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Colorectal cancer: role of commensal bacteria and bystander effects

Xingmin Wang[1] and Mark M Huycke[2,3,*]

[1]Department of Radiation Oncology; University of Oklahoma Health Sciences Center; Oklahoma City, OK USA; [2]Department of Medicine; University of Oklahoma Health Sciences Center; Oklahoma City, OK USA; [3]The Muchmore Laboratories for Infectious Diseases Research; Oklahoma City VA Health Care System; Oklahoma City, OK USA

For years the human microbiota has been implicated in the etiology of colorectal cancer (CRC). However, identifying the molecular mechanisms for how aneuploidy and chromosomal instability (CIN) arise in sporadic and colitis-associated CRC has been difficult. In this Addendum we review recent work from our laboratory that explore mechanisms by which intestinal commensals polarize colon macrophages to an M1 phenotype to generate a bystander effect (BSE) that leads to mutations, spindle malfunction, cell cycle arrest, tetraploidy, and aneuploidy in epithelial cells. BSE represents the application of a phenomenon initially described in the radiation biology field. The result of commensal-driven BSE on colon epithelial cells is aneuploidy, chromosomal instability (CIN), expression of stem cell and tumor stem cell markers and, ultimately, malignant transformation. Our findings provide a conceptual framework for integrating the microbiota with aging, cyclooxygenase (COX)-2, and inflammation as risk factors for CRC.

Keywords: Bystander effects, carcinogenesis, colorectal cancer, commensal bacteria

*Correspondence to: Mark M Huycke; Email: markhuycke@ouhsc.edu

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This decade has increasingly witnessed the importance of commensal microbiota in the etiology of CRC.[1,2] However, despite a better understanding of associations between the human microbiota and CRC, much remains to be learned of how commensals initiate cancer. Although several lines of investigation, including our own,[3-8] suggest mechanisms by which wayward commensals, or so-called pathobioants, can damage epithelial cell DNA, large gaps remain in our knowledge of how bacteria induce aneuploidy and CIN in sporadic and colitis-associated CRCs. One example of an intestinal commensal that can lead to DNA damage is *Escherichia coli*. Strains that express hybrid peptide-polyketide or cytolethal distending toxins generate double-strand DNA breaks, induce G_2/M cell cycle arrest, promote invasive carcinoma in mice, and occur at increased frequency in patients with CRC and inflammatory bowel disease.[9] These findings directly implicate one member of the complex and diverse human commensal microbiota in CRC initiation. The genome landscape for CRC includes cancer cells and premalignant precursors that often express highly variable and abnormally structured genomes that consist of non-diploid chromosome numbers (*i.e.*, aneuploidy) that constantly evolve on a background of complex recombinations that include gene duplications, deletions, inversions, and translocations (*i.e.,* CIN).[10,11] Despite recent advances, the role of the microbiota in CRC and origin of initiating events for aneuploidy and CIN remain obscure.

We have reported in several papers a novel mechanism by which the intestinal microbiota induces aneuploidy and CIN in primary colon epithelial cells.[3-5] We termed this mechanism as macrophage-induced BSE (**Fig. 1**). Using a human intestinal commensal, *Enterococcus faecalis*, to test this theory, we linked the polarization of macrophages to the induction of aneuploidy and CIN in chromosomally stable epithelial cells. In addition, we most recently showed that BSE induced stem cell and tumor stem cell markers during malignant transformation.[5]

Plausible theories for CRC should explain the clinical and epidemiological evidence that is strongly associated with these cancers. Any theory should be able to describe the role of cyclooxygenase (COX)-2, inflammation, and aging as risk factors for CRC.[12,13] Merging these

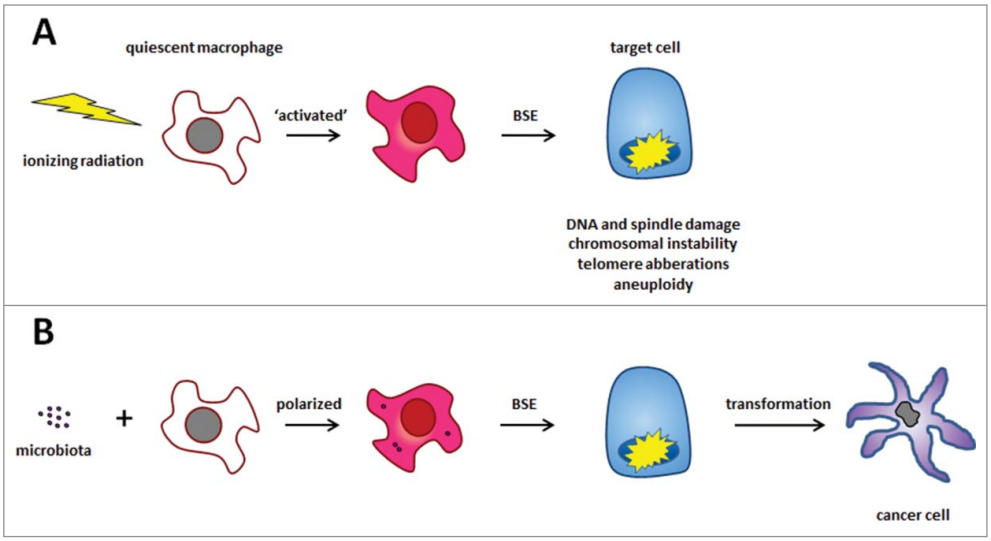

Figure 1. Radiation- and macrophage-induced bystander effects (BSE). (**A**) Ionizing radiation activates cells to generate diffusible clastogens that damage genomic DNA in non-irradiated target cells leading to CIN. (**B**) Similarly, polarization of quiescent macrophages by commensals generates BSE in a fashion similar to irradiation. In our recent paper,[5] we show this causes malignant transformation of primary colonic epithelial cells.

diverse observations into a coherent theory for microbiota-driven CRC carcinogenesis has been challenging, in part, due to the complex ecology of the colon. However, work by our group now provides a theoretical foundation for linking these data. In a series of publications using both *in vitro* and *in vivo* models of colitis-associated CRC,[3,4,6-8] we demonstrated that normally quiescent colon macrophages can be polarized by *E. faecalis*. As a consequence, COX-2 is induced to produce BSE that results in mutations, aneuploidy, and CIN in target cells during malignant transformation.[5] We use colonization of IL-10 knockout mice with *E. faecalis* to polarize colon macrophages *in vivo* and generate molecular signatures for BSE.[7] In this Addendum, we briefly review BSE as a concept and describe how this particular phenomenon, when triggered by commensals, initiates cellular transformation. We also speculate on BSE as a mechanism for endogenous mutagenesis leading to CRC.

Bystander Effects and DNA Damage

BSE was first observed as chromosomal damage in non-irradiated cells that was caused by factors detected in the plasma of irradiated human subjects. The two initial reports described subjects who were accidentally or therapeutically exposed to whole-body radiation.[14,15] Investigators found "substances" in plasma that damaged chromosomes of normal non-irradiated leukocytes. These non-targeted effects of radiation were noted to occur years after exposure. Since the initial descriptions of these findings, a large body of evidence has accumulated that confirm the ability of irradiated cells (typically myeloid cells or fibroblasts) to generate diffusible clastogens (*i.e.*, chromosome-breaking factors) that can damage DNA in non-irradiated cells leading to CIN, telomere aberrations, and micronucleation.[16] Radiation-induced BSE has been observed in plasma from survivors of the atomic bombing of Hiroshima, Japan, and the catastrophic nuclear disaster at Chernobyl.[17,18] It has also been noted *in vitro* using grids to shield target cells,[16] and confirmed *in vivo* using mice in sophisticated congenic sex-mismatch bone marrow transplant studies.[19] Another form of radiation-induced BSE involves gap junction signaling but is not further considered here.

Work by our laboratory and others has shown that ionizing radiation is not the only trigger for BSE. In the 1980s Emerit et al. reported on extracellular superoxide, an anionic radical with a short half-life, as an initiator of cellular events leading to clastogenesis.[20] Investigation by this group suggested that clastogens were <10 kDal in weight. One particular mediator was identified as *trans*-4-hydroxy-2-nonenal (4-HNE), a breakdown product of ω-6 polyunsaturated fatty acids. These findings were important to our later work.

Bystander Effects and Commensal Bacteria

We used these various observations to explore the role of *E. faecalis* in generating genomic DNA damage leading to CRC. Enterococci are Gram-positive cocci that colonize the intestinal tract of animals and often cause opportunistic infections in hospitalized patients.[21] A unique characteristic of this bacterium is the ability to generate extracellular superoxide. Genetic studies by us showed that this radical was generated through the rapid non-enzymatic reduction of dioxygen by membrane-associated demethylmenaquinones.[22] The rate of extracellular superoxide production, when rates were normalized to surface cell membrane area, was comparable to neutrophils undergoing a respiratory burst.[23] This unique physiology is shared by few other intestinal bacteria.[24] However, among humans enterococcal colonization is common if not ubiquitous.[21] In a prospective study of fecal carriage, we found 64 (40%) of 159 samples tested positive for superoxide-producing enterococci.[25] Although we were unable to find any association between enterococcal colonization and the occurrence of large adenomas or CRC, we were surprised to find that colonization with enterococcal strains was not stable over time in this elderly cohort of subjects. This likely confounded our ability to detect an association.

In another study using real-time PCR to quantify fecal bacteria that are potentially associated with CRC, increased

colonization by *E. faecalis* was reported in patients with CRC compared to healthy volunteers.[26] Butyrate-producing bacteria and *Desulfovibrio* spp. were, in contrast, reduced in number or no different than controls. Overall, we were strongly led to further investigate the potential role of *E. faecalis* in CRC given its unique pro-oxidant phenotype that could potentially generate DNA damage in epithelial cells.[4] We believed any such evidence would provide a nexus for microbiota-triggered CRC.

Initially, we used an aromatic hydroxylation assay to measure superoxide production *in vivo* in rats colonized with *E. faecalis*.[27] We detected radical generation but did not observe colon inflammation. This suggested that normal host defenses readily control the oxidant stress generated by colonizing enterococci. However, our study design did not address the question of whether *E. faecalis* could damage the genomic DNA in colon epithelial cells. To initially approach this issue we utilized an ultra-sensitive alkaline single cell gel electrophoresis (comet) assay to measure DNA damage. We again colonized rats with superoxide-producing *E. faecalis* and detected significantly increased levels of DNA damage in colon epithelial cells compared to controls.[28]

These results led us to study host responses to redox stress and DNA damage produced by *E. faecalis*. We acutely exposed the colons of wild-type mice to superoxide-producing *E. faecalis*. As with the rat experiments, we found no histological abnormalities in colon biopsies at 6 hours.[29] However, immunohistochemical staining showed that NF-κB was activated and induced COX-2 in stromal macrophages. Gene array analyses identified one highly significant interaction network that included genes for NF-κB signaling, regulation of apoptosis, and cell cycle control. These results indicated that *E. faecalis* was able to strongly induce proinflammatory responses in colon macrophages. This led to a series of experiments using macrophages that were polarized by *E. faecalis*.

It was at this point that we used DNA damage at a distance due to radiation-induced BSE to guide our thinking. We sought evidence for whether *E. faecalis* could induce CIN through a macrophage-induced BSE. We measured CIN in target cells using a validated A_LN-CD59 complement-lysis assay developed by radiation biologists.[30] This assay uses *CD59* on human chromosome 11 in transformed Chinese hamster ovary cells as targets for mutagenesis. In the presence of anti-CD59 antibody that fixes complement, cells with normal CD59 expression are lysed allowing for positive selection of cells with CD59 mutations.

In a series of experiments, we exposed A_LN cells to *E. faecalis* and found mutations at rates comparable to 2 Gray of ionizing radiation.[4] Importantly, *E. faecalis*-treated A_LN cells were protected from DNA damage by superoxide dismutase, γ-tocopherol, and COX-2 inhibitors, indicating that superoxide and COX-2 contributed to this effect. We developed a dual-chamber tissue culture system using macrophages polarized by *E. faecalis* to mimic BSE with A_LN cells as targets. We found increased mutant fractions in A_LN cells. These fractions decreased when COX-2 was silenced using short interfering RNA. For comparison, we tested *Escherichia coli*, a Gram-negative intestinal commensal that produces negligible extracellular superoxide, and detected only modest increases in mutant fractions. These *in vitro* observations linked the pro-oxidant phenotype of *E. faecalis* to macrophage-induced BSE. Of great significance was the role COX-2 appeared to play in generating DNA damage.

We then more closely examined the genomic damage generated by BSE using the near-diploid HCT116 cell line, derived from a human CRC with deficient mismatch repair, as a cellular target in the dual-chamber system.[3] The average percent of aneuploid cells increased when the multiplicity of infection for macrophages was 1,000, but this effect was not observed at lower levels of infection. Using flow cytometry, double-strand DNA breaks were detected in target cells using antibody to γH2AX. In these experiments we saw anaphase bridging, rapid phosphorylation of ATM—a DNA damage sensor protein[31]—and G_2/M cell cycle arrest. Superoxide dismutase, catalase, and tocopherols attenuated, while caffeine and glutathione synthase inhibitors exacerbated, these aneugenic and cell cycle changes.

To characterize potential mediators for macrophage-induced BSE we focused on 4-HNE, a highly reactive aldehyde arising from lipid peroxidation.[32,33] We purified 4-HNE from macrophages polarized by *E. faecalis* and found that it damaged genomic DNA and promoted CIN.[6] We also noted that colonic epithelial cells exposed to 4-HNE developed cell cycle arrest and a failure of cytokinesis due to mitotic spindle damage. The result of these changes was tetraploidy, a genomic state considered to be an initial step toward aneuploidy and CIN.[34] Studies by other investigators show that 4-HNE can form bulky DNA adducts that preferentially generate CIN, instead of microsatellite instability,[35] and lead to mutations in genes associated with CRC (*e.g.*, *TP53*, *APC*, and *KRAS*).[36] Finally, 4-HNE-protein adducts are a sensitive tissue marker for the production of this aldehyde and were found in colon macrophages from biopsies of interleukin-10 knockout ($Il10^{-/-}$) mice colonized with *E. faecalis*.[6] $Il10^{-/-}$ mice are a useful model for CRC research involving the microbiota because colonization with pathobioants like *E. coli* or *E. faecalis* specifically cause colitis, dysplasia, and cancer.[6,9] When these knockout mice are housed in germ-free or specific-pathogen free environments they fail to develop colon pathology. This demonstrates the key role selected commensals can play in CRC initiation. At this point, our findings indicated that 4-HNE was generated by polarized macrophages and acted as a diffusible endogenous mutagen and spindle poison for epithelial cells.

Next, using specific inhibitors of COX-2 and siRNA, we found that 4-HNE was generated by macrophages that were polarized by *E. faecalis* in a COX-2 dependent manner.[37] Colonization of $Il10^{-/-}$ mice with *E. faecalis* produced 4-HNE-protein adducts in colon macrophages expressing COX-2. These findings reinforced COX-2 as a procarcinogenic enzyme that generated clastogens and damaged DNA in target cells.[37] Immunohistochemical staining of colon biopsies confirmed an M1 polarization of macrophages by *E. faecalis*.[7] M1 cells arise from quiescent macrophages that have been exposed to bacteria, interferon-γ, or tumor necrosis factor (TNF)-α.[38] The phenotype is usually considered microbicidal and/or

tumoricidal but an emerging role in cancer initiation has also been described by us and others.[7,39] In contrast, M2 macrophages are generated by anti-inflammatory factors such as IL-10, IL-13, IL-4, IL-1ra, or transforming growth factor-β. These cells release anti-inflammatory cytokines that induce immune tolerance, angiogenesis, and tissue remodeling and, for existing cancers, promote tumor growth. Depletion of tissue macrophages using encapsulated liposomal clodronate blocked BSE and suppressed COX-2 and TNF-α. 4-HNE-protein adduct formation, colitis, and CRC were no longer detected. These findings provided strong evidence that colon macrophages were key effector cells for commensal-triggered BSE, inflammation, and cancer initiation.

We believe the overall evidence from these studies shows 4-HNE as a potent endogenous mutagen generated by M1 polarized macrophages. 4-HNE is a diffusible mediator for BSE that contributes to carcinogenesis by damaging DNA, inhibiting DNA repair, activating NF-κB, and helping generate its own production by inducing COX-2.[6,40-43] Finally, we take note that *E. faecalis*-polarized macrophages also produce a multitude of other inflammatory cytokines that potentially act as mediators for BSE.[8] For example, TNF-α is classically produced by M1 polarized macrophages and can promote carcinogenesis by activating NF-κB and damaging DNA.[44] Our own work suggests that TNF-α contributes to macrophage-induced BSE by inducing netrin-1 in colon epithelial cells. This anti-apoptotic protein promotes intestinal epithelial cell proliferation and is considered an oncogene for CRC.[8,45] In sum, these observations show the importance of BSE as a mechanism for driving microbiota-triggered colorectal carcinogenesis.

Bystander Effects and Malignant Transformation

We most recently investigated whether repetitive exposure of non-transformed colonic epithelial cells to macrophages that had been polarized by *E. faecalis*, or purified 4-HNE, could lead to heritable CIN and cellular transformation. Using *tsA*58 as a target gene for mutagenesis in YAMC cells—a primary murine colon epithelial cell line—we found that mutant fractions increased significantly after only a few treatments.[5] As noted previously,[3] aneuploidy increased following these treatments with clones becoming malignant after only 10 weeks. In NOD/*scid* mice, 8 of 25 treated clones formed poorly differentiated carcinomas with 3 tumors invading skin and/or muscle. Gene expression profiling of these clones showed strong upregulation of Ly6A/E stem cell markers. Products of these same genes were found by immunohistochemical staining in colon epithelial cells from areas of inflamed or dysplastic tissue in *Il10*$^{-/-}$ mice colonized with *E. faecalis*. These findings provide evidence for macrophage-induced BSE as an endogenous mechanism for commensal-driven malignant transformation in CRC.

Resident colon macrophages are important innate immune effector cells that help maintain immunological tolerance to commensals and resistance to invading pathogens. Because these professional phagocytes are not typically polarized by commensals, any loss of regulatory control over their activation could drive malignant transformation. This might occur if aging leads to significant cellular senescence, loss of regulatory controls over tolerance, or deficiencies in host defense (e.g., as represented by the knockout of IL-10 in our murine model).[46] We have shown that colonization with *E. faecalis* polarizes quiescent macrophages to an M1 phenotype in IL-10 deficient mice. The M1 state is characterized by expression of inducible nitric oxide synthase 2 and TNF-α and the absence of arginase 1 activity.[5,7] Notably, *Helicobacter pylori*, a group 1 carcinogen, polarizes gastric macrophages to an M1 phenotype.[39] In a recent study, we depleted colon macrophages in IL-10 knockout mice colonized with *E. faecalis* and found that this prevented the development of colitis and cancer.[7] These findings strongly suggested that M1-polarized macrophages were primary effector cells that drove inflammation and malignant transformation in our model.

In our *Gut* study[5] we found malignant transformation of a primary colon epithelial cell line associated with changes in "driver" genes that promote carcinogenesis.[10] Treated clones that formed malignant tumors in NOD/*scid* mice each had differential expression of 3 to 7 CRC driver genes. Although we did not investigate events leading to altered expression of these genes in these clones, we speculate that changes likely arose from direct mutations, chromosome copy number variations, epigenetic modifications, and/or altered transcriptional regulation. Several mechanisms for altered gene expression have been noted in our model of macrophage-induced BSE.[3-5]

A longstanding question exists about whether CIN is a driving force for malignant transformation versus a consequence of that transformation. The results from our current study do not settle this issue. Instead, we note that a majority of aneuploid clones, especially clones with higher percentages of aneuploidy, did not form tumors in NOD/*scid* mice. This may reflect a tumor-suppressive effect of aneuploidy that has been described by others.[47] Interestingly, although multiple mutations in *APC*, *SMAD4*, *TP53*, *KRAS*, and *PI3CA* were reported in one study using transformed organoids from normal human colonic epithelial cells, CIN was essential for macrometastases suggesting this phenotype is necessary for cancer invasion.[48]

An unexpected finding from our *Gut* publication concerned the induction of stem cell markers by BSE.[5] We found that Ly6A and Ly6E showed increased expression in transformed clones. In addition, these same proteins were seen by immunohistochemical staining in colon biopsies from *E. faecalis*-colonized *Il10*$^{-/-}$ mice. Ly6A and Ly6E are stem/progenitor cell markers that belong to a multigene family of glycosyl phosphatidylinositol-anchored cell surface proteins.[49] Unlike Ly6E, Ly6A has no human homolog. Overexpression of *LY6E* has been reported in many human malignancies including breast, lung, gastric, ovarian, pancreatic, and colon.[50,51] The potential importance of LY6E as a target for therapy was noted in one model that used antibody-drug conjugates to induce tumor regression.[50]

Surprisingly, doublecortin-like kinase 1 (Dclk1), a newly identified colon cancer

stem cell marker,[52,53] was also strongly expressed in malignant allografts and colon epithelial and stromal cells from *E. faecalis*-colonized *Il10*$^{-/-}$ mice. Analysis of transformed clones failed to identify increased gene expression. We believe this may reflect altered regulation of post-translational processing for this gene product. Under normal physiological circumstances, colon epithelial cells that express Dclk1 are quiescent and show long *in vivo* life spans.[53] This may be important to colon epithelial cells accumulating multiple mutations over time during malignant transformation. Our findings suggest that Dclk-positive cells may be important targets for BSE (**Fig. 2**). Taken together, the increase in expression of stem cell markers by macrophage-induced BSE conforms to a cancer stem cell theory for CRC.[54]

To these findings we would like to add that the transformed clones generated by BSE included differentially expressed genes for stromal cell-derived factor 1 (SDF-1/Cxcl12), insulin-like growth factor 2 (Igf2), pleiotrophin (Ptn), and secreted frizzled-related protein 2 (Sfrp2) (**Fig. 3**). A recent study showed that a combination of SDF-1/Cxcl12, IGF2, PTN, and ephrin B1 led to the differentiation of human embryonic stem cells to neurons.[55] Although ephrin B1 was not altered in our transformed clones, perhaps decreased expression of these growth factors leads to de-differentiation or stem cell enriched states that give rise to tumor stem cells. We found that Sfrp2, a soluble Wnt antagonist, was significantly decreased in all transformed clones. Activation of Wnt signaling by NF-κB induces de-differentiation of intestinal epithelial cells and can help initiate tumorigenesis.[56] Hypermethylation of the Sfrp2 promoter and/or decreases in Sfrp2 expression have been seen in many human cancers including CRC.[57] Sfrp2 is also involved in stem cell fate and cell migration.[58] Speculatively, down-regulation of these or similar genes by macrophage-induced BSE, in a mutagenic environment promoted by macrophage-induced BSE, could lead to tumor stem cell formation. Further work is needed to investigate mechanisms by which BSE contributes to the de-differentiation of colonic epithelial cells.

Finally, we must pause and recognize that the vast majority of intestinal commensals are beneficial symbiotes that co-exist with their hosts to promote health and exclude potentially pathogenic exogenous bacteria.[59] *E. faecalis*, however, appears to be an unusual member of the colonic microbiome with the potential to initiate DNA damage leading to aneuploidy, CIN, and inflammation-associated CRC. Much work remains to be done before this commensal should be considered causally linked to human CRC. Other wayward commensals undoubtedly exist that can also damage epithelial cell DNA and potentially contribute to CRC. *E. faecalis* is just one example and extracellular superoxide one mechanism.

Summary

Radiation- and macrophage-induced BSE appear similar if not identical in consequence on affected genomes, differing only in triggering events and leading to

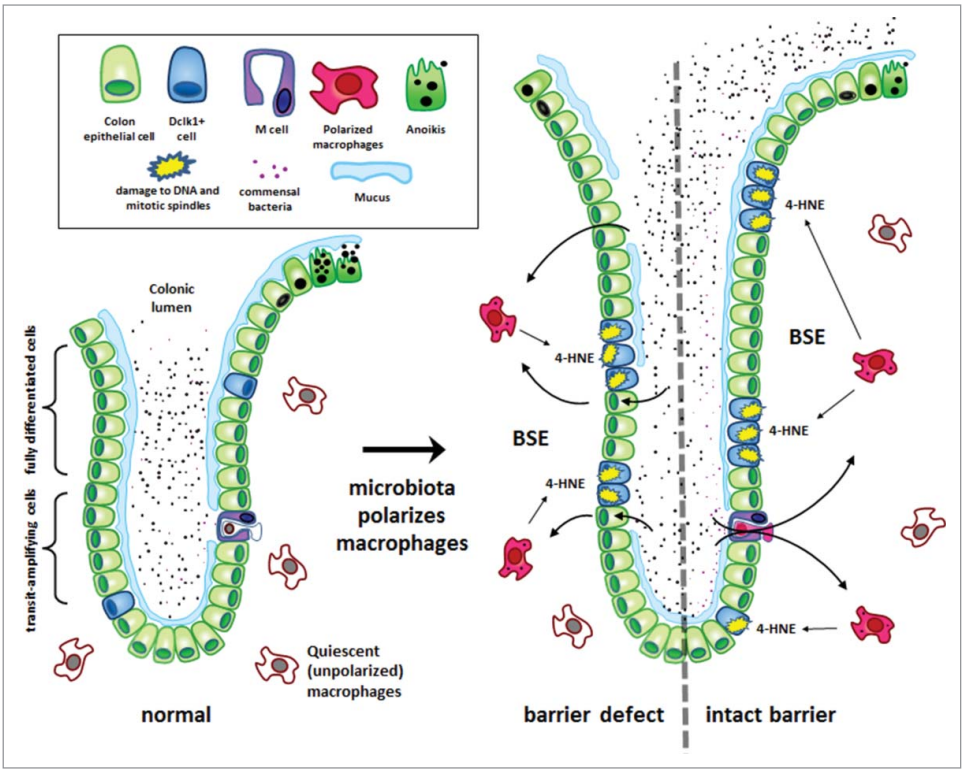

Figure 2. Macrophage-induced bystander effects (BSE) induce colorectal cancer. This is a commensal-triggered model for CRC involving endogenous mutagenesis caused by macrophage-induced BSE that drives cellular proliferation, mutagenesis, transformation, and colorectal cancer. (**Left**) Under normal physiological conditions, mucus layers, intraepithelial tight junctions, and quiescent colon macrophages protect the colonic epithelium from adverse effects of the microbiota. (**Right**) The loss of mucus, disruption of tight junctions, or dysregulation of macrophages can result in chronic macrophage polarization. Commensals initiate polarization by direct invasion when barrier defects are present (*left*) or through M cell portals when mucus layers are intact (*right*). Macrophage polarization generates bystander effects (BSE). One mediator is *trans*-4-hydroxy-2-nonenol (4-HNE), a clastogen and spindle poison (see text for details). Four-HNE diffuses into colon epithelial cells and causes mutations, double-strand breaks, and spindle dysfunction. A consequence of BSE is colon crypt hypertrophy, reduced anoikis, chromosomal instability, and malignant transformation. One potential target for BSE are epithelial cells that express doublecortin-like kinase 1 (Dclk1). These long-lived cells are scattered throughout the crypt, dramatically increase in number during inflammation, and appear to be good candidates for tumor stem cell progenitors. It remains to be determined whether this cell type, and/or others, are proximate target for BSE-mediated DNA damage, aneuploidy, and chromosomal instability leading to CRC.[52,53]

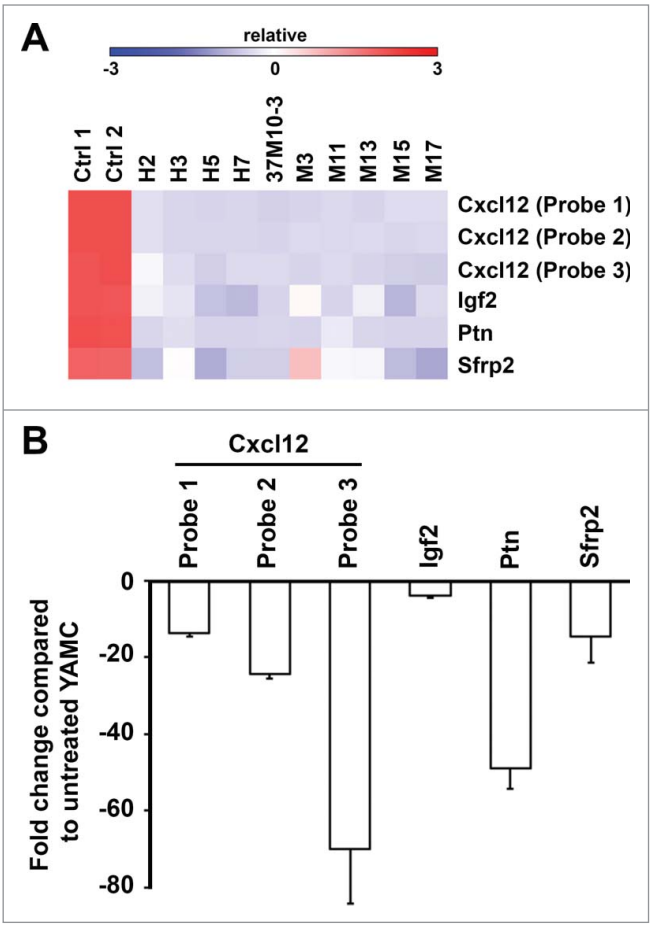

Figure 3. Gene expression of Cxcl12, Igf2, Ptn, and Sfrp2 in transformed clones. (**A**). Heat map of gene expression. (**B**). Fold changes for Cxcl12, Igf2, Ptn, and Sfrp2 normalized to untreated YAMC cells. Expression of these genes is significantly decreased in transformed clones compared to untreated YAMC controls.

pathways involving clastogenesis, DNA damage, aneuploidy, CIN, and malignant transformation. Commensal bacteria represent a trigger for polarizing colonic macrophages in a promising theory for CRC that links the intestinal microbiota to COX-2, inflammation, and, potentially, aging. This represents an integrated etiology for these cancers. We selected *E. faecalis* as a model microorganism for our work because of its unusual redox properties. We still do not know whether *E. faecalis*, or any of the many other members of the microbiota, act singly or in concert to chronically polarize colon macrophages to generate BSE. Despite such gaps in our knowledge, BSE represents an important mechanism for cellular transformation and provides a rich field for hypothesis testing. The results of future investigation in this area should identify new targets for prevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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