Deep Sequencing and Functional Analyses Identify a Role of Fusobacterium Species in Colorectal Tumorigenesis

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Deep sequencing and functional analyses identify a role of 
*Fusobacterium* species in colorectal tumorigenesis

A dissertation presented

by

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Deep sequencing and functional analyses identify a role of *Fusobacterium* species in colorectal tumorigenesis

Abstract

The tumor microenvironment is a complex community consisting of neoplastic cells, surrounding stromal cells, a broad array of immune cells, and a microbiota. By sheer numbers, the microbiota has its greatest manifestation in colorectal cancer (CRC) because the colon contains up to 100 trillion bacteria, outnumbering human cells by a factor of 10 and encoding a gene-content that is 100-fold larger than that of the human genome. Indeed, previous studies using germ-free mice in a variety of genetic backgrounds have demonstrated that the microbiota can impact colorectal tumorigenesis. In addition, specific strains of enterotoxigenic bacteria have been shown to promote colitis-associated cancer in mice. Here, we explore the composition of the tissue-associated microbiota in human CRC and evaluate the role of tumor-enriched microbes in potentiating colorectal tumorigenesis in mice.

Advances in DNA sequencing technology have fueled a renaissance in the microbiome field. Deep sequencing metagenomics enables rapid, culture-independent characterization of a microbial community. We present PathSeq, a highly scalable software tool that performs computational subtraction on high-throughput sequencing data to identify nonhuman nucleic acids. PathSeq makes it possible to analyze sequence datasets as large as human whole-genomes for the purpose of metagenomics and also to discover previously unsequenced microorganisms. We used PathSeq to characterize the composition of the microbiota in human CRC using whole-
genome sequencing on nine tumor/normal pairs and 16S rDNA sequencing on an additional 95 pairs. The genus *Fusobacterium* was highly enriched in tumors, while the Bacteroidetes and Firmicutes phyla were depleted.

We show that in the *Apc<sup>Min<sup>+/</sup></sup>* mouse model of intestinal tumorigenesis, *Fusobacterium nucleatum* increases tumor multiplicity, selectively recruits tumor-infiltrating myeloid cells, and is associated with a pro-inflammatory expression signature that is shared with human fusobacteria-positive colorectal carcinomas. We find that *Fusobacterium* spp. are enriched in human colonic adenomas relative to surrounding tissues and fusobacterial abundance is increased in stool samples from patients with colorectal adenomas and carcinomas, compared to healthy subjects. Collectively, these data support that fusobacteria may be involved in early stages of intestinal tumorigenesis and, through recruitment of tumor-infiltrating immune cells, may generate a pro-inflammatory tissue microenvironment conducive to colorectal neoplasia progression.
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### List of Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACF</td>
<td>aberrant crypt foci</td>
</tr>
<tr>
<td>AOM</td>
<td>azoxymethane</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;Min/+&lt;/sup&gt;</td>
<td>multiple-intestinal neoplasia model of FAP bearing a heterozygous mutation in APC</td>
</tr>
<tr>
<td>BFT</td>
<td>Bacteroides fragilis toxin</td>
</tr>
<tr>
<td>CAC</td>
<td>colitis-associated cancer</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCA</td>
<td>deoxycholic acid</td>
</tr>
<tr>
<td>DEN</td>
<td>diethylnitrosamine</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>EDRN</td>
<td>Early Detection Research Network</td>
</tr>
<tr>
<td>ETBF</td>
<td>Enterotoxigenic Bacteroides fragilis</td>
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<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GF</td>
<td>germ-free</td>
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<tr>
<td>HMP</td>
<td>Human Microbiome Project</td>
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<tr>
<td>HPV</td>
<td>human papillomavirus</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>IEC</td>
<td>intestinal epithelial cell</td>
</tr>
<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
</tr>
<tr>
<td>LDA</td>
<td>Linear Discriminant Analysis</td>
</tr>
<tr>
<td>MALT</td>
<td>mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MAM</td>
<td>methylazoxymethanol</td>
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<tr>
<td>MCA</td>
<td>3’-methylcholanthrene</td>
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<tr>
<td>MetaHIT</td>
<td>Metagenomics of the Human Intestinal Tract</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding domain leucine-rich repeat protein or NOD-like receptor</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediate</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acid</td>
</tr>
<tr>
<td>SFB</td>
<td>segmented filamentous bacteria</td>
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<tr>
<td>SMO</td>
<td>spermine oxidase</td>
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<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>Th1</td>
<td>T helper type 1 T cell</td>
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<td>Th17</td>
<td>T helper type 17 T cell</td>
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<tr>
<td>Th2</td>
<td>T helper type 2 T cell</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13-acetate</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>UC</td>
<td>ulcerative colitis</td>
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<td>WT</td>
<td>wild-type</td>
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INTRODUCTION

Microbes and Inflammation in Cancer

Portions of this Introduction appear in the following publication:

Overview

At the most fundamental level, a tumor is composed of a group of neoplastic cells harboring aberrant genomes that endow it with the ability to proliferate beyond normal means. Hanahan and Weinberg famously described the six hallmarks of cancer that most, if not all, tumors must acquire: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [1]. The focus of cancer research in the decades leading up to this seminal review had been on the cancer cell. But the tumor microenvironment, composed of not only the neoplastic cells themselves but also the surrounding stromal cells and a vast array of immune cells, may be just as important to the tumorigenic process as the cancer cell itself [2]. Malignant cells do not act alone in driving cancer, but rather they influence their surrounding cells to participate in the growth of the tumor. The past decade has seen tremendous advances in our understanding of the contribution of the microenvironment to tumorigenesis, and therefore it may not be surprising that the hallmarks of cancer have been recently updated to include four additional members that emphasize the role of the microenvironment: tumor-promoting inflammation, avoidance of immune destruction, deregulating cellular energetics, and genome instability and mutation [3].

The past decade has also seen a reimagining of the extent of our physiological interaction with our resident microbiota. Recent research has revealed roles for the microbiome in host physiology ranging from angiogenesis [4,5] and skeletal biology [6,7] to lipid metabolism [8,9] and even behavior [10-13]. Likewise, there is an increasing interest in the impact of the microbiota on cancer [14]. The lumen of the human distal gut is one of the most densely populated ecosystems on our planet, and we also harbor several distinct microbiomes on the
other surfaces of our body such as the respiratory and urogenital tracts and the skin. The number of bacterial cells on our body outnumber our own cells by a factor of ten, and from a functional standpoint, the number of genes encoded in our microbiome outnumbers the genes in our genome by a factor of 100. Therefore, the tumor microenvironment, in addition to containing neoplastic cells, stromal cells, and immune cells, should also be considered in the context of an additional component: the tumor microbiota.

In this Introduction I explore the roles of the microbiota and microbiota-associated inflammation in cancer. I use the colon as a model ecosystem for these interactions, although the microbiota and inflammation can have influences on cancer types beyond intestinal cancers.

**The Human Gastrointestinal Microbiota**

**Overview**

The meta’omics revolution in both deep sequencing and big data analytics is fostering an explosion of interest in how the gut microbiome impacts physiology and propensity to disease. Also driving interest in this field is the relatively recent discovery of the causal role of *Helicobacter pylori* in duodenal ulcers and gastric cancers [15,16], the role of the gut microbiota in inflammatory bowel disease [17], a new understanding of the impact of antibiotic use on the microbiota and the emergence of opportunistic pathogens [18,19], and the therapeutic use of fecal transplantation. Although the first successful application of fecal transplantation to *Clostridium difficile*-associated disease was first reported more than 50 years ago, the astounding clinical efficacy of this approach has only recently been demonstrated in a controlled trial [20].

The microbiota is necessary for the maintenance of physiologic homeostasis, but in the context of specific microbial and/or host phenotypes, the microbiota can contribute to disease
pathogenesis. For instance, the type VI secretion system (T6SS) of *Helicobacter hepaticus* directs an anti-inflammatory gene expression profile on intestinal epithelial cells (IECs) to create a tolerogenic immune environment, but *H. hepaticus* T6SS mutants disrupt this balance and promote colitis by driving a T helper type 17 T cell (Th17) response [21]. *H. hepaticus* is an example of what has been termed a pathobiont, a symbiotic microbe that is capable of promoting pathology only when specific host, environmental, or microbial factors are altered [22,23]. Similarly, *Helicobacter pylori* is a human pathobiont that is a resident of the stomach in 50% of the population globally [24] and may be protective against asthma and other allergic disease [18,25,26], but under specific conditions which have yet to be completely defined, *H. pylori* causes gastric cancer [16]. Host genetics also play a role in making a pathobiont pathogenic. For example, germ-free *Il10*<sup>−/−</sup> mice do not show any signs of intestinal inflammation, but when they are colonized by commensal strains of *E. coli* and *Enterococcus faecalis* that do not cause any disease in wild-type mice, these bacteria drive severe colitis in the IL-10-deficient mouse [27]. The emergence of pathobionts can also be influenced by diet [28] and by the use of antibiotics [29].

**The Intestinal Ecosystem: The Flora**

There is significant variability in the density and complexity of the microbiota throughout the body. Within the gastrointestinal tract, we harbor 10<sup>8−10</sup> colony-forming units (CFU) of bacteria per gram (g) of saliva, 10<sup>3</sup> CFU/g of gastric juice, 10<sup>2−4</sup> CFU/g of contents in the duodenum and jejunum of the small intestine, 10<sup>10</sup> CFU/g in the small intestinal ileum, and 10<sup>10−14</sup> CFU/g of colonic content [14]. Interestingly, the higher microbial density in the colon relative to the small intestine correlates with a greater than 12-fold risk of cancer in the colon [30].
human gastrointestinal tract is home to more than 100 trillion bacteria, and the microbiome contains as many as 150 times the number of genes in the human genome [31,32].

The human intestinal microbiota is comprised predominantly of two phyla, the Firmicutes and Bacteroidetes, with a smaller representation of the Proteobacteria and Actinobacteria, and a rare representation of the Fusobacteria, Verrucomicrobia, and Cyanobacteria [30,33,34]. The mouse serves as an excellent model for microbiota studies because its gut microbiota bears striking similarities to ours [35]. The intestinal microbiota is dominated by strict anaerobes, most notably the genera of Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus, and Atopobium, whereas facultative anaerobes are present at 1,000-fold lower levels [14]. Approximately 500 to 1,000 different species comprise the normal colonic microbiota [36].

Comprehensive characterizations of the “healthy” human adult microbiota have been carried out by the European and Chinese-led Metagenomics of the Human Intestinal Tract (MetaHIT) Consortium and the NIH-sponsored Human Microbiome Project (HMP). MetaHIT focused on shotgun metagenomic sequencing of fecal samples from 146 European individuals, uncovering the presence of a minimal gut genome and metagenome, based on taxonomy and gene functionality [37]. MetaHIT reported that individuals, regardless of gender, race, or geography, can be grouped into one of three enterotypes characterized by variation in the level of the Bacteroides, Prevotella, and Ruminococcus genera [38], however recent studies have favored a continuum or gradient of species rather than discrete enterotypes [39-41]. The HMP has, in a series of 16 articles published in parallel (www.plos-collections.org/hmp), produced the largest dataset to-date of the diversity of the human microbiome across body sites on a large number of healthy adults. The HMP carried out both 16S rRNA gene sequencing and metagenomic sequencing on 15 (for males) or 18 (for females) body sites on each of 242 healthy adults in the
United States sampled on three separate visits [42]. The gut and tooth habitats were found to harbor the greatest between-subject microbial diversity but also the lowest between-visit variability, whereas the skin had lower between-subject diversity but much higher between-visit variability [43]. These studies suggest that the composition of the microbiota varies substantially across individuals, though it is temporally stable within a single person, and the functionality of the microbiota at the gene-level is highly conserved across individuals [43].

**The Intestinal Ecosystem: The Host**

Though host-microbiota interactions are bidirectional, direct contact with the epithelium is limited by a thick mucus layer and secreted factors. The mucus layer is principally composed of mucin, a high molecular weight glycosylated protein, and also consists of trefoil peptides, antimicrobial peptides, and secretory IgA [44]. The colon has a 150µm inner mucus layer that is firmly adherent to the epithelium and a variable-thickness outer layer that is loose and non-attached, whereas the small intestine has a single-layer incomplete barrier [45,46]. α-defensins, small antibacterial peptides secreted by Paneth cells of the small intestine, are required for the maintenance of the composition of the luminal microbiota [47]. By contrast, RegIIIγ is a secreted antimicrobial lectin that does not affect the luminal microbiota, rather it is limited to the mucus layer and restricts the access of Gram-positive bacteria to the epithelium [48,49].

**Colorectal Cancer**

Gastrointestinal cancers account for 25% of all cancer incidences and for 9% of cancer deaths globally [36], and there are nearly 1 million new cases of colorectal cancer (CRC) worldwide each year (http://www.who.int/en). Familial CRC accounts for 15-20% of CRC cases [50], which are classified as familial adenomatous polyposis (FAP), MYH-associated polyposis,
hereditary nonpolyposis CRC, hamartomatous polyposis syndromes, or hyperplastic polyposis syndrome [51]. The genes responsible for the majority of these disorders have been uncovered: \textit{MHL1, MSH2, MSH6, PMS2, APC, MYH, STK11, SMAD4, BMPRIA,} and \textit{PTEN} [51]. One of the most widely used mouse models of CRC, the \textit{Apc}^{\text{Min/+}} mouse, bears a heterozygous mutation in the tumor suppressor gene \textit{APC} [52,53], as do patients with FAP [54-56]. The \textit{Apc}^{\text{Min/+}} mouse will develop tens to hundreds of tumors in the small and large bowel [52], whereas FAP patients can develop thousands of adenomas but they are limited to the large bowel [57]. The identification of the germline mutations responsible for familial CRC has been instrumental in understanding the molecular pathways that underlie sporadic (i.e. non-familial) CRC.

CRC tumorigenesis proceeds through a series of genetic alterations, as first proposed by Fearon and Vogelstein [58]. The Wnt—\(\beta\)-catenin signaling pathway is essential for intestinal epithelial cell renewal, and mutations that lead to Wnt—\(\beta\)-catenin activation occur early in the course of tumorigenesis in greater than 90% of sporadic CRC cases; these mutations most commonly occur in \textit{APC} and also \textit{GSK3B}, which encodes a kinase that controls \textit{APC} and \(\beta\)-catenin stability [59,60]. The hyperplasative epithelium will then accumulate additional mutations, most commonly in \textit{KRAS} and \textit{TP53}, as it increases in size, dysplasia, and villous content to form a malignant tumor [58]. Recent large scale cancer genome sequencing efforts have confirmed that \textit{APC}, \textit{TP53}, and \textit{KRAS} are the most commonly mutated genes in non-hypermutated CRC tumors, but mutations in \textit{PIK3CA, SMAD4}, and the F-box protein \textit{FBXW7} are also frequently observed [61], as well as a recently-discovered recurrent \textit{VTI1A-TCF7L2} fusion [62].

Colitis-associated cancer (CAC) is a form of colon cancer that is preceded by clinically detectable inflammatory bowel disease (IBD). IBD is classified into either ulcerative colitis
characterized by a non-transmural mucosal inflammation that is limited to the colon, and Crohn’s disease (CD), which is a transmural inflammation of the mucosa that can affect the entire gastrointestinal tract [63,64]. Patients with CD have an 8% increased cumulative risk of CAC after 30 years of active CD, and for UC patients it is 18-20% [65]. The risk of developing CRC is 10-fold higher for individuals with IBD compared to the healthy population [30], however less than 2% of all CRC cases have a history of IBD [66]. Interestingly, wild-type (WT) mice can reproducibly develop CAC by administering the detergent dextran sulfate sodium (DSS) in the drinking water along with a single injection of the carcinogen azoxymethane (AOM) [67]. In contrast to sporadic CRC, mouse models of CAC develop mutations affecting Wnt—β-catenin signaling relatively late in the tumorigenic process, and develop early mutations in Trp53 and Kras [60,68].

## The Role of Inflammation in Colorectal Cancer

Inflammation is a hallmark of cancer [3]. Chronic inflammation can drive sustained innate immune cell recruitment, tumor growth, and metastasis, and directly promotes malignant cell transformation by inducing chromosomal and microsatellite instability, CpG island methylation, epigenetic alteration, and post-translational modifications [44]. Therefore, inflammation can contribute to all three stages of tumorigenesis: initiation, promotion, and progression [69].

Inflammation contributes to tumor initiation by virtue of its ability to cause mutations. Indeed, mucosal inflammation elicits systemic DNA damage that can contribute to tumoral genetic instability [70,71]. For example, mice deficient in ATM, a kinase involved in DNA double-strand break recognition and repair, show not only elevated DNA damage, but persistent
immune activation and increased sensitivity to DSS-induced colitis [72]. Another integral component of the DNA damage repair machinery, p53, is one of the most commonly mutated genes in CAC, but is also found to be mutated in colitic tissue without signs of dysplasia [73]. Inflammatory cells, particularly activated neutrophils and macrophages, produce significant amounts of reactive oxygen species and reactive nitrogen species that cause oxidative damage and can directly lead to oncogenic mutations in inflamed tissue [60].

Once a tumor has been initiated, there are numerous mechanisms by which inflammation can contribute to tumor promotion (i.e. proliferation) and progression (i.e. malignant transformation, invasion, and metastasis).

**The Role of Inflammatory Responses to the Microbiota in Cancer**

Pattern recognition receptors (PRRs) are the fingers of the immune surveillance system. They are an elegant set of receptors that can identify microbial ligands such as cell wall components or nucleic acids and are expressed on intestinal epithelial cells and mucosal immune cells. PRRs include Toll-like receptors (TLRs), Nucleotide-binding domain leucine-rich repeat proteins or NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) among others [74-76]. TLRs and NLRs recognize the symbiotic microbiota, induce host defense responses against pathogens, and control adaptive immune responses. TLRs are transmembrane proteins containing leucine-rich repeats that play a crucial role in the innate immune response by sensing microbe-associated molecular patterns on bacteria, viruses, or parasites in the extracellular environment (TLRs 1, 2, 4-6, 11) or in endolysosomes (TLRs 3, 7-9, 10).

**MyD88**

MyD88 is an adaptor protein common to IL-1 and IL-18 signaling and to the TLRs (with the exception of TLR3). Thus, loss of MyD88 may be expected to impact a wide range of innate
immune sensing of the microbiota. The absence of MyD88 in the non-obese diabetic mouse strain led to an altered microbiota composition with enriched abundance of Lactobacillaceae, Rikenellaceae, and Porphyromonadaceae [77]. In another recent study, loss of MyD88 was examined from both a microbial ecology and host transcriptome perspective along the length of the small intestine and colon [78]. The small intestinal microbiota of MyD88-deficient mice was notable for an enrichment of segmented filamentous bacteria (SFB) and greater interindividual variation. Recently, SFB have garnered increased attention, as they promote a population of T cells [79], called T helper 17 cells, which function in immunity against extracellular bacteria and fungi. Targeted intestinal epithelial deletion of MyD88 using Villin-Cre X MyD88-Flox mice has revealed that such mice have reduced levels of the polymeric immunoglobulin receptor mucin-2 and antibacterial peptides [80]. MyD88 also regulates of the expression of RegIIIγ, the antibacterial lectin that restricts the localization of bacteria and ensures its proper segregation from the inner mucus layer of the intestinal mucosa [48]. MyD88 has been demonstrated to have roles in promoting cancer as well. MyD88−/− mice formed fewer skin papillomas when exposed to the carcinogens 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetate (TPA), fewer fibrosarcomas when exposed to 3′-methylcholanthrene (MCA) [81], and fewer hepatic tumors in the diethylnitrosamine (DEN)-induced model of liver cancer [82] compared to WT mice. MyD88-deficient mice develop less inflammation and decreased colonic tumorigenesis compared to WT when treated with oxazalone-AOM [83] or DSS-AOM [84]. MyD88 is also instrumental in driving intestinal tumorigenesis in Apc\(^{Min/+}\) mice. Apc\(^{Min/+}\) X MyD88−/− mice have significantly reduced small intestinal and colonic tumors relative to Apc\(^{Min/+}\) mice [85] because MyD88 signaling post-transcriptionally stabilizes the c-myc protein through activation of the kinase ERK, which induces the multiple intestinal neoplasia phenotype [86].
TLRs

There have been several studies on the impact of the TLRs upstream of MyD88. Investigations of TLR5-deficient mice have revealed phenotypes resulting in metabolic syndrome [87] and colitis [88], with coincident alterations in the microbiota and transient elevations in the Proteobacteria and, in particular, enterobacterial species in colitic Tlr5 knockout mice [87,89]. While there have been other observations supporting that alterations in TLR signaling impact the microbiota (e.g., that there is an altered colonic mucosal microbiota in Tlr2−/− mice) [90], other studies have challenged the magnitude of the impact of TLR signaling perturbations on the gut microbiome. A denaturing gradient gel electrophoresis and fluorescence in situ hybridization (FISH)-based investigation [91] and a more recent study using deep 16S rRNA gene surveys both call into question whether TLRs or MyD88 alter the gut microbiota in a genotype-dependent fashion [92]. Ubeda et al. (2012) generated MyD88−, TLR2−, TLR4−, TLR6−, and TLR9-deficient mouse lines from heterozygote X heterozygote breeding strategies. Interestingly, they did not detect statistically significant differences in community composition or diversity in cecal luminal, ileal luminal, or ileal microbial communities in these mice compared with their littermate controls. They also did not detect statistically significant differences in microbial community response after an antibiotic (vancomycin) perturbation. However, differences were observed between WT and TLR-mutant colonies that had been maintained as separate lines from homozygous X homozygous crosses for many years. These results raise awareness about the importance of considering lineage or legacy effects in microbiome studies, and it should be noted that these effects were considered in several of the cited studies [48]. Like its roles in shaping the microbiota, the impact of TLRs on intestinal tumorigenesis is also currently not well understood. Deletion of Tlr4, which is responsible for
detecting lipopolysaccharide of Gram-negative bacteria, protects DSS-AOM-treated mice from developing colon tumors [93,94], but deficiency of TLR2, which recognizes peptidoglycan and lipoteichoic acid among other molecules produced by bacteria and fungi, results in an increased intestinal tumor load [95].

**NLRs and the Inflammasome**

The other major class of PRRs are the NLRs. The NLRs are a central component of the inflammasome, which drives an innate immune response against intracellular pathogens [96,97]. The inflammasome is a multiprotein complex that is composed of several members of the NLR family, Procaspe-1, and the adaptor protein ASC [98]. Inflammasome activation results in Caspase-1 activation and subsequent Caspase-1 proteolytic activation of two proinflammatory cytokines: IL-1β and IL-18 [99]. Mutations in the NLR family member NOD2 increase the risk of Crohn’s disease. Nod2 expression in the intestine is dependent on the presence of a gut microbiota, and *Nod2* knockout mice are more susceptible to colonization by intestinal mouse pathogens [100]. Mice deficient in Nod1 and Nod2 have altered gut microbiota composition as compared with their heterozygous littermates [101]. Nod1 is expressed ubiquitously in intestinal epithelial and immune cells, and recognizes the peptidoglycan of Gram-negative bacteria [102,103]. Nod1 has a protective role against colitis-associated cancer; DSS-AOM-treated *Nod1<sup>−/−</sup>* mice develop more colonic tumors than DSS-AOM-treated WT mice, and *Apc<sup>Min/+</sup> × Nod1<sup>−/−</sup>* mice exhibit increased intestinal tumors than *Apc<sup>Min/+</sup>* controls [104]. NLRP6 is a NLR family member that functions in inflammasome, type 1 interferon, and NF-κB signaling. NLRP6 inflammasome-deficient mice have an altered gut microbiota notable for an expansion of *Prevotellaceae* and the TM7 phylum [105]. Although why this bacterial family expands remains unclear, this microbiota is functionally significant because co-housing and cross-fostering
experiments have revealed that the microbiota and the phenotype are transferable to WT mice, leading to increased colitis severity induced by DSS [105,106]. NLRP6 is a negative regulator of inflammatory signaling, and its loss activates MAP kinase and NF-κB signaling pathways downstream from TLRs and increases circulating monocyte numbers [107]. These cellular and signaling alterations may explain why NLRP6-deficient mice have an increased resistance to a number of pathogenic bacteria and an altered endogenous microbiota [107]. NLRP6 also has a role in suppressing inflammation-induced colon tumorigenesis, because Nlrp6−/− mice have increased colitis and colonic tumors when treated with DSS-AOM compared to WT controls [108,109]. Similar phenotypes are seen in mice deficient in the related NLR family members Nlrp3 [110,111] and Nlrp12 [112,113]. Caspases are cysteine proteases that play wide-ranging roles functioning in apoptosis to inflammation. Caspase-1 cleavage of IL-1β and IL-18 contributes to regulating inflammatory tone in the gut and thus may modulate the microbiome. The gut microbiota of Capsase-1−, Capsase-3−, and Capsase-7-deficient mice were recently evaluated in comparison with WT mice using 16S rRNA gene fecal profiling, and significant differences were observed across several families, including Lachnospiraceae, Porphyromonadaceae, and Prevotellaceae [114]. In keeping with the pivotal role of caspases in inflammasome function and consequently suppression of tumorigenesis, both Caspase-1-deficient [115] and Caspase-12-deficient [116] mice have increased susceptibility to developing colon tumors following treatment with DSS-AOM.

An intriguing insight that is gleaned from these studies is that, while both pathways are similarly involved in microbial detection and innate immune response, TLR/MyD88 signaling promotes the development of CRC, whereas NLR and inflammasome signaling appears to protect the host from CRC. This difference might be dictated by the motif that the PRR
recognizes or the location (i.e. extra- versus intracellular) of the PRR [30].

Defensins

TLRs and NLRs are of course not the only molecules involved in host response to the microbiota that affects tumorigenesis. Defensins are cationic proteins found in both the animal and plant kingdom that have broad antimicrobial activity against bacteria, fungi, and viruses, and the microbiota of mice with altered enteric defensin activity have been investigated. In mice and humans, defensins are principally produced by small intestinal Paneth cells and also by intestinal absorptive enterocytes [117]. Intestinal tissue and luminal samples from transgenic mice expressing one or two copies of the human α-defensin (DEFA5) and knockout mice deficient in the metalloprotease MMP7, which proteolytically activates α-defensin, were analyzed showed several significant differences in the gut microbiota from the phylum to the species level, including a strong reduction in the SFB [118]. Interestingly, α-defensin shows killing activity against *Helicobacter pylori* at low concentrations *in vitro* [119]. While it may seem intuitive that antimicrobial molecules, TLRs, and NLRs would impact the microbiome, the microbiome field is relatively young. Thus, there is a need to define the impact of such genes on the microbiota and clarify whether changes are stochastic or host genotype driven.

**NF-κB Activation and the IL-6—STAT3 Axis**

Inflammatory processes lead to the production of cytokines and growth factors that prevent malignant cells from apoptosis [120,121]. Genetic ablation of components of the NF-κB pathway or factors in STAT3 activation in epithelial cells blocks the expression of anti-apoptotic genes including Bcl-xL and Bcl-2, resulting in increased levels of apoptosis and more severe colitis, but remarkably, decreased tumor load [122-124]. When NF-κB activity was specifically ablated in myeloid cells with the use of IKKβ floxed mice and the LysMcre conditional deleter,
both colon tumor size and multiplicity were significantly reduced [122]. NF-κB activation in myeloid cells controls the expression of multiple inflammatory and tumor-promoting cytokines, including TNF-α and IL-1β [125,126], which are capable of activating NF-κB in epithelial and malignant cells [60].

NF-κB activation in myeloid cells also leads to the production of IL-6. IL-6 is a key cytokine in CAC; IL-6 enhances the proliferation of colonic carcinoma cells in vitro, is required for the survival of IECs and development of CAC in vivo, and interference with IL-6 signaling in the late stages of CAC results in the slowing of tumor growth [124,127,128]. IL-6 can influence the differentiation of Th17 cells (and IL-17 produced by Th17 cells in turn induce the IL-6—STAT3 signaling pathway [129], forming a positive feedback loop), the suppression of regulatory T cells (Tregs), regulate the recruitment of many myeloid cell subtypes, among other functions that influence the immune response [130,131].

IL-6 production by myeloid cells in turn drives STAT3 activation in epithelial cells, which can then also result in the activation of the Ras—Erk and PI3K—Akt pathways [60]. STAT3 leads to the up-regulation of anti-apoptotic genes (Bcl-xL and Bcl-2), cell cycle regulators (Cyclin D1, c-myc), and angiogenic factors (bFGF, VEGF) [121,132]. Conversely, the epithelial inactivation of STAT3 reduces cell survival and proliferation, and results in decreased CAC tumor growth and multiplicity [123,124].

IL-23

IL-23 is a member of the IL-12 cytokine family. IL-23 expression is up-regulated in many cancers including CRC [133,134], and IL-23 receptor (IL-23R) blockade reduces intestinal inflammation and tumor growth in ApcMin/+ mice colonized with enterotoxigenic Bacteroides fragilis [135] (see Associations between Single Bacterial Species and Cancer). IL-23 may be a
key factor in driving intestinal tumorigenesis that results from epithelial barrier defects and
infiltration of luminal bacteria into dysplastic regions [136] (see Intestinal Barrier Defects and
Microbial Infiltration in CRC). IL-23 functions in part by up-regulating the Th17 response, and
IL-23R blockade reduces IL-17A production [135], therefore it is likely that IL-23 imparts its
effects by controlling the expression of IL-6, IL-22, and IL-17, but does not act on cancer cells
directly [60].

COX-2

Cyclooxygenase-2 is an enzyme that converts arachidonic acid to prostaglandins, key
mediators of inflammation [137]. Unlike COX-1, which is a housekeeping gene involved in
producing prostaglandins at basal levels, COX-2 is not normally expressed by most cell types but
can be strongly induced by a variety of different growth factors and pro-inflammatory cytokines
[138]. Approximately 85% of human colorectal carcinomas and 50% of colonic adenomas
exhibited elevated COX-2 expression [139-141], and COX-2 is up-regulated in intestinal
adenomas from Apc^{Min/+} mice [142]. Correspondingly, a daily dose of aspirin or other non-
steroidal anti-inflammatory drug, which block COX-2 activity, over the course of 10-15 years
can reduce the relative risk of developing CRC by up to 50% [143-146] and can reduce colonic
adenoma size and number in FAP patients [147,148].

Mouse Models of Immunodeficiency in Colitis and Cancer

Several genetic mouse models of colitis have been interrogated regarding their
microbiome patterns before, after, and during active inflammation, which can perturb the
microbiota as oxidative stress and antimicrobial molecules increase during inflammation [149].
Mouse models of colitis, including FVB.mdr1a^{−/−} [150], BALB/c.T-bet^{−/−} X Rag2^{−/−} [151,152], and
IL-10R2^{−/−} X TgfbrII^{−/−} [153] mice, have been profiled using multiple methodologies inclusive of
culture-dependent and -independent techniques and revealed distinctive patterns with the gut microbiota. The Enterobacteriaceae Klebsiella pneumoniae and Proteus mirabilis were associated with colitis in T-bet−/− X Rag2−/− [152], while Bacteroides thetaiotaomicron was associated with disease in IL-10R2−/− X TgfbrII−/− mice [153], suggesting that a variety of genes may impact colonization and fitness of specific gut microbes in the setting of an inflamed epithelium and genetic immune perturbations. Remarkably, the colitigenic microbiota of the T-bet−/− X Rag2−/− mice is transmissible to WT mice by co-housing [151,152,154]. The T-bet−/− X Rag2−/− mice develop colonic adenocarcinoma after 6 months of age, but all signs of dysplasia are prevented by keeping the mice on a cocktail of antibiotics [155], suggesting that the microbiota is responsible for driving colonic tumorigenesis in this model.

The Role of the Gut Microbiota in Colorectal Cancer

Associations between Single Bacterial Species and Cancer

The best characterized association between a single bacterial species and cancer is that of Helicobacter pylori and gastric cancer, a discovery that resulted in a Nobel Prize for the co-discoverers Barry J. Marshall and J. Robin Warren [156]. H. pylori is the most common etiologic agent in infection-related cancer, and accounts for 5.5% of all cancers globally [157]. Most individuals that harbor H. pylori, approximately 50% of the world’s population [24], do not develop peptic ulcers or cancer, but there is a significant amount of data to support a causal relationship between H. pylori and gastric cancer based largely on epidemiology and case-control studies [158]. A series of meta-analyses that assessed the association between gastric cancer and H. pylori, despite finding that approximately 50% of studies produced negative results, independently came to the conclusion that the association has an odds-ratio of
approximately 2.0 (range 1.92 to 2.56) [158-164]. Although there is conflicting evidence on whether or not *Helicobacter* eradication therapy reduces incidence of gastric cancer [165], at least one study convincingly demonstrates that successful eradication of *H. pylori* in gastric mucosa-associated lymphatic tissue lymphoma can successfully treat 80% of patients with early stage lymphomas [166]. Beyond epidemiological data, there is evidence for molecular mechanisms that link *H. pylori* infection with cancer. The development of gastric cancer is believed to be a multi-step process which includes superficial gastritis, chronic atrophic gastritis, and proceeds to metaplasia, dysplasia, and then carcinoma [167]. *H. pylori* is involved in the early stages of this process, causing chronic active gastritis and atrophic gastritis (Correa’s hypothesis) [15,16]. Upon bacterial attachment to the epithelial cell, a number of bacterial proteins are secreted into the cell by the type IV secretion system, including CagA and the VacA toxin. VacA induces vacuole formation in the cell that stimulates apoptosis of the epithelial cell [168]. CagA becomes tyrosine-phosphorylated by endogenous kinases and then causes the sustained activation of SHP-2, ERK1/2, and Src kinase [169,170], leading to actin cytoskeletal changes in the cell that may promote proliferation [16].

There is a long-standing clinical observation that links *Streptococcus bovis* (now known as *Streptococcus gallolyticus*) infection with endocarditis and CRC [171]. Patients that present with *S. gallolyticus* endocarditis or septicemia are routinely screened for the presence of colorectal cancer, because 60% of *S. gallolyticus*-infected patients are found to have a concomitant adenoma or carcinoma [172]. The underlying pathophysiology of this strong association is not understood [173-175], but it may be the result of decreased epithelial barrier function at the site of colonic adenomas or carcinomas that allows streptococcal species to enter into the circulation [136].
Growing evidence suggests a role for adherent-invasive strains of *Escherichia coli* in driving CAC. Strains of *E. coli* that carry the polyketide synthase (*pks*) pathogenicity island cause DNA double-strand breaks and activation of the DNA damage checkpoint pathway, leading to cell cycle arrest and cell death *in vitro* [176]. The *pks* island encodes the polyketide-polypeptide genotoxin Colibactin. *E. coli* harboring Colibactin induced phosphorylated H2AX foci in mouse enterocytes *in vivo*, as well as the appearance of micronuclei, aneuploidy, ring chromosomes, and anaphase bridges, suggesting that Colibactin induces breakage-fusion-bridge cycles and chromosomal instability [177]. A recent study showed that monocolonizing germ-free, AOM-treated, *Il10*−/− mice with a *pks*-containing strain of *E. coli* resulted in enhanced tumor multiplicity compared to *Enterococcus faecalis* –monocolonized control mice despite similarly high levels of colitis [178]. Remarkably, intestinal tumorigenesis could be abolished in this model by deletion of the *pks* island in the *E. coli* strain [178]. Although previous studies have observed an association between *E. coli* and CRC [179] and Crohn’s disease [180], *pks*-positive *E. coli* were found to be enriched in both IBD and CRC cohorts [178], suggesting that this DNA-damaging bacterium may be a clinically relevant etiologic agent in human CRC.

Enterotoxigenic *Bacteroides fragilis* (ETBF) secretes the metalloprotease toxin *B. fragilis* toxin (BFT) and is associated with inflammatory diarrheal disease in young children [181]. A stool-based quantitative PCR-based study found that ETBF was found at a higher prevalence in CRC patients compared to healthy individuals in a Turkish cohort [182]. This finding raised the possibility that ETBF could have a role in promoting tumorigenesis in this population, as other studies have shown that ETBF induces c-myc expression and proliferation when co-cultured with a colon carcinoma cell line [183], triggers the cleavage of E-cadherin and enhances β-catenin signaling *in vivo* [184], and that it induces colitis in WT mice [185]. In a seminal study, Cynthia
Sears and colleagues showed that $Apc^{Min/+}$ mice colonized with ETBF developed severe colitis and a significantly increased colonic tumor load compared to control mice colonized with a non-BFT-containing strain of $B. fragilis$ [135]. The colonic lamina propria of ETBF-colonized mice were enriched for Th17 and $\gamma\delta$ T lymphocytes, and antibody-based blockade of IL-17 ameliorated colonic tumorigenesis in this model [135]. Therefore, inflammation as a result of response to the BFT toxin drives inflammation and carcinogenesis. In addition to this inflammatory mechanism, ETBF was found to induce spermine oxidase (SMO), a polyamine catabolic enzyme, leading to the generation of reactive oxygen intermediates, and DNA damage in vitro, and inhibition of SMO reduces ETBF-mediated inflammation in $Apc^{Min/+}$ mice [186]. It has been proposed that ETBF, perhaps like $pks$-containing $E. coli$, is an “alpha-bug,” capable of introducing DNA damage, promoting IEC proliferation, driving tumor-promoting inflammation, and perhaps influencing the microbiota as whole to drive colonic tumorigenesis [187].

**Evidence for a Role of the Microbiota in CRC from Gnotobiotic Mice**

Gnotobiotics is the science of well-controlled microbial environments within and for biological specimens, encompassing the generation and maintenance of both germ-free (GF) and defined microbial community animals [188]. While gnotobiotic mice are discussed here, there are gnotobiotic fish, flies, rats, pigs, and foals. Gnotobiotic techniques have been essential to mechanistically interrogate host–microbiota interactions in mice [189]. Rederivation of any combination of genetic mutant mice is possible via embryo transfer into GF pseudopregnant mice or aseptic harvesting of a gestational uterine package and transfer of the fetuses to a GF foster female. It is important to realize that although GF mice do not harbor live bacteria or archaea, they are not naive to microbial-associated molecular patterns, as they encounter them in their sterilized (by autoclave or irradiation) food, water, and bedding. Microarray-based
comparisons of host tissues from GF mice and their conventional counterparts have been a successful approach to understand the broad impact of the microbiome on physiology [78,190,191]. Microarrays of GF and conventionalized mice even have shown that the gut microbiota modulate host gene expression post-transcriptionally by altering expression of host microRNAs within the small and large intestine [192].

The first evidence that the microbiota may have a role in CRC came from gnotobiotic experiments in GF rats that showed a higher incidence of CRC in conventionally-raised compared to GF rats [193]. Since then, there have been a number of studies in genetically engineered mice predisposed to CRC showing a lower incidence of disease under GF conditions. For instance, T cell receptor beta-chain and p53 double-knockout (Tcrβ−/− X p53−/−) mice showed no incidence of intestinal adenocarcinoma under GF conditions, whereas ileocecal and cecal adenocarcinomas were detected in 70% of the conventionalized group [194]. Tgf-β1−/− mice normally develop CRC, but are free from inflammation, hyperplasia, and carcinoma when reared germ-free, but intestinal lesions re-appear when the mice are exposed to Helicobacter hepaticus [195]. Similarly, Rag2−/− mice, which lack an adaptive immune system, are also free from inflammation and all signs of hyperplasia under GF conditions, but develop inflammation and intestinal carcinoma in the presence of Helicobacter hepaticus [196]. ApcMin/+ mice show a reduction in both small intestinal and colonic tumors under GF versus conventional conditions, and the gut microbiota has been found to trigger the c-Jun—JNK and STAT3 signaling pathways to accelerate tumor growth in this mouse model [197].

There are a number of studies that explore the contribution of the gut microbiota to colitis and CRC in IL-10-deficient mice. IL-10 was initially identified to be produced by T helper type 2 (Th2) cells, B cells, and macrophages to inhibit Th1 cell functions, and was subsequently
found to be a potent suppressor of macrophage activation and generally a strong anti-inflammatory cytokine [198,199]. Consequently, $\text{Il10}^{-/-}$ mice develop chronic colitis [198] and are also predisposed to developing CRC. Germ-free $\text{Il10}^{-/-}$ mice developed significantly less colitis than conventionally raised $\text{Il10}^{-/-}$ mice [200]. $\text{Il10}^{-/-}$ mice mono-associated with $\text{Enterococcus faecalis}$ developed colitis and colonic adenocarcinoma, but both GF mice and mice mono-associated with a number of control strains of bacteria did not develop any signs of carcinoma [200]. IL-10-deficient mice that are treated with the carcinogen AOM develop colitis and CRC under conventional conditions, but do not develop any intestinal inflammation or dysplasia under GF conditions [201]. Two related studies suggest that colonization by $\text{Helicobacter hepaticus}$, a common symbiont of the mouse gut microbiota, specifically is required to drive AOM-induced colon tumors in $\text{Il10}^{-/-}$ mice [202,203]. Similarly, mono-association with a Colibactin-positive, but not Colibactin-negative, strain of $\text{E. coli}$ in AOM-treated, $\text{Il10}^{-/-}$ mice drives CAC [178] (see Associations between Single Bacterial Species and Cancer). These studies indicate that the microbiota is required for the development of colonic tumors in a number of different mouse models and so raise the question: What microbial factors are responsible for contributing to tumorigenesis?

**Microbial Products that Contribute to Tumorigenesis**

Most studies on bacterial contributions to cancer have been approached from the perspective of the inflammatory response to the microbe (see The Role of Inflammatory Responses to the Microbiota in Cancer), but recent research points to possible direct contributions of microbial metabolites to the carcinogenic process.

Early work in rats showed that conventionally raised but not GF animals develop CRC when given the plant glycoside carcinogen cyasin, however GF rats develop colon tumors when
given the downstream active metabolite of cyasin, methylazoxymethanol (MAM) [204]. The conversion of cyasin to its carcinogenic form MAM was found to be imparted by β-glucosidase, an enzyme that is encoded by the microbiota [204]. In subsequent decades, it has been found that the microbiota can convert latent carcinogens into bioactive compounds through the action of a number of bacterial enzymes including β-glucosidase, β-glucuronidase, 7-α-dehydroxylase, azobenzene reductase, and nitroreductase [30,205].

β-glucuronidase

Many compounds that are metabolized in the liver become conjugated to glucuronic acid and then secreted into the small intestine via the bile. Bacterial β-glucuronidase in the colon hydrolyzes these conjugates, releasing the parent compound. For example, one of the most commonly used colonic carcinogens, AOM, is hydrolyzed into the activated procarcinogenic metabolite MAM in the liver, conjugated with glucuronic acid, and then secreted through the bile into the small intestine [206]. When this compound becomes introduced to the colonic microbiota, β-glucuronidase activity converts it to its carcinogenic form by removing the glucuronide group. The activity of additional, yet-uncharacterized bacterial enzymes also metabolize MAM into methyldiazonium and a reactive methyl-carbonium ion [207]. The inhibition of β-glucuronidase in AOM-treated rats significantly reduced colon tumor load [208]. In another example, the colon cancer chemotherapeutic CPT-11 causes severe diarrheal disease when reactivated into the toxic form by β-glucuronidase, and β-glucuronidase inhibitors block removal of the glucuronide group and thereby prevent toxicity caused by this substance [209].

As might be expected, populations at high risk for CRC show high levels of β-glucuronidase activity in stool [210]. Rats fed a high-fat diet, associated with a high risk for CRC, show increased β-glucuronidase activity in cecal and colonic contents [211]. Conversely, diets high in
fiber and bran are associated with decreased risk of CRC and decreased β-glucuronidase activity in rats [212] and in humans [213].

7-α-dehydroxylase

7-α-dehydroxylase is a microbial enzyme that converts the bile acid cholate into deoxycholic acid (DCA). DCA has been shown to cause DNA damage and apoptosis in IECs [214]. High levels of fecal DCA are associated with increased CRC risk [215], and 7-α-dehydroxylase activity is higher in stool from CRC patients relative to healthy control individuals [216]. Bile acids can disrupt the integrity of the colonocyte cell membrane, resulting in the release of arachidonic acid and prostaglandin E2 (PGE2), which can drive proliferation in IECs. Secondary bile acids can also contribute to cell necrosis, hyperplasia, proliferation, DNA damage, and apoptosis [14].

Reactive Oxygen Intermediates

Reactive oxygen intermediates are derivatives of molecular oxygen and include superoxides, hydrogen peroxide, hypochlorous acid, singlet oxygen, and hydroxyl radicals. Some intestinal microbes, for example Enterococcus faecalis, produce substantial amounts of extracellular superoxide and hydrogen peroxide that have been shown to damage DNA and promote chromosomal instability in IECs in vitro [217-219]. The use of superoxide dismutase and γ-tocopherol, which block the production of ROI, in co-cultures of E. faecalis and IECs reduced levels of chromosomal instability [218]. Of course in vivo, activated macrophages and neutrophils [220-222] as well as epithelial cells and pre-malignant cells [223,224] also produce significant amounts of ROI, and therefore it can be difficult to determine how much ROI is contributed by the host versus the microbes themselves. During chronic inflammation, the DNA damage caused by ROI leads to mutations, deletions, and sister chromatid exchanges, which all
contribute to chromosomal instability and drive the carcinogenic process [14].

Approaches aimed at reducing the production of ROI or the inhibition of nitric oxide synthase, which produces reactive nitrogen species, have been successful at reducing DNA damage and preventing tumorigenesis [221,223-225]. Mice that are deficient in both glutathione peroxidase enzymes Gpx1 and Gpx2, the major enzymes responsible for reducing hydroperoxides in the intestine, develop cancer with a penetrance of 25% under conventional conditions, less than 9% under specific pathogen-free conditions, and no tumors were observed under GF conditions [226].

**N-nitroso Compounds, Heterocyclic Amines, and other Products**

Nitrate in the diet is converted to nitrite by the intestinal microbiota [14], and nitrite reacts with amines, amides, and methylurea to produce nitric oxide compounds, carcinogenic DNA-alkylating agents [227,228]. Furthermore, experiments using germ-free rat have demonstrated that N-nitrosation in the colon is dependent on the gut microflora, the products of which have been shown to have carcinogenic properties [229,230]. Heterocyclic amines that are produced when meat is cooked at high temperatures have been implicated in a number of gastrointestinal cancers [30]. Recent studies have shown that the production of the active carcinogenic form of these compounds is dependent upon the enzymatic activity of the intestinal microbiota [231-234].

Certain members of the microbiota that have been demonstrated to promote tumorigenesis produce carcinogenic compounds. Specifically, enterotoxigenic *B. fragilis* produces *B. fragilis* toxin, a metalloproteinase that cleaves E-cadherin [185] and contributes to colonic tumorigenesis in a mouse model [135], strains of *E. coli* that encode the *pks* pathogenicity island can induce DNA double strand breaks in eukaryotic cells and promote CRC
and Helicobacter pylori encodes CagA and VacA among other secreted proteins that contribute to carcinogenesis in gastric adenocarcinoma [15,164,235-237] (see *Associations between Single Bacterial Species and Cancer*).

**Bacteria that Show Protective Effects in CRC**

Just as certain members of the gut microbiota promote carcinogenesis, other bacteria show evidence of having a protective effect against cancer. For example, the daily administration of *Lactobacillus acidophilus* to *Apc^Min/+* mice results in decreased tumor multiplicity and size [238]. Similarly, AOM-treated rats fed *L. acidophilus* had decreased aberrant crypt foci (ACF) [239], *L. acidophilus* in combination with *Bifidobacterium longum* inhibited colonic tumorigenesis induced by dimethylhydrazine (a derivative of AOM) [240,241], and administration of *Lactobacillus rhamnosus* with *Bifidobacterium lactis* in AOM-treated rats decreased colonic tumor load [242]. Some bacteria can inhibit the activity of enzymes that produce carcinogens. For example, AOM-treated rats that were fed *B. longum* and an inulin derivative had decreased colonic ACF and reduced β-glucuronidase activity [243]. The co-administration of *L. acidophilus* and *Lactobacillus casei* can decrease the enzymatic activity of β-glucuronidase, azoreductase, and nitroreductase in rats [244] and in humans [245]. *L. casei* can prevent DNA damage and tumorigenesis induced by the carcinogen methylnitronitrosoguanidine [246], and can metabolize a number of heterocyclic amines [247], and thereby prevent DNA damage in the colon and liver of rats [248]. Similarly, *B. longum* has been shown to have protective effects in rats against heterocyclic amine-induced colon, mammary, and liver carcinogenesis [249]. The high-potency probiotic preparation CSL#3 reduces signs of colitis and delays dysplasia in a rat model of CAC [250,251], and administration of the probiotic combination of *L. rhamnosus* GG and *B. lactis* Bb12 improved clinical biomarkers in human
colon cancer patients [252]. In addition to the above *in vivo* studies, there are many *in vitro* studies demonstrating anti-proliferative effects of specific bacteria: *Bifidobacterium adolescentis* can inhibit the production of TNF-α and the activity of β-glucuronidase and β-glucosidase, and reduce the proliferation of several human cell lines [253]; *Bacillus polyfermenticus* adheres to Caco-2 cells and confers a dose-dependent inhibition of proliferation [254]; Strains of lactic acid bacteria (LAB) reduce the growth and viability of HT-29 cells [255]; A number of *Bifidobacterium* species and *Lactobacillus* species can inhibit the growth of the MCF7 breast cancer cell line [256].

**Probiotics**

Probiotics are living microbes that can contribute to the health of the host. The species of probiotics that are most frequently used are *Lactobacillus* spp., *Bifidobacterium* spp., and LAB [36] and, in fact, most of the bacteria that show evidence of a protective effect against cancer are commonly-used strains of probiotics. Generally, probiotics do not become established members of the microbiota, but rather only persist during the time of dosage and shortly thereafter [257]. There are several mechanisms by which probiotics might impart a protective effect against carcinogenesis: the binding or degradation of carcinogens; the inactivation of microbial enzymes involved in procarcinogen activation; the production of anti-tumorigenic compounds; direct inhibitory effects on proliferation; the enhancement of the anti-tumor immune response; the production of biofilms that prevent the attachment or invasion of pathogenic bacteria; out-competing pathogenic bacteria [36,258].

A principle metabolic product of probiotic bacteria, and the gut microbiota as a whole, is the short chain fatty acid (SCFA), which includes butyrate, propionate, and acetate. These are produced by the fermentation of dietary fiber and starch. SCFA serves as the primary energy
source for colonocytes [259], influences immune responses and protects against inflammatory disease [260], and is involved in recovery from intestinal injury [259,260]. SCFA promotes cell cycle arrest and apoptosis in cultured IECs [261] by the up-regulation of Bak and down-regulation of Bcl-xL through the mitochondrial pathway of apoptosis [262,263]. Butyrate induces histone hyperacetylation [264] by inhibiting histone deacetylase activity [265,266], thus explaining in part its pro-apoptotic effects [267]. Therefore, SFCA is likely a key molecule that contributes protective effects against cancer by probiotics.

There is a 15 to 20 year time interval from the appearance of pre-cancerous lesions to the development of carcinoma in colon cancer, so although much work remains to determine the full extent of its efficacy, probiotics may be an effective therapy to prevent or delay CRC in patients that are predisposed to the disease [36,268-270].

**Intestinal Barrier Defects and Microbial Infiltration in CRC**

A unique feature of dysplastic intestinal lesions is the local loss of intestinal barrier function. This is explained in part by the lack of mucus production because of decreased differentiation into goblet cells, and also by the loss of tight junctions between epithelial cells. As a result, dysplastic lesions are prone to infiltration by the luminal bacteria of the intestine. Indeed, disruption of the mucus layer renders mice susceptible to intestinal inflammation [45,271,272], which progresses to CAC [273] and the accelerated adenoma growth that is seen in the context of the *Apc<sup>Min/+</sup>* genotype [274]. Interestingly, the absence of local barrier function at tumor sites in *Apc<sup>Min/+</sup>* mice results in the translocation of microbial products across the epithelium and increased IL-23 expression [136]. IL-23 is a key cytokine in driving tumorigenesis in response to microbial infiltration, because its inactivation results in decreased tumor number and size and reduced levels of other pro-inflammatory cytokines [136].
Thesis Summary

This thesis reports on the characterization of the colorectal tumor microbiota and describes the functional contribution of members of the microbiota to the tumor microenvironment. In Chapter 1, we introduce PathSeq, a computational tool that is designed to analyze deep-sequencing datasets such as human whole-genomes and transcriptomes for the presence of microorganisms. PathSeq removes human sequences by computational subtraction before identifying microbe-derived sequences, and therefore is capable of identifying the presence of any microorganism including previously unsequenced, novel microorganisms. PathSeq also includes a module that performs a metagenomic analysis by calculating the relative abundance of each bacterial taxon present in the sample. In Chapter 2, we use the metagenomic analysis module of PathSeq to characterize the colorectal cancer microbiome using whole-genome sequencing of CRC tumor and adjacent-normal tissue pairs. As a result of this analysis, we discovered a strong enrichment of Fusobacterium species in CRC tumors. Finally, in Chapter 3, we introduce Fusobacterium species and other members of the CRC microbiome to Apc\textsuperscript{Min/+} mice, and find that Fusobacterium induces an NF-\kappaB inflammatory response in the tumor microenvironment and accelerates intestinal tumorigenesis.
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CHAPTER 1

PathSeq: software to identify or discover microbes by deep sequencing of human tissue

This Chapter is a reproduction of a published manuscript:

Author Contributions:
Abstract

Many human diseases are believed to be caused by undiscovered pathogens [1,2]. The advent of next-generation sequencing technology presents an unprecedented opportunity to identify pathogens in hitherto idiopathic diseases. Here we present PathSeq, a highly scalable software tool that performs computational subtraction on high-throughput sequencing data to identify non-human nucleic acids that may indicate candidate microbes. PathSeq exhibits high sensitivity and specificity in its ability to discriminate human from non-human sequences using both simulated and experimental transcriptome and whole-genome sequencing data. PathSeq is implemented in a cloud-computing environment making it readily accessible by the scientific community.

Introduction

Previously our group and others have developed a computational approach to pathogen discovery, sequence-based computational subtraction [3-6]. This method is based on the premise that infected tissues contain both human and microbial nucleic acids and that novel pathogen-derived sequences can be detected after subtracting human sequences. This unbiased approach to pathogen discovery is an advance over targeted PCR or pan-microbial array methods because it requires no sequence information ab initio about the organism being sought. However, performing computational subtraction at any significant scale was initially cost-prohibitive as this method requires a large number of input sequences, given that any pathogen present is likely to have low nucleic acid representation relative to that of the human host.
The recent development of next-generation sequencing methods [7,8], however, has made computational subtraction-based pathogen discovery a viable option. For example, massively parallel pyrosequencing combined with computational subtraction has resulted in the discovery of novel viruses in human disease: Merkel cell polyomavirus in Merkel cell carcinoma [9] and a novel Old World arenavirus in a cluster of patients with fatal transplant-associated disease [10]. Indeed, the past few years have seen steep drops in price and increases in throughput for next-generation sequencing technologies, and these trends are expected to accelerate in the near future [7,8]. However, this advancement in technology brings with it new computational challenges. Analyzing sequence data using the computational subtraction method is computationally expensive relative to most other next-generation sequencing analyses because it requires subtractive alignments to several large reference databases using local alignment algorithms such as BLAST.

Here we present PathSeq, a comprehensive computational tool for the analysis of the non-host portion of resequencing data that is capable of detecting the presence of both known and novel pathogens as well as any resident microorganisms. PathSeq runs efficiently on sequence datasets of any size in a scalable and completely reproducible fashion because it is developed on a parallel computing architecture and is implemented in a cloud-computing environment. The PathSeq software package is available for public use in the form of a machine image for cloud computing, which can be launched and monitored using no more than a basic laptop computer. We believe that PathSeq opens the way for a new large-scale effort in pathogen discovery by any researcher with access to deep sequencing data from human tissue.
Results and Discussion

The PathSeq process begins with a subtractive phase in which input reads are subtracted by alignment to human reference sequences (Fig. 1-1a), and continues with an analytic phase in which the remaining reads are aligned to microbial reference sequences and assembled *de novo*. The input reads are first filtered to remove low quality, duplicate, and repetitive sequences. The initial subtractive alignments are performed using the rapid short read aligner MAQ [11] against five reference human sequence databases, including both genomic DNA and transcriptome references (see Material and Methods). At the end of each subtractive alignment step, mapped reads are discarded and unmapped reads are subjected to further subtractive analyses. In the final steps, the residual reads are aligned to two additional human reference databases first using the Mega BLAST algorithm and then BLASTN. This identifies alignable reads with additional mismatches and/or short gaps that are not aligned by MAQ. The set of reads which remain unmapped after the subtractive phase are candidate non-human, pathogen-derived reads. A similar schema may be used for other host organisms by substituting the appropriate reference genome databases.

The analytic phase of PathSeq is composed of several steps that are performed in parallel (Fig. 1-1b). To identify previously sequenced microbes, all unmapped reads are aligned to reference viral, bacterial, and fungal sequence databases by BLASTN and BLASTX. To assess the bacterial composition of a sample containing a rich microbiome, PathSeq performs a metagenomic analysis by aligning all unmapped reads to the complete collection of currently sequenced whole bacterial genomes and quantifying bacterial representation by a measure of both the total number of aligned reads and the bacterial genome coverage (see Materials and
Figure 1-1. The PathSeq workflow. (a) Conceptual workflow of the subtractive phase of PathSeq. The size of the read set (orange bars) is proportional to the number of reads at the indicated step in a typical run of the method. The black dots in the bars represent pathogen-derived sequences which become progressively concentrated. The steps in this conceptual workflow have been reordered for concision (see Materials and Methods for actual ordering). (b) Conceptual workflow of the analytic phase of PathSeq. The asterisk indicates the unmapped readset that is carried over from the subtractive phase.
Methods, Supplementary Tables 1-1 and 1-2). To increase the likelihood of discovering a novel organism, all unmapped reads are \textit{de novo} assembled using the short read assembler Velvet [12]. The formation of large contigs composed of several unmapped reads that do not possess significant alignment similarity to any sequence in the reference databases may be suggestive of a previously undetected organism.

To demonstrate the utility of PathSeq, we used simulated data to assess the ability of the method to (i) efficiently subtract human-derived sequences and (ii) minimize the subtraction of microbe-derived sequences (Fig. 1-2a). We created a simulated sequence dataset by combining sequences generated from a reference human transcriptome database and several virus genomes (Supplementary Fig. 1-1). Twenty million 100-mers were randomly generated from the reference transcriptome. The simulated virus reads were generated from twelve viral genomes; each viral genome was substitutionally mutated randomly at twelve distinct rates (0, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90 percent), to simulate unknown viruses at different evolutionary distances from known viruses, producing 1,000 reads per mutated genome for a total of \((12 \times 12 \times 1,000)\) 144,000 virus reads.

After the subtractive steps of the PathSeq pipeline, all 20 million human transcriptome-derived reads were correctly identified as human, and only 1,122 (0.78%) virus genome-derived reads were subtracted (Fig. 1-2a). Of these 1,122 reads, 1,120 were identified as repetitive sequences and the remaining 2 reads were subtracted because of alignment similarity to the human genome (Supplementary Table 1-3).

To model the performance of PathSeq on low-quality sequence data, we introduced “sequencing errors” into this same readset based on the distribution of actual Illumina sequencing errors and found that this did not significantly affect the performance of PathSeq.
Figure 1-2. PathSeq performance on simulated and experimental sequence data. (a) Reads were generated by sampling random 100-mer sequences from a human transcriptome database to produce 20 million reads, and from a set of twelve virus genomes each substitutionally mutated at twelve distinct rates, generating 144,000 reads (see Supplementary Fig. 1-1). The blue bars represent the number of human reads remaining after the indicated step in the PathSeq workflow, and the red squares connected by a line represent the remaining viral reads. (b) We applied whole-genome sequencing data from a human ovarian tumor and (c) one lane of total-RNA transcriptome sequencing from HeLa cell lines to PathSeq. The inset in panel c shows that the 30,790 reads remaining after the subtractive phase of PathSeq are predominantly composed of HPV-18 sequences.
(Supplementary Fig. 1-2, Supplementary Table 1-4, Materials and Methods). With the exception of human rhinovirus A, which contained many repetitive sequences, greater than 97% of all non-mutated, virus-derived reads were correctly identified by alignment to the viral nucleotide database, and over 50% were still correctly identified at a substitutional mutation rate of over 20% (Supplementary Fig. 1-3a). Sequence alignment following de novo assembly allowed the identification of sequences with an even higher mutation rate (Supplementary Fig. 1-3b). We note that the presence of large, unidentifiable contigs in experimental sequence data could suggest the presence of a novel microbe lacking sequence homology to known microbes, and propose that such a result should justify follow-up by PCR, 3’- or 5’-RACE, and Sanger sequencing.

This notion of identifying the presence of microbes by contig formation prompted us to ask how many reads are required to form sufficiently large contigs. The probability of forming contigs from reads originating from a single genome is a function of two variables: (i) the size in base-pairs of the genome in question; and (ii) the number of reads derived from the genome. We simulated the ability of Velvet, the short read assembler that is used in PathSeq, to form contigs that are at least 1.75 times the size of the input reads from a genome by randomly generating reads from genomes of varying length (Supplementary Fig. 1-4). We found that there is a >75% chance of forming contigs from genomes as large as 20kb when only 20 reads are derived from the genome (using 100bp reads). This suggests that relying on contig assembly to indicate the presence of a novel genome may be a practical approach.

We then tested the performance of PathSeq on a set of sequences representing many-fold coverage of the whole genome of a serous ovarian carcinoma tumor that was sequenced as part of The Cancer Genome Atlas (see Material and Methods). Starting with slightly more than 1.7
billion reads of 101 bp each, we first removed mapped reads following initial alignment to the reference human genome, and then performed stringent quality filtering to yield 26.3 million reads. We ran these sequences on PathSeq and were left with a final set of 13,019 reads after subtraction, less than 0.001% of the original reads (Fig. 1-2b). The analytic phase of PathSeq did not yield any evidence that the remaining reads were derived from a pathogen; rather they likely represented yet-uncharacterized regions of the human genome or sequencing artifacts. This substantial subtraction efficiency, 99.9992%, demonstrates that the performance of PathSeq on simulated data can indeed be extended to real, human whole-genome sequencing.

We next generated sequence data from HeLa cervical cancer cell lines with the expectation of finding human papillomavirus (HPV) type18. We sequenced a cDNA library generated from total RNA isolated from HeLa cells on a single lane of Illumina sequencing, generating 10.3 million quality- and purity-filtered 76-bp reads. We applied this sequence data to PathSeq (Fig. 1-2c). Human-derived reads were efficiently subtracted during the subtractive phase such that 0.30% of input reads remained unmapped. Out of these 30,790 reads, 25,879 were identified as HPV-18, leaving 4,911 non-human, non-HPV-18 reads. We then collected all of the HPV-18 reads and searched for those whose pair-mate aligned to the human reference genome. This allowed us to identify the integration site of the viral genome in a region of chromosome 8q24 between positions 128,300,300 and 128,310,400 just upstream of the MYC oncogene.

One longstanding goal of computational subtraction is the identification and characterization of every read in a dataset. Although the sensitivity and specificity data for PathSeq are impressive, it still leaves 0.00076% of the ovarian whole-genome sequence reads and 0.013% of the HeLa RNA-Seq reads unaccounted for. This shortfall might be explained by
error-ridden sequences passing the quality filter, reads that map to the splice junction of rare splice variants in RNA-Seq data, or reads that map to regions of the human genome that have not yet been characterized. A number of groups have recently reported novel human genome sequences by de novo assembly of next-generation whole-genome sequence data [13,14] or by using a fosmid end-sequence pair mapping approach [15]. Ideally, these new sequences could form a subtractive database for PathSeq and help reduce the total number of unaccounted reads. However, by performing a simple Mega BLAST alignment of these sequences to microbial databases we find that many sequences from all three above-mentioned studies have perfect matches to known bacteria, fungi, and viruses, raising the question of whether they may contain novel microbes as well. Therefore, an essential next step in the improvement of computational subtraction-based methods is the creation of a reliable database of human genome scaffolds that extend the current human reference genome.

Taken together, our results demonstrate the ability of PathSeq to identify both known and novel microorganisms in high throughput human resequencing data. Just as current metagenomic surveys of the world’s oceans and soils are yielding remarkable new organisms, so too do we expect to reveal new viruses, bacteria, and fungi in human tissue with important medical implications. We are making PathSeq available for public use at http://www.broadinstitute.org/software/pathseq/, and it is our hope that investigators will use this tool to join our efforts in pathogen discovery.

Materials and Methods

RNA-seq library construction from HeLa cells and sequencing
RNA was extracted from cultured HeLa cells according to the RNeasy Kit (Qiagen) protocol. cDNA sequencing library construction was performed as described previously [16], with noted modifications below. The cDNA library was sequenced on the Illumina Genome Analyzer II (GAII) platform. The mean fragment length was approximately 350 base pairs. One lane of paired-end, 76 base pair sequencing was performed, producing 38.5 million purity filtered reads, which yielded 10,304,513 high quality reads following quality filtering.

**Modifications made to the Illumina RNA-Seq protocol**

Total RNA (500 ng) was heated at 98°C for 100 min in THE RNA Storage Solution (1 mM sodium citrate, pH 6.4; Ambion/ABI, AM7000) to fragment the RNA to a mean size of ~500 nucleotides. Quality of RNA fragmentations was assessed on a Bioanalyzer 2100 (Agilent). First-stand cDNA synthesis was performed by adding random hexamers (Invitrogen, 48190-011) to the RNA and heating at 70°C for 10 min, and then immediately incubating at 50 ºC for 1 h upon addition of Superscript III reverse transcriptase (Invitrogen). Second-strand synthesis was carried-out with *E. coli* DNA ligase and *E. coli* DNA polymerase I (Invitrogen) for 2.5 h at 16 ºC. cDNA was purified using the MiniElute PCR Purification Kit (Qiagen) and evaluated using Bioanalyzer. End-repair, addition of adenine to the 3’ end of the DNA fragments, and adapter ligation was performed as described in Guttman *et al.*, except a 2:1 molar ratio of adapter to DNA fragment was used during adapter-ligation. The resulting adapter-ligated fragments were purified on a 4% SeaKem LE agarose gel (Lonza) and a 400-500 base pair band was cut out of the gel and purified using the MiniElute kit. PCR was performed with Phusion DNA polymerase (Finnzymes) and adapter-specific primers using the following conditions: 2 min at 98 °C; [10 s at 98 °C, 30 s at 65 °C, 30 s at 72 °C] for 13 cycles; 5 min at 72 °C. Following PCR, a second round
of gel extraction was performed as described above, and the product was submitted for Illumina sequencing.

The PathSeq workflow and cloud implementation

The PathSeq pipeline is designed using the Apache Hadoop implementation of the MapReduce programming framework (http://hadoop.apache.org/mapreduce) and can be run on the Amazon Elastic Compute Cloud (EC2) (http://aws.amazon.com/ec2/) [17]. The workflow is comprised of three modules: pre-subtraction, subtraction, and post-subtraction. The pre-subtraction module is simply a quality filtering step and is run on the user’s local machine, whereas the subtraction and post-subtraction modules are executed on a Hadoop-based cluster (19 worker nodes and 1 master node) built using the Amazon Elastic Compute Cloud (Amazon EC2).

Amazon’s Simple Storage Service (S3) file system (http://aws.amazon.com/s3/) is used to store the reference sequences and readset, and the config files and scripts are distributed across all nodes on the cluster using the Hadoop Distributed File System (HDFS). The reference sequences are continuously updated on the PathSeq system and users are given the option of substituting any built-in database with a database of their choice; however we provide data download dates for reference sequences used in experiments reported in this paper below.

All processes are run on the Hadoop cluster in multiple map phases. The subtraction module comprises of two mappers. First, subtractive alignments are performed with MAQ (Release 0.5.0, default settings) against a set of six human sequence databases: the 1000 Genomes Project female reference (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/technical/reference/, downloaded 2009-04-11), the Ensembl
*Homo sapiens* cDNA database (ftp://ftp.ensembl.org/pub/current/fasta/homo_sapiens/cdna/, downloaded 2009-04-22), the human genome and transcriptome BLAST database (ftp://ftp.ncbi.nih.gov/blast/db/, downloaded 2009-05-25), and the set of three assembled human genomes available on NCBI (hs_alt_Celera, hs_alt_HuRef, hs_ref_GRCh37, ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/Assembled_chromosomes/, downloaded 2009-06-19). The next map phase is composed of three steps: RepeatMasker (http://www.repeatmasker.org/), MegaBlast, and BLASTN. First, the reads are applied to RepeatMasker (version open-3.2.8, libraries dated 2009-06-04), and any reads with three or more masked nucleotides are discarded. Subtractive alignments are next performed using MegaBlast (Blast Tools version 2.2.23, cut-off expect value $10^{-7}$, word size 16) to two human sequence databases: the NCBI *Homo sapiens* RNA database (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/RNA/, downloaded 2009-11-20), and the Ensembl human genome reference (ftp://ftp.ensembl.org/pub/current/fasta/homo_sapiens/dna/, downloaded 2009-10-22). The final set of subtractive alignments are then performed with BLASTN (Blast Tools version 2.2.23, cut-off expect value 10-7, word size 7, nucleotide match reward 1, nucleotide mismatch reward -3, gap open cost 5, gap extension cost 2) to the same two databases. A reduce phase gathers all remaining reads into one consolidated file which serves as input to the post-subtraction module.

The post-subtraction module is also comprised of two mappers. The first mapper is a set of BLASTN (parameters as above) and BLASTX alignments (Blast Tools version 2.2.23, cut-off expect value 10-4, word size 3, matrix: BLOSUM62, gap open cost 11, gap extension cost 1) to viral (downloaded from NCBI Nucleotide (http://www.ncbi.nlm.nih.gov/nucleotide) using search term “‘viruses’[porgn:__txid10239]” on 2010-02-26), fungal (downloaded using the term “‘fungi’[porgn:__txid4751]” on 2009-11-23), bacterial and archaeal
(ftp://ftp.ncbi.nih.gov/genomes/Bacteria/, downloaded 2010-03-30), and non-redundant protein
also performs a \textit{de novo} assembly (Velvet 0.7.31, \textit{k}-mer size 21) on the full set of reads
remaining from the previous map phase. The complete post-subtraction BLAST output files as
well as the full set of unmapped reads and contigs are then uploaded and stored on the S3 storage
system.

The Amazon Machine Image (AMI) required to build the PathSeq Hadoop cluster is
accessible from Amazon Web Services (http://www.broadinstitute.org/software/pathseq/). PathSeq is implemented in Python, Java, C++
and C shell, and has been tested on a Linux 2.6.18-194.8.1.e15 X86_64 system.

**PathSeq runtime and performance**

PathSeq analysis was performed using a cluster of 19 worker nodes and 1 master node,
which were EC2 Large CPU instances (7GB of memory and 2 processor cores). Full analysis of
HeLa cell RNA-Seq data described in this report was performed in approximately 13 hours (wall
clock time) for a total price of $89 USD. The CPU time for this analysis was approximately 270
hours. Actual runtime and cost may vary depending on congestion on the Amazon EC2, Internet
traffic, and the method of data upload. Because of its parallel architecture, PathSeq can analyze
substantially larger datasets in a similar timeframe simply by increasing the cluster size.

**Metagenomic analysis**

The metagenomic analysis module of PathSeq reports the relative abundance of bacteria
and archaea. This analysis begins with a MegaBlast alignment of the readset against the
complete set of fully sequenced bacterial and archaeal genomes (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/, downloaded 2010-03-30), reporting all hits with >90% sequence identity and >90% query coverage. The top 30 hits for each read are reported. Using these alignment results, classifications of each read are attempted at the phylum, then genus, then species level. If a given read cannot be classified uniquely at a given classification level (i.e. it has multiple hits to different reference sequences with equivalent E-values), then it is considered ambiguous and discarded from analysis at that level. Using species-level classifications, the fraction-genome-coverage is calculated for each species that received a hit, and this metric is used to quantify the relative abundance of a given species, normalized by the genome size.

Generation and analysis of simulated sequencing data

*Simulated human transcriptome and virus sequence data.* Twenty million 100-mers were randomly generated from a reference human transcriptome (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/RNA/, downloaded 2009-11-20). Simulated virus reads were derived from twelve virus sequences: NCBI Nucleotide accession AY740741, CY000455, EU643590, FJ356716, FJ464337, GQ290462, GQ415051, NC_000883, NC_001405, NC_001806, NC_005179, and NC_007815. For each of these sequences, substitutional mutations were introduced at a frequency of 0%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%. In this process, nucleotides along the sequence are chosen at random with replacement (i.e. the same nucleotide can be chosen twice at random) and converted to a different nucleotide (for example, A is converted to C, G, or T). This produces 144 sequences (12 input sequences, each mutated at 12 frequencies). For each of these 144 sequences, 1,000 “reads” of length 100bp were produced at random. The resulting 144,000
simulated reads were pooled with the 20 million simulated human reads and analyzed on PathSeq.

Contig formation simulations. In this experiment, “genomes” of size 200bp to 20,000bp in increments of 200bp were generated from the Human herpesvirus 5 genome (accession GQ466044). For each of these 100 “genomes”, two to twenty 100-mer sequences (“reads”) were generated at random. This process was performed eleven times. For each “genome” size, “read” number pair, Velvet assembly with k-mer size 21 was performed. The frequency with which contigs of at least 175bp were generated was recorded.

Introduction of “sequencing errors” into simulated sequence data

Sequencing errors were introduced into the simulated reads based on quality scores seen in a whole-genome sequence dataset of a glioblastoma multiforme (GBM) primary tumor (sequenced as part of The Cancer Genome Atlas; data available via the NCBI Short Read Archive (SRA) identifier SRX010704). The average quality score for each base along the length of the reads was calculated across the dataset and offset by -5 (Supplementary Fig. 1-3a). This was converted into a probability value and used to “mutate” our simulated reads (i.e. for a sequence error probability of 0.001, there is a 0.1% chance that the base will be converted to a different base).

Human whole-genome ovarian tumor sequencing data

The human ovarian tumor whole-genome sequencing dataset was sequenced as part of The Cancer Genome Atlas, and the data is available via the NCBI SRA identifier SRX010747.
This is a 101 base pair, paired-end sequence dataset with a nominal fragment length of 264 base pairs.

References


CHAPTER 2

Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma

This Chapter is a reproduction of a published manuscript:

Author Contributions:
Abstract

The tumor microenvironment of colorectal carcinoma is a complex community of genomically altered cancer cells, non-neoplastic cells, and a diverse collection of microorganisms. Each of these components may contribute to carcinogenesis, however the role of the microbiota is the least well understood. We have characterized the composition of the microbiota in colorectal carcinoma using whole genome sequences from 9 tumor/normal pairs. *Fusobacterium* sequences were enriched in carcinomas, confirmed by quantitative PCR and 16S rDNA sequence analysis of 95 carcinoma/normal DNA pairs, while the Bacteroidetes and Firmicutes phyla were depleted in tumors. Fusobacteria were also visualized within colorectal tumors using FISH. These findings reveal alterations in the colorectal cancer microbiota; however, the precise role of fusobacteria in colorectal carcinoma pathogenesis requires further investigation.

Introduction

Malignant tumors are complex communities of oncogenically transformed cells with aberrant genomes, associated non-neoplastic cells including immune and stromal cells, and sometimes microbes, including bacteria and viruses. Several viruses that can integrate into the human genome directly cause cancer, such as human papillomavirus in cervical cancer [1] and Kaposi’s sarcoma-associated herpesvirus in Kaposi’s sarcoma [2]. In other cases, microorganisms lead indirectly to cancer through chronic inflammatory responses—a mechanism by which *Helicobacter pylori* contributes to both gastric cancer and MALT lymphoma [3,4].
In the human distal gut, where microbial cells outnumber host cells nine-to-one [5], the microbiome can impart both beneficial and detrimental effects on host physiology contributing to health or disease susceptibility. Gut microbial communities (microbiota) may also influence the development of colorectal carcinoma [6-8]. Sears and Pardoll have recently introduced the concept of the “alpha-bug” – wherein select members of a microbial community, in addition to possessing virulence and pro-carcinogenic features, are capable of remodeling the microbiome as a whole to drive pro-inflammatory immune responses and colonic epithelial cell transformation leading to cancer [9].

We postulate that if the microbiota play an active role in the pathogenesis of colorectal carcinoma, then these microbes will be found within the tumor microenvironment, and the composition of the tumor microbiome will differ from that of adjacent non-neoplastic tissue. We have undertaken unbiased, sequence-based approaches, followed by cytological analysis, to probe the differences in the microbial composition of the colorectal carcinoma tumor microenvironment relative to adjacent non-neoplastic tissue. We now report an association of *Fusobacterium* with the colonic mucosa of colorectal carcinoma.

**Results**

To determine the microbial composition of human colorectal cancer, we analyzed whole genome sequences of nine colorectal cancers and matched normal colons [10] using PathSeq, a computational subtraction pipeline that culls out candidate microbial sequences [11]. These presumed bacterial sequences were identified by alignment to known sequenced microbial genomes (Fig. 2-1a, Supplementary Fig. 2-1, Supplementary Table 2-1). PathSeq analysis
also identified the presence of virus sequences in these specimens including human herpesvirus 7, however no significant differences in viral sequence levels were detected between tumor and normal DNA (Supplementary Table 2-2). Hierarchical clustering analysis of the species-specific relative abundances of microbial sequences revealed that the microbial communities of a tumor and matched non-cancerous colon from a given patient were more similar to each other than are tumors or non-affected colon samples from different patients (Fig. 2-1b). This finding suggests that a patient’s intestinal ecosystem may be more significant in shaping the microbiota than the generic microenvironment of a colon tumor or normal colonic tissue.

To identify bacterial species whose sequences are more abundant in colorectal tumors than in the matched, non-cancerous colorectal tissue, we applied a metagenomic biomarker discovery approach, LEfSe (Linear Discriminant Analysis (LDA) coupled with effect size measurements), which performs a non-parametric Wilcoxon sum-rank test followed by LDA analysis to assess the effect size of each differentially abundant taxon [12]. Using LEfSe, we found that Fusobacterium sequences were significantly enriched in the colorectal cancer metagenomes as were sequences from the family of Streptococcaceae (Fig. 2-1c, Fig. 2-1d, Supplementary Fig. 2-2).

As our initial screen was performed on a sample size of 9 cases, we next examined a larger cohort of 95 paired specimens of colon cancer and normal colonic DNA to survey the colon cancer microbiome and validate the tumor-specific enrichment of Fusobacterium. We amplified ribosomal 16S rDNA by PCR using consensus primers from 95 tumor/normal pairs, followed by pyrosequencing to assess the relative abundance of DNA from bacterial species
Figure 2-1. Whole-genome sequencing analysis of the colorectal cancer microbiome. (a) Schematic of experimental and computational whole-genome sequencing analysis workflow. (b) Hierarchical clustering of phylotype relative abundance measurements demonstrates that microbial composition of tumor/normal pairs within individuals is more highly correlated than tumor/tumor pairs or normal/normal pairs from different individuals. Normal samples are shown in green, tumors are shown in purple. (c) Linear Discriminant Analysis (LDA) coupled with effect size measurements identifies *Fusobacterium* as the most differentially abundant taxon in colon tumor versus normal specimens by whole-genome sequencing in 9 individuals. Tumor-enriched taxa are indicated with a positive LDA score (black), and taxa enriched in normal tissue have a negative score (gray). Only taxa meeting an LDA significant threshold of 1.8 are shown. (d) Percent relative abundance for the genus *Fusobacterium* is depicted across all samples in the order of the labels in (b), demonstrating a tumor-enrichment in most individuals.

(Fig. 2-2a). Overall, as was the case in our whole-genome sequence data, tumor/normal pairs from the same individual are much more highly correlated than tumor/tumor pairs or normal/normal pairs from different individuals (Fig. 2-2b). Colorectal tumors were associated with broad phylum-level changes including the depletion (i.e. reduced relative abundance) of
Figure 2-2. 16S rDNA sequencing analysis of the colorectal cancer microbiome. (a) Schematic of experimental and computational 16S rDNA sequencing analysis workflow. (b) Beta-diversity distances calculated using phylotype relative abundance measurements between all pairs of samples demonstrate that the microbial composition of tumor/normal pairs within individuals is more highly correlated than tumor/tumor pairs, normal/normal pairs, or tumor/normal pairs from different individuals. (c) Linear Discriminant Analysis (LDA) coupled with effect size measurements identifies *Fusobacterium* as the most differentially abundant taxon in colon tumor versus normal specimens by 16S rDNA sequencing in 95 individuals. Tumor-enriched taxa are indicated with a positive LDA score (black), and taxa enriched in normal tissue have a negative score (gray). Only taxa meeting an LDA significant threshold of 4.2 are shown. (d) A cladogram representation of data in (c). Tumor-enriched taxa are indicated in red, and taxa enriched in normal tissue are blue. The brightness of each dot is proportional to its effect size.
Firmicutes and Bacteroidetes, most prominently the Clostridia (Fig. 2-2c, Fig. 2-2d, **Supplementary Fig. 2-3**); however the overall diversity in the tumors relative to adjacent tissue was not significantly different (**Supplementary Fig. 2-4a and Supplementary Fig. 2-4b**). Consistent with our whole-genome sequencing results, the relative abundance of *Fusobacterium* was highly enriched in the population of tumor versus normal samples (Fig. 2-2c and Fig. 2-2d). However a tumor-enrichment for *Streptococcaceae* was not reproduced, most likely due to small sample-size in our initial whole-genome sequencing results. In addition, we analyzed patient metadata to identify correlations or possible confounding effects (including patient age, gender, ethnicity, tumor anatomic location, tumor purity, inflammation, necrosis, and vascularization), but only found a modest correlation with patient geographic location (**Supplementary Fig. 2-5**), as well as a correlation of higher microbial diversity with tumors of higher histological stage or grade (**Supplementary Fig. 2-4c**). The correlation of fusobacteria abundance with geographic location may either indicate a real geographic effect or else a confounder introduced by slightly differing sample collection protocols at the collection sites, for example time between surgery and freezing (see Materials and Methods).

As we have shown that *Fusobacterium* species are enriched in colorectal cancer DNA and tissue, we sought associations that might suggest that fusobacteria are required for the survival or maintenance of colorectal cancer cells. Because *Fusobacterium* species can invade colonic epithelial cells [13], we examined colorectal cancer cell lines and hepatic and lymph node metastases for evidence of fusobacterial DNA. Quantitative PCR analysis of 59 human colorectal cancer cell line DNAs revealed no significantly detectable *Fusobacterium* DNA, however these *in vitro* passaged cell lines are often cultured in the presence of antibiotics (**Supplementary Table 2-3**). Strikingly, however, when we examined surgically resected
colorectal cancer metastases, *Fusobacterium* was detected in 2 out of 11 cases (Supplementary Table 2-4).

Given the increased abundance of *Fusobacterium* sequences in colon cancer DNA, we next asked whether *Fusobacterium* could be detected in histological sections of colon cancer, and if so, where. To address this question, we used 16S rDNA fluorescence *in situ* hybridization (FISH) oligonucleotide probes on colonic biopsy sections. Employing probeBase consortium 16S rDNA probes that detect the majority of bacteria (EUB338) and members of the genus *Fusobacterium* (FUSO) [14,15], we performed FISH analysis on frozen (9 cases) and formalin-fixed paraffin embedded (12 cases) tissue sections from colorectal cancer and normal colon. The *Fusobacterium* probes detected bacteria in the colorectal cancer and normal tissue sections and were quantitated within the lamina propria and mucus (Fig. 2-3a); z-section stacks suggest that some of the imaged bacteria may reside intracellularly (data not shown). Consistent with the analysis of *Fusobacterium* DNA described above, FISH-detected fusobacteria were enriched in the colorectal cancer compared to the normal samples (Fig. 2-3b, Supplementary Fig. 2-7; see Supplementary Fig. 2-8 for a comparison of *Fusobacterium* quantitation across all 4 methods), in contrast to total bacteria counts which were more evenly distributed (Supplementary Fig. 2-7).

Finally, we sought to assess the specific *Fusobacterium* species that are enriched in colorectal carcinomas. Based on the 16S ribosomal DNA sequences, 5 out of a total of 409 operational taxonomic units (OTUs, a proxy for species) identified in our samples were classified as members of the *Fusobacterium* genus. By performing multiple sequence alignments using our 5 OTUs along with 16S rDNA sequences from a reference set of 31 *Fusobacterium* species and constructing maximum likelihood trees, the OTUs were identified as most closely related to
Figure 2-3. Fluorescence *in situ* hybridization (FISH) detects enrichment of fusobacteria in colorectal tumors. 

(a) FISH using an Oregon-Green 488-conjugated “universal bacterial” 16S rDNA-directed oligonucleotide probe (EUB338, green) (top left) and Cy3-conjugated *Fusobacterium* (FUSO, red) (top right and bottom center) 16S rDNA-direct oligonucleotide probe demonstrates the presence of bacteria and *Fusobacterium* within in the colonic mucosa of colorectal tumor samples. Representative images are shown with a 10 µm scale bar in the lower corner of each panel; white arrowheads mark bacteria. Epithelial cell nuclei were stained with DAPI. 

(b) To determine whether *Fusobacterium* was enriched in tumor versus normal pairs, 3 random 40X fields were chosen for scoring by an observer blinded to tumor/normal status, using selection criteria of mucosal tissue depth and a minimum of 5 bacteria visualized by the EUB338 probe per field. Each dot represents data from either a tumor or normal sample from 9 tumor/normal paired cases. The mean, SEM, and p-values (calculated by a Wilcoxon matched-pairs signed rank test) are shown.
Fusobacterium nucleatum, Fusobacterium necrophorum, Fusobacterium mortiferum, and Fusobacterium perfoetens (Fig. 2-4a). The percent relative abundance in colorectal tumors versus normal colons of the two most abundant OTUs is shown in Fig. 2-4b, demonstrating that for most patients these OTUs are enriched in the tumor. Strikingly, only a subset of the cancers showed dramatic enrichment of Fusobacterium species, accounting for up to 89% of total bacterial DNA in some specimens; this result suggests that fusobacteria may be uniquely related to pathogenesis of subsets of colorectal cancer. The OTU with the greatest similarity to F. nucleatum was the most dominant phylotype identified within cancers, however some tumors contain more than one dominant species (Supplementary Fig. 2-9).

Discussion

In summary, genomic analysis of the microbiome of colorectal carcinomas reveals a significant enrichment of Fusobacterium species in these cancers, especially phylotypes with the greatest similarity to F. nucleatum, F. mortiferum, and F. necrophorum. This enrichment is confirmed by histological analysis of tumor tissue, and also the identification of Fusobacterium DNA in colon tumor metastases. Our analysis also reveals broader changes in the tumor environment such as the depletion of the Bacteroidetes and Firmicutes phyla, most notably the order Clostridiales. Fusobacterium species may have a fitness advantage in the evolving tumor microenvironment resulting in an altered microbiota in accordance with the “alpha-bug” hypothesis.

Interestingly, Fusobacterium species may be associated with inflammatory bowel diseases (IBD) including both ulcerative colitis and Crohn’s disease.
Figure 2-4. Phylogenetic analysis identifies several *Fusobacterium* species in human colon cancer tissues. (a) Approximately-maximum-likelihood phylogenetic trees were constructed on the V3-V5 region of the 16S rDNA gene using 31 reference *Fusobacterium* species along with the five most prominent OTUs identified in colon cancer specimens (indicated in red). Nodes that have bootstrap support above 50% and 75% are indicated with a white and black dot, respectively. The mean percent relative abundance in tumor (T) and normal (N) of each OTU is indicated in parentheses. The full names of the reference strains appear in Supplementary Table 2-5. (b) The abundance of the indicated OTU relative to all other phylotypes in a given specimen is shown for the two most abundant *Fusobacterium* OTUs in tumors (x-axis) and normal colon tissue (y-axis); each point represents tumor and normal abundance data for a different individual. The lower-right quadrant of the graph highlights the substantial proportion of patients for whom the *Fusobacterium* abundance is >10% in tumors but <10% in the matched normal.

[13,16,17], and IBD is a known risk factor, indeed one of three highest risk factors, for colorectal cancer. Furthermore, consistent with our findings in colorectal carcinoma, others have reported that several *Fusobacterium* strains were associated with IBD, however the majority (69%) were specifically associated with *F. nucleatum* [13]. Therefore it is worth further exploration of a causal link between *Fusobacterium* spp. with inflammatory bowel disease and colorectal carcinoma pathogenesis.
*F. nucleatum* and other *Fusobacterium* species can elicit host pro-inflammatory response [18] and possess virulence characteristics that promote their adhesiveness to host epithelial cells [19,20] and their ability to invade into epithelial cells [13,21]. Therefore, our findings of a tumoral enrichment of *Fusobacterium* spp. in colorectal carcinoma suggest the possibility that these organisms may contribute to tumorigenesis, perhaps in a limited subset of patients, most conceivably by an inflammatory-mediated mechanism. Alternatively, it is possible that fusobacteria accumulate in the tumor microenvironment in the late stages of tumorigenesis and therefore do not have a significant role in tumor development. Our results do not prove a causal relationship between *Fusobacterium* and colorectal cancer; the establishment or repudiation of such a relationship will require further studies of colorectal cancer in both human subjects and animal models of the disease. Additionally, case-control studies comparing tumor microbiota to that of colonic epithelial tissues from healthy individuals will serve to demonstrate whether *Fusobacterium* species are more prevalent in individuals with colon cancer relative to the general population.

In summary, our findings reveal species-specific alterations in the colorectal cancer microbiota, which may lead to microbiota-directed prevention, diagnostic, prognostic, and treatment strategies for these cancers.

**Materials and Methods**

Sample collection and preparation
Colorectal adenocarcinoma and adjacent non-affected tissue was obtained from the Vall d’Hebron University Hospital in Barcelona, Spain and Genomics Collaborative Inc. (GCI), using the sample collection protocols detailed below:

Vall d’Hebron University Hospital

All Frozen tissue samples were collected following the Standard Operating Procedures at the site. Participants were enrolled in the study prior to surgery, and informed consent was obtained by the surgeon. After arterial ligation and surgical removal of the tissues, the specimens were immediately transferred from the Operating Room to the Pathology suite and subsequently evaluated by the pathologist and, if possible, one fragment of healthy tissue and one of tumor were chosen and placed in a cryotube and frozen immediately in liquid nitrogen (time interval between specimen removal and freezing: maximum= 30 min.; median= 22 min.). The frozen tissue was stored at -80 °C until DNA extraction. All clinical data, captured on Case Report Forms, was double-data entered in to a clinical database.

Genomics Collaborative Inc.

All Frozen tissue samples were collected following the same Standard Operating Procedure at all collection sites, both within the US and in Vietnam. Participants were enrolled in to the study prior to surgery, and informed consent was obtained. After arterial ligation and surgical removal of the tissues, the tissue was transferred from the Operating Room to the Pathology suite. The time taken from surgical removal of the specimen until the time it was received for processing in the pathology suite, as well as the temperature at which it was transferred (room temp, or on ice) were recorded. The SOP required the Tissue Transmittal time (from removal to freezing of the sample) to ideally be between 30 to no more than 45 minutes. Upon dissection in the pathology suite, samples were cut into approximately 1g pieces and placed into pre-labeled cyrovials.
supplied in the sample kits. Samples were immediately frozen in liquid nitrogen vapor phase in charged vapor-shippers located at each site. An H&E slide was cut from the adjacent face of each tissue sample and sent to GCI along with the frozen tissue samples. Upon receipt at GCI, the samples were qualified by pathologist review of the H&E slide, and all samples were stored in Liquid N2 vapor freezers until requested for research. All clinical data, capture on Case Report Forms, was double-data entered into a clinical database.

**DNA extraction, whole genome sequencing, and analysis**

DNA was extracted from colorectal carcinoma tumors and adjacent non-affected tissues and whole genome sequencing was performed as described previously [10]. Initial alignments to the human reference genome were performed as described [10]. All unaligned sequencing reads were (1) analyzed on PathSeq and (2) aligned to the complete set of fully sequenced bacterial and archaeal genomes (ftp://ftp.ncbi.nih.gov/genomes/Bacteria, downloaded 2010-10-07) by MegaBlast (Blast Tools version 2.2.23, word size 16, match reward 1, mismatch reward -2, gap open reward -5, gap extension reward -2). The top 30 sequence matches with >90% sequence identity and >90% query coverage were reported for each read (i.e. query). Classifications were performed at the domain, then phylum, then genus, then species level requiring unique alignments (i.e. reads with equivalent E-values to multiple taxa were removed from analysis). At the species level, relative abundance (RA) for each organism was calculated as follows: $RA = \left( \frac{\# \text{unique alignment positions in genome} \times 1,000,000}{\# \text{total alignable reads} \times \text{genome size}} \right)$. The RA values were then per-sample normalized such that the total relative abundance for each sample sums to one. The resulting normalized RA matrix was analyzed on LEfSe [12].
Amplification and 454 sequencing of 16S gene

The 16S gene dataset consists of 454 FLX Titanium sequences spanning the V3 to V5 variable regions obtained for 190 samples (95 pairs). Detailed protocols used for 16S amplification and sequencing are available on the HMP Data Analysis and Coordination Center website (http://www.hmpdacc.org/tools_protocols/tools_protocols.php). In brief, genomic DNA was subjected to 16S amplifications using primers designed incorporating the FLX Titanium adapters and a sample barcode sequence, allowing directional sequencing covering variable regions V5 to partial V3 (Primers: 357F 5'-CCTACGGGAGGCAGCAG-3' and 926R 5' CCGTCAATTCTMTTTRAGT-3'). Polymerase chain reaction (PCR) mixtures (25 µl) contained 10 ng of template, 1x Easy A reaction buffer (Stratagene, La Jolla, CA), 200 mM of each dNTP (Stratagene), 200 nM of each primer, and 1.25U Easy A cloning enzyme (Stratagene). The cycling conditions for the V3-V5 consisted of an initial denaturation of 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 40 sec, annealing at 50°C for 30 sec, extension at 72°C for 5 min and a final extension at 72°C for 7 min. Amplicons were confirmed on 1.2% Flash Gels (Lonza, Rockland, ME) and purified with AMPure XP DNA purification beads (Beckman Coulter, Danvers, MA) according to the manufacturer and eluted in 25 µL of 1X low TE buffer (pH 8.0). Amplicons were quantified on Agilent Bioanalyzer 2100 DNA 1000 chips (Agilent Technologies, Santa Clara, CA) and pooled in equimolar concentration. Emulsion PCR and sequencing were performed according to the manufacturer’s specifications.

Processing of 16S sequence data

Resulting sequences were processed using a data curation pipeline implemented in mothur [22], complimented by abundantOTU [23], and custom PERL scripts. Sequences were
removed from the analysis if they were <200 nt or >600 nt, had a read quality score <25, contained ambiguous characters, had a non-exact barcode match, or did show more than four mismatches to the reverse primer sequences (926R). Remaining sequences were assigned to samples based on barcode matches, after which barcode and primer sequences were trimmed and reads were oriented such that all sequences begin with the 5’ end according to standard sense strand conventions. All sequences were aligned using a NAST-based sequence aligner to a custom reference based on the SILVA alignment [22,24]. Chimeric sequences were identified using the mothur implementation of the ChimeraSlayer algorithm [25]. Quality filtered and chimera-free sequences were clustered into Operational Taxonomic Units (OTU's) using abundantOTU [23]. Representative sequences per OTU were classified with the MSU RDP classifier v2.2 [26], maintained at the Ribosomal Database Project (RDP 10 database, version 6).

Quantitative PCR analysis

Quantitative real-time PCR was performed as described [27] using pan-*Fusobacterium* probe-primer sets as described [27]. *Fusobacterium* quantitation was measured relative to human endogenous 18S (Applied Biosystems TaqMan® Ribosomal RNA Control Reagents, Hs99999901_s1 (part number 4331182)).

Microbial FISH analysis

Frozen sections were fixed in Carnoy’s solution overnight and embedded in paraffin, and 5 mm thick sections prepared and hybridized as previously described [15]. The sequences of the following FISH probes were obtained from probeBase (http://www.microbial-ecology).
net/probebase/) [14]: the “universal” bacterial probe-EUB338 (pB-00159), Fusobacterium
targeted probe (pB-00782). Slides were imaged on an Olympus B40 microscope, digitally
photographed using IP Lab. Three random fields per sample were chosen by an observer blinded
to tumor/normal status, using selection criteria of mucosal tissue depth and a minimum of 5
bacteria visualized by the EUB338 probe per field. Composite z-stacks were assembled in IP Lab
and composite photomicrographs were assembled in Adobe Photoshop.

Data access

The 16S sequence data from this study have been submitted to the NCBI Sequence Read

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CHAPTER 3

*Fusobacterium nucleatum* potentiates intestinal tumorigenesis and modulates the tumor immune microenvironment

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Abstract

Deep-sequencing metagenomic analyses have revealed that *Fusobacterium* spp. are associated with human colorectal carcinoma. Here we show that in the *Apc<sup>Min/+</sup>* mouse model of intestinal tumorigenesis, *Fusobacterium nucleatum* increases tumor multiplicity, selectively recruits tumor-infiltrating myeloid cells, and is associated with a pro-inflammatory expression signature that is shared with human fusobacteria-positive colorectal carcinomas. However, in contrast with recent studies of other bacteria in colorectal carcinoma, *F. nucleatum* does not exacerbate colitis or colitis-associated colorectal carcinoma. We find that *Fusobacterium* spp. are enriched in human colonic adenomas relative to surrounding tissues and fusobacterial abundance is increased in stool samples from patients with colorectal adenomas and carcinomas, compared to healthy subjects. Collectively, these data support that fusobacteria may be involved in early stages of intestinal tumorigenesis and, through recruitment of tumor-infiltrating immune cells, may generate a pro-inflammatory tissue microenvironment conducive to colorectal neoplasia progression.

Introduction

There is accumulating evidence that members of the gut microbiota contribute to colorectal cancer, the second most incident cancer worldwide with over 1.2 million new cases per year [1]. The majority of studies have focused on a small subset of colorectal cancers, colitis-associated colorectal cancers, and employed rodent pre-clinical models. Antibiotic-treatment or absence of the gut microbiota reduced tumor incidence in several mouse colitis-associated colorectal cancer models [2-4]. Recently, two bacterial pathogens have been identified that
promote colitis-associated colorectal cancer. Enterotoxigenic *Bacteroides fragilis* induces colitis and colonic tumors in *Apc<sup>Min</sup>* mice by triggering a T helper type 17 (Th17) inflammatory response [5], while adherent-invasive *Escherichia coli* strain NC101 promotes colitis-associated colorectal cancer in monocolonized, azoxymethane-injected *Il10<sup>−/−</sup>* mice [6]. However, the majority of human colorectal cancers do not arise in the setting of inflammatory bowel disease.

The gut microbiota may be a driver in colorectal cancers that are not associated with colitis. Metagenomic analyses using whole-genome sequencing [7], transcriptome sequencing [8], or bacterial 16S ribosomal RNA gene DNA sequencing [7,9] have shown enrichment of *Fusobacterium* species in colorectal cancers relative to adjacent normal tissue. However, to date it has been unclear whether these findings represent an indirect association, or whether *Fusobacterium* spp. functionally contributes to colorectal cancer (CRC) tumorigenesis [10].

Here, we analyze the impact of *Fusobacterium nucleatum* on colorectal cancer progression and on tumor inflammation in mouse models, and assess the association of *F. nucleatum* in stool and colonic tissue from patients with colorectal adenomas and adenocarcinomas. Our data are consistent with the possibility that *F. nucleatum* potentiates non-colitis associated colorectal tumorigenesis.

**Results**

*Fusobacterium nucleatum* promotes intestinal tumorigenesis

The enrichment of *Fusobacterium* spp. in colorectal cancers observed in three independent human cohort studies [7-9] prompted us to examine whether *Fusobacterium* could accelerate tumorigenesis in mouse models. We began with pilot experiments in mice which have
a propensity to develop intestinal tumors because of a T to A transversion of nucleotide 2549 in one copy of the tumor suppressor gene \(Apc\) (C57BL/6 \(Apc^{Min/+}\)) or because of genetic defects resulting in chronic intestinal inflammation (BALB/c \(Il10^{-/-}\) and BALB/c \(T\)-\(bet^{-/-}\) \(X\) \(Rag2^{-/-}\)). We introduced a human clinical isolate of \(Fusobacterium\) \(nucleatum\), several human clinical isolates of \(Streptococcus\) species (\(S.\) \(anginosus\) [4 mice]; \(S.\) \(parasanguinis\) [4 mice]; and \(S.\) \(sanguinis\) [4 mice]), or tryptic soy broth to \(Apc^{Min/+}\) mice and \(F.\) \(nucleatum\) or tryptic soy broth to \(Il10^{-/-}\) and \(T\)-\(bet^{-/-}\) \(X\) \(Rag2^{-/-}\) mice starting at 6 weeks of age. \(Streptococci\) were used as control strains because of the longstanding association of streptococcal species with occult colonic malignancies [11].

Introduction of \(F.\) \(nucleatum\) into \(Apc^{Min/+}\) mice was associated with an accelerated onset of colonic tumors. \(Apc^{Min/+}\) mice fed \(F.\) \(nucleatum\) developed a significantly higher number of colonic tumors at 3.5 months of age as compared to \(Apc^{Min/+}\) mice fed \(Streptococcus\) (\(Strep.\)) spp., or tryptic soy broth control \((P < 0.001, P < 0.0001)\) (Fig. 3-1a-c). However, \(F.\) \(nucleatum\) did not induce colitis (Fig. 3-1a-c) in contrast with enterotoxigenic \(Bacteroides\) \(fragilis\), which causes colitis and accelerates tumorigenesis in \(Apc^{Min/+}\) mice [5]. Also, \(F.\) \(nucleatum\) neither exacerbated intestinal inflammation nor accelerated tumorigenesis in the two mouse models of colitis-associated colorectal cancer, \(Il10^{-/-}\) and \(T\)-\(bet^{-/-}\) \(X\) \(Rag2^{-/-}\), examined (Fig. 3-1a).

In addition to increased colonic tumor numbers, \(Apc^{Min/+}\) mice fed \(F.\) \(nucleatum\) had a higher count of small intestinal adenomas versus \(Strep.\) spp \((P = 0.0002)\), or soy broth \((P = 0.02)\) and small intestinal adenocarcinomas versus \(Strep.\) spp \((P = .0017)\) or tryptic soy broth \((P = .0082)\) (Fig. 3-1d, e). Consistent with prior findings in human colorectal carcinoma [7,8], \(F.\) \(nucleatum\) was culturable from \(Apc^{Min/+}\) tumors and enriched in tumor tissue relative to adjacent normal tissue in \(F.\) \(nucleatum\) fed \(Apc^{Min/+}\) mice as assayed by qPCR (Fig. 3-1f). Furthermore, \(F.\)
Figure 3-1. *Fusobacterium nucleatum* promotes intestinal tumorigenesis and is enriched in tumor tissues of *Apc*<sup>Min/+</sup> mice. (a) Gross colon tumor counts and histologic colitis scores from *Apc*<sup>Min/+</sup>, *Il10<sup>-/-</sup>* and *T-bet<sup>-/-</sup>* *X Rag2<sup>-/-</sup>* mice fed *F. nucleatum* (*F. nuc*), *Streptococcus* spp., or soy broth control. Mice were started on the 8-week, daily...
(Figure 3-1 Continued) feeding regimen at 6 weeks of age. (** P < 0.0001, ** P < 0.001, * P < 0.01). (b) Representative images of gross colons (ruler numbers in cm) and (c) colonic histological analysis of \(Apc^{Min/+}\) mice (100 micrometer scale bar). (d) Histopathologic small intestinal adenoma and adenocarcinoma counts in \(Apc^{Min/+}\) mice. (e) Representative sections of rolled small intestines from \(Apc^{Min/+}\) mice (ruler numbers in cm). (f) \textit{Fusobacterium} abundance in matched tumor (T) versus normal (N) tissues from colons of \(Apc^{Min/+}\) mice fed \(F.\ nucleatum\) measured by quantitative PCR. (g) Representative FISH images of tumor and matched normal tissue from an \(Apc^{Min/+}\) mouse fed \(F.\ nucleatum\) using a \textit{Fusobacterium} 16S rDNA-directed probe (50 micrometer scale bar).

\(F.\ nucleatum\) had a broad distribution within tumor tissue when visualized with fluorescence \textit{in situ} hybridization using a \textit{Fusobacterium} 16S rRNA-directed probe (Fig. 3-1g). Together, these results indicate that \(F.\ nucleatum\) may accelerate tumorigenesis in the absence of colitis, or macroscopic inflammation, in \(Apc^{Min/+}\) mice.

\textbf{\textit{F. nucleatum} selectively expands myeloid-derived immune cells}

Immune cells and their effectors have been found to be key components of the tumor milieu that promote neoplastic progression [12]. This type of inflammation has been referred to as intrinsic inflammation, as it is intrinsic to the tumor, in contrast with the extrinsic inflammation of IBD and colitis-associated colorectal cancer [12]. To address whether \(F.\ nucleatum\) contributes to tumorigenesis by an intrinsic inflammatory mechanism, we characterized and quantified the tumor infiltrating immune cells from the intestinal tumors of \(Apc^{Min/+}\) mice that were fed \(F.\ nucleatum\) or \textit{Streptococcus sanguinis} for 8 weeks, or were not fed bacteria over the same time frame. Small intestinal rather than colonic tumors were used, because controls did not develop sufficient numbers of colon tumors within the experimental timeframe.

We observed a striking increase in infiltrating cells of the myeloid lineage in the tumors from \textit{Fusobacterium}-treated mice. CD11b\(^+\) myeloid cells (mean 3.4X higher in cell number, mean 4.0X higher in % population) and CD11c\(^+\)MHC class II\(^+\) dendritic cells (DC) (mean 3.1X higher in cell number, mean 2.7X higher in % population) increased in the tumors of \(Apc^{Min/+}\).
Figure 3-2. *F. nucleatum* selectively expands myeloid-derived immune cells, but not lymphoid immune cells in the intestinal tumor microenvironment. Flow cytometric analyses: (a) Percentage of intratumoral myeloid cells (upper panel) and lymphoid cells (lower panel). CD11b⁺ myeloid cells, CD11c⁺MHCII⁺⁺ dendritic cells, Gr-1⁺ CD11b⁺ granulocytic neutrophils, CD3⁺CD4⁺ T cells, or CD3⁺CD8⁺ T cells (y-axes) vs forward scatter (FSC). Mean percentages ± s.e.m are shown within each plot. n= 6, 4, or 15 for not treated, *S. sanguinis*, or *F. nucleatum*, respectively. (b) Cell number/gram tumor for myeloid and lymphoid cells from the treatment groups. (c) Cell number/gram tumor for TAM (CD45⁺CD11b⁺F4/80⁺). (d) Left panel, cell number/gram tumor for MDSC (CD45⁺CD11b⁺Gr-1⁺). Right upper panel, cell number/gram tumor of monocytic MDSC (CD45⁺CD11b⁺Gr-1⁺Ly6C⁺) and lower panel, granulocytic MDSC (CD45⁺CD11b⁺Gr-1⁺Ly6C⁻). (e) Left panel, cell number/gram tumor of classical myeloid DCs (CD45⁺CD11c⁺MHCII⁺⁺CD11b⁺) and right panel, cell number/gram tumor of tolerogenic DCs (CD45⁺CD11c⁺MHCII⁺⁺CD103⁺). Each symbol represents data from an individual mouse. *P* values are shown where significant.
mice fed invasive *F. nucleatum* as compared to controls (Fig. 3-2a, b). In contrast, numbers of intratumoral CD11b<sup>+</sup>Gr-1<sup>-</sup> granulocytic neutrophils and T lymphocytes, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells, in mice fed *F. nucleatum* were not significantly different from controls (Fig. 3-2a, b).

Substantial experimental data from clinical and pre-clinical studies indicate that tumor-associated macrophages (TAM) promote tumor progression and metastasis [13-15]. We further characterized the intratumoral myeloid populations and found an enrichment of TAMs (CD11b<sup>+</sup>F4/80<sup>+</sup>) in *F. nucleatum* fed mice as compared to controls (mean 4.1X increased cell number) (Fig. 3-2c). Myeloid-derived suppressor cells (MDSCs) represent an additional population of tumor-permissive myeloid cells with potent immune suppressive activity [16-19]. MDSCs (CD11b<sup>+</sup>Gr-1<sup>-</sup>) were enriched (mean 4.9X increased cell number) in *F. nucleatum* fed mice vs controls. There are two principal MDSC subsets, monocytic and granulocytic, both of which increased in the tumors of *F. nucleatum* fed mice (monocytic MDSCs (CD11b<sup>+</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup>), mean 9.1X and granulocytic MDSCs (CD11b<sup>+</sup>Gr-1<sup>hi</sup>Ly6C<sup>low</sup>), mean 11.6X increased cell number) compared to controls (Fig. 3-2d).

Within tumors, dendritic cells (DC) can either dampen or promote anti-tumor immunity [20]. The goal of several current tumor immunotherapy efforts is to re-program and activate DCs within tumors, as intratumoral DCs can assume tumor permissive phenotypes [21]. The intestine possesses a specific subset of tolerance promoting DCs. These tolerogenic DCs express the cell surface integrin CD103 and promote the expansion of Foxp3<sup>+</sup> regulatory T cells, a CD4<sup>+</sup> T cell subset that suppresses cytotoxic and effector T cells and thus dampen anti-tumor immunity [22-24]. Having identified an expansion of DCs in *F. nucleatum* fed mice, we further characterized the DC populations and found increases in classical myeloid DCs (CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+</sup>) and
CD103+ tolerogenic DCs (CD11c+MHCIIhiCD103+) (mean 3.5X and mean 2.8X increased cell number, respectively) compared to controls. (Fig. 3-2e). To determine whether the changes in TAMs, MDSCs, and DCs were impacting specific subsets of CD4+ T cells implicated in colon tumorigenesis [5,25], we examined both intratumoral T-helper 17 (Th17) cells and CD4+ Foxp3+ regulatory T cells. While there was a trend towards an increase in Foxp3+ regulatory T cells (Treg) and Th17 cells in *F. nucleatum* fed mice, there was significant heterogeneity within this group, that did not correlate with intratumoral *F. nucleatum* abundance, and differences were not statistically significant (Supplementary Fig. 3-1). Additionally, there was no trend between Th17 and Treg cell numbers in the same individual mice. Collectively, these data support that *F. nucleatum* modulates the tumor immune microenvironment and results in expansion of selective myeloid-derived immune cell types that have been well-described to promote tumor progression [14,26,27].

A *Fusobacterium*-associated human colorectal cancer gene signature shared and validated in mice

Given our findings of *F. nucleatum*-induced myeloid-derived cell expansion in mouse intestinal tumors, we asked if there would also be a similar immunological profile in human *Fusobacterium*-associated colon tumor transcriptomic data. We utilized a data set of deep transcriptome sequencing (i.e. RNA-Seq) of 133 colon tumors generated by The Cancer Genome Atlas [28]. All non-human sequencing reads were applied to PathSeq, a computational tool that identifies and quantifies the abundance of all bacteria present in each tumor [29]. By calculating the Spearman’s rank correlation coefficient of the relative abundance of *Fusobacterium* spp. transcripts with host gene expression in this dataset, we identified a *Fusobacterium*-associated human CRC gene expression signature. We found a correlation of immune cell marker genes
associated with TAM (CD209, CD206/MRC1, IL-6, IL-8, and CXCL10), MDSC (CD33 and IL-6), and DC (CD11c/ITGAX, CD209, TNF, CD80) with *Fusobacterium* abundance in human

*Fusobacterium*-associated tumors by RNA-Seq, similar to our findings with *Fusobacterium* in mouse flow cytometry experiments (Fig. 3-3a). Analysis using the Ingenuity IPA gene ontology module revealed that the *Fusobacterium*-associated human CRC gene expression signature was highly enriched for the inflammatory response gene ontology category (corrected $P < 1 \times 10^{-33}$; Ingenuity analysis) as well as other categories also related to immune and inflammatory disease (Supplementary Table 3-1). To test the specificity of this correlation, we performed the same analysis with the other top-four highly abundant genera besides *Fusobacterium* (Bacteroides, Escherichia, Streptococcus, and Propionobacterium), but none of these other genera had enrichment for inflammation-related gene functions, nor did they have comparably high gene ontology enrichments in any other functional categories (Supplementary Fig. 3-2). Relative abundance of *Fusobacterium spp.* transcripts are shown for each of the 133 TCGA colon tumors with scaled expression values for the top 50 ranked genes denoted as the row Z-score in a heat map (Fig. 3-3b). Many of the these top ranked genes, PTGS2 (COX-2), IL1β, IL6, IL8, and TNF (TNF-α), have not only been investigated in colorectal carcinogenesis but also are induced by *Fusobacterium* in co-culture with human and mouse cell lines in vitro [30-32].

The expression signature was suggestive of a NF-κB-driven pro-inflammatory response [33]. As NF-κB has been identified as a central link between inflammation and cancer [34], we assessed whether there was a correlation between increased NF-κB activation and *Fusobacterium* abundance in human colorectal cancers. We obtained freshly resected human colorectal cancer samples and generated nuclear extracts from these samples. After stratifying the samples by their abundance of *Fusobacterium* spp., we performed western blots on the
Figure 3-3. A *Fusobacterium*-associated human colorectal cancer gene signature shared and validated in mice. (a) Immune cell types enriched in *Fusobacterium*-associated mouse tumor are shown with the human marker gene utilized to determine their abundance in the TCGA CRC RNA-seq data set. Spearman’s rank correlation coefficient of the relative abundance of *Fusobacterium* spp. transcripts and *P* values are shown next to each marker gene. (b) Ingenuity Pathway Analysis Biological Function Gene Ontology categories that are enriched for *Fusobacterium* abundance-correlating gene sets are shown. (c) Relative abundance of *Fusobacterium* spp. transcripts are plotted for each of the 133 TCGA colon tumors (upper panel and lower panel x-axis) and scaled expression values for the top 50 ranked genes denoted as the row Z-score (y-axis) are shown in a heat map (lower panel) with a purple (low expression)–yellow (high expression) color scale. (d) Western blot of nuclear extracts from human colon cancer with a high or low *Fusobacterium* relative abundance (see Methods) using an anti-NF-κB p65 antibody and the nuclear marker lamin B1. (e) qPCR analysis of a selection of the top 50 ranked genes in (B) in colon and small intestinal tumors from *F. nucleatum* vs tryptic soy broth fed *Apc*Min/+ mice. Tumors from 6-9 mice per group were used. Means and s.e.m. are plotted. *P* values are shown where significant.
nuclear extracts from *Fusobacterium* high and low tumors to examine NF-κB activation. NF-κB was indeed more activated (increased nuclear translocation of the p65 NF-κB subunit) in tumors with a high vs low *Fusobacterium* abundance (Fig. 3-3c, upper panel).

We found that most of the human *Fusobacterium*-associated pro-inflammatory genes with mouse homologs including: *Ptgs2* (COX-2 mouse homolog), *Scyb1* (IL8 mouse homolog), *Il6*, *Tnf* (*TNFa*), and *Mmp3* were also more highly expressed in both small intestinal and colonic tumors from mice that were treated with *F. nucleatum* vs tryptic soy broth (Fig. 3-3e).

**Fusobacteria are enriched in colonic adenomas and in stools samples from patients with adenomas and colorectal carcinomas**

Colonic adenomas are neoplastic epithelial lesions that have the potential to become malignant and are believed to be the precursors of the majority of sporadic colorectal cancers. Given the enrichment of *Fusobacterium* spp. in colorectal tumor versus adjacent normal tissue, we examined if there would be a similar enrichment in adenomas, suggesting the involvement of *Fusobacterium* in neoplastic initiation or progression, prior to the establishment of carcinoma. A few recent studies have performed case control studies of colorectal adenomas using 16S rRNA gene surveys with one noting fusobacterial enrichments in their patient cohort [35-38]. We measured *Fusobacterium* spp. abundance in paired adenoma tissue versus adjacent normal tissue from the same patient drawing samples from several geographic locations and registries: The Cooperative Human Tissue Network (Eastern, Southern, and Western divisions of the United States), Massachusetts General Hospital (Boston, MA), and University of Aberdeen School of Medicine (Aberdeen, United Kingdom). We found that *Fusobacterium* was detectable by qPCR in 48% of adenomas (n=29), and in those cases that were positive, *Fusobacterium* was enriched in adenomas relative to surrounding tissue (*P* < 0.004) (Fig. 3-4a and Supplementary Table 3-
Figure 3-4. *Fusobacterium* is enriched in adenoma versus adjacent normal tissue and detected at a higher abundance in stool from CRC and adenoma cases than from healthy controls. (a) *Fusobacterium* abundance for normal tissue (x-axis) vs adenoma (y-axis) is plotted. Samples with no difference in fusobacterial abundance between adenoma and normal within a single patient are plotted on the diagonal line. 29 matched adenoma normal tissues pairs were tested. Each symbol represents data from one patient (adenoma and normal tissue). (b) Fecal *Fusobacterium* abundance from healthy subjects (n=30), subjects with colorectal adenomas (n=29), and colorectal cancer (n = 27).
These data suggest that *Fusobacterium* begins to accumulate at early stages of colonic tumorigenesis in some groups of patients.

Next, we determined if *Fusobacterium* spp. are enriched exclusively in tumor and adenoma tissue, or whether fusobacteria have a higher overall abundance in the fecal microbiota of CRC patients relative to healthy controls in a case-control experiment. We compared the levels of *Fusobacterium* spp. to universal Eubacteria 16S by quantitative qPCR of stool. These subjects provided their stool samples to the Early Detection Research Network (EDRN) prior to bowel preparation and screening colonoscopy and had no prior history of colorectal cancer or gastrointestinal disease (Fig. 3-4b and Supplementary Table 3-4). We found that *Fusobacterium* spp. were enriched in CRC patients ($P < 1 \times 10^{-5}$). Because of the *Fusobacterium* enrichment in adenomas (Fig. 3-4a), we examined the *Fusobacterium* spp. abundance in stool from patients who provided stools to the EDRN and who were subsequently diagnosed with colonoscopy-confirmed colonic adenomas (Fig. 3-4b and Supplementary Table 3-4). We found that *Fusobacterium* spp. were also enriched in subjects with adenomas as compared to healthy control individuals ($P < 5 \times 10^{-3}$). These results indicate that increased abundance of *Fusobacterium* species in the gut microbiota may be a general feature of colonic tumorigenesis.

### Discussion

Entry of microbes and microbial products into the evolving tumor microenvironment potentiates tumor growth by eliciting tumor-promoting immune cell responses [25,39]. Our results demonstrate that *Fusobacterium* spp., rare gut microbiome constituents in the healthy human population [40], are found at increased abundance in the stool of patients with adenomas
and colorectal cancer, and are enriched in adenomas and adenocarcinomas relative to non-involved colonic tissues. Introduction of *Fusobacterium nucleatum* to Apc\textsuperscript{Min/+} mice resulted in accelerated small intestinal and colonic tumorigenesis, infiltration of specific myeloid cell subsets into tumors, and an NF-κB pro-inflammatory signature. This pro-inflammatory signature was shared with human colorectal cancer tissue with a high *Fusobacterium* abundance.

In contrast to other bacterial-driven models of intestinal tumorigenesis [5,6], we have found a specific bacterial strain that accelerates intestinal tumorigenesis in the absence of colitis. While Apc\textsuperscript{Min/+} mice fed *F. nucleatum* exhibited enhanced intestinal tumorigenesis, neither Il10\textsuperscript{-/-} nor T-bet\textsuperscript{-/-} X Rag2\textsuperscript{-/-} mouse models of colitis showed accelerated tumorigenesis or exacerbated colitis upon introduction of *F. nucleatum*. This may suggest that the tumorigenic effects of fusobacteria operate downstream of the loss of the tumor suppressor *APC* and the resulting intestinal dysplasia that occurs in Apc\textsuperscript{Min/+} mice. This is relevant to most cases of human CRC, as only 2% of CRC cases are linked to colitis, but greater than 80% of non-hypermutated CRC tumors bear *APC* mutations [28]. Our *Fusobacterium* findings are also relevant to adenomas because mutations in *APC* are among the earliest molecular alterations that occur in an epithelium as it transitions to become an adenoma [41]. Therefore, early tumor-initiating somatic mutations likely precede the tissue enrichment of *Fusobacterium* spp. These mutations may contribute to the development of epithelial barrier defects, featuring the loss of tight junctions, cell-to-cell contacts, epithelial polarity and the mucus layer [25]. Intestinal barrier defects at local sites of dysplasia may promote the infiltration of *Fusobacterium* spp., among other bacteria and microbial products, allowing fusobacteria to take up residence in the tumor environment. This may represent a crucial stage in colorectal neoplasia wherein myeloid cell-mediated
immune responses provide the driving force for inflammatory genotoxic and epigenetic changes that lead to cancer.

Although barrier defects expose the intestinal mucosa to the entire luminal microbial milieu, *Fusobacterium* spp. become the most highly enriched bacterium in colorectal tumors relative to adjacent tissue [7,8,10]. This enrichment may be attributable to the strong adhesive and invasive abilities of fusobacteria to epithelial cells [42,43]. The tumor enrichment of fusobacteria may also result from the growth advantage it provides to the tumor by eliciting pro-tumorigenic responses from myeloid immune cells. Alternatively, fusobacterial metabolic specializations may endow it with a competitive advantage in the evolving tumor milieu. *Fusobacterium nucleatum* is an asaccharolytic bacterium so, unlike the *Enterobacteriaceae*, it will not compete for glucose, a preferred substrate for tumor metabolism [44]. Instead fusobacteria can utilize amino acids and peptides as nutrient sources in the tumor microenvironment. Products of amino acid metabolism generated by fusobacteria, including formyl-methionyl-leucyl-phenylalanine and short chain fatty acids, are myeloid cell chemoattractants, which may explain the intratumoral myeloid cell expansion we observed and interconnect tumor metabolism, bacterial metabolism, and immune cell function within the tumor microenvironment.

In addition, *F. nucleatum* strains, unlike many strict anaerobes of the intestinal lumen, possess a rudimentary electron transport chain, endowing them with a limited ability to respire oxygen [45]. Thus, *F. nucleatum* may be able to persist and slowly replicate in the hypoxic tumor microenvironment. Adhesive molecules that contribute to invasivity in *F. nucleatum* can promote bacterial aggregation and biofilm formation that also enhance oxygen tolerance [46]. Products of fusobacterial metabolism may make the tumor microenvironment more tumor-
permissive over time by directly promoting tumor cell proliferation, blood vessel growth, or immune cell infiltration.

We have shown that, in both human and mouse intestinal tumors, the pro-inflammatory gene expression signature associated with *Fusobacterium* features the up-regulation of *PTGS2* (*COX-2*). Epidemiological and clinical data suggest that non-steroidal anti-inflammatory drugs (NSAIDs) may be effective as a primary and secondary preventative measure in colorectal neoplasia [47]. Our findings on *Fusobacterium* spp. and the intrinsic inflammation it elicits may further explain why anti-inflammatory strategies such as NSAIDs are an effective colorectal cancer prevention strategy.

If our results demonstrating that fusobacteria potentiate tumorigenesis can be extended to human CRC, then targeting *Fusobacterium* populations in the oral cavity where it is most abundant [48], or in the gastrointestinal tract may work to delay or prevent tumor progression in patients at increased risk for CRC. We show that *Fusobacterium* is absent, or present at low basal levels, in the stool of healthy individuals, but is significantly more abundant in patients with a colonoscopy-confirmed adenoma or CRC. These findings suggest a need for epidemiological studies to evaluate whether *Fusobacterium* abundance can be utilized as a prognostic or diagnostic factor in colorectal cancer.

**Materials and Methods**

**Bacterial strains and culturing**

*Fusobacterium nucleatum* (EAVG_002; 7/1) [43] was a gift from the laboratory of Emma Allen-Vercoe (U. Guelph). *Streptococcus* species were isolated from freshly resected human
colon tissues and identified by a combination of Gram stain and morphology, catalase test, 
hemolysis on Difco™ Tryptic Soy Blood Agar Base No. 2 (BD, Sparks, MD) supplemented with 
5% defibrinated sheep blood (Northeast Laboratory, Waterville, ME), and Sanger sequencing on 
16S rDNA positions 27-1492.

Bacteria were stored at -80°C in autoclaved freezing media, consisting of 1% DMSO, 1% 
glycerol, and 12% w/v skim milk powder in distilled water. *F. nucleatum* strains were plated on 
Fastidious Anaerobe Agar (Neogen, Lansing, MI) supplemented with 5% defibrinated sheep 
blood (Northeast Laboratory, Waterville, ME) and grown overnight in Bacto™ Tryptic Soy 
Broth (BD, Sparks, MD) (TSB) supplemented with 5µg/mL hemin, 1µg/mL menadione (Sigma 
Aldrich, St. Louis, MO), at 37°C in an anaerobic cabinet. *Streptococcus* strains were plated on 
TSB with 1.5% w/v agar (Neogen, Lansing, MI) supplemented with 5% defibrinated sheep blood 
(Northeast Laboratory, Waterville, ME), and grown overnight in TSB at 37°C in 5% CO₂. 
Colony forming units (CFU) were measured by serial dilution and plating.

**Mice**

All mice were maintained in a specific pathogen free barrier facility at the Harvard 
School of Public Health (HSPH) and all experimentation was carried out in accordance with 
institutional guidelines. Female *Apc<sup>Min/+</sup>* mice were ordered from Jackson Laboratory (Jackson 
strain C57BL/6J-*Apc<sup>Min/J</sup>*)) and received by 5 weeks of age and *Apc<sup>Min/+</sup>* were bred at HSPH. 
BALB/c *Il-10<sup>−/−</sup>* and *T-bet<sup>−/−</sup>* *Rag2<sup>−/−</sup>* mice were maintained and bred within the HSPH barrier 
facility.  

Bacterial feeding experiments were performed for a period of 8 weeks, beginning at 6 
weeks of age. Bacteria were fed to mice by pipette at 10<sup>8</sup> CFU per day, at a maximum volume of 
100µL. Sham treatment consisted of 100µL of TSB.
**Histopathology**

Colons and small intestines were prepared for histologic analysis and assessed for colitis as previously described [49]. Adenomas (polyps with low-grade or high-grade epithelial dysplasia but no invasion) and invasive adenocarcinomas were counted in colons and small intestines submitted in their entirety.

**Human specimen collection**

*Colonic adenocarcinoma samples*: Patients were identified from upcoming operative cases by co-investigator T.C. in the Department of Surgery at Brigham and Women’s Hospital. Inclusion criteria were patients who had biopsy-confirmed colorectal cancer and were undergoing hemicolectomy. Exclusion criteria included a known synchronous cancer diagnosis or other cancer diagnosis within five (5) years of the operation. The attending surgeon is a study investigator and obtained written informed consent preoperatively. Once the specimen was removed, a portion of the tumor was allocated to members of the study and the remainder submitted for standard processing by the Pathology Department. No antibiotics were given preoperatively. Written informed consent was obtained from all participants.

*Colonic adenoma samples*: Patients were identified and samples were collected by co-investigator P.L. (U. of Aberdeen), D.C. (Massachusetts General Hospital), or by the Cooperative Human Tissue Network (U. of Pennsylvania, U. of Virginia Health System, Vanderbilt U. Medical Center). Written informed consent was obtained from all participants.

*Stool samples*: Samples were collected by the National Cancer Institute Early Detection Research Network. Written informed consent was obtained from each study participant. Colonic adenocarcinoma was confirmed by biopsy; colonic adenoma was confirmed by colonoscopy. All samples were collected pre-operatively. The samples were collected by subjects in their homes.
using a “hat” or specimen collection container that fit under a toilet seat, then stool was packed into glass vials and placed on ice packs until shipped overnight and then stored at -80°C.

**DNA preparation and bacterial quantification by qPCR**

Human and mouse tissues or stools (20-100mg) were digested overnight in 0.7mL molecular grade lysis buffer (100mM TrisHCl pH 8.5, 5mM EDTA pH 8.0, 0.2% SDS, 200mM NaCl, 1 mg/ml proteinase K) at 55°C with rotation. The samples were centrifuged at 20,000xg for 5min then the liquid portion was moved to equal volume isopropanol. The precipitated DNA was recovered and resuspended in 0.4mL TE buffer. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed by inversion, centrifuged at 20,000xg for 5min, then the aqueous phase was transferred to a fresh tube. If aqueous phase was milky, the phenol:chloroform:isoamyl alcohol step was repeated. 0.1 volume of 3M sodium acetate pH 5.2 and 2 volumes of 100% ethanol was added, incubated at -20°C for 1hr, and centrifuged at 20,000xg for 15min. The pellet was washed 2 times by adding 0.5mL of 70% ethanol, centrifuging at 20,000xg for 7min, and discarding supernatant. The DNA pellet was resuspended in molecular grade water. For stool samples, an extra purification step was performed at this stage using the QIAquick Gel Extraction Kit (QIAGEN).

8ng of DNA was used in each 20µL KAPA SYBR® FAST qPCR (Kapa Biosystems, Woburn, MA) reaction, performed in triplicate, and analyzed on the Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA). The following primer sets were used: *Fusobacterium* spp. (Fwd 5’-GGATTTATTGGGCGTAAAGC-3’; Rev 5’-GGCATTCCTACAAATATCTACGAA-3’) [50], and universal Eubacteria 16S (Fwd 5’-GGTGAATACGTTCCCGG-3’; Rev 5’-TACGGCTACCTTGTTACGACTT-3’). Relative abundance was calculated by the ΔC<sub>T</sub> method.
RNA preparation and gene expression by qPCR

Total RNA was extracted using the RNeasy® kit (QIAGEN), DNase treated with the DNA-free™ kit (Ambion), and cDNA was generated using the iScript™ cDNA Synthesis kit (Bio-Rad). All primers were ordered from Sigma Aldrich. Relative gene expression was calculated using the $\Delta\Delta C_T$ method. Primers were designed using MGH primerbank:

pga.mgh.harvard.edu/primerbank/

Microbial FISH analysis

Microbial FISH was performed as described previously [7].

RNA-Seq processing and analysis

All primary sequence data on colonic adenoma (COAD) samples (https://tcga-data.nci.nih.gov/docs/publications/coadread_2012/) were downloaded from The Cancer Genome Atlas via dbGAP and the Data Coordinating Center (http://cancergenome.nih.gov/). At the time of analysis, 133 complete COAD RNA-Seq datasets were available. All datasets were analyzed on PathSeq [29] to calculate microbial relative abundance at the genus level. Relative abundance was defined as the number of uniquely-mapped microbial reads to a given genus, normalized for the total number of uniquely-mapped microbial reads per sample. Host gene expression was calculated using RPKM as described previously [28]. The Spearman’s rank correlation coefficient was calculated for each genus-host gene pairing.

Detection of NF-κB activation

Fresh or fresh-frozen colon tumor tissue (20-80mg per sample) was processed for nuclear protein isolation with the NE-PER® Nuclear and Cytoplasmic Reagents Kit (Thermo Scientific, Rockford, IL). Protein lysates were resolved using SDS-PAGE and transferred to PVDF membrane using a Bio-Rad wet transfer apparatus. Blots were probed with antibodies directed
against p65 and lamin B1 (Cell Signaling Technology, Danvers, MA). Samples were stratified as *Fusobacterium*-high (qPCR *Fusobacterium* abundance greater than 40.0 [calculated as $1.8^\Delta Ct^{Fusobacterium} - Ct^{<Eubacteria>)*1000}$], or 16S sequencing *Fusobacterium* abundance greater than 0.25 [calculated as described previously [7]]) or *Fusobacterium*-low (qPCR *Fusobacterium* abundance less than 1.0, or 16S sequencing *Fusobacterium* abundance less than 0.015).

**Isolation of intestinal tumor infiltrating cells**

Small intestine was removed, cut longitudinally and washed with Ca$^{+}$- and Mg$^{+}$-free Dulbecco’s phosphate buffered saline (DPBS). Tumor nodules were dissected away from normal tissue, weighed, and then finely minced and incubated in Ca$^{+}$- and Mg$^{+}$-free Hank’s balanced salt solution (HBSS) with 0.1 mg/ml collagenase D (Roche) and 50 U/ml DNase I (Roche) for 30 min at 37°C on a shaking platform. The solution containing digested tumors was filtered through a 70 µm cell strainer and centrifuged at 400 x g for 10 min. Isolated tumor infiltrating cells were resuspended in cell staining solution (PBS with 2% FCS) for flow cytometry analysis.

**Flow cytometry**

For cell surface staining, cells were incubated with Fc blocking antibody (BioLegend) for 15 min, and stained with fluorochrome-conjugated monoclonal antibodies of cell surface markers. Following antibodies were used and purchased from BioLegend or eBioscience: CD45 (clone 30-F11), CD11b (clone M1/70), Gr-1 (clone RB6-8C5), CD11c (clone N418), MHC class II (clone M5/114.15.2), CD3ε (clone 145-2C11), CD4 (clone RM4.5), CD8α (clone 53-6.7), F4/80 (clone BM8), Ly6C (cloneHK1.4), CD103 (clone 2E7), and CD16/CD32 (clone CD16/CD32). For intracellular cytokine or Foxp3 staining, cells were stimulated for 4 h in RPMI complete medium with 50 ng/mL phorbol 12-myristate 13-acetate (PMA)(Sigma) and 500
ng/mL ionomycin (Sigma) in the presence of 5ug/mL Brefeldin A (BioLegend). Then, cells were stained with cell surface markers, fixed with Fix/Perm buffer (BioLegend), permeabilized with Perm buffer (BioLegend) according to the manufacturer’s recommendations and stained with anti-IL-17A (clone TC11-18H10.1, BioLegend) or anti-Foxp3 (clone FJK-16s, eBioscience) antibodies. Cells were stained in parallel with the respective control isotype antibodies. Stained cells were acquired using BD LSRII flow cytometry (BD Biosciences) and analyzed with FlowJo9 software (Tree Star).

**Statistical analysis**

Generally, data is displayed in dot-plot format, with the center-line indicating the mean and the standard error of the mean represented by the error bars. All two-group comparisons were performed using the non-parametric Mann-Whitney U test, with the exception of Fig. 3-4a for which the non-parametric Wilcoxon matched-pairs signed rank test was performed. For RNA-Seq data, genus relative abundance was correlated to host gene expression using the Spearman’s rank correlation coefficient, and corrected P-values were obtained by correcting for multiple hypothesis testing using the False Discovery Rate method. Statistical analysis for **Supplementary Table 3-2** was performed using the Fisher’s exact test.

**References**


CONCLUSION

Perspectives on the Study of the Microbiota in Colorectal Cancer
This thesis describes the composition of the colorectal tumor microbiome and explores the functional consequences of some of its constituents to tumorigenesis. First, we describe the development of new computational tools that were used in this analysis. PathSeq builds on the conceptual framework of computational subtraction, and for the first time, makes it technically feasible to analyze human whole-genome sequence datasets for the purpose of pathogen discovery. Second, we make use of whole-genome sequencing using PathSeq to perform the first large-scale study of the colorectal cancer microbiome. In performing this analysis, we identified a striking enrichment of the \textit{Fusobacterium} genus in tumors. Although \textit{Fusobacterium} is a rare gut constituent in healthy individuals, almost every colon tumor sequenced was positive for \textit{Fusobacterium}. We then introduced an invasive human gut isolate of \textit{Fusobacterium nucleatum} into \textit{Apc}^{Min/+} mice and observed accelerated intestinal tumorigenesis as well as a tumor-infiltrating myeloid cell inflammatory signature.

The value of these findings remains to be determined. An inherent limitation of any microbiome study that compares the composition of healthy versus diseased tissues or subjects is that the conclusions are correlative; such studies cannot address causation. A long-term prospective cohort study can be more informative than a case-control study because it allows for the identification of a particular shift in microbial composition at the time of disease onset. However, such a study design is significantly more complex, and it still cannot reveal whether the shift in microbial composition has a causal component or is merely a consequence of the disease state. An experimental animal-based study such as the one we have performed does address causation, however there are significant limitations in extrapolating results from animal studies to human disease. Therefore, taking the case of \textit{Helicobacter pylori} and gastric cancer as an example, to assess whether \textit{Fusobacterium} species can have a causal role in human colorectal
cancer we must combine results from epidemiological and case-control studies with mechanistic animal-based studies and build evidence in a cumulative fashion.

If *Fusobacterium* species do contribute to human colorectal tumorigenesis then this could have significant clinical implications. An exciting aspect of understanding the role of the microbiota in disease is that the microbiome, unlike the human genome, is extremely malleable. For example, the development and application of narrow-spectrum antibiotics that target the Fusobacteria may prevent or delay the onset of colon cancer in patients that are at high risk of developing the disease. Furthermore, although this route will require significantly more investigation, it is possible that altering the composition of the microbiota with the use specific probiotic formulations and even fecal transplants could deplete *Fusobacterium* species from the gut microbiota.

The enrichment of *Fusobacterium* species in colorectal tumors, regardless of its functional contribution to tumorigenesis, may serve as a clinically relevant biomarker. We have demonstrated that *Fusobacterium* spp. can be detected higher levels in stool from patients with colorectal carcinoma, and also in patients with pre-malignant polyps, relative to healthy control individuals. Therefore, such an assay may be of use as a non-invasive clinical diagnostic that could lead to the early detection of disease in some patients.
APPENDIX 1

Supplementary Materials
Supplementary Figure 1-1. Generation of artificial shotgun sequencing reads. (a) 20 million reads were generated from a human transcriptome database by randomly selecting 100-mer sequences. (b) A set of twelve virus genomes were selected. For each genome, substitutional mutations were introduced at twelve distinct mutation rates, and for each of these mutated genomes 100-mer sequences were chosen at random to produce the read set.
Supplementary Figure 1-2. Applying a sequencing error distribution model to artificially generated reads. (a) Average quality score plot for a typical set of reads generated by the Illumina GAII Sequencer. The Q-value is defined as $Q = 10 \times \log_{10}(p/(1-p))$, where $p$ is the probability that the corresponding base call is incorrect. (b) The error distribution was applied to the complete set of reads generated as shown in Supplementary Figure 1. The plot shows the proportion of the read set containing a substituted base at the indicated position. (c) The proportion of the read set is shown as a function of the number of sequencing errors per read.
Supplementary Figure 1-3. Identification of virus-derived reads by sequence similarity. (a) Reads were generated as indicated in Supplementary Figure 1-1b. The proportion of reads identified as a virus sequence (MegaBlast alignment, E-value < 10e-10) is shown as a function of the substitutional mutation rate of the mutated genome. (b) Independent assemblies were performed on all reads from each of the 144 genomes. The proportion of reads incorporated into contigs that were identified as a virus sequence (MegaBlast alignment, E-value < 10e-10) is shown.
Supplementary Figure 1-4. Probability of contig formation for sequences randomly generated from a genome of varying size. This figure shows the probability of forming contigs from a set of simulated, virus-derived reads. Reads were generated by selecting random 100-mers from a series of genomes ranging in size from 100bp to 20,000bp, producing between 2 and 20 reads per genome. The heatmap indicates the frequency among 11 replicates with which a contig of at least 175bp was formed from these reads by de novo assembly.
Supplementary Figure 2-1. Phylum-level microbial classifications of whole-genome sequencing on nine colon tumor and normal tissue pairs. Phylum-level classification demonstrates that most specimens are dominated by the Bacteroidetes and Proteobacteria, collectively making up greater than 80% of all phyla in some samples.
Supplementary Figure 2-2. Whole genome sequencing identifies the *Streptococcaceae* family as significantly enriched in colon tumors. The relative abundance of sequences classified in the *Streptococcaceae* family is shown for nine colon tumor/normal paired samples by whole-genome sequencing. The *Streptococcaceae* family was found to be significantly enriched in tumors. Compare with Fig. 2-1d which shows the relative abundance of *Fusobacterium* sequences in the same set of samples.
Supplementary Figure 2-3. Phylum-level relative abundance of the microbiota by 16S rDNA sequencing on 95 colon tumor and normal tissue pairs. Median relative abundance measurements at the phylum level are reported across all 95 tumor and normal sample pairs, demonstrating a relative depletion of the Bacteroidetes and Firmicutes in the tumor.
Supplementary Figure 2-4. Microbial diversity in the colon cancer microbiome. Comparison of the microbial diversity in tumor and normal tissues by rarefaction analysis on the OTUs detected by 16S rDNA sequencing (a) across all samples and (b) on a sample-by-sample basis by using the QIIME package chao1 metric (see Materials and Methods), a species richness indicator. (c) Correlation of species richness with tumor stage by the chao1 metric (red: normal; blue: stage I; orange: stage II; green: stage III; purple: stage IV) demonstrates that higher stage tumors exhibit greater species richness.
Supplementary Figure 2-5. *Fusobacterium* load correlates with geography. 16S rDNA sequencing shows an increased mean relative abundance of *Fusobacterium* OTUs in tumors from patients in Barcelona, Spain versus GCI samples, which are a composite collection consisting of samples from the United States and Vietnam. P-value calculated using a two-sample t-test.
Supplementary Figure 2-6. Validation of the tumor-enrichment of *Fusobacterium* by quantitative PCR. 

(a) Absolute quantification of *Fusobacterium* DNA relative to a standard curve produced using *Fusobacterium nucleatum* genomic DNA and quantitative PCR probe specific to the *Fusobacterium* genus. 19 data points are beyond the y-axis limits but are reflected in the mean and standard error of the mean indicated on the plot. *P*-value calculated by a Wilcoxon matched-pairs signed rank test (non-parametric). (b) Quantification of *Fusobacterium* in tumor relative to normal by use of the ΔΔCt method quantitated against a probe targeting human endogenous 18S.
Supplementary Figure 2-7. Fluorescence in situ hybridization (FISH) detects enrichment of fusobacteria in colorectal tumors. (a) Total bacterial count data using an Oregon-Green 488-conjugated “universal bacterial” 16S rDNA-directed oligonucleotide probe (EUB338) is shown for 3 random 40X fields chosen for scoring by an observer blinded to tumor/normal status. Total bacterial counts were not enriched in either tumor or normals (P = 0.0517). This corresponds to Fig. 3B, which shows Fusobacterium counts. FISH was also performed on formalin-fixed, paraffin-embedded (FFPE) colorectal tumor and normal tissues using 5 random 40X fields with the Cy3- conjugated Fusobacterium-directed (FUSO) probe (enriched in tumor; p = 0.014). Epithelial cell nuclei were stained with DAPI (b) and the “universal bacterial” EUB probe (no enrichment; p = 0.1024) (c). All p-values were calculated using a Wilcoxon matched-pairs signed rank test (non-parametric).
Supplementary Figure 2-8. Quantitation of *Fusobacterium* across analytic methods. A comparison of *Fusobacterium* quantitation is shown for the first 9 sample pairs by whole-genome sequencing relative abundance, 16S rDNA sequencing relative abundance, absolute *Fusobacterium* DNA levels by quantitative PCR, and *Fusobacterium* cell counts by fluorescence *in situ* hybridization. “N.D.” indicates “not determined.”
Supplementary Figure 2-9. Relative abundance measurements for *Fusobacterium* OTUs in human colon cancer tissues. The relative abundance of each of the five major OTUs in colon tumors (left panel) and normal tissue (right panel) is represented on a per-patient basis (each row represents a different individual). The percent relative abundance is denoted by a color gradient indicating relatively low abundance in blue (0-10%), very high abundance in dark red (>80%), and a range of other colors to specify intermediate abundance. The percentage signifies the abundance of the indicated OTU relative to all other phylotypes in
that specimen.

Supplementary Figure 3-1. *F. nucleatum* does not affect intratumoral Th17 nor Foxp3+ Treg cell numbers. Flow cytometric analyses: Cell number/gram tumor of (a) CD3+CD4+ IL-17+ T cells and (b) CD3+CD4+ Foxp3+ T cells. Each symbol represents data from an individual mouse.
Supplementary Figure 3-2. Human colon tumor RNA-Seq analysis shows enrichment for inflammatory functions in genes correlated with *Fusobacterium* abundance but not the abundance of other genera. Spearman’s rank correlations of genus-level microbial relative abundance with host gene expression was used to weight Gene Ontology enrichment using Ingenuity Pathway Analysis software for the top 5 most abundant genera. Gene-set intersect indicates the number of genes that are shared in the enriched gene sets between *Fusobacterium* and each of the other genera.
Supplementary Table 1-1. Bacterial genomes used to construct an artificial sequence dataset to test the metagenomics module of PathSeq. Shown is the set of bacterial genomes that were each used to create a set of 10,000 random 100-mers. The species were chosen to represent both evolutionary relatedness and divergence to test the ability of the metagenomics module to properly assess the microbial representation of a mixed sample.

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<th>Definition</th>
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<td>Bacteroides fragilis NCTC 9343, complete genome.</td>
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<tr>
<td>NC_009614.1</td>
<td>Bacteroides vulgatus ATCC 8482, complete genome.</td>
</tr>
<tr>
<td>NC_013316.1</td>
<td>Clostridium difficile R20291, complete genome.</td>
</tr>
<tr>
<td>NC_009615.1</td>
<td>Parabacteroides distasonis ATCC 8503, complete genome.</td>
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<td>NC_004663.1</td>
<td>Bacteroides thetaotaomicron VPI-5482, complete genome.</td>
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<td>NC_010816.1</td>
<td>Bifidobacterium longum DJO10A, complete genome.</td>
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<td>NC_008618.1</td>
<td>Bifidobacterium adolescentis ATCC 15703, complete genome.</td>
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<td>NC_012781.1</td>
<td>Eubacterium rectale ATCC 33656, complete genome.</td>
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<td>NC_012778.1</td>
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<td>NC_010655.1</td>
<td>Akkermansia muciniphila ATCC BAA-835, complete genome.</td>
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<td>NC_011353.1</td>
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<td>NC_004668.1</td>
<td>Enterococcus faecalis V583, complete genome.</td>
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Supplementary Table 1-2. Metagenomic analysis on a sequence dataset constructed from a set of twelve bacterial genomes. Shown is the number of reads that were identified as matching to the indicated bacterial genome (actual number of reads is 10,000 for each species).

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<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Number of Reads</th>
<th>Fraction Genome Coverage</th>
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<tr>
<td>Eubacterium</td>
<td>eligens</td>
<td>9903</td>
<td>0.368491132</td>
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<tr>
<td>Bifidobacterium</td>
<td>longum</td>
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<td>0.333497209</td>
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<tr>
<td>Akkermansia</td>
<td>muciniphila</td>
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<td>0.310035802</td>
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<tr>
<td>Enterococcus</td>
<td>faecalis</td>
<td>9900</td>
<td>0.264391797</td>
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<tr>
<td>Eubacterium</td>
<td>rectale</td>
<td>9953</td>
<td>0.249987753</td>
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<tr>
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<td>difficile</td>
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<td>0.208571056</td>
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<td>distasonis</td>
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<td>0.186090932</td>
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Supplementary Table 1-3. PathSeq performance on artificially generated sequence data. Reads were generated by sampling random 100-mer sequences from a human transcriptome database, generating 20 million reads, or from a set of twelve virus genomes each substitutionally mutated at twelve distinct rates, generating 144,000 reads. The rows represent the number of reads remaining at each step in the PathSeq workflow.

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<thead>
<tr>
<th>Stage in workflow</th>
<th>Human reads remaining</th>
<th>Virus reads remaining</th>
<th>Virus reads subtracted at each step</th>
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<tr>
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<td>0</td>
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</table>
Supplementary Table 1-4. PathSeq performance on artificially generated sequence data with introduced sequencing errors. Reads are the same as in Supplementary Table 1-3 except that “sequence errors” were introduced into the reads according to a sequencing error distribution model.

<table>
<thead>
<tr>
<th>Stage in workflow</th>
<th>Human reads remaining</th>
<th>Virus reads remaining</th>
<th>Virus reads subtracted at each step</th>
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Supplementary Table 2-1. PathSeq run-statistics and microbial classifications using colon cancer human whole-genome sequencing data. Shown is the total number of reads, total number of quality-filtered (QF) reads, and reads identified as microbial sequences following PathSeq analysis (see Materials and Methods) for each of the 9 colorectal tumor/normal pairs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Reads</th>
<th>Total QF Reads</th>
<th>Microbial reads</th>
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**MEAN** 1562833440 108540197.7 156490.2778  
**MEDIAN** 1506351982 68713798 100796
**Supplementary Table 2-2. Identification of viruses in colon cancer human whole-genome sequencing data by PathSeq analysis.** Virus sequences were identified among the remaining non-human reads following analysis with PathSeq.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Virus name (Number of reads matching virus sequence)</th>
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<tbody>
<tr>
<td>CRC-0001-N</td>
<td>Norwalk virus (7), parvovirus B19 (2)</td>
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<tr>
<td>CRC-0001-T</td>
<td>Human herpesvirus 4 (2)</td>
</tr>
<tr>
<td>CRC-0002-N</td>
<td>Human herpesvirus 7 (8), Human herpesvirus 4 (1), parvovirus B19 (5)</td>
</tr>
<tr>
<td>CRC-0002-T</td>
<td>Human herpesvirus 5 (16), parvovirus B19 (3), Torque teno virus (3)</td>
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<tr>
<td>CRC-0003-N</td>
<td>Human herpesvirus 6 serotype B (333), Human herpesvirus 7 (14)</td>
</tr>
<tr>
<td>CRC-0003-T</td>
<td>Human herpesvirus 6 serotype B (1)</td>
</tr>
<tr>
<td>CRC-0004-N</td>
<td>Human herpesvirus 7 (2)</td>
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<td>CRC-0004-T</td>
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<tr>
<td>CRC-0009-T</td>
<td>Human herpesvirus 7 (105), Human herpesvirus 6 serotype B (216)</td>
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**Supplementary Table 2-3. Quantitative PCR for Fusobacterium in colon cancer cell lines.** Quantitative PCR was performed using a *Fusobacterium*-directed probe compared against a human endogenous 18S-directed probe on both DNA and RNA extracted from each of the indicated colon cancer cell lines. The qPCR signal was strong for the endogenous control target but completely absent for the fusobacterial target. All experiments were performed in triplicate.

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Supplementary Table 2-4. Quantitative PCR for *Fusobacterium* in metastases from colorectal carcinoma. Quantitative PCR using a probe targeting a region of 16S rDNA conserved among fusobacteria (see Methods) shows that 2 of 11 colon metastases to the liver or lymph nodes have a positive signal. All positive results were positive in three replicates.

<table>
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<th>Avg Ct</th>
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### Supplementary Table 2-5. Reference strain identification for Fusobacterium phylogenetic analysis.

Abbreviated names used in the phylogenetic tree in Fig. 2-4a are specified using complete Greengenes strain names.

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<td>F. russii</td>
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<td>F. simiae</td>
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Supplementary Table 3-1. Sample list of all colonic adenomas assessed for *Fusobacterium* abundance.

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Supplementary Table 3-2. *Fusobacterium* is detected at a higher abundance in stool from CRC and adenoma cases than from healthy controls.

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