proliferation [144]. A retrospective cohort showed that antibiotic exposure during bevacizumab therapy reduced mortality rates in male metastatic CRC patients [145]. Additionally, analysis of three clinical data sets showed that antibiotic use during 5-FU chemotherapy is correlated with longer progression-free and overall survival among metastatic colorectal cancer patients [146]. These data suggest that antibiotic treatment could limit the metastatic capacity in CRC patients when administered during chemo- or immunotherapy. Another study showed that the combination of antibiotic use and a host with a western diet can cause colonisation of the intestine by collagenolytic microbes, like *E. faecalis* promoting tumour recurrence [101]. The colonisation of collagenolytic bacteria cause an altered gut barrier, which, in turn, leads to the facilitation of liver metastasis [147, 148].

Therapeutics neutralising the effect of the bacterial factors could drastically decrease the prevalence of metastatic disease in CRC. For example, local phosphate can suppress the growth of pathogenic bacteria in biofilms via inhibition of quorum sensing, which mediates the formation of the biofilms [149]. Thus far, bacteria themselves have been used in cancer therapy as inducers of the immune response against tumour tissue, oncolytic agents and using bacterial spores to carry tumoricidal agents [150].

Despite the recent advantages in technology allowing for clarifications of mechanisms for EMT, molecular mechanisms of EMT are often poorly understood. For example, the mechanism for the loss of function of the E-cadherin-β-catenin complex is well-known. However, the mechanism by which the complex contributes to invasion and metastasis remains unclear.

To date, mainly animal and in vitro models have been used to demonstrate the interplay between host and microbiota in CRC progression. However, the mechanism for tumour invasion and the microbiota are dependent on the host species and the virulence factor of the bacterial strain [151, 152]. Therefore, more human models are needed to study

the mechanisms that promote progression and mimic invasion and migration of CRC. Current human studies mainly focus on establishing an association between the presence of a particular species and tumorigenesis. Some of these studies also associate the presence of a particular species with prognosis, which is in-depth reviewed by Messaritakis et al. [105]. With the increasing insights on the considerable influence that the microbiome has on the body, the amount of evidence on the mechanisms of gastrointestinal bacteria that contribute to tumour formation has drastically increased in the last few years. However, the amount of evidence on the mechanisms of gastrointestinal bacteria that contribute to tumour progression is still limited. More in-depth studies into the underlying mechanisms will prove a substantial addition to the growing list of significant molecular host-microbe interactions affecting health and disease.

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Declarations

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