

The Role of *Fusobacterium nucleatum* in the Tumor Microenvironment

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ABSTRACT

Systematic characterization of microbes in several tumors including colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) has revealed the presence of multiple species of intracellular bacteria within tumors. However, there is limited knowledge on how these bacteria colonize tumors, how they survive inside host cells, how they modulate host cell phenotypes, and if their elimination should complement cancer therapy. This is, in part, due to the lack of representative animal models, challenges in co-culture of host epithelial cells and bacteria, and limited resolution of available analytical techniques to study host-microbial interactions. I have addressed these challenges by harnessing multiple technologies from microbiology, genetic engineering, tissue engineering, and microfluidics, in order to investigate the role of an emerging oncomicrobe, *Fusobacterium nucleatum*, in the tumor microenvironment (TME). *F. nucleatum* is a Gram-negative, anaerobic bacterium that is normally found within the oral cavity. However, its selective enrichment in CRC and PDAC tumors is correlated with poor clinical outcomes. My work along with collaborators in the Verbridge, Slade, and Lu labs at Virginia Tech has revealed a multifactorial impact of *F. nucleatum* in influencing cancer progression. First, in CRC, we discovered that *F. nucleatum* infection of host cancer cells induced robust secretion of select cytokines that increased cancer cell migration, impacted cell seeding, and enhanced immune cell recruitment. In PDAC, we uncovered additional cytokines that were secreted from both normal and cancerous pancreatic cell lines upon infection with *F. nucleatum* that increased cancer cell proliferation and migration via paracrine and autocrine signaling, notably in the absence of immune cell participation. In order to examine the contribution of a hypoxic TME on infection dynamics, we used a multi-omics approach that combined RNA-seq and ChIP-seq of H3K27ac to determine epigenomic and transcriptomic alterations sustained within hypoxic CRC cells upon infection with *F. nucleatum*. Our findings revealed that *F. nucleatum* can subvert host cell recognition in hypoxia and can modulate the expression of multiple cancer-related genes to drive malignant transformation. Insights gained from this research will pave the way for future studies on the impact of the tumor microbiome in cancer and will identify novel targets for therapy and clinical intervention to control bacteria-induced exacerbation of cancer.

The Role of *Fusobacterium nucleatum* in the Tumor Microenvironment

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GENERAL AUDIENCE ABSTRACT

Colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) are the second and third leading causes of cancer death in the United States, respectively. Recent systematic characterization of various tumor types revealed the presence of distinct bacteria within tumors. However, there is limited knowledge on how these bacteria colonize tumors, how they survive inside host cells, how they modulate host cell phenotypes, and if their elimination should complement cancer therapy. This is, in part, due to the lack of representative animal models, challenges in developing host cell-microbe co-culture models, and limited resolution of available analytical techniques to study host-microbial interactions. I have addressed these challenges by harnessing multiple technologies from microbiology, genetic engineering, tissue engineering, and microfluidics, in order to investigate the role of an emerging cancer-associated microbe, *Fusobacterium nucleatum*, in the tumor microenvironment (TME). *F. nucleatum* is a microbe commonly found within the oral cavity. However, clinical studies revealed that selective enrichment of *F. nucleatum* in CRC and PDAC tumors significantly correlated with poor prognosis. My work along with collaborators in the Verbridge, Slade, and Lu labs at Virginia Tech has revealed a multifactorial impact of *F. nucleatum* in influencing cancer progression. First, in CRC, we discovered that *F. nucleatum* invasion of host cancer cells induced the secretion of select proteins called cytokines that cells use to signal and communicate with each other. These cytokines directly stimulated the cell migration of host cancer cells which is usually associated with increased cancer aggressiveness. In PDAC, *F. nucleatum* infection induced the secretion of additional cytokines from both cancer cells and normal cells that, in addition to cell migration, impacted the proliferation of cancer cells, another feature of aggressive cancers. *F. nucleatum* usually thrives in a low oxygen environment that is prevalent in cancer tissue and hence, we examined how a low oxygen environment can influence infection dynamics using sequencing technologies that probe the genomic constitution within cells. Our findings revealed that *F. nucleatum* can escape recognition in low oxygen environments and can modulate the expression of multiple cancer-related programs within the cell to drive cancer progression. Insights gained from this research will pave the way for future studies on the impact of the tumor-associated microbes in cancer and will identify novel targets for therapy and clinical intervention to control bacteria-induced exacerbation of cancer.

Dedication

*This work is dedicated to my parents, my brother, my wife,
and to all my peers and colleagues working on therapies and treatments for cancer.*

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1 Introduction

Some sections of this chapter have been adapted from:

Microfluidics in Microbiome and Cancer Research

Barath Udayasuryan, Tam T. D. Nguyen, Daniel J. Slade, Scott S. Verbridge

Book Chapter. Nanotechnology and Microfluidics. Wiley Publications. (2019)

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Research into the role of the microbiome in human health is a burgeoning field of study that is poised to create a major paradigm shift in the way we approach the treatment and prevention of a vast number of diseases. With the discovery that the microbiome can profoundly influence digestion, metabolite production, drug absorption and metabolism, pharmacokinetics, and immunomodulation, efforts have been directed towards understanding the molecular mechanisms microbes use to regulate these processes. A stable host-microbiome interaction is requisite for normal gastrointestinal physiology and its alteration leads to a plethora of undesirable consequences including opportunistic infections, inflammatory diseases, cardiovascular disease, and even disruptive effects on the brain and endocrine systems. However, a significant bottleneck in this field is a lack of mechanistic understanding of individual microbiota and how they function holistically in a complex and diverse community, as seen within the gut epithelium. Additional evidence implicates the role of specific bacteria in cancer initiation and progression through the effects of immunomodulation and chronic inflammation. Studies investigating these key links between the microbiome and cancer are being brought together through interdisciplinary research bridging advances in biomaterials, tissue engineering, genetic engineering, systems biology, microfluidics, and microbiology. Microfluidic technologies combining these disciplines hold the promise of delineating

host-microbe crosstalk and community dynamics, as well as unravelling the cellular and molecular mechanisms and interactions initiated by these bacteria.

The microbiome can be leveraged to develop novel diagnostic and prognostic tools to help prevent and manage disease. Probiotics are already available as dietary elements or prescriptions and fecal microbial transplantations (bacteriotherapy) appear to be promising therapies for certain dysbiosis-associated diseases (e.g. *Clostridium difficile* infections)¹ and recently have also been shown to help patients on cancer immunotherapy drugs (PD-1 blockers)². Alterations in the gut microbiome have also been speculated to play a pathophysiological role in brain diseases and psychological disorders^{3,4}. Furthermore, influences of the mycobiome and virome add further complexity to this intricate ecosystem that exists within our body^{5,6}. Thus, there is tremendous opportunity to exploit the microbial functionalities to benefit human health, and the first step towards that is to identify and understand their basic cellular and molecular interactions amongst the microbes and with the host.

There is a growing consensus that bacteria have an active role to play within the tumor microenvironment (TME). The systematic characterization of the tumor microbiome has revealed the presence of multiple and specific species of bacteria within the tumor compared to their adjacent healthy tissue in multiple tumor types. However, the roles and functions of these microbes within the tumor remains poorly understood. Furthermore, it is unclear how normally commensal bacteria, such as *Fusobacterium nucleatum*, can turn pathogenic and influence cancer progression. This dissertation aims to shed light on some of the mechanisms that *F. nucleatum* utilizes to specifically bind to and invade tumor cells and induce alterations in cytokine secretion and modulate gene expression at the transcriptomic and epigenomic level to impact cell signaling, alter cell motility, and ultimately exacerbate tumor progression in colorectal cancer and pancreatic cancer. Furthermore, it aims to understand how specific features of the TME such as low-oxygen environments (hypoxia) impact tumor phenotype and consequently, the response to *F. nucleatum* infection.

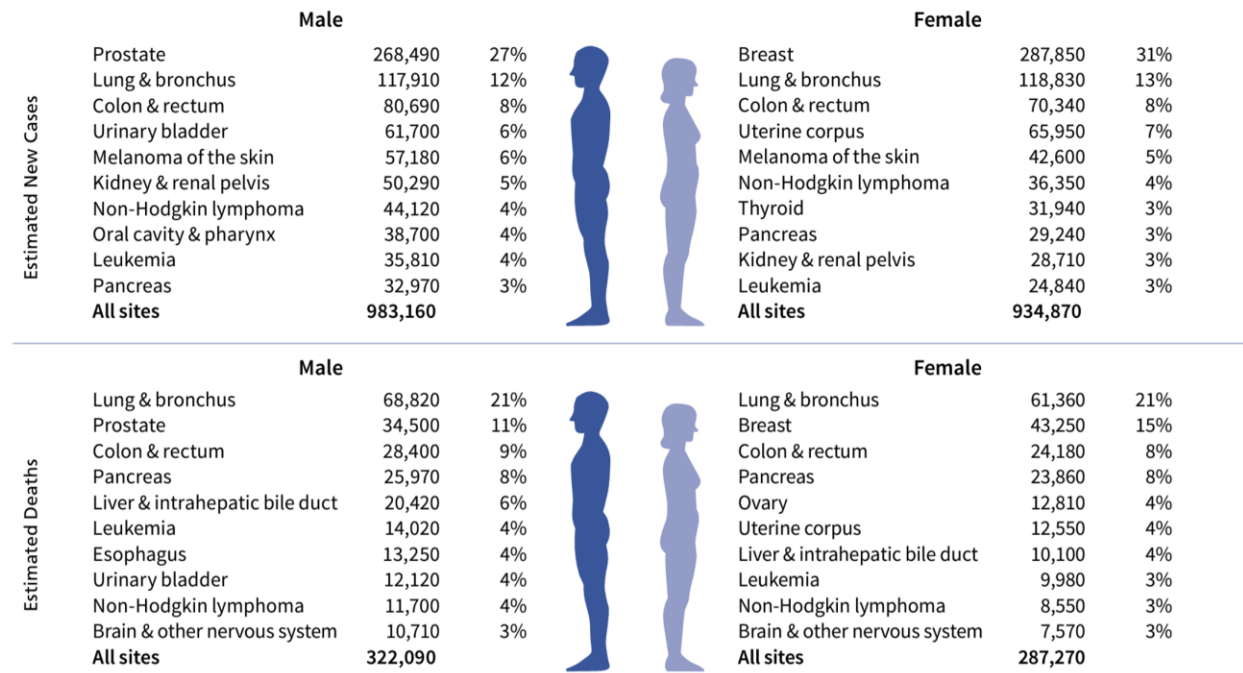
This chapter will provide an introduction to the role of microbiome in human health and disease and introduce the scope and layout of the dissertation.

1.1 Cancer – By the Numbers

The National Institutes of Health (NIH) predicts that in 2022, an estimated 1,918, 030 new cases of cancer will be diagnosed in the United States and 609,360 people are projected to die from the disease⁷. Despite significant advancements in early detection and treatment, mortality rates still remain staggeringly high: 181.4 per 100,000 men and 131.1 per 100,000 women. Furthermore, the NIH approximates that an astounding 39.5% of men and women will be diagnosed with cancer at some point during their lifetimes. Thus, it is crucial to understand this disease at every scale, from macroscopic to molecular and genetic levels, and to identify potential targets for therapeutic intervention.

The specific cancers that are pertinent to this dissertation are colorectal cancer (CRC) and pancreatic cancer. CRC is the third leading cause of death of both men and women in the United States⁸. It is predicted that 151,030 individuals will be newly diagnosed with CRC in 2022 and there will be an estimated 52,580 deaths this year alone (**Figure 1.1**). The 5-year survival rate upon diagnosis for CRC is 65% (**Figure 1.2**). Pancreatic cancer is the seventh leading cause of cancer-related deaths worldwide⁹. In the United States, it is predicted that there will be 62,210 new cases and 49,830 deaths in 2022 (**Figure 1.1**). It continues to be highly lethal since patients exhibit symptoms only at an advanced stage by which time the cancer may have metastasized and it becomes difficult to treat. It had caused 432,242 deaths worldwide in 2018 and its 5-year survival rate is only 11% (**Figure 1.2**).

Leading Sites of New Cancer Cases and Deaths – 2022 Estimates



Estimates are rounded to the nearest 10, and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder. Estimates do not include Puerto Rico or other US territories. Ranking is based on modeled projections and may differ from the most recent observed data.

Figure 1.1 The leading sites of new cancer cases and deaths in 2022 in the United States.

This table presents data on the estimated new cancer cases and deaths in the United States based on cancer type in males and females in 2022. Used with permission from⁷.

Five-year Relative Survival Rates* (%) by Stage at Diagnosis, US, 2011-2017

	All stages	Local	Regional	Distant		All stages	Local	Regional	Distant
Breast (female)	90	99	86	29	Oral cavity & pharynx	67	85	68	40
Colon & rectum	65	91	72	15	Ovary	49	93	75	30
Colon	64	91	72	14	Pancreas	11	42	14	3
Rectum	67	90	73	17	Prostate	98	>99	>99	31
Esophagus	20	46	26	5	Stomach	32	70	32	6
Kidney†	76	93	71	14	Testis	95	99	96	73
Larynx	61	78	46	34	Thyroid	98	>99	98	53
Liver‡	20	35	12	3	Urinary bladder§	77	70	38	6
Lung & bronchus	22	60	33	6	Uterine cervix	66	92	58	18
Melanoma of the skin	93	99	68	30	Uterine corpus	81	95	69	18

*Rates are adjusted for normal life expectancy and are based on cases diagnosed in the SEER 18 areas from 2011-2017, all followed through 2018. Rates by stage reflect Combined Summary Stage 2004+ except for testicular cancer, which is based on Combined Summary Stage 2000 (2004-2017). †Includes renal pelvis. ‡Includes intrahepatic bile duct. §Rate for in situ cases is 96%.

Local: an invasive malignant cancer confined entirely to the organ of origin. **Regional:** a malignant cancer that 1) has extended beyond the limits of the organ of origin directly into surrounding organs or tissues; 2) involves regional lymph nodes; or 3) has both regional extension and involvement of regional lymph nodes. **Distant:** a malignant cancer that has spread to parts of the body remote from the primary tumor either by direct extension or by discontinuous metastasis to distant organs, tissues, or via the lymphatic system to distant lymph nodes.

Sources: SEER*Explorer, National Cancer Institute, 2021. Available from <https://seer.cancer.gov/explorer/>. Testicular cancer survival by stage was calculated using SEER*Stat software (version 8.3.9), National Cancer Institute, 2021.

Figure 1.2 The five-year survival rates of cancer by stage at diagnosis in the United States.

This table presents the five-year relative survival rates of various types of cancer based on its stage at diagnosis (local, regional, distant). Used with permission from⁷.

1.2 What is the Microbiome?

The microbiota consists of the entire collection of microbes present in the myriad tissues of the body including the skin, mammary glands, placenta, seminal fluid, uterus, ovarian follicles, lung, saliva, oral mucosa, conjunctiva, biliary and gastrointestinal tracts. The microbiome refers to the complete genomic constitution of these microbiota, although these terms are sometimes used interchangeably. Among these, 97% are within the colon and the remaining 2-3% are extracolonic¹⁰. Current estimates show that at least 3.8×10^{13} microorganisms spanning over 3000 species inhabit the human intestines and form their own micro-ecosystem¹⁰. Incredibly, the number of human cells is estimated to be around 3.0×10^{13} cells which means that the microbes outnumber the human cells 1.3:1. These microbes have a strong influence on human health and disease¹¹. Most of these organisms are commensal and maintain a symbiotic relationship with the host, however opportunistic pathogens can also be present under certain microenvironmental conditions¹². Additionally, this complex ecosystem includes species that are not present anywhere else in nature possibly indicating co-evolution with the host over several millennia¹³.

The gut microbiota is often collectively thought of as the “forgotten organ” and live in mutually advantageous harmony with their human host, a state termed as eubiosis. However, a number of factors can lead to dysbiosis, perturbing the equilibrium, which, in turn, has been found to potentiate the development and progression of neoplasms by a number of mechanisms including damaging DNA, activating oncogenic signaling pathways, producing tumor-promoting metabolites, suppressing anti-tumor immunity, and modulating responses to cancer therapy¹⁴⁻¹⁷. The compositional perturbation due to dysbiosis has been associated with disease states such as obesity, diabetes, colorectal cancer and allergies¹⁷.

Realization of the association between bacteria and tumors is not new and has been reported in patients for decades¹⁸. Several species of bacteria have been implicated in tumorigenesis including

Clostridium sp., *Salmonella sp.*, *Bifidobacterium sp.*, *Campylobacter jejuni*, *Citrobacter rodentium*, *Escherichia coli*, *Vibrio cholerae*, and *Listeria monocytogenes*¹⁹. However, there is still a lack of substantial direct evidence that microbiota modulate carcinogenesis in humans and their role is quite complex as bacteria have been shown to exhibit both pro- and anti-carcinogenic effects¹³. Evidence of microbial presence within CRC and pancreatic cancer show a correlative role with reduced patients' survival^{20,21}. In fact, pathological imbalance of the gut microbiome in CRC results in dysbiosis and may contribute to tumorigenesis by altering metabolite secretion and modulating the host-immune response. The most prominent among the CRC-associated bacteria are *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Parvimonas*, *Bacteroides*, and *Gemella*²².

1.3 *Fusobacterium nucleatum* and Colorectal Cancer

Fusobacterium nucleatum has been found to play a significant role in the progression of CRC. *F. nucleatum* is an adhesive bacterium and normally co-aggregates with various microbial species in the oral cavity and is usually associated with gingivitis and periodontitis²³. Its initial link to cancer was discovered when highly abundant *F. nucleatum* gene signatures were found in biobanked colorectal carcinoma (CRC) using metagenomics methods^{16,23}. They were highly enriched in human CRCs compared to adjacent normal tissue. Further research showed that *F. nucleatum* accelerates CRC in pre-clinical models using both *in vitro* and *in vivo* systems²³. It has been shown that in *Apc^{Min/+}* mice (adenomatous polyposis coli (APC) multiple intestinal neoplasia (Min) murine model), there was increased carcinogenesis with the introduction of *Fusobacterium nucleatum* or *Bacteroides fragilis*^{24,25}. These studies, however, have not been confirmed by observational studies in humans.

The mechanism of enrichment of *F. nucleatum* is not clear. However, a few proteins have recently been discovered that appear to facilitate localization and internalization of this bacterium into the host. This bacterium encodes several adhesins for interspecies interactions, including Fap2, RadD, FadA, and Aid1. It binds to and invades a variety of mammalian cells including epithelial and

endothelial cells, monocytes, erythrocytes, keratinocytes, fibroblasts, HeLa cells and NK cells, and interacts with host molecules such as salivary macromolecules, extracellular matrix proteins, human IgG, and cadherins^{23,26}. Most recently, it was shown that *F. nucleatum* is present in distant hepatic and lymph node metastases of CRC suggesting that this pathogenic bacterium could potentially migrate along with colorectal cancer cells to metastatic sites in the human body²⁷.

Adherence and invasion are believed to be essential mechanisms for colonization, dissemination, evasion of host defense, and induction of host response. It was found that binding interactions between Fap2 and the host lectin Gal-GalNAc (D-galactose- β (1-3)-N-acetyl-D-galactoseamine), which is overexpressed in human colorectal adenocarcinoma, may provide an explanation for localization. Intravenously injected *F. nucleatum* localizes to mouse tumor tissues in a Fap2-dependent manner²⁵. Fap2 also mediates binding to erythrocytes possibly confirming a hematogenous route to the tumor. Another protein, the fusobacterial FadA protein, binds to host E-cadherin, activates β -catenin signaling, and differentially regulates the inflammatory and oncogenic responses. This leads to suppression of anti-tumor immunity and inhibits tumor killing by natural killer (NK) cells. In addition, FplA (Fusobacterium phospholipase autotransporter) from *F. nucleatum* is a Type Vd autotransporter phospholipase was found potentially play a role in altered host signaling and evasion of autophagy²⁶. These Type V secreted autotransporters are large Gram-negative specific bacterial virulence that *F. nucleatum* can use to bind to and invade host cells. Once intracellular, FplA could play critical roles in phagosomal escape, subversion of autophagy, or eicosanoid mediated inflammatory signaling, and it was shown that FplA binds with high affinity to host phosphoinositide signaling lipids critical to these processes²⁶. Characterization of these secreted autotransporters and other virulence factors is currently an area of active research²⁸.

Apart from microbial associations in gastric cancer and colorectal cancers, there have been studies linking gastrointestinal microbiota in cancers present in other organs including the pancreas^{29,30}, liver³¹, prostate³², and breast tissue^{33,34}. For example, *Helicobacter hepaticus* along with *Helicobacter*

pylori play a role in toxin and virus induced hepatocellular carcinoma³⁵ and *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, members of the oral microbiome, have been associated with an increased risk of pancreatic cancer³⁶. *Fusobacterium nucleatum* and *Granulicatella adiacens* were found to be enriched in the intraductal papillary mucinous neoplasms (IPMNs) of patients³⁷. IPMNs are pancreatic cysts which can progress to invasive pancreatic cancer and further investigation is needed to understand the role of microbes in these cancers. Interestingly, *Porphyromonas gingivalis* was recently identified in the brain of Alzheimer's disease patients giving rise to new questions about bacterial invasion and activity in disease progression generally thought to be independent of microbe involvement³⁸.

1.4 Objectives

Very few studies have investigated the role of the tumor microbiome in cancer initiation and progression. There is likely a multitude of roles played by these microbes within the TME, however unraveling individual mechanisms of action needs to be systematically studied. Using *Fusobacterium nucleatum* as a representative example, I investigated the downstream effects of microbial presence on cancer progression to substantiate its characterization as a hallmark of cancer. Favorable features of *F. nucleatum* that makes it tractable for in vitro experiments include: It is an anaerobic bacterium, hence amenable to long-term co-culture with host epithelial cells without over-proliferation in normoxic experimental conditions, the availability of selective antibodies to target this bacterium, and the recent creation of a galactose kinase markerless gene deletion system to create strains with unlimited gene deletions³⁹. Additionally, since this organism is anaerobic, we hypothesized that the hypoxic environment within the TME may favor bacterial infection and survival.

Various approaches can be used to study the microbiome and host-microbiome interactions (**Figure 1.3**). The majority of early findings in this field have been correlative and associative^{40–43}. To obtain actionable insights to develop therapeutic interventions and precision diagnostics leveraging aspects

of the microbiome, it is important to tailor the experiments to the level of complexity and resolution that is required. One can study the macroscopic community diversity and dynamics or zoom into the level of individual bacterial species and its spread within the body or still further probe the molecular interactions of individual proteins and downstream signaling cascades. We have to ability to delve even further to study the impacts on epigenetics with <100 cell resolution and transcriptional alterations that could lead to long-term imprints on the cells. A comprehensive understanding of the role of this microbe will enable the development of therapeutics targeting the specific phenotypes induced by *Fusobacterium* and preventing and controlling further exacerbation of cancer.

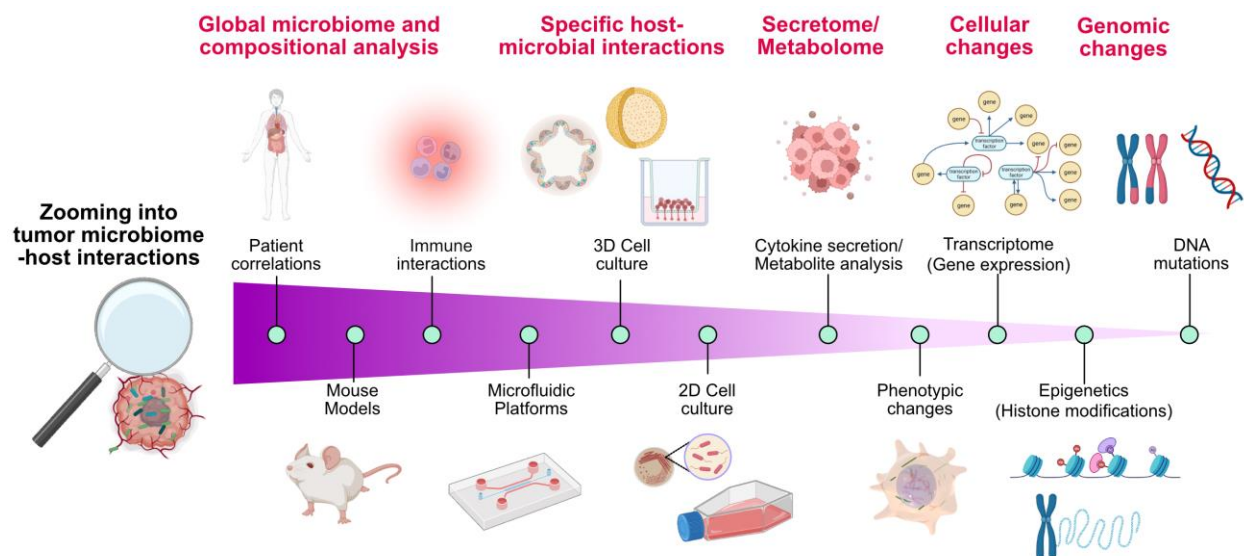


Figure 1.3 Approaches used to study host-microbe interactions

Our overarching hypothesis is that these *oncomicrobes within the TME actively hijack host intrinsic signaling pathways based on the host phenotype for their own survival and dissemination, which in turn leads to adverse reactions by the host in the form of cancer modulation.*

The main objectives that this dissertation sought to examine were:

- Characterize *F. nucleatum* infection of CRC and PDAC in order to understand its contribution towards poor clinical outcomes
- Characterize cytokine secretion and downstream signaling responses elicited by microbe – host interactions in diverse CRC and pancreatic phenotypes
- Understand how hypoxia within the tumor microenvironment can influence infection dynamics
- Identify specific transcriptomic and epigenomic alterations elicited by *F. nucleatum* infection to identify long-term genetic imprints that can impact cancer progression

The main assumptions and limitations that impact these studies include studying host-microbial interactions in isolated systems. The tumor microbiome consists of multiple interacting species of bacteria which may influence the overall physiological observations. However, we only study the impact of this single bacterium and only specific virulent strains that have been previously characterized. Furthermore, we do not incorporate the role of immune cells including neutrophils and macrophages in the current stage of the study which may impact cytokine signaling pathways within the tumor and greatly impact infection dynamics. However, in order to systematically interrogate the varied aspects of host-microbial interactions, it is imperative to build-up individual interactions and in the future, scale-up to multi-species studies as well as increase complexity by incorporating relevant immune cells. We do touch upon some of these aspects, as we continue future work on spheroids and the development of biomimetic tissue platforms that can better recapitulate

the TME. These foundational studies in investigating the tumor microbiome aims to galvanize the research community to focus on further research in this rapidly evolving field.

1.5 Document Organization

This chapter has detailed the main premise of this dissertation and the objectives that I had set out to investigate through the course of my studies. The following chapters dive deeper into the outcomes of my research:

Chapter 2 provides a comprehensive review of the tumor microbiome and the current models that have been used to study the role of microbes in the TME. The aim of this chapter is to provide the reader a high-level understanding of how microbes can play a role in cancer and highlight knowledge gaps in the field. The chapter also describes the tissue-engineered approaches currently used to study host-microbial interactions and perspectives on their continued development. Some of these tools were used in my own work in investigating how *F. nucleatum* impacts cancer progression.

Chapter 3 details my first study on the role of *F. nucleatum* in impacting cell signaling within CRC cells that drives cell migration, a common feature of malignant transformation. We uncovered the role of specific cytokines secreted by host cells upon *F. nucleatum* infection. I show how a specific bacterial lectin, Fap2, can predicate binding and invasion and how intracellular invasion is crucial to explain the observed response of the host cells. I show that immune cells, in contrast, secrete specific cytokines in response to infection, but this response is Fap2-independent. We also explore possible routes of inhibiting binding with small molecules are explored for therapeutic intervention.

Chapter 4 details my second study that focusses on the role of *F. nucleatum* in pancreatic ductal adenocarcinoma (PDAC). I describe how additional cytokines secreted by pancreatic cancer cells and normal pancreatic epithelial cells can influence cancer cell proliferation and migration. I had identified a new role for the cytokine GM-CSF in directly stimulating proliferation of pancreatic cancer cells. Furthermore, I show that infection of normal pancreatic cells also leads to host cytokine

secretions that influence tumor cells. This investigation further implicates a detrimental role of *F. nucleatum* in pancreatic cancer and continues to emphasize the significant role of the tumor microbiome in cancer progression.

Chapter 5 details my final study that ventures into gene expression and epigenomic changes within host cells upon *F. nucleatum* infection. This chapter details how hypoxia (low oxygen environments) within tumors can play a profound role in influencing infection dynamics and the resulting downstream response of the cells. Our findings reveal that *F. nucleatum* is able to subvert host recognition in hypoxic conditions that ensures its survival and sustained activity. This study implores future investigation of host-microbial interactions in TME relevant contexts.

Chapter 6 describes additional related studies investigating *F. nucleatum* infection of spheroids and in exploring bioelectric variations within *F. nucleatum* infected cells that can be exploited for cell separation.

Chapter 7 concludes with a summary of the research and how it connects to the broader field of cancer and tumor microbiome research. I further identify existing knowledge gaps in the field that require investigation and how understanding the tumor microbiome can facilitate cancer diagnosis and the development of therapies.

2 Interrogating host-microbiota crosstalk in cancer

This chapter has been adapted from:

Harnessing Tissue Engineering Tools to Interrogate Host-Microbiota Crosstalk in Cancer

Barath Udayasuryan, Tam T. D. Nguyen, Daniel J. Slade, Scott S. Verbridge.

iScience, Cell Press. (2020) (PubMed ID: 33344921)

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2.1 Summary

The “Hallmarks of Cancer” paradigm has provided a powerful conceptual framework to better understand complex cancer dynamics. This framework has succeeded in laying the groundwork for innovative therapies that target these conserved high-level cancer phenotypes. However, we believe that any enumeration of cancer hallmarks would be incomplete without emphasizing the role of tumor-associated microbiomes. Infection and intracellular localization of these microbes can lead to a number of downstream effects, including inflammatory responses and cytokine release, that may aggravate tumor burden to enhance metastasis and contribute to drug resistance. Recently published systematic characterizations of tumor-specific microbiomes have revealed the presence of distinct combinations of intracellular microbial residents across multiple tumor types. However, the causal relationships and evolution of these microbiotic ecosystems across tumor type and stage remain poorly understood. In order to advance this field and enable targeting of these microbiomes for therapeutic purposes, many challenges remain to be overcome. These include difficulty in isolating any dominant “driver” microorganisms (or microbiome signatures) and separating causation from correlation in complex tumor microenvironments (TMEs). Feasibility of long-term co-cultures with host cells also remains a limitation for the majority of tumor-resident microbes. Because host-

microbe interactions should be studied in a physiological tissue context to accurately reproduce normal as well as pathological processes, animal models have been largely preferred for these studies. Yet these models are often poor mimics of the human TME and are furthermore expensive and challenging to control and interrogate with high spatiotemporal resolution. We believe that *in vitro* three-dimensional tissue engineered platforms could provide a powerful intermediate approach that combines the high-resolution of *in vitro* studies with at least some of the physiological relevance of animal models. This review will focus on tissue engineered approaches to study host-microbe interactions and to establish their role in malignancy as an emerging hallmark of cancer.

2.2 Introduction

The involvement of tumor-specific bacteria, collectively termed the *tumor microbiome*, has garnered significant attention as a key potential regulator of the well-established “Hallmarks of Cancer”. These hallmarks include deregulated proliferation, replicative immortality, genomic instability, evasion of growth suppression, avoidance of immune surveillance, chronic inflammation, angiogenic induction, and the activation of metastatic pathways¹. Bacteria and their secreted metabolites have been implicated in influencing most, if not all, of these host factors². While many *in vitro* models have helped elucidate mechanisms related to tumorigenesis, there are only limited models that are amenable to directly investigate host-microbe interactions, and far fewer in the context of cancer. Similarly, though animal models have been an indispensable tool in microbiome studies associated with cancer, they exhibit significant variability in their resident bacterial species and immune profiles when compared to humans. To address these issues, we believe that tissue engineering provides a unique opportunity to more accurately model and analyze these host-microbe interactions in the tumor microenvironment (TME) that are so critical for tumor progression and therapy response.

Tissue engineered platforms have been developed to study host-microbiome interactions, predominantly in the gut, which harbors the majority of microorganisms in the human body³. In this

review, we highlight the latest insights derived from 2D, 3D, and organ-on-chip platforms that have been used to investigate the interactions within the host-microbial consortia, and explore the potential for adapting these platforms to advance our understanding of the tumor microbiome. Each tissue type has its own specific engineering challenges (i.e., spatial constraints, physical forces, biochemical cues, and growth and differentiation capacities) and hence a personalized, experimentally-tailored approach is preferred for these studies. Furthermore, it is now possible to incorporate advanced sensors and imaging modalities for real-time monitoring of oxygen, pH, barrier permeability, and other biological parameters to recapitulate and analyze host-microbial interactions.

2.3 The Tumor Microbiome

The human microbiome, consisting of trillions of microorganisms co-existing within the human body, has an enormous impact on maintaining health and normal physiology^{4,5}. A dysbiotic microbiome adversely impacts homeostasis which leads to a number of unfavorable outcomes including inflammatory diseases, cardiovascular disease, obesity, diabetes, and can even potentiate cancer initiation and progression^{6,7}. Studies of the microbiome often characterize microbial compositional alterations in disease conditions⁸. However, the inherent complexity and variability in these experiments makes it challenging to derive meaningful conclusions on the microbes' direct impact on cancer progression. This is further compounded by the fact that the gut microbiome can play a dual role by being both tumor-promoting and tumor-restricting. A striking recent example within a mouse model revealed that p53 disabling mutations exhibited divergent effects based on the location of the cells within the gut and its spatially-segregated microbial composition, behaving oncogenic distally, but tumor-suppressive proximally⁹. That microbiotic residents may regulate the action of such an archetypal tumor suppressor gene as p53 suggests that we are only scratching the surface of the role of tumor localized microbiomes in cancer.

Our understanding of the role of microbes in tumor progression has been advanced by next-generation sequencing (NGS), specifically 16S rRNA sequencing, which has uncovered reproducible microbial signatures within a multitude of tumors. Most recently, Nejman et al, identified distinct intracellular bacteria within cancer and immune cells in 1526 tumor samples and their adjacent tissues of seven cancer types: breast, lung, ovary, pancreas, melanoma, bone, and brain tumors¹⁰. The number of bacterial species detected in most of the tumors averaged ~ 9. Interestingly, breast tissue was identified as having the most diverse tumor microbiome with an average of 16.4 species per sample (**Figure 2.1 A-C**). In our own work, we previously examined the impact of molecules secreted by a bacterium that had been identified as present in the breast TME¹¹. This more recent finding by Nejman et al. provides further evidence that local tumor microbiomes may play a critical role in multiple cancer types. However, critical interactions and modes of tumor regulation have largely only been studied in the context of gastrointestinal cancers.

2.3.1 The Role of Specific Microbes in Tumorigenesis

Many seminal studies have shown that individual microbial species play a role in the onset and progression of multiple cancers. Well known examples include *Helicobacter pylori* in gastric cancer^{12,13} and MALT lymphoma¹⁴, *Salmonella typhi* in gallbladder cancer¹⁵, *Streptococcus bovis* in colon cancer¹⁶, and *Chlamydia pneumoniae* in lung cancer¹⁷. Several associations including the discovery of *Fusobacterium nucleatum* within CRC¹⁸, high abundance of *Acidovorax temporans* in lung cancers with TP53 mutations¹⁹, and variations in the oral and gut microbiome of melanoma patients undergoing PD-1 immunotherapy²⁰, indicate a correlative role of the human microbiome with cancer. In addition, intracellular organisms can also directly impact chemotherapy regimens. For example, Gammaproteobacteria within pancreatic ductal adenocarcinoma (PDAC) can metabolize the chemotherapeutic drug gemcitabine²¹. The most dramatic consequence is the lowering of patient survival with the presence of these bacteria in the tumor.

From prior work largely on colorectal cancer (CRC) it is known that bacteria within tumors can cause chronic inflammation or produce and release toxins that impact the cell cycle and induce DNA damage that leads to tumor initiating or promoting mutations²². Microbes in the TME can also influence tissue remodeling and deregulate mucosal immunity, creating a favorable niche for tumor cells to expand and migrate²³. Moreover, bacteria can induce epigenetic alterations upon gaining intracellular access that can activate dormant tumor-promoting genes²⁴.

As a prototypical oncomicrobe that has received significant attention, *Fusobacterium nucleatum*'s involvement in CRC has been extensively characterized in recent years and serves as a prime example to highlight the multiple mechanisms pathogens can use to impact cancer progression. High *Fusobacterium* levels in tumors correlate with decreased patient survival in CRC^{25,26} and pancreatic cancer²⁷. An oral commensal microbe, it has been associated with periodontitis, gingivitis, and multiple extra-oral diseases. Its surface lectin, Fap2, targets the host carbohydrate Gal/Gal-NAc^{28,29} that is overexpressed on many cancers³⁰ and may explain how these bacteria are found in higher abundance in CRC compared to the adjacent healthy tissue. Strikingly, it was found that *Fusobacteria* can travel intracellularly within a migrating host CRC cell leading to bacterial seeding at distant sites such as the liver³¹(**Figure 2.1D**), yet it was unclear if these bacteria were active or passive participants in this process. We more recently provided an early clue as to the answer to this latter question, demonstrating that the cytokines IL8 and CXCL1 are specifically secreted upon *F. nucleatum* invasion of HCT116 CRC cells and contribute to enhancing cancer cell migration directly³² (**Figure 2.1E**). This bacterium is able to induce alterations even at the epigenomic level, where it was discovered that *F. nucleatum* infection, in conjunction with *Hungatella hatheway*, induces the hypermethylation of tumor suppressor gene promoters in colonic epithelial tissue³³ (**Figure 2.1F**).

2.3.2 Unanswered Questions

These observations have generated a number of fundamental questions that we believe should be a broad focus of researchers across multiple cancer types beyond the gut, including:

- *Are bacteria seeded early on in tumorigenesis, thereby actively contributing to tumor initiation, or do they arrive at later stages?*
- *Are specific features of the TME favorable for bacterial localization or survival/proliferation?*
- *How do the bacteria modify the TME?*
- *Are there cooperative relationships among multiple bacteria types within the TME, or between tumor and bacteria?*
- *What factors govern the bacterial interactions with tumor-associated immune cells?*
- *Does elimination of the internalized microbes reverse their effect on the tumor?*
- *Can microbial signatures be used to identify the type or stage of the tumor?*
- *Why do some tumors host more diverse microbiota than others?*
- *Is tissue or niche-specific bacterial colonization useful to deliver therapies?*

The consequences of these microbe-microbe and host-microbe interactions may materialize over long time-scales. Nejman et al. suggest that there may be a low level of bacteria in every tissue and bacterial translocation increases after disruption of the epithelial barrier and increased vascular permeability¹⁰. Furthermore, evidence of alterations in metabolic profiles of host cells and the internalized bacteria³⁴, as well as the secretion of inflammatory cytokines, that may dramatically impact the hallmarks of cancer, anticipate challenges in unraveling the effects of individual secreted factors. Answers to these questions will help identify novel therapeutic targets and reshape current cancer treatment procedures. However, many of these questions have yet to be addressed directly due to a lack of representative models to study tumor-resident bacteria.

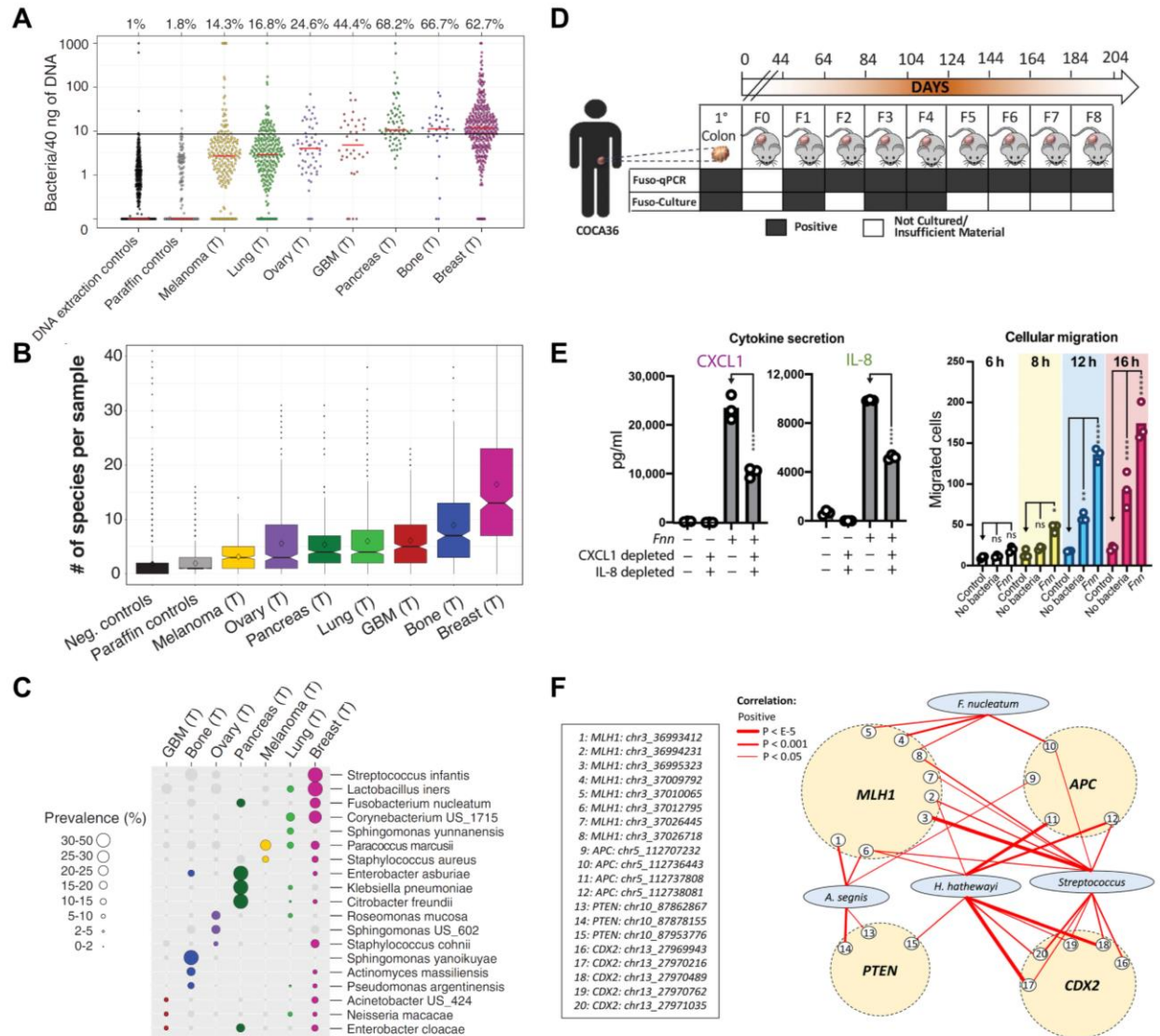


Figure 2.1 Characterization of the Tumor Microbiome

(A) Nejman et al. characterized the cancer microbiome profile of 1526 human tumors across seven different tumor types. The presence of bacteria was assessed by bacterial 16S rDNA qPCR. (B) There is high diversity of microbial species across the tumor types. (C) Nejman et al. also characterized the prevalence of 19 bacterial species across the different tumor types. Reproduced from Nejman et al., 2020 (D) Bullman et al. demonstrated persistence of *F. nucleatum* in patient-derived xenografts over a period of 204 days. Reproduced with permission from Bullman et al., 2017 (E) Casasanta et al. showed *Fusobacterium nucleatum* induced IL-8 and CXCL1 secretion from HCT116 colorectal cancer cells, driving cell migration *in vitro* using *F. nucleatum* conditioned media and that could be blocked by inhibiting bacterial internalization. Reproduced with permission from Casasanta et al., 2020. (F) Xia et al. showed that increased positive correlation of interactions involving *Fusobacterium nucleatum* and *Hungatella hathewayi* and the genes MLH1, APC, PTEN, and CDX2 in colorectal cancer (CC-BY-4.0 (<https://creativecommons.org/licenses/by/4.0/>)).

2.4 Methods to Study Host-Microbial Interactions

To systematically interrogate host-microbe interactions, targeted questions must be defined in order to select appropriate experimental protocols³⁵. Experiments in animals and humans have generally been limited to overall population/compositional studies via 16S ribosomal RNA gene sequencing and shotgun metagenomics, due to difficulties in isolation and sampling and downstream culture of bacteria³⁶. Although focusing on mechanistic studies of individual microbes may appear as a low-hanging fruit, microbes exhibit contrasting behaviors when studied within a multi-species community. In fact, many metabolites are only produced in the presence of other microbes³⁷. The secreted metabolites themselves may directly affect the tumor viability and proliferation. For example, we have previously shown that *Pseudomonas aeruginosa* found in breast cancer tissue secretes N-(3-oxododecanoyl)-L-homoserine lactone which modulates viability of MDA-MB-231 and MCF-DCIS.com cells differently depending on the specific culture microenvironment¹¹.

Paradigmatic changes in experimental and conceptual approaches are needed in order to develop a comprehensive understanding of all the factors that influence host-microbiome interactions in cancer. Challenges that have hindered this goal include difficulties in:

- Isolating causal microorganism(s)
- Preventing over-proliferation of single species in a multi-species model
- Accurately replicating *in vivo* physiological geometry and biochemical cues
- Limiting variability in organoid structures and batch-to-batch extracellular matrix (ECM) composition
- Developing cell culture medium supportive of all non-microbial cells within the model
- Culturing anaerobic bacteria in oxygenated models.
- Real-time monitoring of host and microbial cells and their associated biochemical parameters

Overcoming these bottlenecks is crucial to develop technologies to effectively dissect microbial interactions with the host and to target these therapeutically.

2.4.1 Limitations of Animal Models

Animal models are frequently employed due to the availability of powerful genetic tools and physiological relevance to humans. Moreover, with recent advances in whole-animal editing, animal models are becoming increasingly “humanized”, and are ideal for long-term compound studies³⁸. However, current animal models (i.e., xenograft tumor mice models) are poor representatives of human biology since they exhibit distinct immune profiles compared to humans³⁹. Furthermore, animal studies are expensive and not as scalable and accessible as other *in vitro* models. More specifically, when it comes to manipulating signaling molecules or growth factors, there is much less experimental control, making it challenging to add or tune elements that are necessary to mimic a physiological environment. Therefore, it is beneficial to utilize *in vitro* technologies that can provide valuable insights at a fraction of the cost of transgenic animals, and can reduce dependence on animal experimentation at earlier screening or discovery stages of research, or to help dissect specific mechanism in later stages of study.

2.4.2 Embracing Tissue Engineered Models

Tissue engineering evolved as a strategy to build a tissue from the ground up and can prove to be a viable tool to reconstruct physiologically relevant *in vitro* models. These techniques start from seeding cells in decellularized scaffolds, or ECM based hydrogels. The use of stem cells and induced pluripotent stem cells (iPSCs) catapulted this field with organoid technology. Precise and tunable control with microfluidic devices and microelectromechanical systems have added further control and interrogation options to these technologies. Moreover, engineering principles guide the use of mechanical articulation to simulate biophysical cues which influence the differentiation of cells. However, a number of challenges still remain. While developing tissue engineered models, it is essential to recreate the natural homeostatic environment as well as the tumor-specific microenvironment, specifically for tumor-microbiome studies. Some challenges to this include

developing the normal or physiological environment first, before incorporating the pathological tumor element.

2.4.3 Design Considerations for a Complex Tumor Microenvironment

There are a host of factors within the tumor microenvironment that need to be considered for disease modeling. These factors markedly influence the type of microbes that colonize and infect the tumor.

Figure 2.2 exemplifies the microenvironmental parameters to simulate the gut. There exists complex chemical, pH, nutrient, and oxygen gradients throughout the length of the gut that, for certain bacterial species, determine if the colonies are aerobic or anaerobic. The intestinal walls are composed of several different cell types including enterocytes, enteroendocrine cells, Paneth cells, goblet cells, M cells and Tuft cells, each with unique functions. These cells help establish the epithelial barrier. The barrier itself has a high turnover rate and any compromise to barrier may lead to microbial invasion and dissemination. Vascular and lymphatic networks skirt the walls of the intestine. In addition, cancer associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), stromal cells, and myriad immune cells create a highly intricate and dynamic microenvironment. Immune-host interactions are compartmentalized along the length of the intestinal tract which additionally influences microbial diversity. A major factor to consider is mucus secreted by goblet cells which significantly contributes to bacterial spatial aggregation⁴⁰. Biomechanical cues arising from peristalsis of the gut and mucociliary flow invariably influence host cell differentiation and the distribution of bacterial colonies. To further add to the tumor milieu, are the microbes themselves, their virulence proteins, and synthesized metabolites, and a medley of host secreted factors, cytokines, and gradients of soluble factors.

Similar complex microarchitectures can be described for cancers of the breast⁴², pancreas⁴³, lung⁴⁴, and other organs. Tissue engineering strategies endeavor to recreate these complex architectures and organ-type specificities to more accurately recapitulate host-microbial interactions in cancer.

However, it is essential to note that not all components may be required to study a specific interaction and may additionally impact the reproducibility of studies.

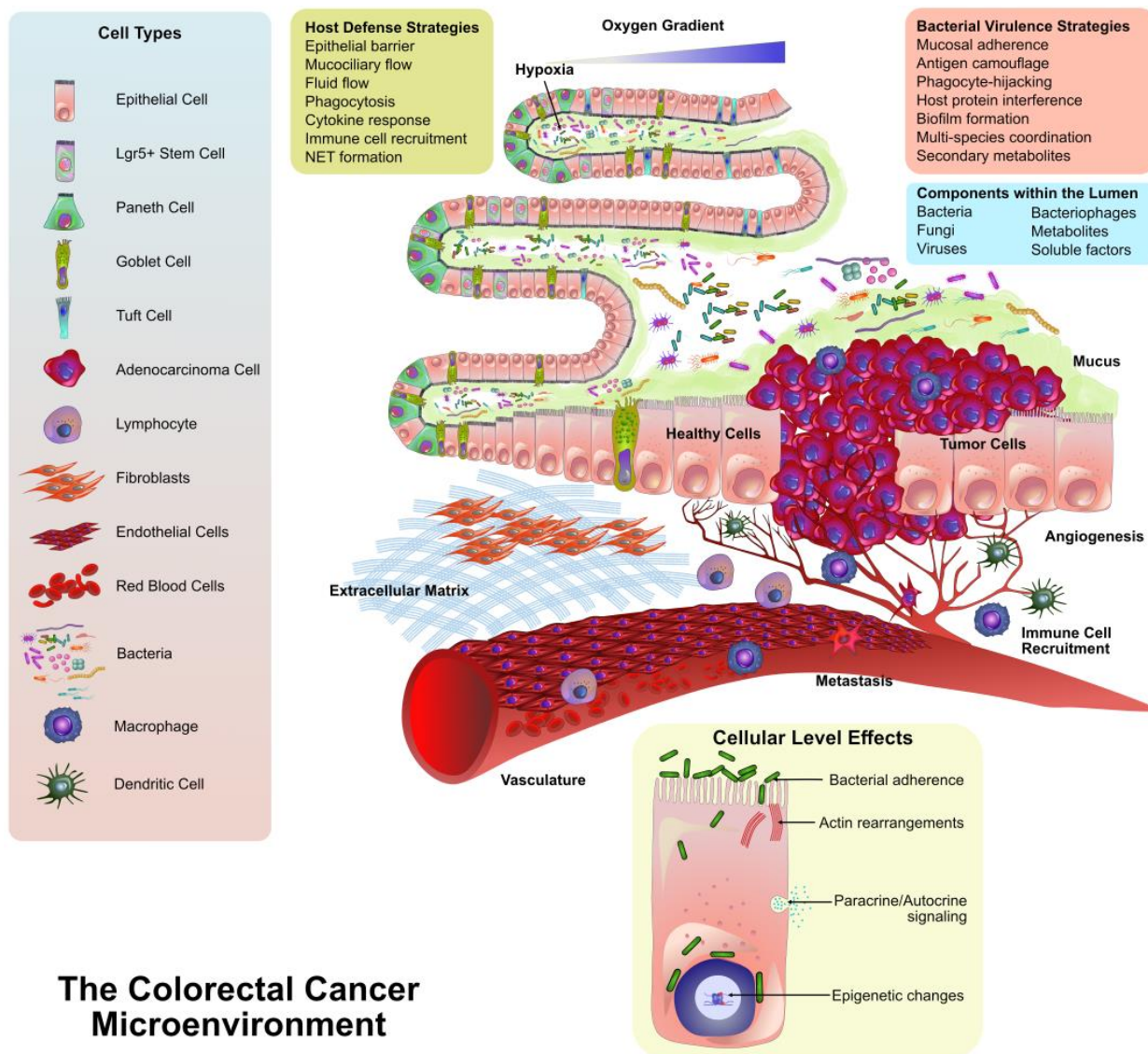


Figure 2.2 A multitude of interacting factors within the complex tumor microenvironment in CRC.

The gut microenvironment is composed of several different cell types including epithelial, endothelial, and immune cells. Thousands of microbial species co-exist within the microenvironment. The host cells maintain the barrier integrity through multiple host defense strategies whereas the microbes utilize multiple virulence strategies to target the host. There are variations in oxygen levels within the folds of the microvilli and the tumor specific microenvironment can exhibit changes in local ECM composition. Vascular networks can be hijacked by tumor cells via angiogenic signaling which can enable cancer cell metastasis. Furthermore, many alterations can occur upon bacterial intracellular invasion that could contribute to tumor formation. (Figure panels adopted from⁴¹)

2.5 In vitro Models

There has been a logical evolution of *in vitro* models of increasing complexity that have enabled the interrogation of host-microbial crosstalk. Despite limitations present in two-dimensional (2D) and 3D cell culture systems, both strategies offer fundamental advantages to explore specific aspects influencing the host-microbiome dynamics. Far from comprehensive, the selected models highlight key features and findings in this field.

2.5.1 2D and 2.5D Models

The simplest model used to study host-microbial interactions consists of human cell lines grown as 2-dimensional (2D) monolayer and inoculating microbes within the culture medium. An advancement to this system is the hanging basket model which suspends a coverslip consisting of a pre-grown multi-species biofilm of pathogenic bacteria over a monolayer of epithelial cells to study gingival inflammation⁴⁵ (**Figure 2.3A**). Using a similar approach to simulate an aerobic-anaerobic interface naturally occurring in the gut, the 'Human oxygen-Bacteria anaerobic' (HoxBan) system⁴⁶ utilized 50 mL culture tubes with host Caco-2 cells attached to a coverslip and positioned over a bacterial culture medium containing *Faecalibacterium prausnitzii* (**Figure 2.3B**).

Transwell-based approaches (sometimes referred to as 2.5D) are widely used to assess migratory and invasive responses of the host cells^{32,47}. This culture format consists of host cells seeded on top of a porous membrane or ECM-deposited membrane, with microbes most commonly introduced to the apical side or conditioned media obtained from infected cells to the lower chamber (**Figure 2.3C**). An advantage of Transwells is that they can be used to produce polarized, differentiated, multi-layer epithelial cultures. Moreover, since an epithelial monolayer harbors apical and basolateral compartments, this feature enables independent analysis of secretomes per direction, explaining this model's popularity.

Recently, Li et al. introduced a 96-deep well plate-based culturing model (MiPro) that conserved the functional and compositional profiles of individual gut microbiomes⁴⁸. The MiPro setup consists of microbiome samples cultured in a 96-deep well plate in which the plate is covered with a silicone-gel cover and shaken at 500 rpm on a digital shaker (**Figure 2.3D**). The authors demonstrated the applicability of this model for high-throughput drug-microbiome interaction studies. More importantly, the MiPro system can be optimized for investigation of host-microbe crosstalk.

Though 2D models offer simple low-cost maintenance and high reproducibility, they fundamentally fail to mimic most of the natural 3D structures of tissues, resulting in the inability to induce complete cell differentiation and recapitulate key physicochemical parameters. More specifically, control of cellular bioactivities is insufficient due to cellular over-proliferation and lack of proper nutrient and oxygen gradients which greatly affects the analysis of host-microbe dynamics. These limitations are similar to those that have contributed to the disappointing track record of 2D culture for screening of cancer drugs, due in part to the non-physiological hyperactive metabolism of cells cultured on hard plastic surfaces⁴⁹.

2.5.2 3D Models

3D cell culture systems provide a relatively young but rapidly maturing approach to addressing the inability of 2D models to reconstitute *in vivo* host-microbiome interactions. Representative examples include the rotating wall vessel, hydrogel scaffolds, organoids and tumor spheroids. While these have been in relatively wide use in tissue engineering broadly, and tumor engineering specifically, we believe there is still great potential to leverage as well as to advance previously developed approaches for tumor-microbiome interaction studies. In this section we will outline some of the more traditional approaches that have already been used to study host-microbe interactions.

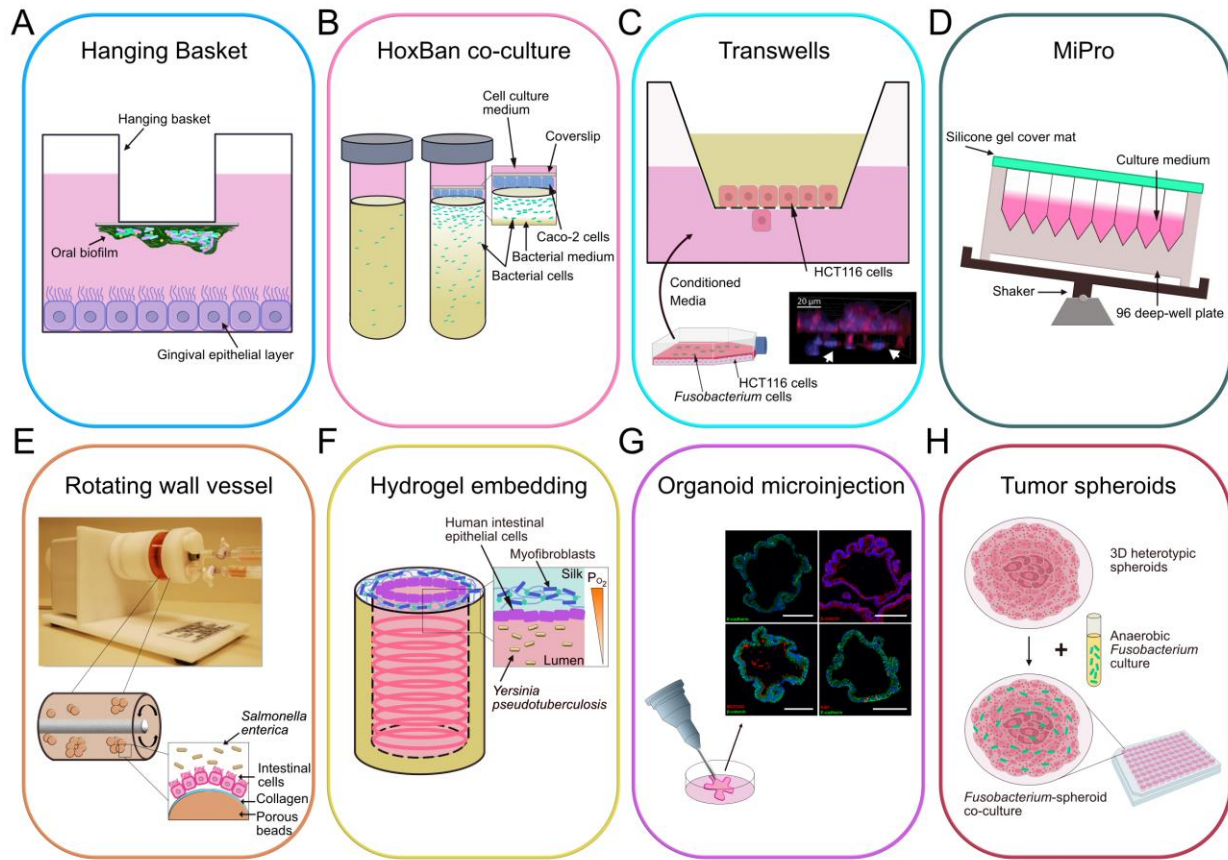


Figure 2.3 Techniques used to study host-microbiome crosstalk.

2D in vitro static models include: (A) the Hanging Basket model used to study gingival inflammation⁴⁵, (B) the HoxBan model which co-cultured *Faecalibacterium prausnitzii* in medium overlaid with Caco-2 cells attached to a coverslip⁴⁶, (C) the Transwell model that studied the migration of host HCT116 cells in response to conditioned media from *Fusobacterium nucleatum* infected HCT116 cells³² (Image reproduced with permission from Casasanta et al., 2020), and (D) The MiPro model utilizes a shaking 96-deep well microplate format to culture microbiome samples. 3D host-microbe culture systems include: (E) the rotating wall vessel (RWV) that induces continuous fluid rotation, ultimately enabling formation of 3D aggregates to model *Salmonella enterica* infection⁵¹, (F) hydrogel embedding, where a 3D porous silk scaffolds was used to reconstitute human intestinal model⁵³, (G) 3D organoid models to mimic host-pathogen interactions⁵⁹. CC-BY-4.0 (<https://creativecommons.org/licenses/by/4.0/>), and (H) tumor spheroids, used to co-culture intra-tumor *Fusobacteria* with colorectal tumor spheroids in a microplate platform³⁴.

The rotating wall vessel (RWV) is a horizontally rotating cell culture chamber with suspended cells. These cells are usually adhered to ECM-coated microcarriers, and rotation results in cell aggregation. This construction induces continuous surface shear stress on the host cells to mimic physiological fluid forces⁵⁰ (**Figure 2.3E**). Radtke et al. implemented this culture method to provide relevant

pathological insights into human enteric *salmonellosis*⁵¹. Using the RWV, Ilhan et al. established a human endometrial epithelial cell (EEC) model. By incubating 13 *Prevotella* clinical strains isolated from the endometrium, vagina, amniotic fluid, and oral cavity with the EEC model, the authors explored species-specific effects of *Prevotella* on physiological and host defense responses in the human endometrial epithelium⁵².

By leveraging biocompatible hydrogel materials and 3D bioprinting technologies, host tissue matrix and functionality can be rebuilt with increased accuracy and robustness. Indeed, recent work has shown successful replication of human intestinal epithelium microarchitecture with months-long functions *in vitro*⁵³. Silk protein was employed to construct a 3D hollow lumen and to house human intestinal epithelial cells which were supported and nourished by surrounding myofibroblasts (**Figure 2.3F**). The authors also showed the applicability of this model to microbiome studies by using it to model *Yersinia pseudotuberculosis* and *Lactobacillus rhamnosus* infections.

Organoid technology has greatly advanced the field of tissue engineering. Since organoids more accurately reproduce the complexity of multi-cellular tissue, these systems provide a more precise picture of the host-microbiome interface. Organoids are generated from multiple sources including adult/fetal tissues, embryonic stem cells, induced pluripotent stem cells, and recently, patient-derived cells^{54–56}. As organoids are typically embedded in extracellular matrices, they can receive matrix cues to facilitate self-organization and specific lineage commitment, resulting in production of near-native epithelial cell clusters⁵⁷. This technology has been used to develop infectious models of pathogenic *Helicobacter pylori* by microinjection into the organoid's lumen^{58,59} (**Figure 2.3G**). Recently, Pleguezuelos-Manzano et al. exposed human intestinal organoids to genotoxic *pks*⁺ *Escherichia coli* by repeated luminal injection over five months⁶⁰. The authors were able to identify that colibactin secreted by *pks*⁺ *E. coli* directly caused distinct mutations in host epithelial cells; potentially putting individuals that harbor this *E. coli* strain at an increased risk of CRC. Combining

organoid technology with the RWV, Barrila et al. demonstrated that incorporation of phagocytic macrophages into this 3D co-culture model revealed the contribution of distinct cell types during host-pathogen interactions of infection⁶¹.

Finally, tumor spheroid models rely exclusively on cellular aggregation of either homotypic or heterotypic cells (**Figure 2.3H**), making this a non-scaffold-based culture method⁶². Several techniques are available for spheroid production including liquid overlay, hanging drop, U-bottom microplates, microfluidic-based assembly, and spinner flasks⁶³. Kasper et al. has introduced a tumor spheroid model that promotes the growth of anaerobic bacteria³⁴. By directly co-culturing 28 *Fusobacterium* clinical isolates, the authors presented a unique model to study intra-tumor anaerobic bacteria and analyze subsequent effects including cancer-related gene expression and metabolomics.

While 3D models offer a middle ground between 2D cell cultures and in vivo models and exhibit more accurate physiological response and can recapitulate native cell-cell interactions, their applicability can be limiting due to limited differentiation capacities, the inability to provide biomechanical cues, constraints in real-time monitoring of host-microbial interactions, lack of oxygen control, and the long-term maintenance of a stably sustained host-microbiome ecosystem.

2.6 Microfluidic and Organ-on-chip Models

Microfluidic organ-on-a-chip models are proving invaluable to dissect microenvironmental factors governing interactions between microbial and human cells, particularly via secreted soluble factors. Fluids are easily manipulated at the microscale allowing for precision tunability and the development of reproducible chemical gradients⁴¹. At this scale, diffusive forces dominate over convective mixing which enables laminar flow profiles to regulate the subtle balances of chemicals and metabolites during infection. The basic template inscribing the fluid flow profile for these devices are constructed through soft lithography techniques predominantly using PDMS (polydimethylsiloxane) polymers.

More sophisticated layered structures can be constructed using microporous membranes. Culture medium is typically perfused through the device using a syringe or peristaltic pump to maintain controlled flow rates that are used to manipulate fluid shear stress on cells, which has been shown to directly impact their differentiation and morphogenesis. Air and pressurized gas chambers have also been incorporated within these devices to recreate the physical cyclic compression of the tissue.

2.6.1 The HuMiX model

The HuMiX device (Human-microbial crosstalk) mimics the human gut, and allows for the analysis of molecular crosstalk between the microbiome and human colorectal adenocarcinoma enterocytes⁶⁴. This system consists of three co-laminar microchannels for medium perfusion, human epithelial cell culture, and bacterial consortia. By providing a proximal 0.5-1 mm partition for human and microbial microchambers across a nanoporous membrane, this perfusion bioreactor reproduces a healthy intact epithelial barrier. Furthermore, the HuMiX setup integrates one dedicated inlet and outlet per microchannel, oxygen sensors, and a commercial chopstick style electrode for precise control of physicochemical parameters, accurate monitoring of oxygen concentrations, and valid measurement of transepithelial electrical resistance (TEER). Moreover, Shah et al. demonstrated how the HuMiX enabled recapitulation of transcriptional, metabolic, and immune responses in human Caco-2 cells after co-culturing with probiotic *Lactobacillus rhamnosus* GG.

2.6.2 Gut-on-a-chip models

Gut-on-a-chip models have advanced the field by incorporating mechanical stimuli to boost cell differentiation and permit real-time monitoring and assessment of microbial contribution to intestinal disease exacerbation. One such representative biomimetic system⁶⁵ contains a microfabricated porous elastic membrane sandwiched between an upper and lower chamber to house intestinal Caco2 cells and endothelial cells (**Figure 2.4 A-B**). The device is equipped with two

hollow lateral vacuum chambers lining the chambers. By applying a cyclic suction, the design effectively induces peristaltic-like motions and trickling-like flow on the intestinal cells. Most importantly, this cyclic strain induced spontaneous villus morphogenesis of the Caco2 cells and differentiated into four lineages of small intestinal cells (absorptive, goblet, enteroendocrine, and Paneth). Using this platform, the authors demonstrated that the addition of lipopolysaccharide (LPS) induced the secretion of the cytokines IL-1 β , IL-6, IL-8, and TNF α into the microvascular chamber replicating chronic inflammatory diseases⁶⁵. In a follow-up study, this system was co-cultured with a living microbiome and maintained viability for a week with a mixed population of eight different facultative or obligate anaerobic, probiotic bacteria (*Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*, and *Streptococcus thermophiles*)⁶⁶. Employing the 'Gut chip'. Grassart et al. revealed that *Shigella* in effect hijacked the host intestinal microarchitecture and mechanical forces to maximize its infectivity⁶⁷ (**Figure 2.4C**).

The Gut-Chip platform demonstrates high adaptability and feasibility to study specific microenvironmental parameters that may influence host-microbe interactions. For example, to support a more diverse microbial community, the Gut Chip was modified to enable precise oxygen control. In the 'Anaerobic-intestine-on-a-chip', Jalili-Firoozinezhad et al. established an oxygen gradient across the endothelial and epithelial interface⁶⁸. Six oxygen quenched fluorescent particles were embedded within the system to monitor oxygen levels. This enhancement increased the duration for a complex microbiota co-culture with patient-derived intestinal epithelium to at least five days. Furthermore, the introduction of *B. fragilis* to the chip was found to enhance the barrier function of the intestinal lining. Using a complementary approach, the Anoxic-Oxic Interface-on-a-chip (AOI) incorporated the simultaneous flow of anoxic and oxic culture medium through dedicated microchannels to recreate an oxygen gradient within the chip⁶⁹ (**Figure 2.4D**). The authors employed TEER and platinum dendrimer-encapsulated nanoparticles to quantify barrier permeability and monitor oxygen gradient, respectively. The AOI chip was used to demonstrate increased viability of

two obligate anaerobic bacteria (*Bifidobacterium adolescentis* and *Eubacterium hallii*) in co-culture with the gut epithelium. The Gut Chip has also been used to model the colonic mucus layer structure and function with the Colon-on-a-chip⁷⁰ (Figure 2.4F) and has been used to study drug transport and metabolism in the Duodenum Intestine chip⁷¹ (Figure 2.4E).

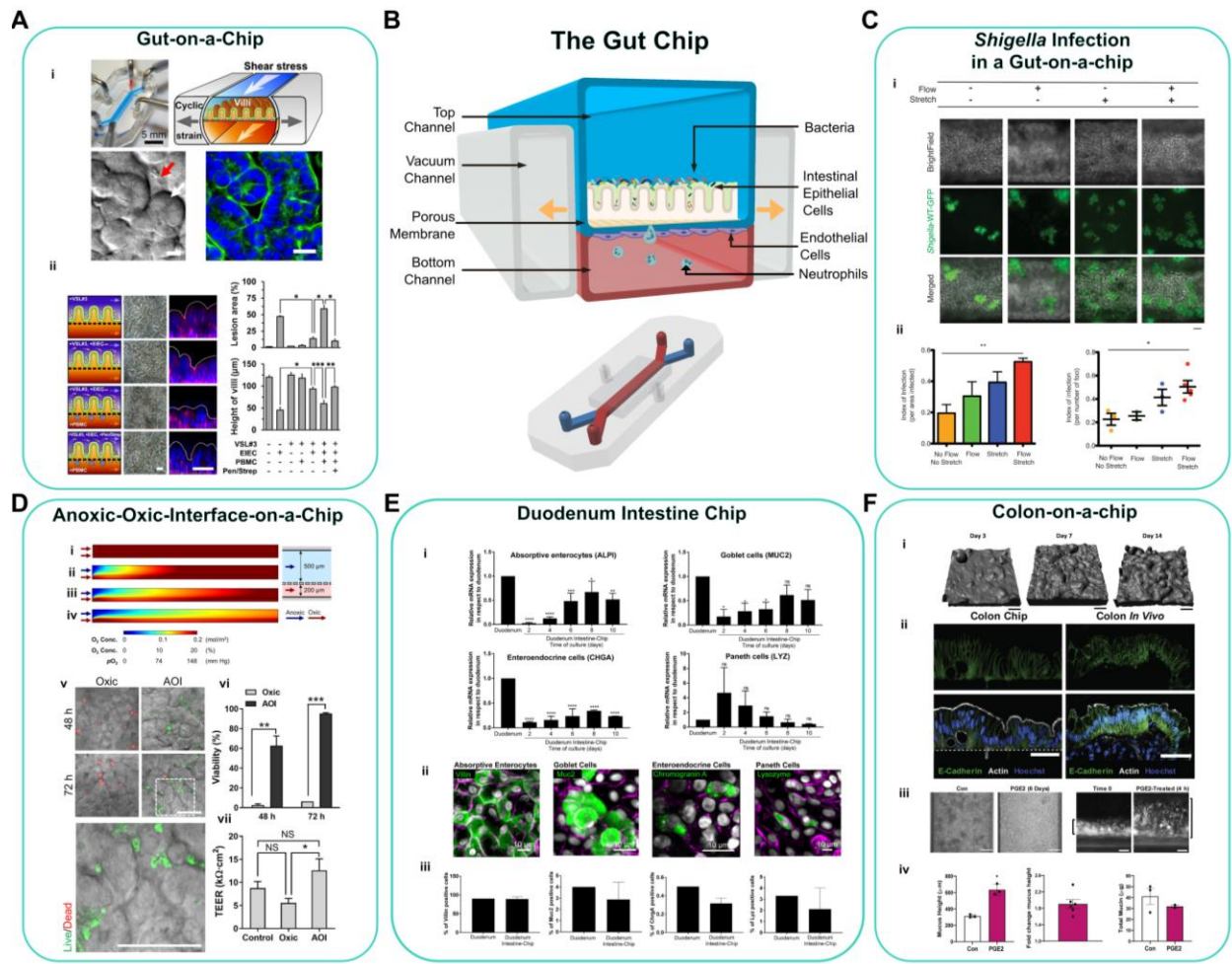


Figure 2.4 The Gut Chip and models derived from it.

(A) The first human-gut-on-a-chip was used to demonstrate that probiotic gut microbiota can protect against enteroinvasive *E. Coli* (EIEC)-induced, immune cell-associated injury on chip with the presence of PBMCs (immune cells) (Reproduced with permission from⁶⁵. (B) Schematic of the Gut Chip. (C) *Shigella*-WT-GFP (green) infections in the Intestine Chip is dependent upon flow and stretch (Reproduced with permission from Grassart et al., 2019). (D) The Anoxic-Oxic interface (AOI) on a chip by generating an oxygen gradient by balancing the flow rates of anoxic and oxic culture medium. The authors co-cultured *B. adolescentis* with the 3D epithelial cells on the AOI Chip which show significantly increased viability and demonstrate enhanced barrier integrity quantified using TEER

(Shin et al., 2019) CC-BY-4.0 (<https://creativecommons.org/licenses/by/4.0/>). (E) The Duodenum Intestine-chip shows multi-lineage differentiation the human intestine and show the expression of markers specific for each differentiated intestinal type (Kasendra et al., 2020) CC-BY-4.0 (<https://creativecommons.org/licenses/by/4.0/>). (F) The Colon-on-chip was used to study PGE2-induced mucus layer swelling on the chip (Sontheimer-Phelps et al., 2020) CC-BY-4.0 (<https://creativecommons.org/licenses/by/4.0/>).

2.6.3 Other Microphysiological Models

Notably, microphysiological developments of gut-on-chip models also give rise to unique physiome-on-chip platforms such as '10-MPS' and 'OrganoPlate' systems^{72,73}. Edington et al. developed microphysiological systems (MPS) supporting '4-way' (liver/immune, lung, gut/immune, and endometrium), '7-way' (4-MPS supplemented with brain, heart, and pancreas), and even '10-way' (7-MPS with kidney, skin, and skeletal muscles) interactions for weeks-long functional relevance. Together, their multi-MPS systems have enabled high-content preclinical drug screening pipeline, and exhibit increasing appeal for studying the dynamics at host-microbe interface. The OrganoPlate, developed in 2017, showed, for the first time, a comprehensive approach to interrogate culture-perfused epithelia tubules that are exposed to an ECM. The OrganoPlate setup consists of 40 microfluidic channel networks integrated in the bottom of a 384 well-plate format, wherein epithelial cells are introduced to a collagen ECM-housing lane adjacent to culture medium lanes. Lanz et al. demonstrated the translational utility of this model through therapy response testing of breast cancer⁷⁴. Kramer et al. took it a step further by placing the plate on a tilted rocking platform which creates height difference, subsequently reproducing microfluidic interstitial flow to model intratumoral pressure in pancreatic ductal adenocarcinoma⁷⁵.

2.6.4 Tumor-on-a-Chip Models

While tissue or organ-microbiome interaction models have shed light on normal physiological processes, tumor-specific microbiomes have not been widely studied in such platforms. Tumor-on-chip models based on organ-on-chip biomimetic principles hold great potential for recreation of human tumor microenvironments and adoption to study host-microbiome crosstalk which may

ultimately reveal both similarities as well as divergences among different tumor types and stages⁷⁶. In this section, we will outline some models that have aimed to recapitulate the classical hallmarks of cancer, including angiogenic induction, immune interactions, biophysical alterations within the TME, and cell migration and metastasis. While we refer the reader to several excellent review topics that go into more depth^{49,76-79}, here we will focus on more recent developments.

Vascularized multi-tissue organ models were the first to incorporate an endothelial layer juxtaposed with an epithelial layer, combined with mechanical stretch. The earliest application of this model was in the design of a lung-on-a-chip that accurately reconstituted the alveolar-capillary interface and its surrounding microenvironment⁸⁰. This biomimetic lung model expanded the capabilities to model other organs including the gut, breast, and pancreas, as well as human cancers. Chips that recreated vasculogenesis and angiogenesis have helped elucidate the molecular mechanisms of angiogenic sprouting and serve as a foundation for future vascularized mechanical, biochemical, and cellular studies^{81,82}. For instance, the in vitro vascularized microtumors (VMT) system encapsulates some of the complexity of the TME by the addition of an ECM and stromal cells with nutrients perfused through microvessels⁸³. Microvasculature embedded within 3D hydrogels are also commonly used in the construction of these devices⁸⁴.

Immune interactions are ubiquitous within the TME and may influence microbial residents. In a multicellular tumor-on-a-chip platform, Aung et al. demonstrated that cancer cell-monocyte interactions increased T cell recruitment⁸⁵. Other platforms have studied the effects of macrophages and neutrophils migration and extravasation⁸⁶

Several microphysiological devices have also emphasized the importance of the TME in influencing cancer progression and treatment. 3D microengineered models of breast cancer, have revealed insights into how the TME could contribute to an invasive phenotype⁸⁷. Using an orthotopic lung-on-a-chip, Hassell et al. identified that physical cues from breathing motions could influence lung cancer

cell growth, invasion, and response to therapy⁸⁸. In addition, the HepaChip® integrated microfluidics and dielectrophoresis to better recapitulate the 3D microenvironment of pancreatic cancer and revealed that higher doses of Cisplatin are needed to reduce the viability of PANC1 cells when cultured in a more physiological context⁸⁹. Other models have investigated starvation-induced tumor cell adaptations and resulting metabolic profiles that influence the development of necrotic cores in large tumors⁹⁰. Platforms such as the Colorectal-tumor-on-a-chip have further enabled studies in nanoparticle distribution in precision nanomedicine⁹¹.

Finally, recent studies that implicate microbes impacting the metastatic potential of tumor makes chips that study this phenomenon highly relevant⁹². Specific examples include devices that monitor *in vitro* metastatic breast and brain tumors and their extravasation to secondary tumor sites^{93,94}.

Taken together, these microfluidic platforms are proving valuable to model intricate interactions to help better understand the development and progression of cancer. With increased relevance of the tumor microbiome in impacting these models, we believe that the development of an integrated tumor microbiome-on-chip will be especially crucial to advance future studies in this field (**Figure 2.5**).

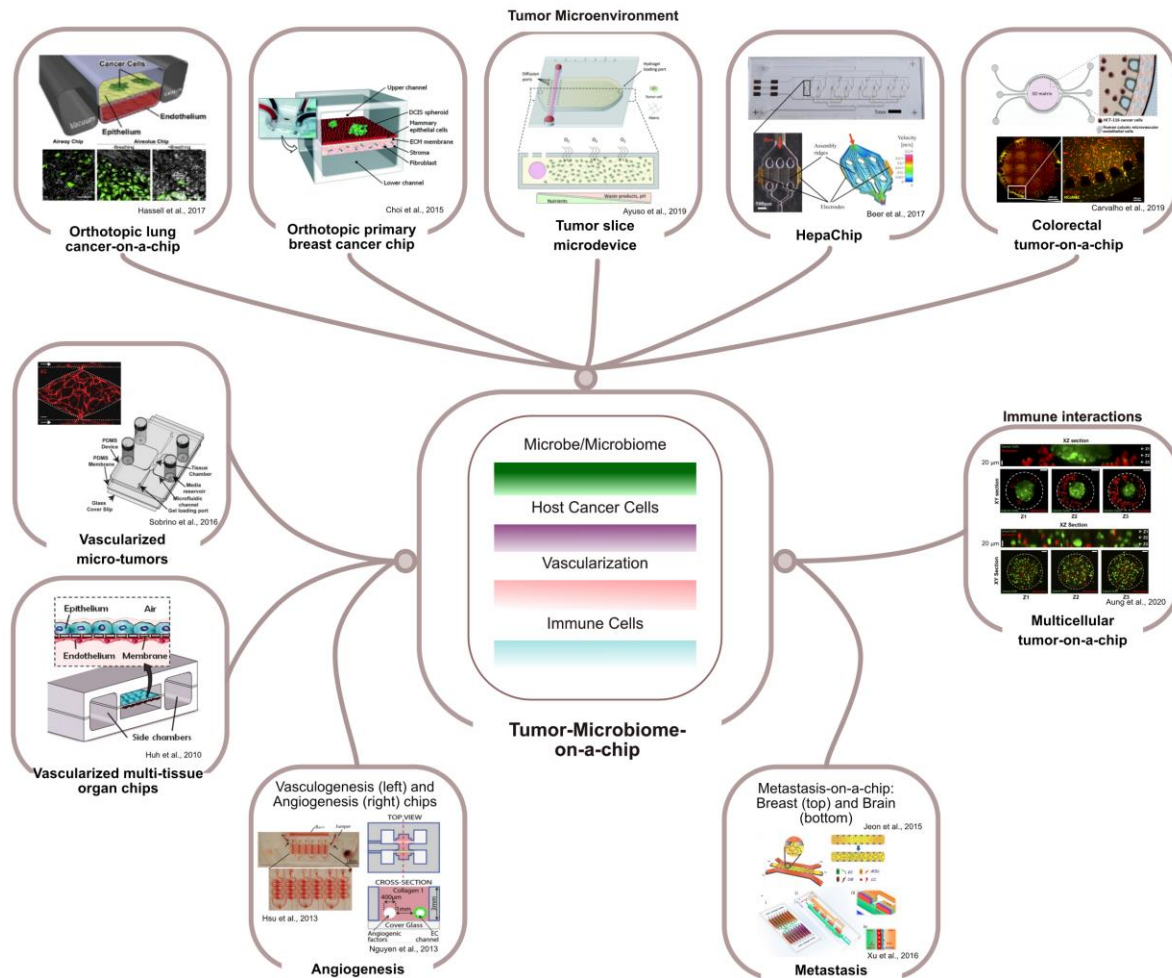


Figure 2.5 Incorporating salient features from Tumor-on-a-chip models to develop a Tumor-Microbiome-on-a-chip Model.

Several tumor-on-a-chip platforms investigate the different aspects of the hallmarks of cancer. The technologies used to build these devices can be adopted to create a tumor-microbiome-on-a-chip that incorporates the necessary elements to characterize tumor-associated microbes.

2.7 Future Perspectives

We believe that the future of host-microbiota studies in the context of cancer should focus on the development of next generation platforms to overcome current challenges including stable culturing of user-defined bacterial communities, advancements in precise differentiation and patterning of cells, improved perfusion capabilities, and incorporating immune cells.

Multiplex devices merging engineering and biology will be critical in this field. Advances on the engineering front will go hand-in-hand with new biological insights. The development of effective biomaterials and scaffolds is critical for recreating physiologically relevant tumor microbiome niches *in vitro*. For example, by making key advancements in collagen hydrogel biomimetic platforms and 3D printed platforms, multiple biomaterials and cell types can be patterned^{95,96} which could include tumor microbiotic participants. Gradients of soluble factors, and even bacteria can be spatially patterned. However, some challenges to overcome are the development of practical bioinks with desired properties, as well as improved mechanical extrusion methods as these can harm cells. Alternative polymers may be used to address the limitations of using PDMS for hypoxic studies.

Modular approaches need to be compatible with different analytical techniques, and modularity can enable the combination of multiple devices. Automated biosensors can be integrated to continually monitor and measure microenvironmental parameters. Furthermore, the commercialization of these technologies will accelerate scale-up, improve robustness, refine usability, and greatly reduce costs⁹⁷. Advancements in analytical techniques including mass spectrometry for proteomic and metabolite analysis, and epigenetic profiling of $< \sim 100$ cells is now possible (MOWChIP)^{98,99} and will complement research in understanding the direct effects of bacteria on tumorigenesis. With respect to visualization, innovative genetic tools are needed to create bacterial mutant strains that express fluorescent proteins for visualization for live microscopy. Advanced imaging technologies such as holographic imaging and light sheet microscopy can improve resolution while live imaging in 3D.

Although complexity can be limitlessly extended and features added, the strengths of simpler 2D and 3D models shouldn't be overlooked. To recapitulate the large number of variables and features to develop a complex, multi-dimensional TME is a daunting task. However, preliminary and pilot studies based on 2D and 3D culture can inform experimentation in more sophisticated platforms. Population models inevitably will have to be performed with animal models. Observations from individual bacterial species need to be connected to multi-species infection models which may vastly alter the metabolome and infection dynamics. Organoids are increasing in complexity and relevance and will play a huge role in studying immune effects on infected organoids as they are a highly tractable system to study immune regulation ⁵⁷.

Hypoxia, angiogenesis, metastasis, and immune dysfunction are some of the leading hallmarks of cancer that are impacted by bacterial presence. The relatively immunosuppressed environment within the TME can define multiple cancer stages and therapy response. Without a vascular interface and immune modulation, the engineered models function in isolation greatly diminishing their physiological relevance. Shear stress, pressure, flow rate, oxygen gradients, vascular permeability, and tissue topography greatly affect bacterial localization and pathogenesis. Lymphatic vessels and interstitial fluid pressure are emerging themes in cancer¹⁰⁰ and provide a route for host cell dissemination. Many microbes are thought to follow a hematogenous route to the tumor and thus, the incorporation of a microvascular and/or lymphatic network is critical to study host bacterial localization and homing. Endothelialized blood vessel models have already been developed for cancer studies which need to be integrated into host-microbial platforms¹⁰¹. The ability to develop and maintain hypoxic gradients is especially crucial to study the pathogenic mechanisms used by anaerobic bacteria in tumor cores and deep within the villus structure of the intestine. Real-time monitoring of oxygen concentrations will assist in validation of the mechanistic insights derived from these anaerobic models. The subsequent challenge will be co-culturing both aerobic and anaerobic species within the same platform.

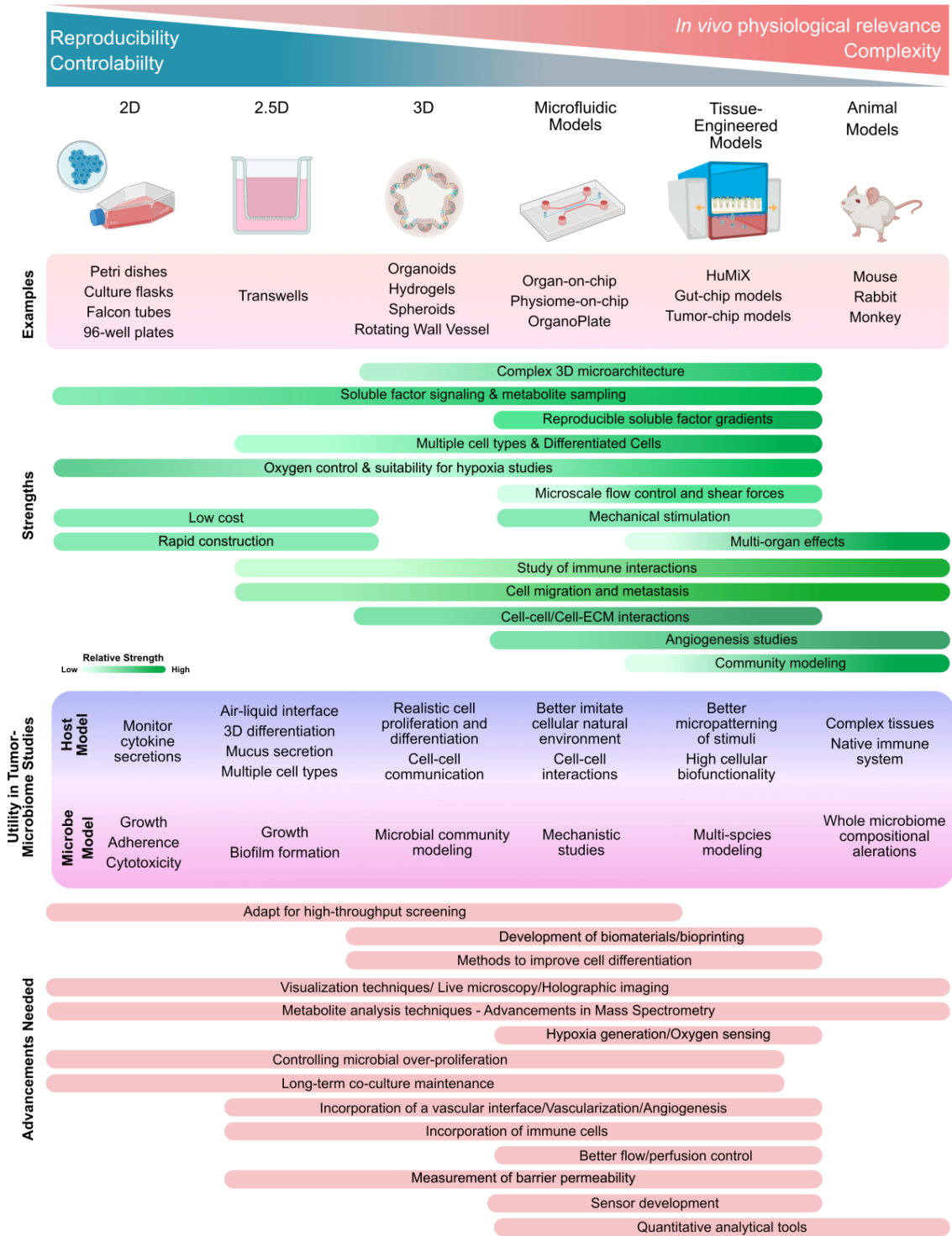


Figure 2.6 Summary of current models.

The strengths of current models, their utility in tumor-microbiome studies, and advancements needed to augment their current capabilities.

As a final note, more advanced platforms and high-throughput technology yield enormous amounts of data that must be efficiently excavated. Here, computational techniques, bioinformatics, and mathematical models can help coalesce disparate data and define experimental parameters. Artificial intelligence and machine learning technologies could be more broadly applied to tissue engineering studies where the degree of complexity may be substantial yet defined in a fully deterministic manner¹⁰². New methods may need to be imported from disparate fields such as ecology and evolutionary biology which may have useful quantitative analytical tools to make sense of such complex interacting systems ¹⁰³.

Ultimately, just as the Hallmarks of Cancer paradigm has led to promising approaches for targeting these hallmarks as therapy, e.g., targeting the altered tumor metabolism, better understanding of tumor-microbe interactions may also reveal new targetable tumor hallmarks. Bacteria can be harnessed to target cancers directly using a Trojan horse approach to deliver drugs to the cells in bacteriotherapy¹⁰⁴. While antibiotic therapy has shown promise for inhibiting tumor growth in animal models³¹, improved models need to be designed to advance such innovations to use for human patients. Important questions must still be answered, such as will targeting and killing intracellular bacteria help control a tumor once it has advanced beyond a certain point, or are there irreversible phenotypic changes driven by these bacteria that occur early in the history of a given tumor? It is likely that the concept of the tumor microbiome could be too intimately connected to the other hallmarks to be truly considered as distinct. In this case, it may be helpful instead to focus on how tumor microbiota influence each of the other hallmarks. For example, in our own work we have made key observations into mechanisms by which *F. nucleatum* may directly drive metastatic phenotypes³².

2.8 Concluding Remarks

The systematic characterization of the tumor microbiome and mounting evidence implicating the role of ‘oncomicrobes’ in cancer indicate a need to revise our current understanding of the hallmarks of cancer. Shifting from broad integrated microbiome studies to more focused studies that characterize the multiple mechanisms that individual or cohorts of pathogens employ to infect cells requires a conceptual shift to develop versatile experimental techniques to dissect host-microbe crosstalk. Despite significant progress in cancer-focused tissue engineering, current technologies do not completely recreate physiologically relevant systems and hence there is still a preference for expensive and sometimes poorly representative animal models. However, with progress in microfluidic and tissue engineered devices there remains much promise in this field. In summary, recent tissue engineering advances in cancer have resulted in exciting new technologies and biomimetic platforms to characterize host-microbial interactions, thereby opening avenues of thought that could give rise to new paradigms of research and precision medicine.

2.9 Acknowledgements

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3 *F. nucleatum* infection in colorectal cancer stimulates cancer cell migration

This chapter has been adapted from:

Fusobacterium nucleatum host cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell line migration.

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3.1 Abstract

Fusobacterium nucleatum is implicated in the acceleration of colorectal cancer (CRC) and is found within metastatic CRC cells in patient biopsies. Here, we found that bacterial invasion of CRC cells and cocultured immune cells induced a differential cytokine secretion that may contribute to CRC metastasis. We used a modified galactose kinase markerless gene-deletion approach to reveal that *F. nucleatum* invaded HCT116 CRC cells in culture by binding to the surface adhesin Fap2. In turn, Fap2-dependent invasion induced the secretion of the pro-inflammatory cytokines IL-8 and CXCL1. These cytokines, which are strongly associated with CRC progression, promoted HCT116 cell migration. Two-chamber migration assays revealed that conditioned media from *F. nucleatum*-infected HCT116 cells caused naïve cells to migrate; however, depleting CXCL1 and IL-8 from the conditioned medium blocked migration. Inhibiting *F. nucleatum* host-cell binding and entry using galactose sugars, L-arginine, neutralizing membrane protein antibodies, or deletion of the *fap2* gene, attenuated HCT116 cytokine secretion and cellular migration. *F. nucleatum* is also known to induce the mobilization of

immune cells in the tumor microenvironment; however, in neutrophils and macrophages the bacterial-induced secretion of cytokines—specifically, CCL3, CXCL2, and TNF α — was Fap2-independent. Thus, our findings show that *F. nucleatum* both directly and indirectly modulates immune and cancer cell signaling and migration. Because increased production of IL-8 and CXCL1 in tumors has been associated with increased metastatic potential and cell seeding, poor prognosis, and enhanced recruitment of tumor-associated macrophages and fibroblasts, we propose that inhibition of host cell binding and invasion, potentially through vaccination or novel galactoside compounds, could be an effective strategy for reducing *F. nucleatum*-associated CRC metastasis.

3.2 Introduction

The role of bacteria and viruses in the onset and progression of diverse cancers is well established¹⁻³. Recent studies on the oral, anaerobic, gram-negative bacterium *Fusobacterium nucleatum* in the acceleration of CRC pathogenesis have revealed as many questions as they have answered for the mechanisms and proteins this bacterium uses to potentiate disease⁴⁻⁹. An overarching question in the field is: How does *F. nucleatum* enter and reside in tumors after likely arriving via the bloodstream from its native oral cavity? In addition, a second theme of pathogenesis that remains understudied is: Are these bacteria capable of leaving the primary tumor on or within immune or cancerous cells to seed and accelerate metastatic cancer sites¹⁰? Answering these questions will be key in understanding both the host and bacterial mechanisms at play in microbe-accelerated cancers. Two studies reported that *F. nucleatum* directly induces cancer cell metastasis through nuclear factor- κ B (NF- κ B)-mediated increased expression of keratin 7 (KRT7)¹¹, as well as increased expression of caspase activation and recruitment domain 3 (CARD3), and downregulation of E-cadherin¹². Our study here provides additional insights regarding the upstream bacterial binding as well as downstream chemokine/cytokine signaling that contribute to *F. nucleatum*-induced cellular migration.

Chemokines/cytokines play a crucial role in tumor initiation, progression, and metastasis¹³. Initially discovered as chemotactic mediators of leukocytes, they are now known to be secreted by several cell types and can be expressed constitutively or induced by inflammatory stimuli, including bacterial infections, and function in a variety of roles including cell survival, proliferation, angiogenesis, and cell migration. In cancer, chemokines mainly function in regulating angiogenesis, activating tumor-specific immune responses, and directly stimulating the tumor through autocrine or paracrine mechanisms¹³. In our study presented here, the results indicate that direct binding and invasion of host cancer and immune cells by *F. nucleatum* induces the secretion of the proinflammatory and prometastatic cytokines IL-8 and CXCL1, and that conditioned media from *F. nucleatum*-infected HCT116 CRC cells cause non-Fusobacterium exposed cells to chemotactically migrate as well.

The cytokines interleukin-8 (IL-8, also known as C-X-C motif chemokine ligand 1 (CXCL8)) and CXCL1 (also known as growth-regulated oncogene α (GRO α)) play a pivotal role in CRC progression. Multiple studies have characterized their role in influencing CRC invasiveness. Increased expression of CXCL1 (measured by immunohistochemistry in tissue microarrays) is correlated with cancer progression and metastasis and ultimately poor prognosis in patients with CRC, thus indicating its potential as a biomarker for CRC¹⁴. CXCL1 is an autocrine growth factor that binds to the receptor CXCR2 with high affinity. Many colorectal adenocarcinoma cell lines (LS174T, KM12L4, KM12C, SW480) constitutively express CXCL1, and it is well known that high levels of this cytokine increase invasive potential¹⁵. Additionally, antibodies to CXCL1 or CXCR2 have been shown to inhibit colon cancer cell proliferation¹⁶. It is relevant for these studies that non-metastatic Caco2 and low-metastatic HT29 cell lines expressed lower levels of CXCL1 than the highly metastatic cancer cell line LS147T. In another study, Ogata et al. showed that CXCL1 increases the number of invasive DLD-1 and LoVo cells, and that these effects are quenched in the presence of anti-CXCL1 antibody¹⁷. Furthermore, there is emerging evidence to indicate that CXCL1 participates in premetastatic niche formation in

liver tissue, which in turn recruits CXCR2-positive myeloid-derived suppressor cells (MDSC) to support liver metastases of CRC¹⁸.

IL-8 is a ubiquitously prevalent cytokine in CRC, where it has been characterized as the most potent chemoattractant and activator of neutrophils in both in vivo and in vitro studies. IL-8 expression at both the mRNA and protein levels is substantially upregulated in pathological colorectal tissues liver metastases¹⁹, and the abundance of both IL-8 and its receptor CXCR2 are increased in colon cancer²⁰. Additionally, increased levels of IL-8 in the serum and the tumor microenvironment enhances the growth of human and mouse colon cancer cells in vivo and promotes the dissemination of cancer cells to the lung and liver²¹. IL-8 can bind to both CXCR1 and CXCR2, but it exerts different effects upon binding to either receptor²²; binding to CXCR1 induces neutrophil migration, whereas binding to CXCR2 modulates angiogenic activity¹³. The angiogenic effect of IL-8 promotes tumor growth by providing access to oxygen and nutrients, as well as an opportunity to metastasize.

Our initial goal was to investigate the role of outer membrane adhesins in *F. nucleatum* direct binding and invasion of cancer cells to determine if this was critical for altered cell signaling. Multiple adhesins have been characterized in *F. nucleatum* binding and signaling, with important roles for the small multimeric adhesin FadA^{6, 23} as well as the large, outer membrane autotransporter adhesin Fap2^{23–25}. Fap2 docks with host cells through Gal/GalNAc sugar residues, which are overexpressed on CRC cells, as well as through protein-protein interactions with the inhibitory receptor TIGIT (T-cell Ig and ITIM domain) on natural killer (NK) cells²⁶. However, aside from these two adhesins, most outer membrane proteins of *F. nucleatum* have not been characterized in the bacterium's interaction with cancer cell. Here, we expanded upon these analyses by developing a new, modified version of a galactose kinase markerless gene deletion system capable of creating strains with unlimited gene deletions. We implemented this system to functionally characterize the role of FadA, Fap2, and multiple uncharacterized Type 5c trimeric autotransporter adhesins (CbpF, FvcB, FvcC, FvcD)^{27, 28}. Our studies reveal that the invasion of HCT116 cells by *F. nucleatum*—which we found can be

inhibited by small molecules, antibodies, or adhesin gene deletions—is critical to the induction of pro-inflammatory signaling that promotes CRC cell motility.

3.3 Results

3.3.1 *F. nucleatum* 23726 outer-membrane adhesins are critical for the binding and invasion of HCT116 CRC cells.

Previous studies established that *F. nucleatum* is highly invasive and can undergo a non-obligate intracellular life stage within epithelial, endothelial, keratinocytes, and potentially immune cells^{29–32}. We first confirmed the invasive potential of *F. nucleatum* subsp. *nucleatum* [American Type Culture Collection (ATCC) strain 23726] into HCT116 CRC cells using fluorescence microscopy (**Figure 3.1, A-C**), imaging flow cytometry (**Figure 3.1D**), and classic flow cytometry (**Figure 3.1E**) by following surface bound and intracellular *F. nucleatum* labeled with a fluorescent green lipid or membrane antisera to detect intracellular and surface-bound bacteria (**Figure 3.1A-C**). To then gain a deeper understanding of how *F. nucleatum* may contribute to the acceleration of CRC, we investigated the importance of direct interactions between *F. nucleatum* and cancer cells and verified bacterial-cancer cell interactions, which are driven by bacterial outer-membrane adhesins. Our goal was to confirm the roles of FadA and Fap2 in the bacterium's binding to, and induction of signaling in, HCT116 cells as well as characterize multiple Type 5c trimeric autotransporter adhesins that have a well-established role in the virulence potential of other gram-negative bacteria, such as *Yersinia*^{33, 34}. In previously reported bioinformatics, we identified five Type 5c adhesins in the strain *F. nucleatum* 23726, with four of these (CbpF, FvcB, FvcC, FvcD) containing all the classic domains that make up a complete adhesin²³. The fifth protein (FvcE) lacks a 'head' domain that is predicted to coordinate adhesion²³ and was therefore not explored here. To characterize these outer membrane adhesins, we developed completely markerless, single-gene knockouts of *fadA*, *fap2*, *cbpF*, *fvcB*, *fvcC*, *fvcD* in *F. nucleatum* 23726, as well as multiple gene deletions per strain using a new version of a galactose

kinase (GalK) genetic system (**Figure 3.7**) previously used in *F. nucleatum*³⁵, as well as many classical studies in *Clostridium*³⁶.

This optimized genetic system relies on a base strain lacking the galK and galT gene operon, thereby rendering it sensitive to growth in the presence of galactose (**Figures 3.8, 3.9**). The *F. nucleatum* Δ galKT strain (Hereby called *Fnn*) has no growth defects (**Figure 3.9**), as well as not being attenuated for binding or invasion with colorectal cancer cells (**Figure 3.1E**). Target gene deletion is initiated by construction of a chloramphenicol/thiamphenicol resistant plasmid containing a constitutively active galK gene, as well as fused regions of homology up- and downstream of the target gene (750 bp). Electroporation of this plasmid into *F. nucleatum* Δ galKT and selection on thiamphenicol results in single-crossover homologous recombination onto the chromosome at the target gene. Next, colonies are then selected for double crossover gene deletions that remove the target gene and plasmid containing thiamphenicol resistance and galactose sensitivity by selecting on galactose containing media (**Figure 3.10**). We verified that all genes are accurate, complete gene deletions, and show no polar effects of gene deletion via RT-PCR of up and downstream genes (**Figure 3.11**). We then established that the deletion of galKT, which remains in all of the subsequent mutant strains, did not affect the proliferation of HCT116 cells (**Figure 3.9 H**).

We next found that deletion of *fap2* (*Fnn* Δ *fap2*) significantly attenuated binding to HCT116 cells (**Figure 3.1F**), but that *Fnn* Δ *fadA* did not result in such a decrease in HCT116 interactions. While *FadA* has been shown to drive interactions with cancer cells, we note that these studies were done in the strain *F. nucleatum* 12230^{31, 37, 38}. Upon bioinformatic analysis, we found that *F. nucleatum* 23726 contained four *fadA* ortholog genes (*fadA2*, *fadA3a*, *fadA3b*, *fadA3c*), and *F. nucleatum* 12230 encoded for only one orthologue²³. This could mean that a single deletion of the *fadA* gene in *F. nucleatum* 23726 remains adhesive, and that multiple *fadA* family proteins are contributing to binding and invasion. In addition, a *Fnn* Δ *fap2* *fadA* double mutant did not further reduce binding when compared to *Fnn* Δ *fap2* (**Figure 3.1F**). Concurrently, we analyzed single mutants of the trimeric

autotransporter adhesins cbpF, fvcB, fvcC, fvcD, as well as a strain lacking all four genes (*Fnn Δcbpf fvcBCD*). FvcB, FvcC, and FvcD contribute to invasion, albeit less potently than Fap2. Notably, *Fnn Δfap2 cbpf fvcBCD* quintuple mutants showed no significant decrease in their invasion of HCT116 cells when compared to the single *Fnn Δfap2* gene deletion strain. We note here that the insignificant binding loss we report for the *Fnn ΔcbpF* mutant could be due to low levels of CEACAM1 expression on HCT116 cells⁷⁶, as CEACAM1 was previously identified as the receptor for *F. nucleatum* CbpF (CEACAM binding protein of Fusobacterium)²⁷. The proportion of intracellular *Fnn* and *Fnn Δfap2* determined by antibiotic protection assays correlates with the flow cytometry data, indicating that the trypsinization used to prepare infected HCT116 cells likely removes the majority of surface bound bacteria. Together, these data indicate that host cell binding and invasion is largely driven by Fap2.

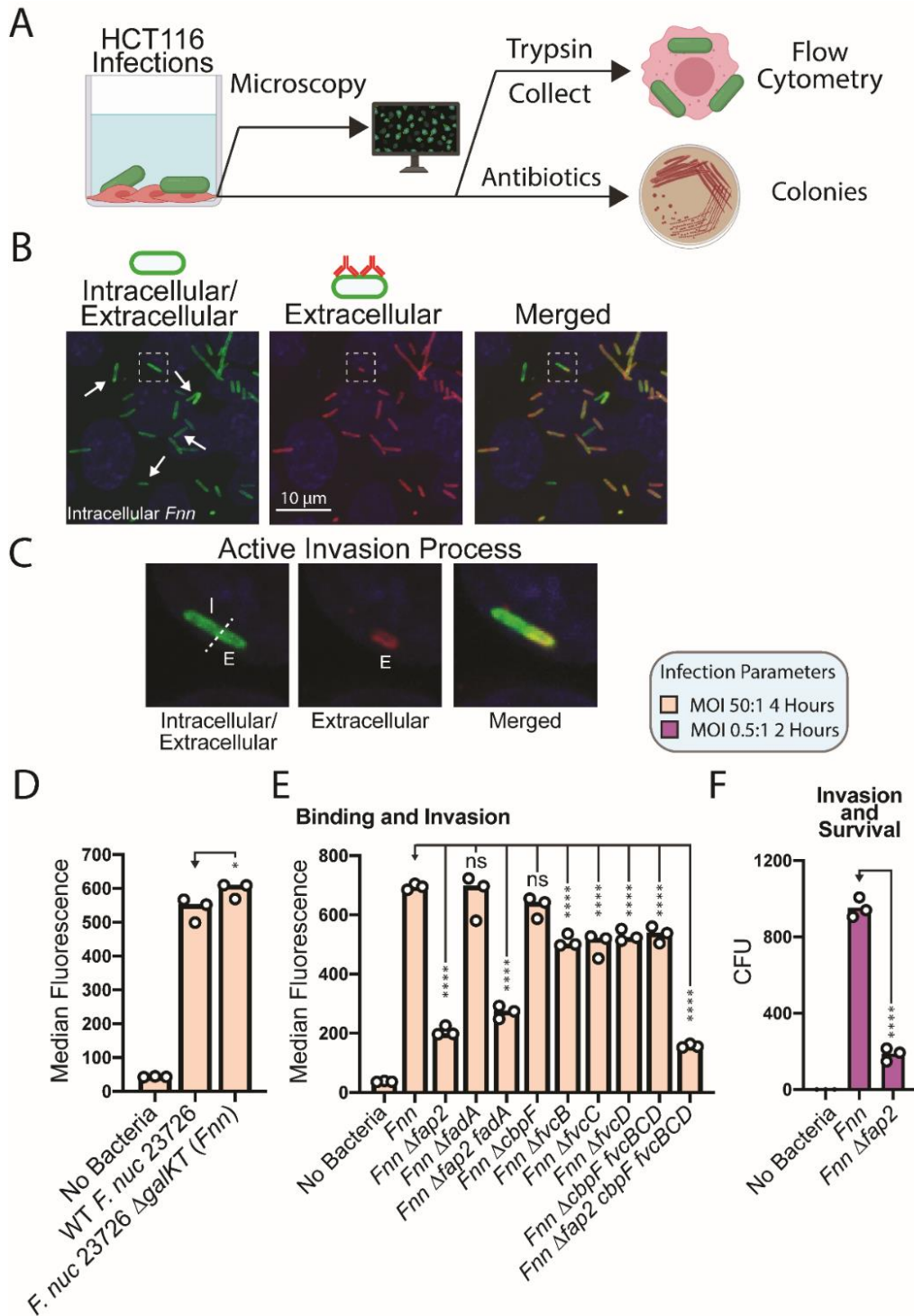


Figure 3.1. *F. nucleatum* binds to and invades HCT116 cells.

(A) Overview of experiments used to analyze binding and invasion of *Fnn* and adhesin mutants in HCT116 CRC cells. (B) Cocultures of HCT116 and *Fnn* (2 hour, MOI 50:1 *Fnn*:HCT116) stained for intra- and extracellular *Fnn*. *Fnn* was labeled with the fluorescent green membrane-intercalating dye FM 1-43FX to detect intracellular and extracellular bacteria, and labelled with a pan-*Fusobacterium*-

membrane antisera (Alexa Fluor 594 goat anti-rabbit antibody) for extracellular bacteria. Host cell nuclei are stained with DAPI (blue). Arrows indicate HCT116 cells that have migrated across the transwell barrier towards *Fnn* conditioned media. (C) *Fnn* bacterium that is half intracellular, half extracellular in HCT116 cells. (D) Comparison of HCT116 binding and invasion by wild-type (WT) *F. nucleatum* 23726 with *F. nucleatum* 23726 $\Delta galKT$ (*Fnn*) (n=3 independent flow cytometry experiments). (E) Binding and invasion analysis of *Fnn* and adhesin gene deletion strains using flow cytometry (n=3 independent flow cytometry experiments). (F) Invasion and survival of *Fnn* and *Fnn* $\Delta fap2$ in HCT116 cells (n=3 independent antibiotic protection assays). CFU, Colony Forming Units. In (E and F), infection parameters were 50:1 MOI *Fnn*:HCT116 for 4 hours (tan), 0.5:1 MOI *Fnn*:HCT116 for 2 hours (purple). ^{ns}*P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; refer to Methods.

3.3.2 *F. nucleatum* host-cell binding and invasion is critical for inducing the secretion of pro-inflammatory and pro-metastatic cytokines from cancer cells

After confirming the importance of outer membrane adhesins for invading HCT116 cells, we analyzed the ability of *F. nucleatum* to induce the secretion of cytokines from both human cancer cells. Using cytokine arrays and ELISA (**Figure 3.2A-C**), we found that *F. nucleatum* 23726 interactions with HCT116 cells induced the secretion of high concentrations (~1000 pg/mL after four hours) of the CXC family cytokines IL-8 and CXCL1 into the culture medium (**Figure 3.2D**). To determine if direct binding and invasion is necessary for induced cytokine secretion, as well as to rule out whether an unidentified secreted protein or molecule induces this phenomenon, we compared *Fnn* to *Fnn* $\Delta fap2$. Our results show that the ability of *Fnn* $\Delta fap2$ to induce cytokine secretion from HCT116 cells is significantly attenuated. This correlates well with reduced invasive potential and confirms direct interaction is needed to alter host cells (**Figure 3.2D**).

To test if this phenomenon was specific to strain *F. nucleatum* 23726, we compared the secretion of IL-8 and CXCL1 from HCT116 cells in response to *F. nucleatum* 25586 and 7_1, which cover subspecies *nucleatum* and *animalis* (*Fna*) respectively. We found that these strains also induce comparable, substantial levels of cytokine secretion (**Figure 3.2E**). We note that both *F. nucleatum* 25586 and 7_1 contain the *fap2* gene, potentially making their induction of cytokines also largely driven by Fap2 binding to host cells. Lastly, we compared the 4-hour secretion levels to those at 24-

hours in response to *F. nucleatum* 23726 and report an increase in CXCL1 and IL-8 to ~ 7000 pg/mL and ~2500 pg/mL respectively.

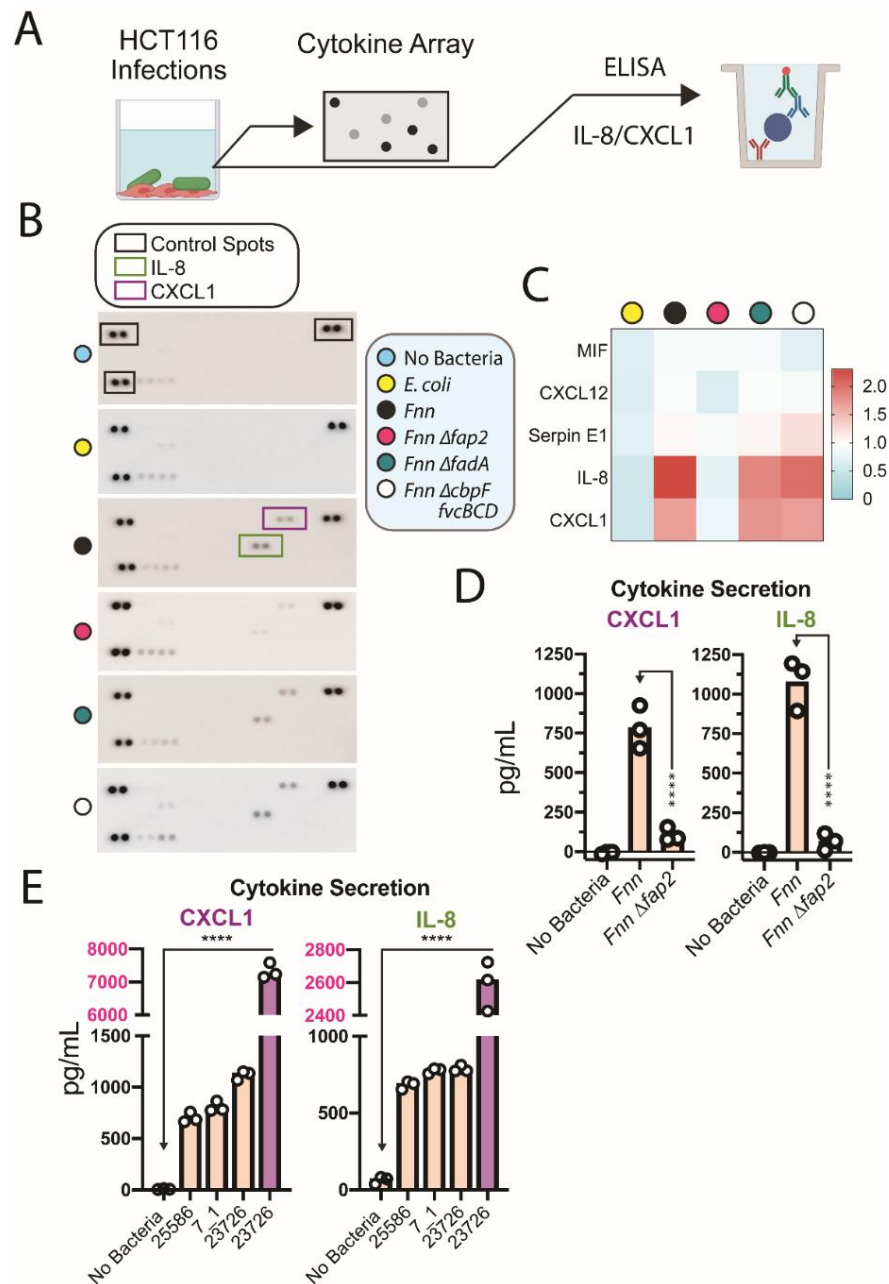


Figure 3.2 *F. nucleatum* induces cytokine secretion from HCT116 cells.

(A) Overview of experiments used to analyze *Fnn* induced cytokine secretion from HCT116 CRC cells. (B) Broad cytokine array dot blots (36 proteins) analyzing *Fnn* and *Fnn* adhesin deletion strains. Control spots indicate successful Western blots and provide densitometry controls for quantitation in panel C. (C) Heatmap of fold increased dot blot intensity reveals an increase in IL-8 and CXCL1

secretion from HCT116 infected with invasive *Fnn*, and decreased cytokine levels for *Fnn Δfap2*. (D) IL-8 and CXCL1 ELISA to quantitate cytokine secretion from HCT116 cells induced by *Fnn* and *Fnn Δfap* (n=3 independent ELISAs) 2. (E) IL-8 and CXCL1 ELISA to quantitate and compare cytokine secretion from HCT116 cells induced by *F. nucleatum* subsp. *nucleatum* 25586, *F. nucleatum* subsp. *nucleatum* 23726, *F. nucleatum* subsp. *animalis* 7_1 (*Fna*) (n=3 independent ELISAs). In (D and E), infection parameters were 50:1 MOI *Fusobacterium*:HCT116 for 4 hours (tan), 50:1 MOI *Fusobacterium*:HCT116 for 24 hours (purple). pg/ml, picograms per milliliter. ^{ns}*P* >0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

3.3.3 *F. nucleatum* induced cytokine secretion in neutrophils and macrophages is not Fap2 dependent

Because bacterial-induced immune cell alterations have been implicated in a number of cancer processes^{1,2,4} we next characterized bacterial-induced cytokine secretion from mouse immune cells using a mouse-specific cytokine array (**Figure 3.3A, Figure 3.13**), similar to the process described for HCT116 cells. We first concurrently tested neutrophil cytokine secretion (**Figure 3.3B**) and validated the bacterium's direct binding to and invasion of neutrophils using fluorescence microscopy (**Figure 3.3C**). *F. nucleatum* induces the secretion of CCL3, CXCL2, and TNFα, and high amounts of CCL3 and CXCL2 were secreted from neutrophils, as measured by ELISA (**Figure 3.3D**). In mouse macrophages, *F. nucleatum* also induces robust CCL3, CXCL2, CCL5, and TNFα secretion (**Figure 3.3E**), as well as additional cytokines when compared to either uninfected (no bacteria) or *E. coli*-infected controls. CCL3 stimulates lymphocyte recruitment in early metastatic CRC⁽³⁹⁾, and CXCL2 promotes angiogenesis in the CRC microenvironment⁴⁰. Notably, our data that shows elongated extracellular neutrophil DNA (**Figure 3.3C**) revealed that *F. nucleatum* could be inducing the formation of neutrophil extracellular traps (NETs); an alternative pathway for cytokine secretion⁴¹. *F. nucleatum* and other oral bacteria were previously shown to degrade NETs, which is a potential escape mechanism from neutrophil-mediated death⁴². In stark contrast to the Fap2-driven cytokine secretion seen in HCT116 cancer cells, *Fnn Δfap2* did not cause a significant decrease in the amount of CCL3 and CXCL2 secreted from immune cells, as analyzed by cytokine arrays and ELISA (**Figure 3.3D** and **Figure 3.13**). Therefore, we conclude that Fap2 selectively enables the bacterium's

binding and invasion of cancer cells, which is further supported by a lack of Gal/GalNAc sugar on immune cells. Finally, because a major role of immune cells is to engulf and clear invading microorganisms, these cells have acquired the ability to recognize a broad range of bacterial ligands as endocytosis targets. Given that Fap2 does not drive immune cell interactions, our data indicates that the proteins or macromolecules on *F. nucleatum* and immune cells that coordinate these interactions remain unidentified.

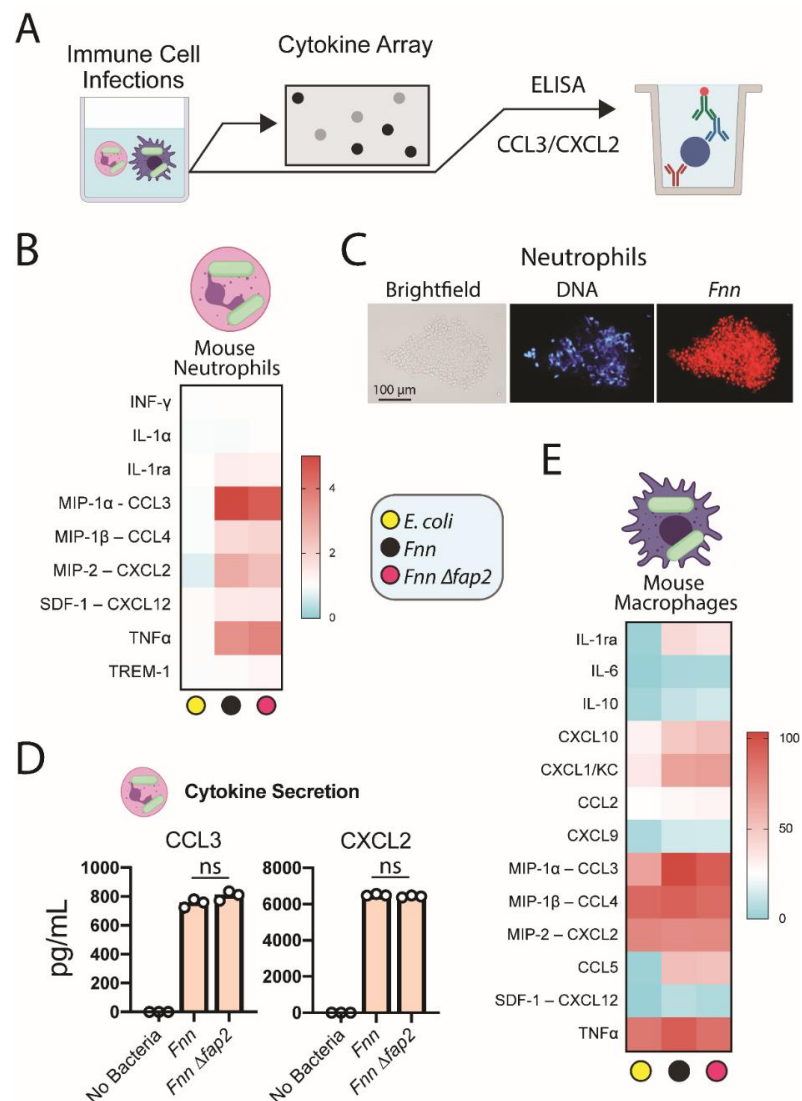


Figure 3.3 Cytokine secretion analysis from mouse neutrophils and macrophages.

(A) Overview of experiments used to analyze *Fnn* induced cytokine secretion from immune cells. (B) Mouse cytokine array shows *Fnn* induced secretion of CCL3, CXCL2, and TNFα from neutrophils.

Infection parameters were 50:1 MOI *Fnn*:neutrophils for 4 hours. (C) Fluorescence microscopy of *Fnn* interacting with mouse neutrophils. DNA was detected using DAPI, and *Fnn* was labeled with the fluorescent red membrane-intercalating dye FM 4-64FX. (D) ELISA confirming *Fnn* induction of CCL3 and CXCL2 and assessing the effect of deletion of *fap2* on cytokine secretion in neutrophils (n=3 independent ELISAs). (E) Cytokine array assessing the effect of *Fnn* infection on the secretion of several cytokines from mouse macrophages when compared to that of *E. coli* and controls. In (B to E), infection parameters were 50:1 MOI *Fusobacterium*:neutrophils/macrophages for 4 hours.

3.3.4 Inhibition of bacterial invasion blocks cytokine secretion

Because Fap2 is a Gal/GalNAc sugar-binding lectin, and this sugar is overrepresented on the surface of CRC cells, we next tested a panel of galactose and non-galactose containing sugars for their ability to inhibit bacterial binding to HCT116 cells and consequent IL-8 and CXCL1 secretion. We found that galactose, GalNAc, and lactose (galactose disaccharide) potently inhibited the bacterium's binding of and invasion into HCT116 cells, whereas control sugars—including glucose and maltose—did not (**Figure 3.4A**). In addition, we tested L-arginine as an inhibitor because the *F. nucleatum* surface adhesin RadD, which is predominantly thought to coordinate interspecies bacterial interactions in the oral biofilm, is inhibited by L-arginine⁴³⁻⁴⁵. We found that L-arginine inhibited the invasion of *F. nucleatum* into HCT116 cells, but not to the extent achieved by galactose sugars (**Figure 3.4A**). All of the tested sugar molecules and L-arginine led to reduced secretion of IL-8 and CXCL1, with the most potent being galactose-containing sugars (**Figure 3.4B**) as also seen in the binding and invasion assay. We believe the addition of 10 mM glucose and maltose results in less potent and non-specific inhibition of cytokine secretion or could be binding to an unidentified *F. nucleatum* lectin that may affect the host intracellular response after infection. Next, we developed a pan-*Fusobacterium*-membrane antisera (FMAS; DJSVT_MAS1) with the goal of blocking HCT116 cell invasion, cytokine secretion, and migration. The pan-*Fusobacterium* membrane antisera significantly decreased cancer cell binding (**Figure 3.4C**) and cytokine secretion (**Figure 3.4D**). However, *Fnn* Δ *fap2* was still more highly attenuated for invasion and cytokine secretion when compared to the effect of the pan-

Fusobacterium membrane antisera, indicating the antisera was unable to inhibit all Fap2-HCT116 docking at the concentrations used.

Additionally, we tested the autophagy and endosome maturation inhibitor chloroquine for its ability to inhibit bacterial invasion and consequent cytokine signaling (**Figure 3.14**). This experiment was performed because the two previous studies reporting that *F. nucleatum* induces metastasis showed that chloroquine inhibited CRC cell migration^{11, 12}; thus, we set out to test if this might be due to reduced cytokine levels that we observed. However, we found that chloroquine does not inhibit bacterial invasion when applied at 10 μ M, and only slightly decreases invasion when applied at 50 μ M (**Figure 3.14 A**). Curiously, we saw the reverse effect of chloroquine (50 μ M) on cytokine signaling, with a slight increase in secreted IL-8 and CXCL1 induced by *Fnn Δ fap2* (**Figure 3.14 B**). We elaborate on this finding below in our discussion of the results, but certainly the mechanism of chloroquine's effects – and, seemingly separately, the intracellular mechanism(s) of cytokine induction observed here – in modulating *F. nucleatum*-associated CRC cell motility and metastasis requires further investigation.

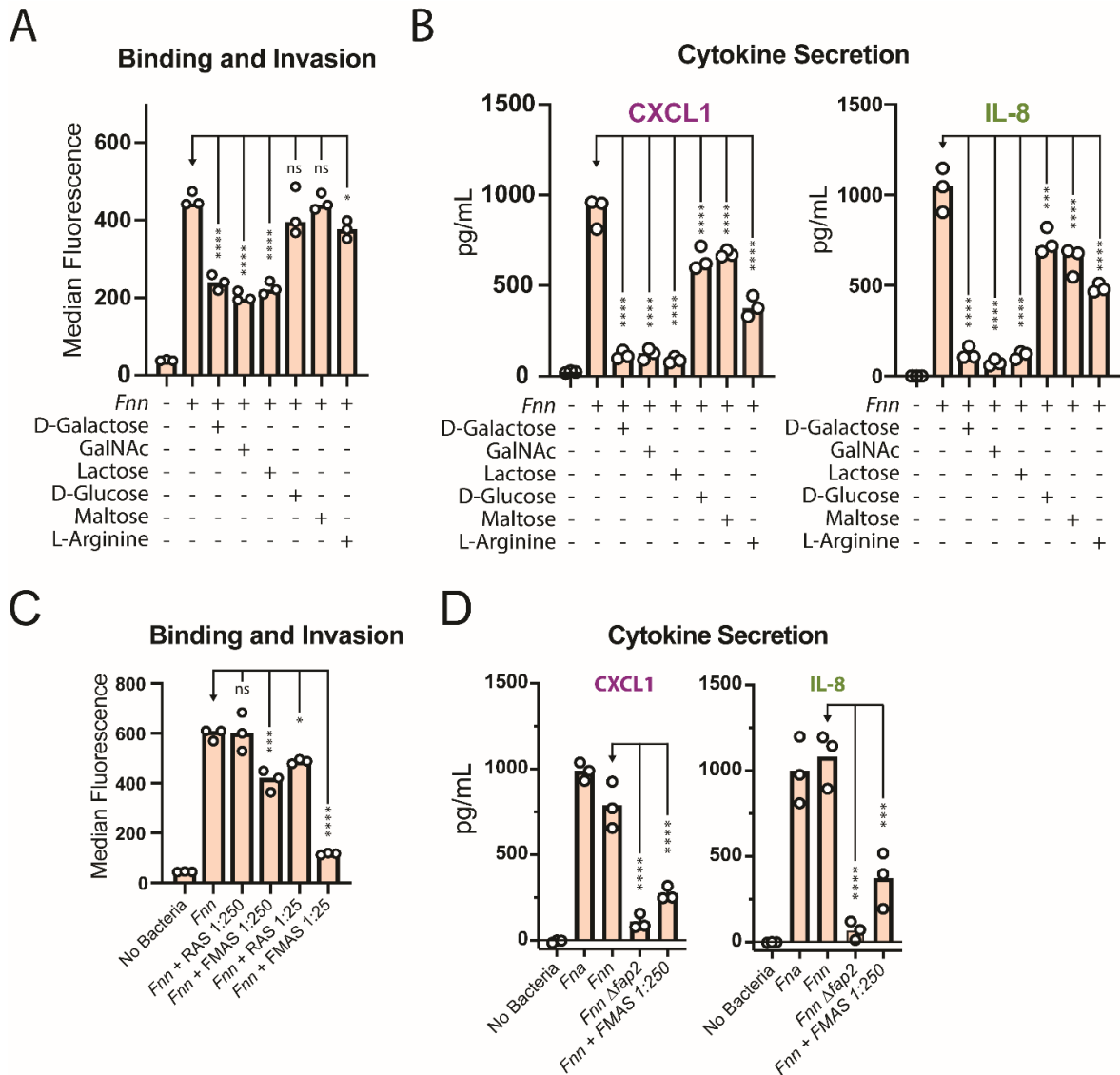


Figure 3.4 Inhibition of *Fnn* binding to and invasion of HCT116 cells using small molecules and membrane antibodies.

(A) *Fnn* invasion is significantly inhibited by galactose containing sugars and L-arginine (10 mM) (n=3 independent flow cytometry experiments). (B) Effect of sugars and L-arginine (10 mM) on the secretion of IL-8 and CXCL1 from HCT116 cells. (n=3 independent ELISAs). (C) *Fnn* invasion in the presence of *Fusobacterium* membrane antisera (FMAS) versus control rabbit antisera (RAS). (n=3 independent flow cytometry experiments). (D) Effect of FMAS and *Fnn* Δfap2 on secretion of IL-8 and CXCL1 from HCT116 cells (n=3 independent ELISAs).

3.3.5 *F. nucleatum* conditioned supernatants from HCT116 infections are enriched in IL-8 and CXCL1 and cause cancer cell migration

We first tested if *F. nucleatum* conditioned culture media from HCT116 infections could cause non-infected cells to migrate. We show through transwell assays (**Figure 3.5A-C**) that *F. nucleatum* conditioned HCT116 culture media significantly induces non-Fusobacterium exposed cancer cells to migrate starting at 8 hours, with increasing significance through 16-hour incubations with conditioned media (**Figure 3.5D**). Not only did *F. nucleatum* subsp. *nucleatum*-conditioned media induce cellular migration, but *F. nucleatum* subsp. *animals* (*Fna*) also induced significant migration in HCT116 cells (**Figure 3.5E**). We subsequently found that conditioned medium from cells infected with *Fnn Δfap2*, which led to lower levels of IL-8 and CXCL1 secretion, resulted in significantly reduced cell migration compared to that induced by *Fnn*-infected cell-conditioned media (**Figure 3.5E**). We were able to reduce cellular migration by adding the pan-Fusobacterium membrane antisera at 1:250 dilution to the *Fnn* conditioned media, but this concentration did not result in a significant decrease in migration. This could be due to insufficient antibody concentrations added to the assay, or since the anti-sera was developed with 11 strains of Fusobacterium, its specific inhibitory effects on *F. nucleatum* 23726 have been distributed across non-*Fnn* proteins.

Finally, we found that adding purified cytokines IL-8 and CXCL1 stimulated HCT116 cellular migration (**Figure 3.5F**), whereas bead-conjugated antibody-mediated depletion of IL-8 and CXCL1 (**Figure 3.5G**) from *F. nucleatum* conditioned culture media significantly reduced the number of migrated cells (**Figure 3.5H**). Because the depletion of IL-8 and CXCL1 did not completely abolish *Fnn*-induced cell migration, we hypothesize that *F. nucleatum* modulates multiple additional host proteins and pathways involved in cellular migration and metastasis.

Fnn-conditioned media containing high CXCL1 and IL-8 concentrations (n=3 independent transwell migration assays). (E) Effect of deletion of *fap2* (*Fnn Δfap2*) and *Fusobacterium* membrane antisera (FMAS) on bacterial-induced HCT cellular migration (n=3 independent transwell migration assays). (F) Effect of the addition of purified IL-8 and CXCL1 to culture media within the lower transwell chamber on HCT116 cell migration (n=3 independent transwell migration assays). (G) Analysis of CXCL1 and IL-8 levels after concentration, with and without antibody depletion of cytokines and before loading into the bottom chamber of the transwell (n=3 independent ELISAs). (H) HCT116 cellular migration upon depleting CXCL1 and IL-8 from media vs controls (n=3 independent ELISAs).

3.4 Discussion

Studies have illuminated a strong correlation between the presence of *Fusobacterium nucleatum* within colorectal tumors and increased tumor microenvironment inflammation, enhanced oncogenic signaling, and poor patient prognosis^{49–52}. To address the role of *F. nucleatum* in tumor microenvironment signaling, we set out to characterize potential signaling pathways that drive cellular migration and immune cell-driven cancer acceleration. Our data show that direct *F. nucleatum*-cellular interactions with cancer and immune cells, largely coordinated in CRC cells (minimally the HCT116 line used here) by the outer membrane adhesin Fap2, drive host cells to define a locally pro-metastatic, inflammatory microenvironment. The ability of this bacterium to cause selective induction of the proinflammatory and prometastatic cytokines IL-8 and CXCL1 from CRC cells complements prior studies in which these gross phenotypes of oncogenic acceleration from human patients and mouse models are consistently reported^{10, 53, 54}. In addition, our findings add to a growing understanding that *F. nucleatum* directly induces metastasis through cytokine release, increased NF-κB expression and subsequent expression of KRT7¹¹, increased CARD3, and downregulation of E-cadherin¹². These studies have now shown active roles for *F. nucleatum* in mouse models of metastasis, which complement the seminal study by Bullman et al. first reporting viable *F. nucleatum* within human CRC cell metastases in the liver (**Figure 3.6**)¹⁰.

CXCL1 and IL-8 both play roles in immune cell recruitment, particularly neutrophil recruitment and programming. Through paracrine signaling, *F. nucleatum* interactions with CRC cells could be

releasing factors that create not only a metastatic environment, but one that provides pro-tumor inflammation. There is the potential for recruited neutrophils to be activated to tumor-associated neutrophils (TANs) that could further accelerate tumor progression. In addition, if *F. nucleatum* or the tumor microenvironment then induces neutrophil extracellular trap (NET) formation, these structures have been shown to increase metastasis and sequester circulating tumor cells to promote reseeding⁵⁵⁻⁵⁷. Finally, it was shown that high intra-tumor loads of *F. nucleatum* resulted lower CD3+ T-cell density in CRC, providing another layer to the complexity of *F. nucleatum* immune cell regulation⁵⁸. To address these potential scenarios, a priority should be placed on investigating the potential for this bacterium to induce the formation of additional pro-oncogenic cell types including cancer-associated fibroblasts (CAFs)⁵⁹, tumor-associated macrophages (TAMs)⁶⁰, and TANs⁵⁵.

Bacteria such as *Streptococcus gallolyticus* are known to induce the secretion of IL-8 in colon tumor cells⁶¹. It has also been shown that increased expression of IL-8 leads to a significant resistance to the cytotoxic effects of oxaliplatin, a platinum based chemotherapeutic drug⁴⁶. As *F. nucleatum* was previously shown to induce chemoresistance through the induction of autophagy and subsequent inhibition of apoptosis⁵¹, our report of the induction of IL-8 and CXCL1 by this bacterium adds another dimension to this story.

Our data definitively shows the importance of *F. nucleatum* binding and invasion of human cancer cells in producing pro-metastatic cytokines. We and others have proposed that the development of small molecule compounds or proteins that are able to block *F. nucleatum* docking to host cells could be effective strategies to reduce cancer and immune cell signaling that accelerates metastasis from the colon. A starting point could be the selective inhibition of Fap2, which we have now shown drives binding and cytokine secretion. Fap2 was previously characterized as a lectin and shown to bind to Gal/GalNac sugars that are in abundance on the surface of most cancer cells, especially colorectal⁶². The potential for targeting of bacterial lectins for disease treatment has been validated in urinary tract infections by using mannoside sugar derivative compounds that block the uropathogenic *E. coli*

lectin FimH from binding to host receptors, thereby resulting in depletion of this pathogenic bacteria from the gut⁶³.

Two studies have reported that chloroquine blocks metastasis by lowering *F. nucleatum*-induced NF- κ B-driven expression of KRT7, a protein linked to increased cancer cell motility and invasive potential^{64, 65}, and overexpression of CARD3, a pro-metastatic kinase previously characterized in breast cancer⁶⁶. We were therefore interested in whether the IL-8 and CXCL1-regulated cell migration we have observed might be mitigated by chloroquine administration. Our results show that chloroquine treatment actually increased the secretion of IL-8 and CXCL1, suggesting that multiple pathways may be at play in regulating *F. nucleatum*-driven metastasis. This outcome is also the opposite of the effect chloroquine has on reducing the amount of IL-8 secreted upon *Campylobacter jejuni* infections⁶⁷. Together, these observations could mean the IL-8 and CXCL1 secretion we observed is not caused by a Toll-like receptor (TLR) maturation-dependent mechanism, which chloroquine regulates^{67, 68}, but is still NF- κ B dependent. In addition, inhibiting autophagy could lead to increased intracellular survival of *F. nucleatum*. This should be characterized further before these inhibitors are considered for use as more than chemical tools to understand host biological pathways.

Finally, because the consensus belief is that *F. nucleatum* leaves the oral cavity and traverses the human body through the blood and potentially lymph, it could be advantageous to use a vaccine-based strategy whereby antibodies block and clear this bacterium prior to leaving the bloodstream, thereby preventing downstream tumor interactions. Our data using a pan-Fusobacterium-membrane antisera show effective blocking of *F. nucleatum* entry into cancer cells, but this strategy needs refinement to increase strain specific potency. Hence, interfering with *F. nucleatum* interactions in the human body could be a targeted approach that provides an alternative to using non-specific antibiotics. Finally, we hypothesize that future studies characterizing how *F. nucleatum*

intricately influences multiple cell signaling pathways during cancer progression and metastasis will lead to targeted approaches for controlling additional oncomicrobes in cancer.

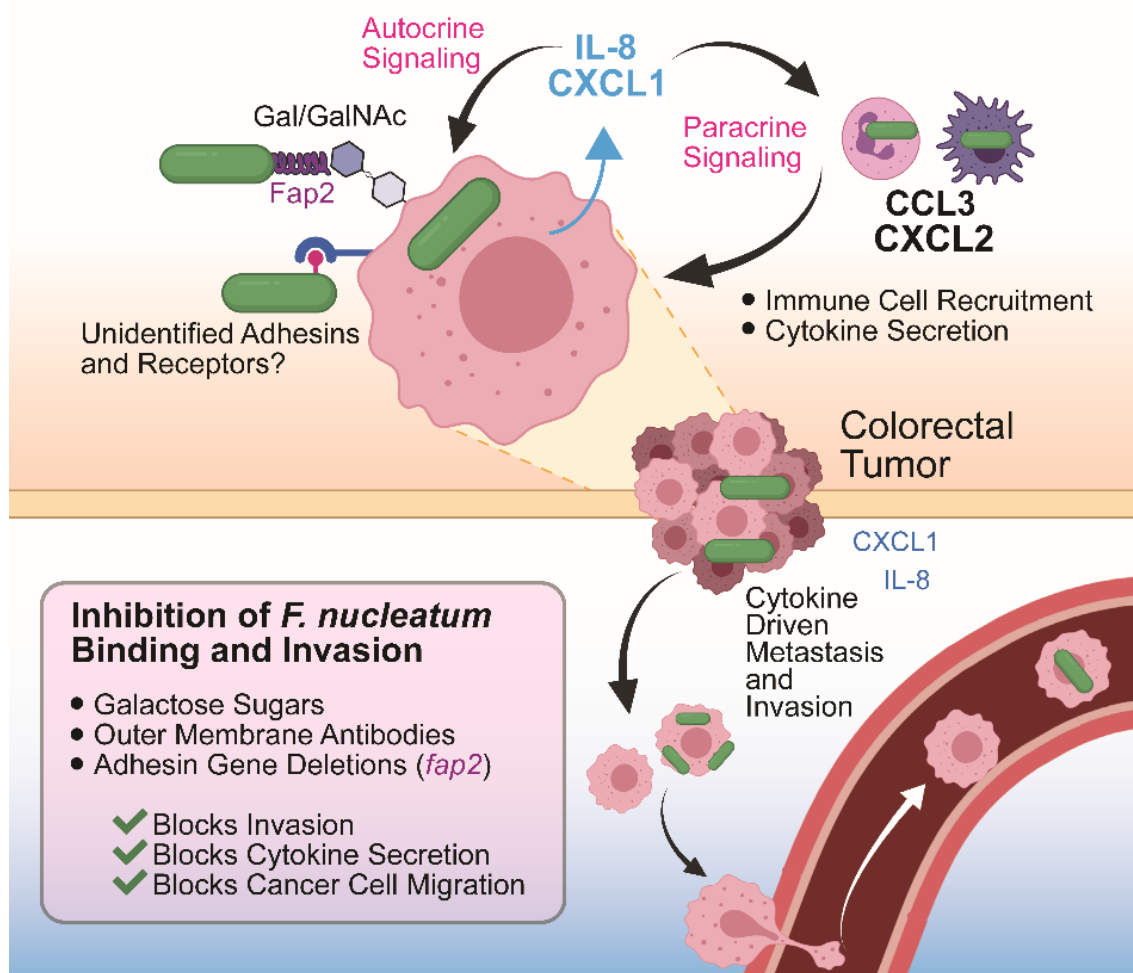


Figure 3.6 A model of *F. nucleatum* induced metastasis through cytokine signaling.

Model of our findings, that Fap2-dependent *F. nucleatum* invasion into CRC cells induces IL-8 and CXCL1 secretion, and both cytokines have been characterized as key players in cancer metastasis and subsequent downstream cell seeding. HCT116-derived IL-8 and CXCL1 can participate in autocrine signaling back to cancer cells as a metastatic signal, as well as paracrine signaling to recruit neighboring immune cells, which further secrete their own cytokine signatures (CXCL2, CCL3, TNF α) that alter the tumor microenvironment through metastatic, inflammatory, and immune cell programming. Our data shows that Fap2 drives colorectal cancer cell docking, which is a key step in initiating the pro-metastatic cytokine cascade but is not necessary for immune cell signaling.

3.5 Materials and Methods

3.5.1 Culturing *Fusobacterium nucleatum*

Fusobacterium nucleatum subsp. *nucleatum* ATCC 23726, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, and *Fusobacterium nucleatum* subsp. *animalis* 7_1 (*Fna*) were cultured on solid agar plates made with Columbia Broth (Gibco) substituted with hemin (5 µg/mL) and menadione (0.5 µg/mL) (CBHK) under anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) at 37 °C. Liquid cultures started from single colonies were grown in CBHK media under the same conditions. For infections, overnight cultures were back diluted 1:1000 and grown to mid-exponential phase (~0.5 OD₆₀₀) prior to subsequent experiments unless otherwise noted.

3.5.2 Culturing HCT116 CRC cells

HCT116 (ATCC CCL-247) cells were purchased from ATCC and grown on tissue culture treated plates and flasks in McCoy's 5A media supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Cells were grown to no more than passage 15 at 37 °C with 5% CO₂. For maintenance between infections, cells were passaged by gentle trypsinization and reseeding. For infection experiments, cells were grown to >90% confluency in 6- or 24-well plates. Unless otherwise indicated, all experiments were conducted with HCT116 media supplemented with 10% FBS.

3.5.3 Molecular cloning of a galactose selectable gene deletion system.

We have developed a new iteration of a galactose-selectable gene deletion system in *Fusobacterium nucleatum* ATCC 23726 (*Fnn*) capable of deleting an unlimited number of genes in a single strain. We showcase the power of our genetic system by developing strains containing multiple gene deletions from ~300bp-12kb, as well as chromosomally complementing tagged versions of these virulence factors for detection and purification. The plasmids used for these genetic mutations are all new, developed in house. This system is different than a previously reported system in that it relies on the deletion of both the galactose kinase (*galK*) and galactose-1-phosphate uridylyltransferase (*galT*) in

the Leloir pathway (**Figure 3.7**); ultimately creating a system that allows for the selection of target gene deletions on solid media containing galactose and not the more expensive 2-Deoxy-D-galactose. The first step in creating the chloramphenicol and thiamphenicol selectable plasmid pDJSVT1 was to PCR amplify the *catP* gene from a pJIR750 backbone (*Clostridium* shuttle vector) followed by using overlap extension PCR (OLE-PCR) to fuse a pMB1 *E. coli* origin of replication from pUC19 (**Figure 3.8A**) (see table S1 for primers). This linear product, which contains a multiple cloning site with GC rich DNA restriction sites (XhoI, NotI, KpnI, and MluI) that are optimized for cloning in DNA from AT rich bacteria such as *Fusobacterium* (~75% AT), was cut with NotI and ligated to circularize the plasmid. This final high copy pDJSVT1 construct is small (~1800 bp), which likely enhances transformation efficiency.

pDJSVT1 is the base vector to create multiple additional vectors to complete the genetic system. To delete the *galK* and *galT* genes in *F. nucleatum* 23726 to make the base strain DJSVT02 (**Figure 3.9 A**), 1000 bp directly up and downstream of the *galKT* gene cluster were amplified separately, then fused using OLE-PCR. This 2kb PCR product was then digested with KpnI/MluI and ligated into pDJSVT1 digested with the same enzymes. This final vector is pDJSVT13. This vector was electroporated (1-3 µg DNA, 2.5 kV, 50 uF capacitance, 360 OHMS resistance) into competent *F. nucleatum* 23726 and selection on chloramphenicol (single chromosomal crossover), followed by selection on solid media containing 1% 2-Deoxy-D-galactose to select for either *galKT* gene deletions, as the absence of the *galT* gene makes 2-Deoxy-D-galactose non-toxic to *F. nucleatum* (**Figure 3.7**).

The next plasmid is for targeted gene deletion in the *F. nucleatum* 23726 Δ *galKT* (Fnn: strain DJSVT02) background. This vector, pDJSVT7 (**Figure 3.8 B**), contains a FLAG::*galK* gene under the control of a *Fusobacterium necrophorum* promoter. Transformation allows for initial chromosomal integration and selection with thiamphenicol, followed by selection for double crossover gene deletions on solid media containing 3% galactose. For this study, this vector was used to create gene deletions in *fap2*, *fadA*, *cbpF*, *fvcB*, *fvcC*, and *fvcD* in *F. nucleatum* 23726 Δ *galKT*. As shown in **Figure**

3.10, 750 bp directly up and downstream of a target gene for intra-bacteria homologous recombination were amplified by PCR, making complementary fragments fused by OLE-PCR. This product (**Figure 3.10 A**) is ligated into pDJSVT7 using KpnI/MluI restriction sites. This vector is then electroporated (2.5 kV, 50 uF capacitance, 360 OHMS resistance) into competent *F. nucleatum* 23726 Δ galKT and selected on chloramphenicol (single chromosomal crossover), followed by selection on solid media containing 3% galactose which produces either complete gene deletions or wild-type bacteria revertant. Gene deletions are verified by PCR and sequencing, and we show this system has been accurate down to the single base level (**Figure 3.11**). For creating multiple gene deletions in a single strain, additional vectors are created and mutants made in sequential fashion, as gene deletion leaves no trace of vectors, including excision of the chloramphenicol and galactose selection genes.

The third vector in the suite, pDJSVT11 (**Figure 3.8 C**; not used in this study), is to create single copy chromosomal complementation at a static chromosomal location within the *arsB* gene, which is only necessary during times of high arsenic exposure and therefore doesn't change the phenotype of Fnn under any conditions tested (**Figure 3.1E** and **Figure 3.9 H**).

3.5.4 RNA Extraction and RT-PCR

F. nucleatum cultures were grown to stationary phase and pelleted by high-speed centrifugation (12,000 g for 2 min at room temperature). TRIzol Extraction Isolation of total RNA was performed following manufacturer's instructions (Invitrogen). Briefly, cell pellets were resuspended in 1 mL of TRIzol reagent (Invitrogen) and 0.2 mL of chloroform was added. Solution was centrifuged for 15 min at 12,000 g at 4°C. The RNA-containing aqueous phase was collected, and the RNA precipitated after 500 μ L of isopropanol had been added. The RNA pellet was then washed with 75% ethanol and centrifuged at 10,000 g for 5 min at 4°C. After drying at room temperature for 10 min, the RNA pellet was resuspended in 30 μ L sterile RNase-free water and solubilized by incubating in water bath at 55°C for 10 min. Total RNA was quantified using Qubit™ RNA HS Assay Kit (ThermoFisher).

Before reverse transcriptase (RT)-PCR, RNA samples were subjected to DNase treatment. Briefly, 500 ng of total RNA was incubated with DNase I (Invitrogen) for 2 hours at 37°C. After treatment, DNase I was inactivated using EDTA and heating mixture for 5 minutes at 65 °C. (RT)-PCR was performed using the Takara PrimeScript™ One Step RT-PCR Kit according to the manufacturer's instructions. The PCR conditions consisted of reverse transcription for 30 min at 50°C, initial denaturation for 2 min at 94°C, followed by 30 cycles (30 sec at 94°C, 30 sec at 50-62°C, and 30 sec at 68°C) and elongation at 68 °C for 1 min. The expected bands around 250 bp was confirmed on a 1.5% agarose gel. Specific primers to detect knockout of gene and validate intact genes upstream and downstream of gene of interest (table S1) were used to amplify from RNA extracts.

3.5.5 Development of a pan-Fusobacterium outer membrane antisera

To test if broadly-neutralizing polyclonal antisera against Fusobacterium outer membrane proteins could be effective at blocking cellular binding and entry, rabbits were injected (New England Peptide) with purified total membrane proteins from 11 strains of Fusobacterium that span 7 species (*F. nucleatum subsp. nucleatum* 23726, *F. nucleatum subsp. animalis* 7_1, *F. nucleatum subsp. polymorphum* 10953, *F. nucleatum subsp. vincentii* 49256, *F. periodonticum* 2_1_31, *F. varium* 27725, *F. ulcerans* 49185, *F. mortiferum* 9817, *F. gonidiaformans* 25563, *F. necrophorum subsp. necrophorum* 25286, *F. necrophorum subsp. funduliforme* 1_1_36S). 20 mL of each strain was grown as previously described to stationary phase before pooling. 220 mL of pooled Fusobacterium culture was centrifuged, and the pellet resuspended in 40 mL of phosphate-buffered saline pH 7.4 (PBS) with 2 roche EDTA free protease inhibitor tablets, frozen at -80 °C, and passed through an Emulsiflux C3 at >15k PSI to lyse cells. Lysate was centrifuged at 18000g for 25 minutes to pellet debris and unlysed cells. The supernatant was carefully transferred to ultracentrifuge tubes, and centrifuged in a 50-Ti rotor at 38,000 rpm (~144kG average) for 1 hr and 40 min. The supernatant was discarded, the membrane protein pellet was gently washed with PBS, and 0.5 mL of PBS with 1% BOG was added to each of the two tubes and gently resuspended by stirring overnight at 4 °C. The protein concentration

was determined by BCA prior to shipment to New England Peptide for inoculation into rabbits. The resulting membrane antisera is herein named DJSVT_MAS1, but for clarity is described in Figure legends as FMAS for *Fusobacterium* membrane antisera.

3.5.6 Immunofluorescence sample preparation using pan-*Fusobacterium* membrane antisera

Confluent HCT116 cells on slides were infected with FM -143FX labeled (Green fluorescent lipid) *F. nucleatum* 23726 in wells containing McCoy's 5A media with 10% FBS and no antibiotics at MOI of 50:1 for four hours at 37 °C with 5% CO₂. Post infection, media was removed and thoroughly washed with PBS with gentle orbital shaking. Infected cells were then fixed with PBS/3.2% paraformaldehyde for 20 min at room temperature, followed by washing with PBS. Cells were blocked with Sea Block (Abcam) for 2 hours at 37 °C, followed by the addition of 1:100 dilution of DJSVT_MAS1 overnight at 4 °C. Slides were washed in PBS and incubated for 1 hour with Alexa Fluor 594 goat anti-rabbit antibody diluted in Sea Block. After washing with PBS, cells were permeabilized with PBS/1% Triton X-100 for 20 minutes. Post-permeabilization, cells were washed in PBS and labeled with DAPI for 30 minutes. Following three final PBS washes, coverslips were mounted with 80% glycerol/0.1M Tris pH 8.5 and sealed onto glass slides. Fluorescence microscopy was performed on a Zeiss LSM 800 confocal microscope.

3.5.7 Flow cytometry

Mid-log phase *F. nucleatum* were incubated with 5 ug/mL FM 1-43FX Lipophilic Styryl Dye to stain outer membranes and allow for green fluorescence detection. After washing in PBS, bacteria were resuspended in PBS at 100x concentration (MOI 50:1) before adding to cultures of HCT116 in media containing 10% FBS. Infections lasted four hours prior to removing the culture media (in many cases used for ELISAs as described), cells washed twice with PBS, followed by cell recovery using 0.05% trypsin. After neutralizing trypsin with media containing 10% FBS, cells were pelleted and

resuspended in PBS containing 20 mM EDTA. Cells were loaded onto a Guava easyCyte 5 flow cytometer (Luminex) and 10,000 cells were collected using an initial single cell gate to measure the median green fluorescence induced by intracellular *F. nucleatum* labeled with FM 1-43FX. Post data acquisition, FlowJo 10 software was used to further refine gating to single cells as well as determine median fluorescence of all samples. FlowJo analysis was then transferred to GraphPad Prism for statistical analysis and figure generation. We show intracellular *F. nucleatum* 23726 using imaging flow cytometry on an Amnis ImageStream X Mk II (Fig. 1D). For this experiment, the same protocol for bacterial and HCT116 growth, FM 1-43FX labeling, and infection times were used.

3.5.8 Invasion and survival antibiotic protection assays

HCT116 monolayers were washed one time with PBS and the cells were incubated in media containing 10% FBS and no antibiotics. HCT116 cells were then infected at an MOI of 0.5:1 with exponential phase *F. nucleatum* for 2 hours at 37 °C with 5% CO₂. After infecting cells for 2 hours, the cell culture media was aspirated-off and cells were washed 2 times with antibiotic-containing cell culture media to remove any unbound bacteria. Cells were then incubated in media containing penicillin and streptomycin (readily kills *F. nucleatum* 23726) for 1 hour to kill any remaining extracellular bacteria. At the end of the incubation period, cells were washed twice with PBS. Epithelial cells were then incubated with warm sterile water to lyse HCT116 cells. Lysates were plated on CBHK plates and incubated at 37 °C under anaerobic conditions for 48 hours followed by colony counting.

3.5.9 Inhibition of *F. nucleatum* binding to HCT116 cells and subsequent signaling using chemical compounds and antibodies

F. nucleatum invasion and induced host cell signaling inhibition by chemical compounds and neutralizing antibodies was analyzed using the standard infection conditions described above. Just prior to adding bacteria, with no pre-incubation time, 10 mM of D-galactose, GalNAc, lactose, D-

glucose, maltose, or L-arginine were added to the culture media. For DJSVT_MAS1 *Fusobacterium* membrane antisera, serum was added from between 1:25 to 1:250 dilutions per infection. Chloroquine was added to HCT116 cells at 10 μ M for four hours prior to infection and remained in the culture media during infections. For all compounds, after a 4-hour infection at MOI 50:1, binding and invasion was analyzed using flow cytometry.

3.5.10 Isolation of mouse neutrophils and macrophages

Mouse bone marrow neutrophils were isolated from wild type C57BL/6 mice over a 62.5% percoll gradient with centrifugation at 1100g for 30 min. Purity was >90% as determined by flow cytometry analysis with Ly6G+CD11b+ staining. Neutrophils were used immediately after isolation. For mouse bone marrow derived macrophages, bone marrow cells were cultured for 5 days in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 1% penicillin/streptomycin, and 10ng/mL M-CSF. Fresh medium was replaced every other day. Wild type C57BL/6 mice were bred and maintained in the animal facility at Virginia Tech in accordance with the Institutional Animal Care and Use Committee (IACUC)-approved protocol.

3.5.11 Human and mouse inflammatory cytokine arrays

Exponential phase *F. nucleatum* 23726 or *F. nucleatum* 23726 Δ ap2 were used to infect HCT116 cell monolayers (100% confluency 1.2×10^6 cells/well for 6 well plate) in antibiotic free cell culture media for four hours at a multiplicity of infection of 50 bacteria per human cell (MOI: 50:1). For neutrophils and macrophages, 1×10^6 cells in suspension were used. After four hours all media (1.5 mL) was collected and sterile filtered through 0.2 μ m filters (Millipore Sigma) to remove bacterial and human cells from the sample. The cytokine array membranes (R&D: Proteome Profiler Human Cytokine Array (ARY005B), Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006)) were then blocked for one hour in TBS 3% bovine serum albumin (BSA) at room temperature on a rocking platform. While the membrane was blocking, 1.5 mL of each sample of collected infection

media was incubated with 15 μ L of the human cytokine array detection antibody cocktail at room temperature for 1 hr. After 1 hr, the blocking buffer was poured off and the sample/antibody mixture was incubated with the array membrane at 4 °C overnight. Following incubation with the sample/antibody mixture, the array membrane was washed 3 times with PBS. A streptavidin-HRP conjugate was then added to the membrane and incubated for 30 min on a rocking platform. The array membrane was then washed 3 times with wash buffer to remove any unbound streptavidin-HRP. After the membrane was sufficiently washed it was incubated with a chemiluminescent substrate solution and results were analyzed using the G:Box gel imaging platform.

3.5.12 ELISA

HCT116 cells were seeded to confluence in 24-well plates (2×10^5 cells/well at 100% confluence) and *F. nucleatum* was added to 500 μ L of these wells at a MOI of 50:1. The plates were then incubated at 37 °C, 5% CO₂ for four hours. The media was then collected from the wells, sterile filtered using a 0.2 μ m filter (Millipore Sigma) and diluted to concentrations within the range of the R&D Systems DuoKit ELISA to analyze human IL-8 and CXCL1 concentrations. For ELISAs detecting mouse CCL3 and CXCL2, 1×10^6 fresh neutrophils in suspension were used as described for the HCT116 protocol.

3.5.13 Transwell HCT116 migration assays

Transwell HCT116 migration assays were performed with Corning 8 μ m Transwell inserts in 24-well plates. HCT116 cells were first cultured in McCoy's 5A (ATCC) to 90% confluence, followed by collection with trypsin. Cells were then stained using CellTrackerRed (ThermoFisher) and resuspended at a concentration of 2×10^6 cells/mL in media supplemented with 1% FBS. 100 μ L (2×10^5 cells) of the cell suspension was added to the top chamber of the 8 μ m Transwell insert pre-coated with 100 μ L Matrigel (Corning) (250 μ g/mL). The lower chamber contained 600 μ L of media with 1% FBS in addition to adding the following: 1) chemokines; purified recombinant human IL-8 (ThermoFisher Scientific) and CXCL1 (Sigma-Aldrich), individually or together at a concentration of

100 ng/mL. 2) conditioned and concentrated media obtained from four-hour *F. nucleatum* infections of HCT116 cells. To prepare concentrated media for each sample, three T-75 flasks with confluent HCT116 cells were used. Before infection, the complete media in each flask was replaced with 10mL serum-free and PenStrep-free media. The bacteria resuspended in serum-free media were added at 50:1 MOI, and the flasks were incubated in a hypoxic chamber (1% O₂) for four hours. The media containing the cell secretions was collected and pooled from three flasks, spun down at 3000xG for five minutes and passed through a 0.22µm filter. The samples were then concentrated from 30 mL to 1.5 mL using a 3000 MWCO Amplicon concentrator (Millipore Sigma) at 4 °C. The resulting sample was used for ELISA and Transwell migration assays. The Transwells were incubated at 37 °C, 5% CO₂ for 6-16 hours, after which the cells on the top were removed using a cotton-tipped applicator and migrated cells at the bottom of the membrane were stained with DAPI and imaged on a Zeiss LSM 800 confocal microscope using a 10X objective. ImageJ was used to count the number of cells in 5 representative images per Transwell in biological triplicate⁶⁹.

3.5.14 Depletion of IL-8 and CXCL1 from conditioned media

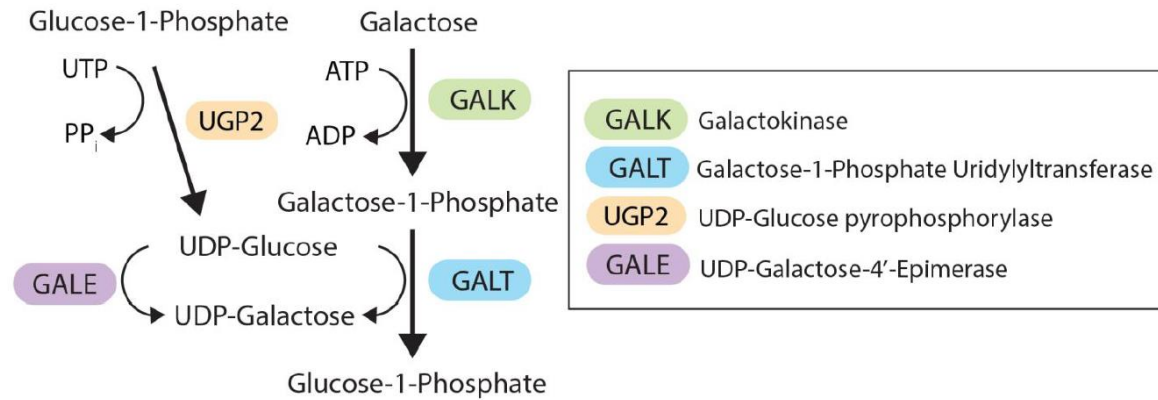
Conditioned and concentrated media was obtained as described. Human IL-8/CXCL8 biotinylated antibody (R&D Systems BAF208) and Human/Primate CXCL1/GROα/KC/CINC-1 biotinylated antibody (R&D Systems BAF275) was added to the media to a final concentration of 40 ng/mL and incubated at room temperature with gentle shaking for 30 minutes. 150 µL of magnetic streptavidin particles (Sigma-Aldrich 11641778001) were first washed twice with PBS and spun down at 1500 xG and added to the solution. The mixture was incubated at room temperature with gentle shaking for another 30 minutes. The samples were then spun down at 1500g, and the supernatant was collected containing the conditioned media with reduced cytokines. An ELISA quantified and confirmed the depletion. This media was subsequently used in transwell migration assays as described.

3.5.15 Statistical analysis

All statistical analysis was performed in GraphPad Prism Version 8.2.1. For single analysis, an unpaired Student's t test was used. For grouped analyses, Two-way ANOVA was used. In each case, the following P values correspond to star symbols in figures: $^{ns}P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. To obtain statistics, all studies were performed as three independent biological experiments. For all experiments in which statistical analysis was applied, an N of 3 independent experiments was used (details in figure legends).

Supplementary Figures

A



B

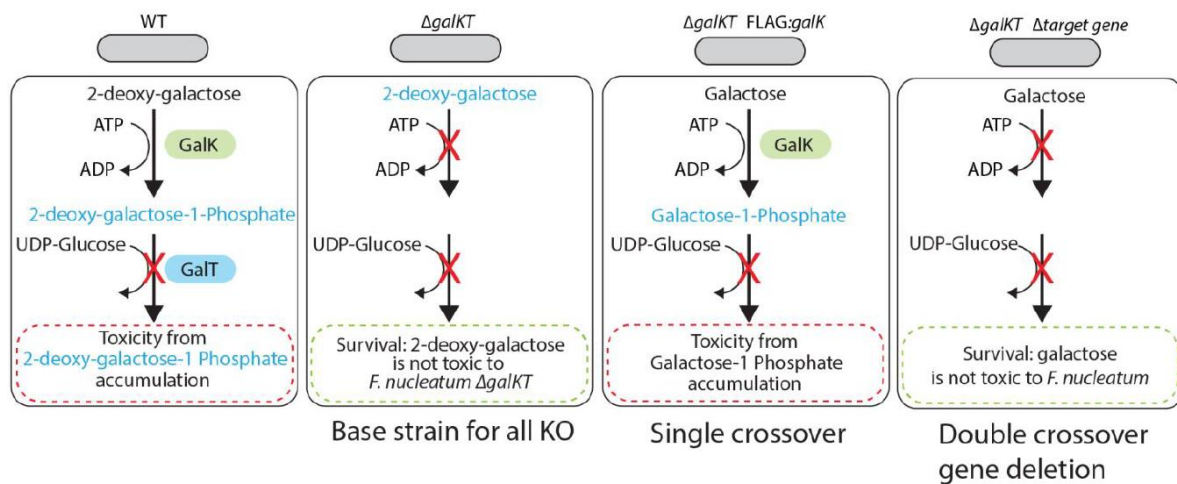


Figure 3.7 Using the Leloir pathway for targeted gene deletions in bacteria.

(A) The Leloir pathway of galactose catabolism. (B) Graphical representation of the different selection stages of *F. nucleatum* gene deletions and how the GalK and GalT affect cellular survival in the presence of galactose and 2-deoxy-galactose.

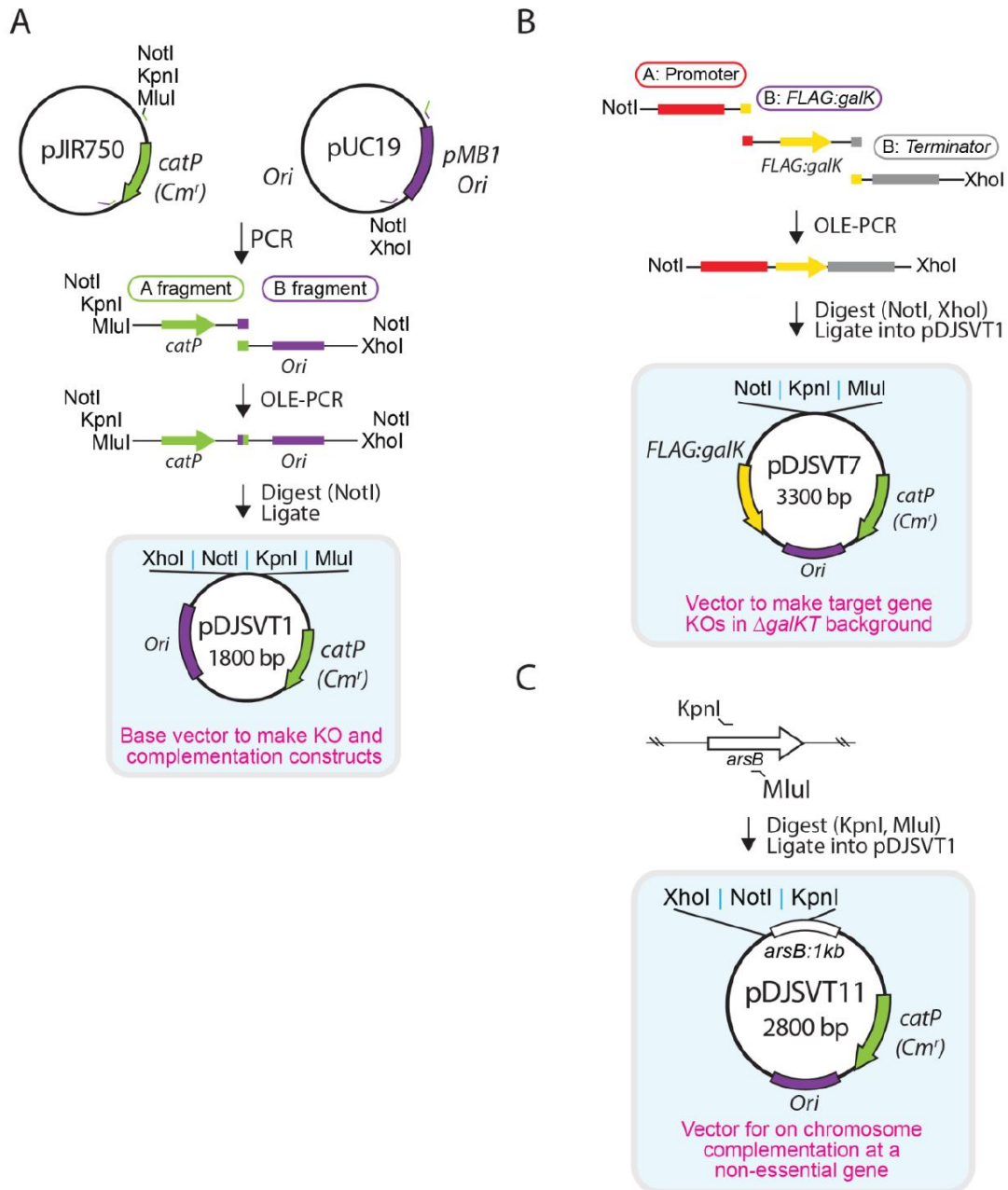


Figure 3.8 Development of vector for markerless gene deletion in *F. nucleatum*.

(A) Method to create pDJSVT1; the base vector for all gene deletion constructs that consists of an *E. coli* origin and chloramphenicol/thiamphenicol resistance and a GC-rich, multiple-cloning site for efficient cloning of AT-rich *F. nucleatum* DNA. (B) pDJSVT7 plasmid incorporation of a constitutively active *FLAG:galk* gene for selection on 2-deoxy-galactose. (C) Development of a chromosomal complementation vector that incorporates the entire pDJSVT11 plasmid onto the chromosome at the *arsB* gene in *F. nucleatum* 23726.

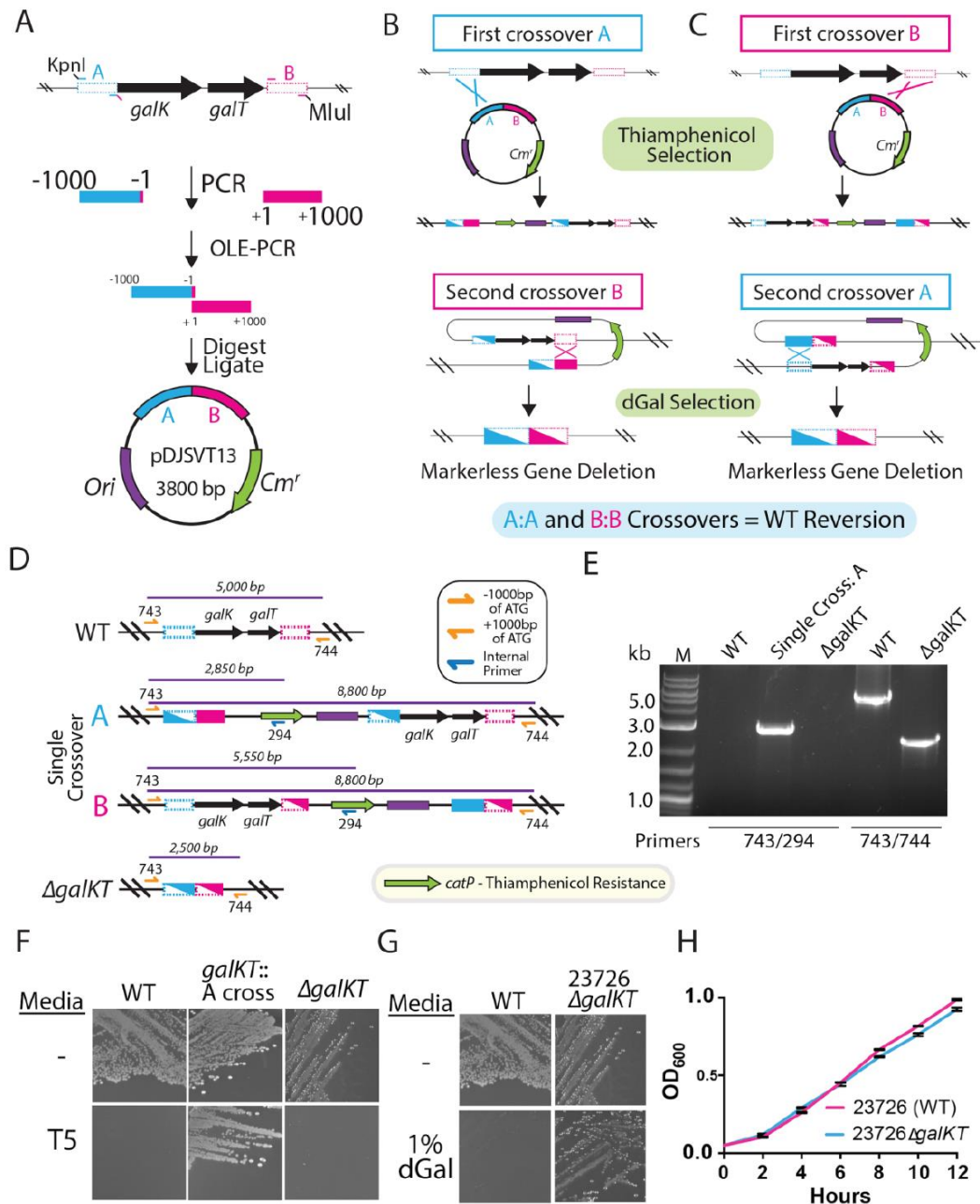


Figure 3.9 Deletion of the *galkT* gene operon in *F. nucleatum* 23726

(A) Development of pDJSVT13, the plasmid for *galkT* gene deletion by inserting 1000 bp *galkT* flanking sequences into pDJSVT1. (B) Representation of single-crossover homologous recombination onto *F. nucleatum* 23726 chromosomes with either the A (upstream) or (C) B (downstream) 1000 bp DNA fragments in pDJSVT13 followed by showing how the double crossover results in complete plasmid excision. (D) Map of chromosomal plasmid incorporation after the initial single-crossover insertion. (E) PCR verification of single-cross pDJSVT13 and subsequent double-crossover deletion of the *galkT* operon. (F) Plating of 5 μg/mL thiamphenicol (single-crossover selection) and 1% 2-

deoxy-galactose (double-crossover), resulting in *galKT* operon deletion. (G,H) Validation that *F. nucleatum* 23726 $\Delta galKT$ grows the same as WT *F. nucleatum* 23726.

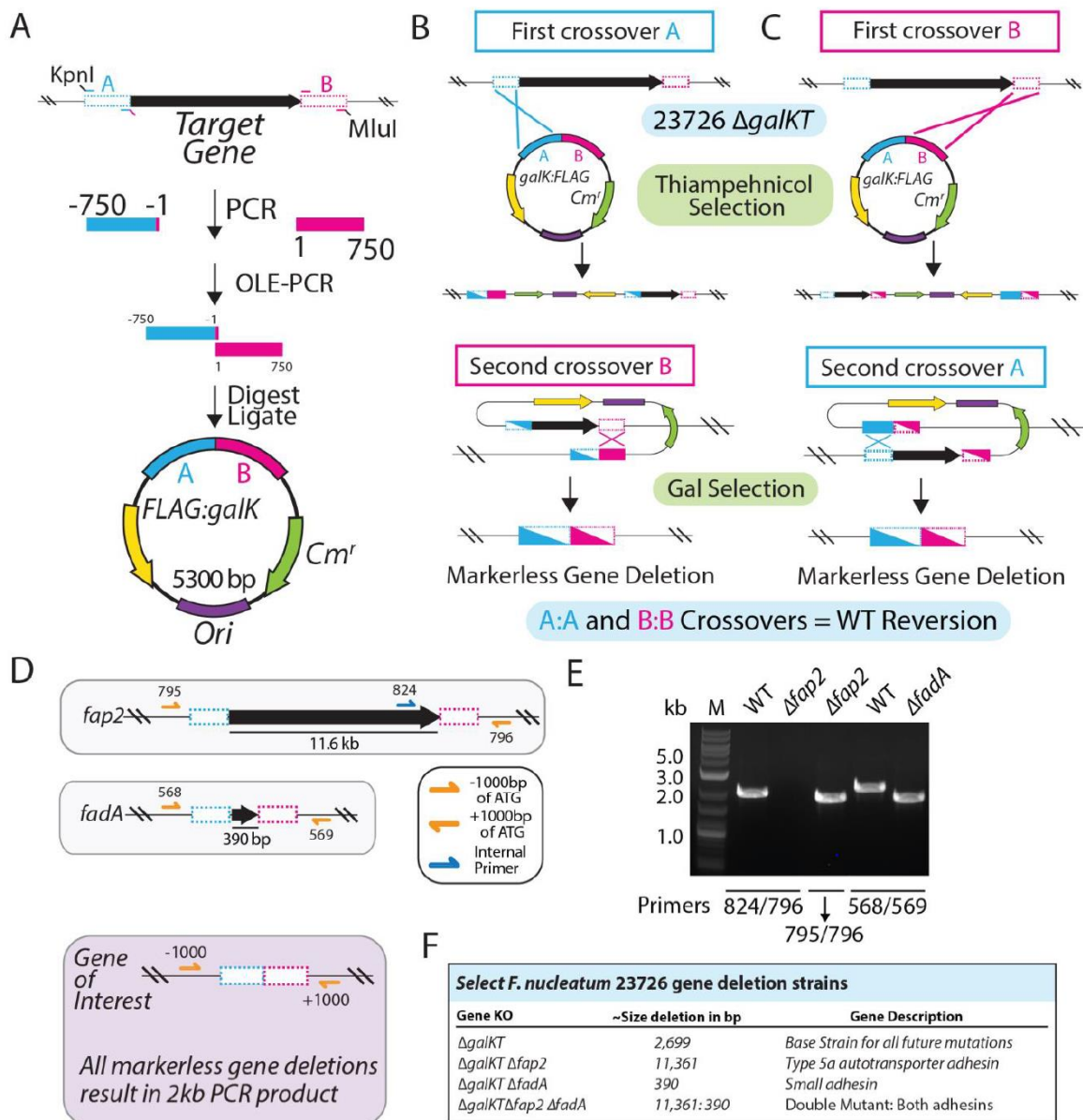


Figure 3.10 Target gene deletion in *F. nucleatum* 23726 $\Delta galKT$ (Fnn)

(A) Development of all target gene deletion plasmids by inserting 750 bp target gene flanking sequences into pDJSVT7. (B) Representation of single-crossover homologous recombination onto the *F. nucleatum* 23726 chromosomes with either the “A” (upstream) or (C) “B” (downstream) 750 bp DNA fragments in followed by a depiction of how the double crossover results in complete plasmid excision. (D) *fap2* and *fadA* as examples of gene excision. KO validation primers are shown flanking genes at -1000 and +1000 bp. This results in 2kb PCR products as shown in (E) for the *fap2* and *fadA* gene deletions. (F) Table of select deleted genes in this study and their function.

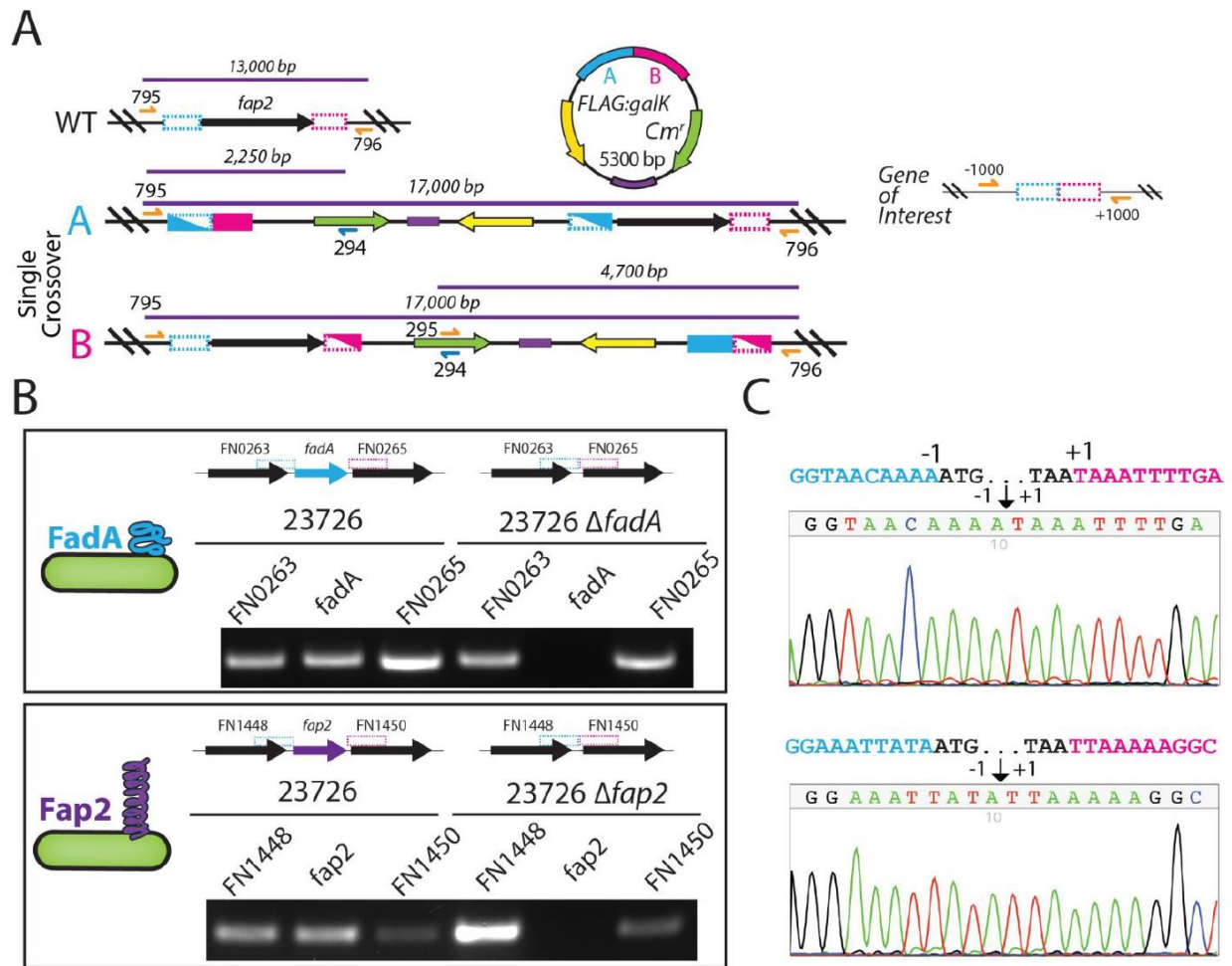


Figure 3.11 Validation of markerless *fap2* and *fadA* gene deletions in *F. nucleatum* 23726 $\Delta galKT$ (Fnn)

(A) Map of chromosomal plasmid incorporation after the initial single-crossover insertion for *fap2*. (B) RT-PCR of *fadA* and *fap2* with their two surrounding genes showing loss of gene transcription due to gene deletion. (C) DNA sequencing with primers sitting -250 and +250 from the start codon of *fadA* and *fap2*. All constructs created are 100% accurate and show perfect excision and recision of the genome to the -1 and +1 base for each gene.

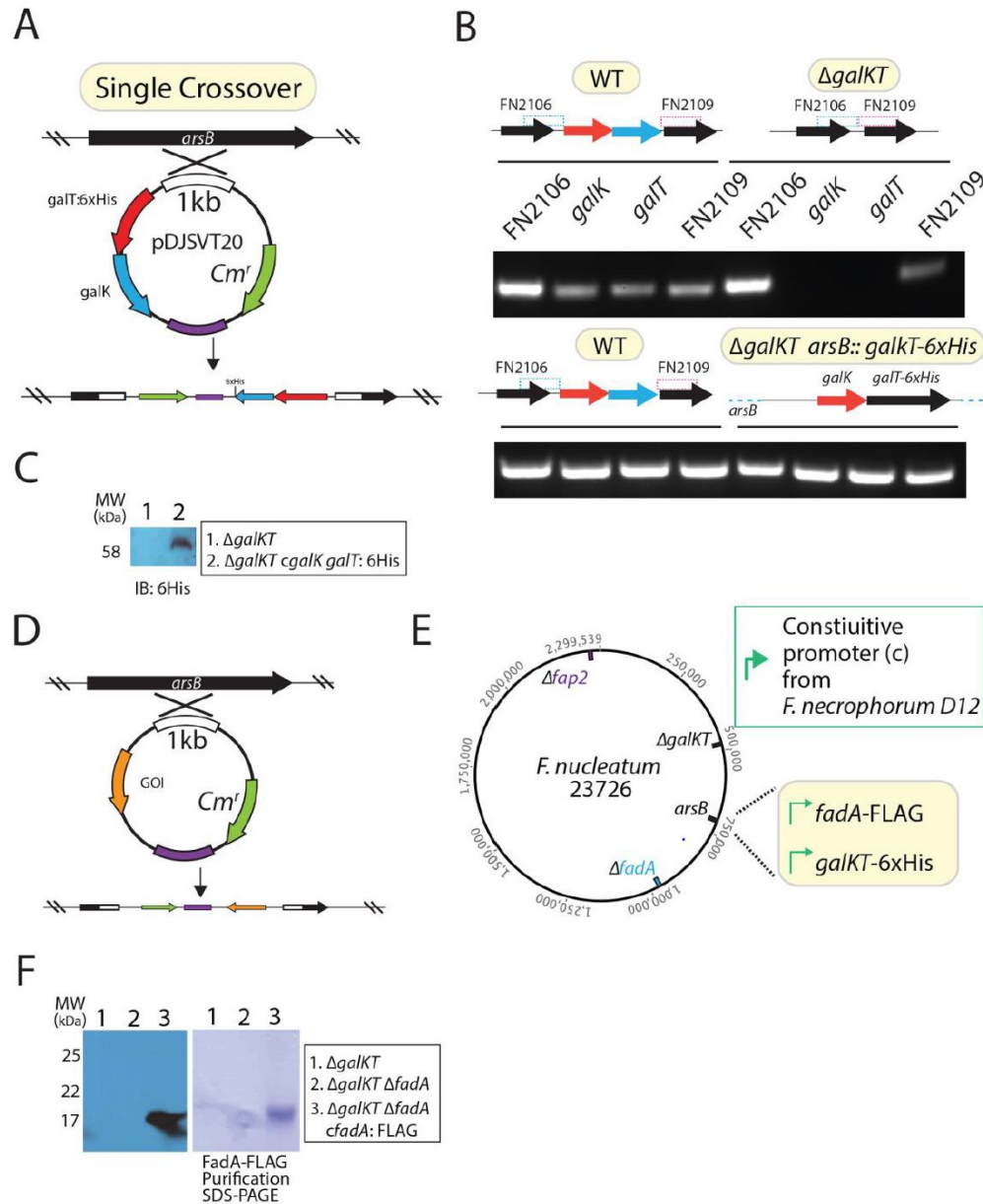


Figure 3.12 Complementation of gene deletions at the static *arsB* gene site in *F. nucleatum* 23726.

(A) pDJSVT20 plasmid created for complementing $\Delta galKT$ with *galK**T*::6xHis. (B) RT-PCR of $\Delta galKT$ and $\Delta galKT$ *galK**T*::6xHis and the two genes flanking the operon (FN2106, FN2109). (C) Western blot verification of *galK**T*::6xHis complementation and constitutive expression of GalT::6xHis. (D) pDJSVT11 base vector depicted with target gene of interest complementation and how it incorporates into the chromosome at *arsB*. (E) Potential sites for complementation of gene deletions used in this study. However, we did not complement the $\Delta fap2$ strain due to the extreme difficulties of complementing large genes (12kb). (F) Complementation of $\Delta fadA$ with *fadA*::FLAG and Western blot verification of constitutive expression of FadA::FLAG.

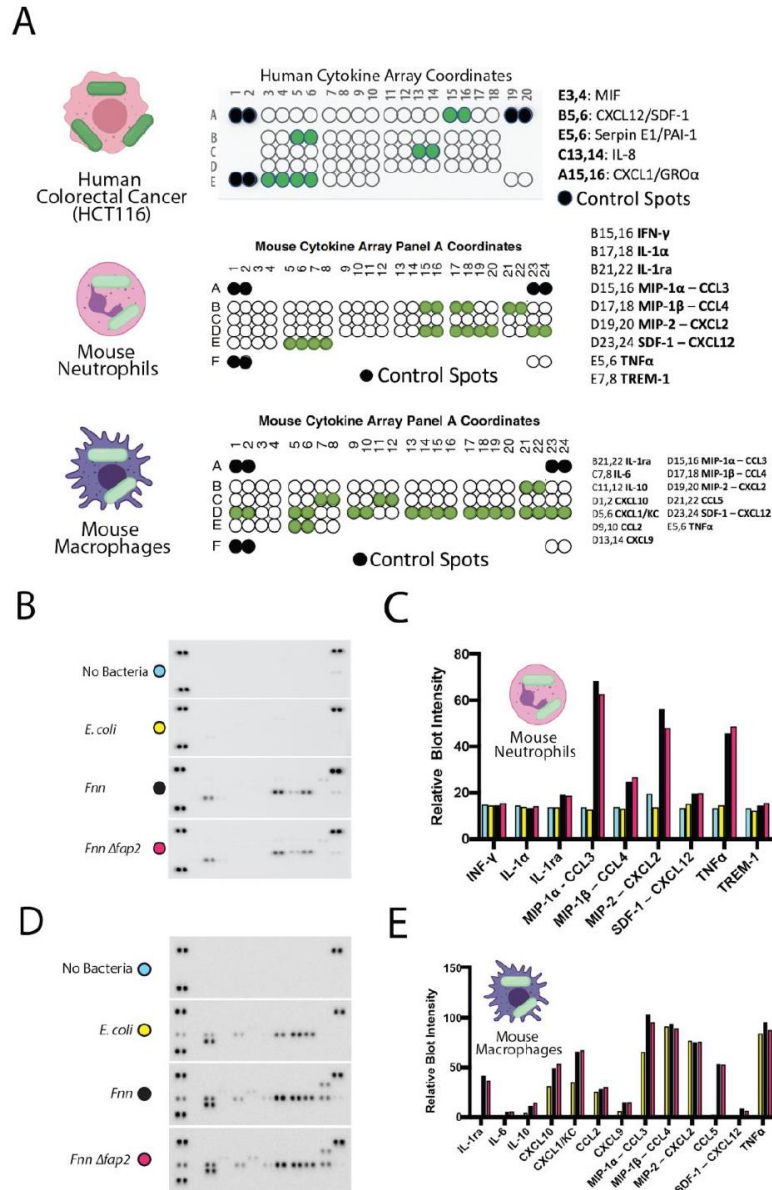


Figure 3.13 Human and mouse cytokine arrays to detect *F. nucleatum*-induced immune cell signaling

(A) Cytokine array coordinates for human and mouse array with green dots representing spots analyzed due to detected expression. Gene names are shown to the right, and full coordinates for all spots can be found at R&D Systems [R&D: Proteome Profiler Human Cytokine Array (ARY005B), Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006)]. (B) Raw blots for mouse cytokines secreted by mouse neutrophils after infection with *F. nucleatum* 23726 at 50:1 MOI for four hours. (C) Quantitation of relative spot intensity compared to the control blot of uninfected neutrophils. (D) Raw blots for mouse cytokines secreted by mouse macrophages after infection with *F. nucleatum* 23726 at 50:1 MOI for four hours. (E) Quantitation of relative spot intensity compared to the control blot of uninfected macrophages. All data are representative of n=3 independent experiments.

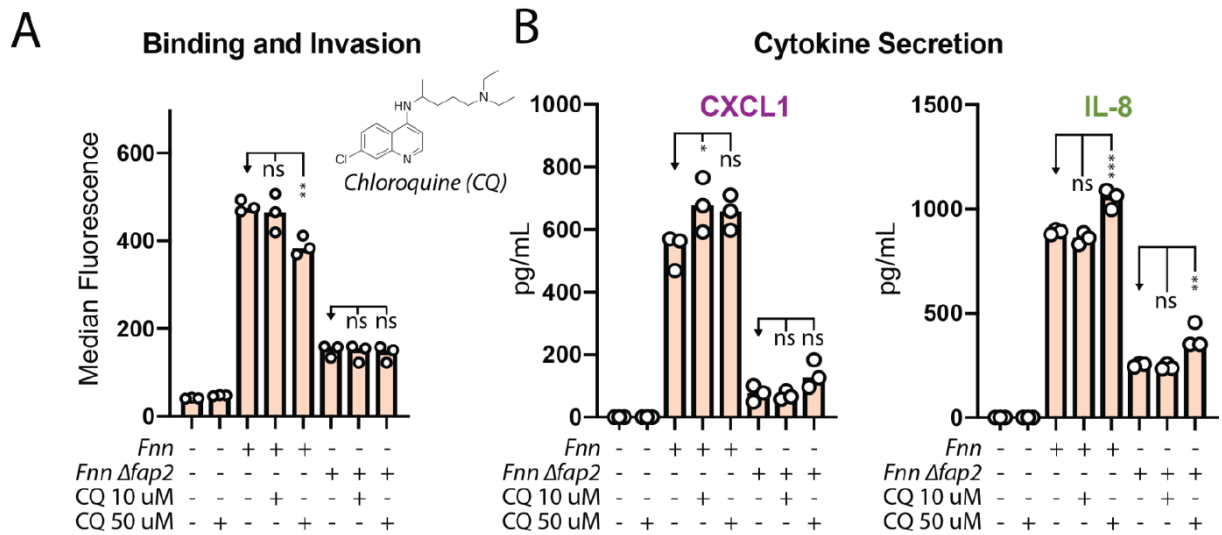


Figure 3.14 Chloroquine does not inhibit *F. nucleatum* cellular invasion and cytokine secretion.

(A, B) Effect of chloroquine, an autophagy and endosome maturation inhibitor (10 or 50 μ M), on Fnn invasion (A) and CXCL1 and IL-8 secretion (B). $n=3$ independent flow cytometry experiments. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by unpaired Student's t test or two-way ANOVA for single or grouped analyses, respectively.

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4 *F. nucleatum* infection in pancreatic cancer stimulates cancer cell proliferation and migration

This chapter has been adapted from:

Fusobacterium nucleatum induces pancreatic cancer cell proliferation and migration by regulating host autocrine and paracrine signaling.

*Barath Udayasuryan, Tam T. D. Nguyen, Ariana Umaña, Raffae N. Ahmad, LaDeidra Monét Roberts, Polina Sobol, Stephen D. Jones, Jennifer M. Munson, Daniel J. Slade, Scott S. Verbridge
BioRxiv. (2022) (Submitted & under review at the time of writing)*

4.1 Abstract

Pancreatic ductal adenocarcinoma (PDAC) harbors a complex tumor microbiome that has been implicated in cancer progression and resistance to chemotherapy. Recent clinical investigations uncovered a correlation between high loads of intratumor *Fusobacterium nucleatum* and decreased patient survival. Here we show that healthy and cancerous pancreatic cell lines harboring intracellular *F. nucleatum* secrete increased levels of cancer-associated cytokines including GM-CSF, CXCL1, IL-8, and MIP-3 α . We report that GM-CSF (granulocyte-macrophage colony stimulating factor) secretion directly increases the proliferation and migration of pancreatic cancer cells via an autocrine mechanism, notably in the absence of immune cell participation. Furthermore, we show that non-cancerous pancreatic epithelial cells do not exhibit increased proliferation or migration in response to these cytokines, but nevertheless, their secreted cytokines stimulate these responses in cancerous cell lines through paracrine signaling. Our results provide evidence that intratumor *F. nucleatum* in the pancreas elicits an infection-specific cytokine secretion profile from both normal and cancerous cells that adversely contributes to cancer progression through autocrine and

paracrine mechanisms. Therefore, these results support the importance of investigating the contributions of both microbiome and host driven processes in pancreatic cancer to guide future therapeutic interventions.

4.2 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy that has a median survival of fewer than 6 months, and a 5-year survival rate of 3-7%¹. Oncogenic *KRAS* mutation is critical in driving disease progression from early, preinvasive precursor lesions to a malignant and invasive adenocarcinoma²⁻⁴. Characterized by extensive stromal remodeling and desmoplasia, the PDAC tumor microenvironment (TME) has limited vascularization and poor immune infiltration which makes it highly intractable to chemotherapy^{5,6}. Furthermore, the TME is enriched with immunosuppressive cells such as T regulatory (Treg) cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs)^{7,8}. This immune suppression is further compounded by tumor-specific microbial species residing within the TME which have been implicated in worse outcomes for patients with PDAC⁹⁻¹¹.

Recent systematic characterization of intratumoral bacteria in multiple cancer types details a diverse bacterial ecosystem within tumors that is not found in adjacent healthy tissue¹². These observations have elicited several fundamental questions on the precise role that microbes play within the TME to impact tumorigenesis, cancer progression, and therapy response^{13,14}. Little is known on how these bacteria reach the tumor, the survival strategies that they employ within the TME, the host tumor cells' response to the infection, and if their elimination could augment cancer therapy. Previous studies have revealed positive associations of certain microbes with the development of PDAC. These include *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Neisseria elongata*, *Streptococcus mitis*^{11,15}, and *Helicobacter pylori*¹⁶. Moreover, the PDAC tumor microbiome has been shown to

influence immune response and can impact chemotherapy¹⁷. For example, Gammaproteobacteria in PDAC tissue regulates drug resistance to a common chemotherapeutic agent, gemcitabine¹⁸. However, there is still limited insight into the individual roles these bacteria play in worsening cancer prognosis. Our work aims to shed light on the role of a specific microbe, *F. nucleatum*, and its impact within the PDAC TME.

Fusobacterium nucleatum is a Gram-negative, anaerobic, rod-shaped bacterium usually found within the oral cavity. It is a commensal microorganism that plays a supportive role as a bridge-species within biofilms connecting primary and secondary microbial colonizers¹⁹. However, in certain disease conditions, it can act as an opportunistic pathogen. In addition to participating in inflammatory diseases within the oral cavity, such as in gingival and periodontal disease, it is a risk-factor in preterm birth and intrauterine infections²⁰. There is also associative evidence of its role in appendicitis²¹, urinary tract infection, endocarditis²², and other respiratory tract infections²³. Moreover, *F. nucleatum* is increasingly recognized as an oncomicrobe due to its disproportionate presence in several cancers and association with a worse prognosis. These cancers include colorectal cancer^{24,25}, esophageal cancer²⁶, pancreatic cancer^{27,28}, and Lauren's diffuse type gastric cancer²⁹.

To date, most mechanistic studies of *F. nucleatum* have focused on its role in colorectal cancer (CRC). *F. nucleatum* uses its surface adhesin, Fap2, to initially bind to host Gal/Gal-NAc³⁰ sugar residues found abundantly in CRC, which then drives entry into host cells and intracellular colonization. In fact, Gal/Gal-NAc is highly expressed in a wide variety of tumors and may act as a key factor in bacterial homing to these sites³¹⁻³³. In addition, the adhesin FadA has been shown to bind to E-cadherin, which activates β -catenin and stimulates downstream signaling that promotes carcinogenesis³⁴. However, there is still a critical knowledge gap in our understanding of how *F. nucleatum* adapts to the TME or continues to influence cancer phenotypes once colonizing the TME, to exacerbate the aggressiveness of both early and late-stage cancers³⁵. Moreover, it has been shown

that *F. nucleatum* may travel within the primary cells to distant tumor metastatic sites³⁶ and could contribute to pre-metastatic niche formation. We have previously shown that *F. nucleatum* infects host cells in a Fap2-driven mechanism, and leads to the secretion of potent cytokines, IL-8 and CXCL1, which we demonstrated to directly increase cellular migration of a colorectal cancer cell line, HCT116³⁷, which may directly facilitate metastatic spread of CRC.

Emerging evidence has tied *F. nucleatum* to increased mortality from pancreatic cancer. Mitsuhashi et al. initially uncovered the association of *Fusobacterium* species in PDAC²⁸. They tested cancer tissue specimens from 283 patients with PDAC and identified an 8.8% detection rate of *Fusobacterium* species and observed highly significant mortality in the *Fusobacterium* positive group. Gaiser et al. identified an enrichment of oral bacterial taxa including *F. nucleatum* and *Granulicatella adiacens* in cyst fluid from intraductal papillary mucinous neoplasms (IPMNs) with high-grade dysplasia (HGD)²⁷. Additionally, Alkharaan et al. identified circulating IgG reactivity to *F. nucleatum* and high salivary IgA reactivity to *F. nucleatum*, and Fap2 of *F. nucleatum* in patients with HGD³⁸. Further corroboration comes from the study by Nejman et al. which identified *F. nucleatum* in pancreatic cancer with a prevalence of 15-20% from the 67 tumor samples that were tested¹². These observations suggest a disproportionate presence of *F. nucleatum* within the pancreatic TME, warranting further research into its function in pancreatic cancer pathogenesis.

A distinguishing feature of PDAC is that tumor cells form only a small fraction of the TME. In fact, studies have shown how PDAC progression and resistance to therapy is considerably driven by its dense stroma, which can constitute up to 80% of the tumor³⁹. It consists of multiple non-oncogenic cell types including cancer associated fibroblasts, pancreatic stellate cells, immune cells, and endothelial cells that shape the behavior of PDAC cells, by remodeling the extracellular matrix, promoting epithelial-mesenchymal transition (EMT), and fostering aberrant cell signaling^{40,41}. The

dense fibrosis and limited vascularization hence formed creates a stiff and hypoxic TME that could provide a favorable niche for anaerobic organisms such as *F. nucleatum*.

Our previous findings from *F. nucleatum* infection of CRC cells showed that bacterial infection can exert local effects on cancer cells without the involvement of immune cells³⁷. We hypothesized that *F. nucleatum* may play an analogous role in PDAC to impact tumor progression. There have been few if any studies to our knowledge that have investigated the phenotype of *F. nucleatum* infected healthy and cancerous pancreatic cell lines, and it is unclear if the infection of these cells is Fap2-driven as in the case of CRC. We therefore sought to investigate the specific role of *F. nucleatum* in pancreatic cancer and identify key secretory factors from host cells that could contribute to worse outcomes in patients harboring this bacterium in their tumors.

4.3 Results

We carried out our studies using four pancreatic cancer cell lines and a primary normal pancreatic epithelial cell line, the details of which are shown in **Supplementary Table 1**⁴². These lines were selected as they were extensively characterized, and exhibit differences in sex, metastasis, and differentiation, which could impact their response to *Fusobacterium* infection. The data from BxPC3 and Panc1 cell lines are shown in the main text, and the data from HPAC and Capan1 cell lines can be found in the Supplementary Material.

4.3.1 *Fusobacterium* binds and invades BxPC3 and Panc1 cells through a Fap2-driven mechanism

We first confirmed that *F. nucleatum* can bind and invade pancreatic cancer cells. Scanning electron microscopy (SEM) imaging enabled direct visualization of the various modes of binding and invasion of *F. nucleatum subsp. nucleatum* ATCC 23726 (which will be subsequently referred to as *Fnn* in this article) into BxPC3 cells (**Figure 4.1A**). We further confirmed the intracellular localization of *Fnn*

within BxPC3 cells through confocal imaging and flow cytometry (**Figure 4.1B**). We sorted the cells based on the intensity of fluorescence observed and imaged them in high resolution z-stacks to visualize the number of intracellular bacteria within each cell. Based on our work and previous research highlighting the importance of Fap2 in *F. nucleatum* binding, we hypothesized an analogous interaction with pancreatic cancer cells. Using flow cytometry, we identified that the Fap2 surface adhesin deletion mutant (*Fnn Δfap2*), previously created and used in our work^{37,43}, infects both BxPC3 and Panc1 cells at a significantly decreased rate compared to the wild-type *Fnn* (**Figure 4.1C**).

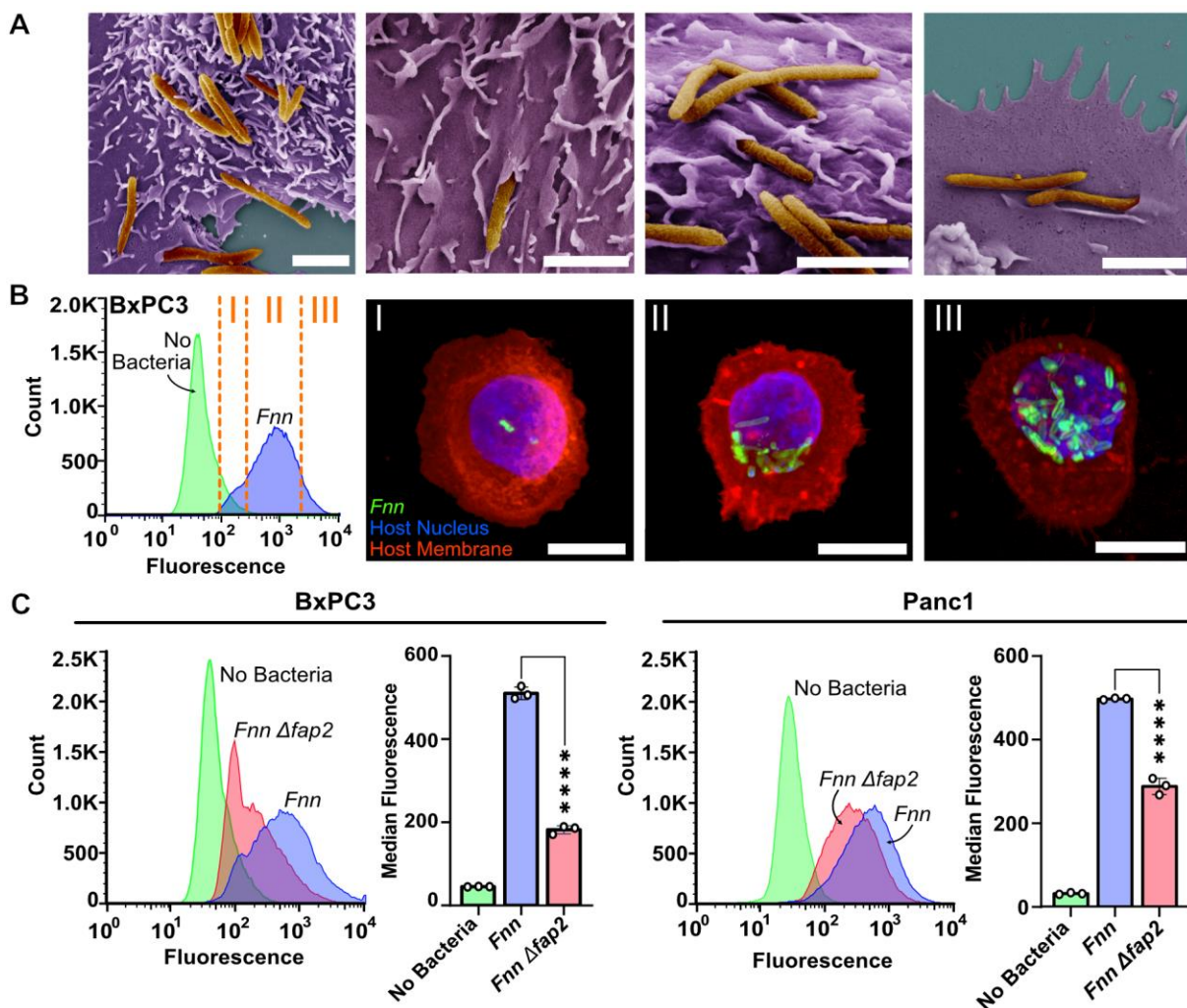


Figure 4.1 *Fusobacterium nucleatum* binds to and invades pancreatic cell lines.

(A) Scanning electron microscopy images of *F. nucleatum subsp. nucleatum* 23726 (*Fnn*) invading BxPC3 pancreatic cancer cells line through multiple points of adherence to the cell membrane.

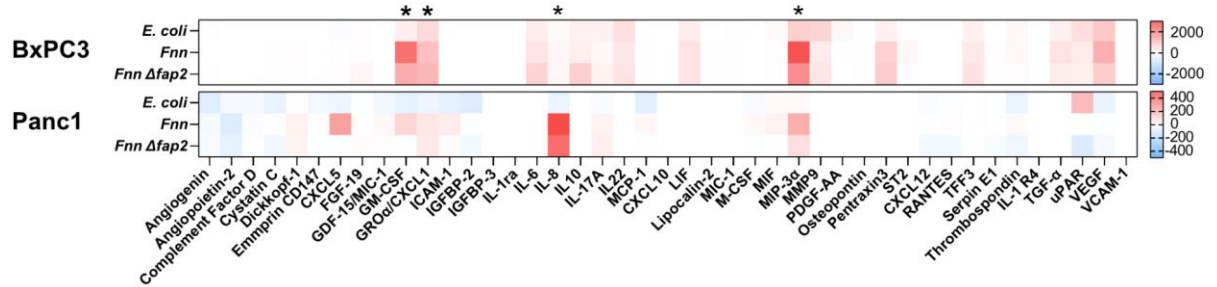
Pseudocolored with: bacteria in orange, host cells in purple (scale bar: 2µm). (B) Flow cytometry analysis of BxPC3 cells without bacteria (No Bacteria) and with infection with *Fnn* at 50:1 MOI demonstrating an increase in fluorescence of infected cells. The cells were further sorted and collected based on three levels of fluorescence intensity (I,II,III), corresponding to increased intracellular bacterial loads. These cells were plated and imaged on a confocal microscope to identify relative levels of infection. Blue: host cell nuclei stained with DAPI; green: *Fnn* membrane stained with FM 1-43 FX; red: host cell membrane stained with MemBrite 568 (Biotium) (scale bar: 10 µm). (C) Flow cytometry analysis of *Fnn* vs *Fnn Δfap2* infection of BxPC3 and Panc1 cells at 1 hour after a 50:1 MOI shows a significantly slower infection of *Fnn Δfap2* indicating Fap2 driven intracellular invasion. N = 3 independent experiments, compared using ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test. P-value significance denoted by ns (not significant) for P>0.05, and **** for P≤0.0001.

4.3.2 Host pancreatic cancer cells secrete specific cytokines upon *Fusobacterium* infection

Once we confirmed host cell binding to *Fnn* and subsequent intracellular invasion into host cells, we then analyzed the host cells' response to infection. Western blot arrays were used to identify infection-induced cytokine secretions from the pancreatic cancer cell lines tested in four conditions: (a) No bacteria (b) infection with *Escherichia coli*, (c) infection with *Fnn* and (d) infection with *Fnn Δfap2*. Heat maps were constructed based on the raw intensities (**Figure 4.7**) and the percent fold change in protein secretion compared to the No Bacteria condition was determined (**Figure 4.2A**). While pancreatic cancer cell lines are highly secretory without infection, we identified *Fnn* infection specific increases in secretion for GM-CSF, CXCL1, IL-8, and MIP-3α. There was minimal or no change in secretion in response to infection by *E. coli*. ELISAs were performed to confirm and quantify the secretion of these cytokines, and significant increases were noted for the four cytokines when compared to the No Bacteria condition for BxPC3 and Panc1 cells (**Figure 4.2B, Figure 4.8**). There was a significant increase in IL-8 and CXCL1 secretion upon infection with *E. coli* for BxPC3 cells but not for Panc1 cells. Furthermore, there was a significant increase in the secretion of GM-CSF in BxPC3 cells upon infection with *E. coli* with respect to No Bacteria, but was significantly lower (p≤0.001)

compared to GM-CSF secretion elicited by infection with *Fnn* and *Fnn Δfap2*. Notably, there was minimal change in response to measured cytokine values between infection with *Fnn* and *Fnn Δfap2*.

A Percent Change in Secretion in Response to Infection



B Specific Secretion of Cytokines

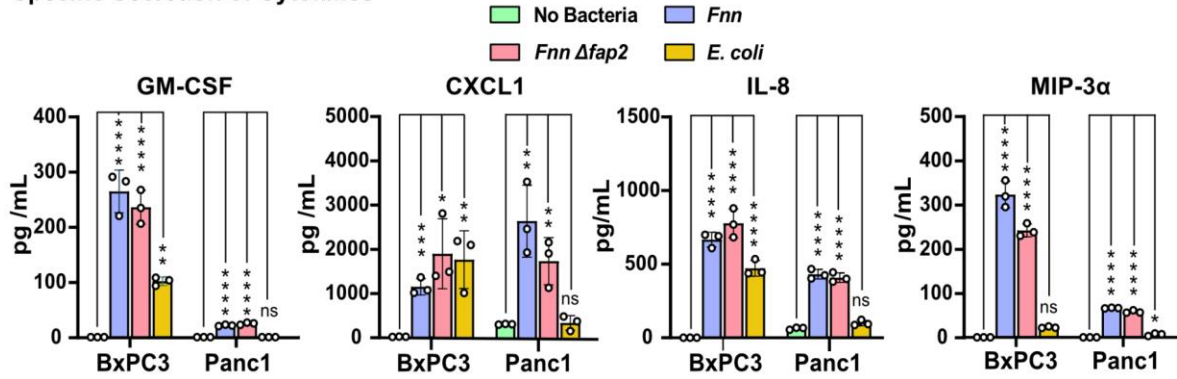


Figure 4.2 *Fnn* infection elicits secretion of specific cytokines from host cells.

(A) Heat maps depicting the percent change in cytokine secretion in response to infection in comparison to the non-infected samples of BxPC3 and Panc1 cell lines tested using the Human XL cytokine array (R&D Systems Inc.) upon infection with *E. coli*, *Fnn*, and *Fnn Δfap2*. Using these results, we identified the specific increase in secretion of GM-CSF, CXCL1, IL-8, and MIP-3α (marked with an asterisk). (B). Measured values of selected cytokines using ELISA confirming specificity to *Fnn* infection. N = 3 independent experiments, compared using ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test. p-value significance denoted by ns (not significant) for P>0.05, * for P≤0.05, ** for P≤0.01, *** for P≤0.001, and **** for P≤0.0001.

4.3.3 GM-CSF increases the proliferation of pancreatic cancer cells

We hypothesized that the four cytokines we identified from the cytokine arrays could be involved in impacting the proliferation of pancreatic cells; a common feature in aggressive cancers. We first observed their impact on proliferation individually on BxPC3 cells using an XTT assay (**Figure 4.9**). From this preliminary assay, we discovered that GM-CSF at a concentration of 200 pg/mL increased the proliferation of BxPC3 cells, while the other cytokines, IL-8, CXCL1, and MIP-3 α , did not. In both BxPC3 and Panc1 cells, we observed increased proliferation of cells when infected with *Fnn*. Furthermore, conditioned media obtained from infected cells after a 4-hour infection was sufficient to induce the proliferation of uninfected cells. This was confirmed by two independent assays to quantify proliferation (BrdU and XTT, **Figure 4.3A-B**). The addition of recombinant human GM-CSF (rhGM-CSF) at 200 pg/mL was sufficient to increase the proliferation of BxPC3 and Panc1 cells (**Figure 4.3C**). To confirm that GM-CSF was primarily responsible for the proliferative effect, we depleted GM-CSF from the conditioned media using biotinylated anti-GM-CSF antibodies to capture free GM-CSF and used streptavidin-coated magnetic particles to capture and spin down the cytokines and observed a concomitant decrease in proliferation (**Figure 4.3D**). Since Panc1 cells showed lower but measurable secretion of GM-CSF, we supplemented 200 pg/mL rhGM-CSF to these cells, and depleted rhGM-CSF from the media once again and confirmed that GM-CSF directly contributed to an increase in cell proliferation of this pancreatic cancer cell line (**Figure 4.3E**). This last assay indicates that exogenous GM-CSF within the TME can directly impact the proliferation of pancreatic cancer cells.

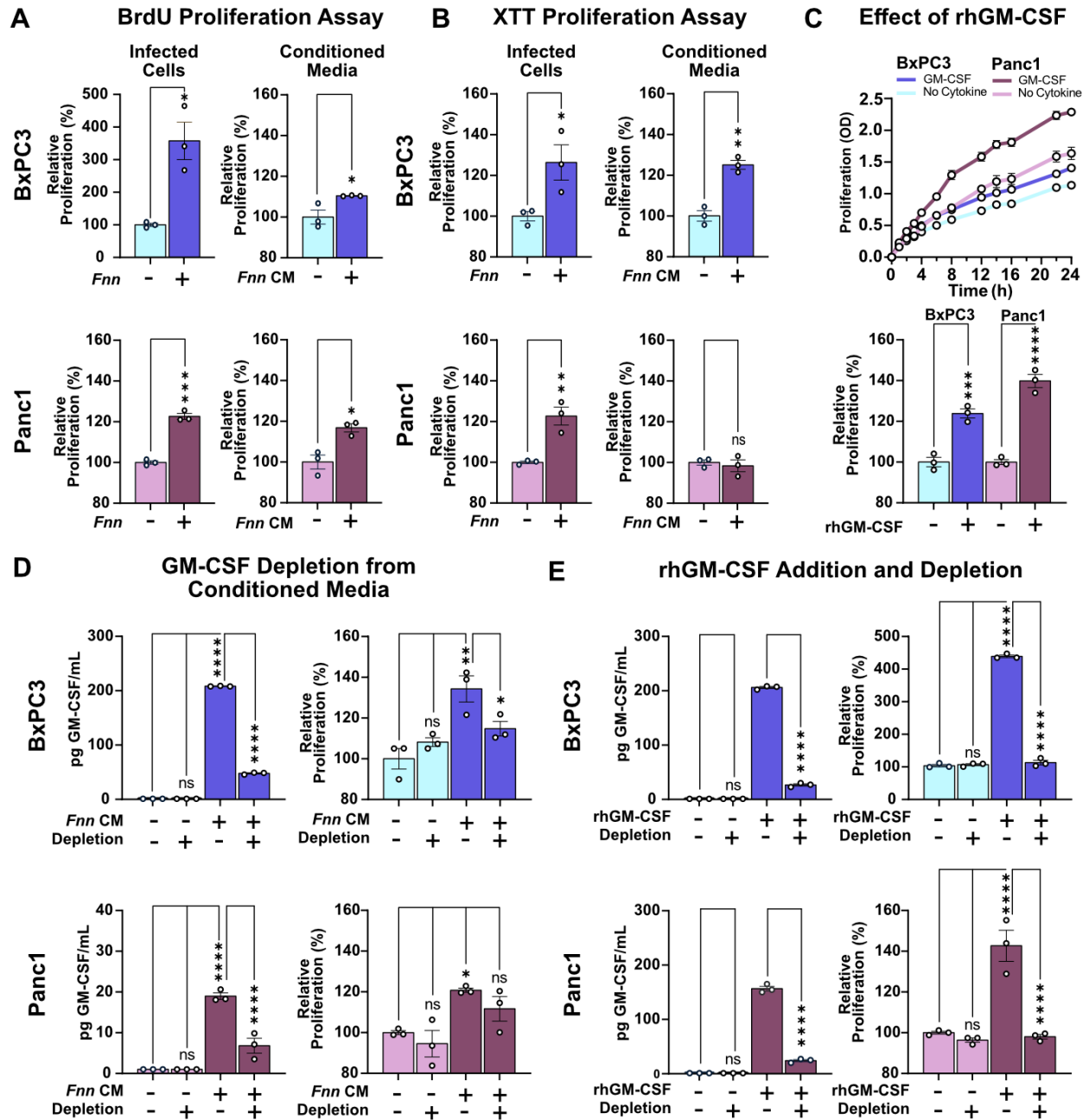


Figure 4.3 GM-CSF induces pancreatic cell proliferation.

(A, B) BrdU and XTT assays both independently confirmed that *Fnn* infected host BxPC3 and Panc1 cells have increased proliferation compared to non-infected cells. This increase in proliferation of non-infected cells is sustained in the presence of conditioned medium (*Fnn CM*) isolated from infected cells. (C) Addition of recombinant human GM-CSF (R&D Systems 200 pg/mL) to BxPC3 and Panc1 cells significantly increases their proliferation. (D) GM-CSF depletion from conditioned media obtained from *Fnn* infected cells significantly reduced the relative proliferation of BxPC3 and Panc1 cells. (E) GM-CSF depletion after supplementing cells with exogenous rhGM-CSF also reduces cell proliferation of BxPC3 and Panc1 cells. N = 3 independent experiments, compared using student's t-

test, ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test. p-value significance denoted by ns (not significant) for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$.

4.3.4 Infected Cells and non-infected cells exhibit increased migration in response to

***Fnn* induced host cell secretion**

Since several studies have implicated the four upregulated cytokines we identified in cell migration, we hypothesized that the *Fnn* induced host secretion would impact pancreatic cancer cell migration. We determined the migration of *Fnn* infected and uninfected host cells in an in vitro Transwell co-culture model. BxPC3 cells were infected with *Fnn* at 50:1 MOI for 4 hours after which they were collected and added to the top chamber of a Transwell with a membrane that consisted of 8µm pores. *Fnn* infected BxPC3 cells trans-migrated across the membrane in a Transwell assay at a significantly increased rate compared to uninfected cells over 16 hours (**Figure 4.4A**). In another assay, we collected conditioned media from the 4-hour infection of host cells with *Fnn* at 50:1 MOI, filtered and concentrated it 16x, then added the concentrated media to the outer (lower) chamber of the transwell. We observed significantly greater migration of uninfected BxPC3 cells from the top of the membrane to the bottom in response to concentrated conditioned media obtained from infected cells than in response to concentrated conditioned media obtained from uninfected cells over 16 hours (**Figure 4.4B**). The short time period of 16 hours and using 1% serum supplemented media restricted the contribution of cellular proliferation in this assay.

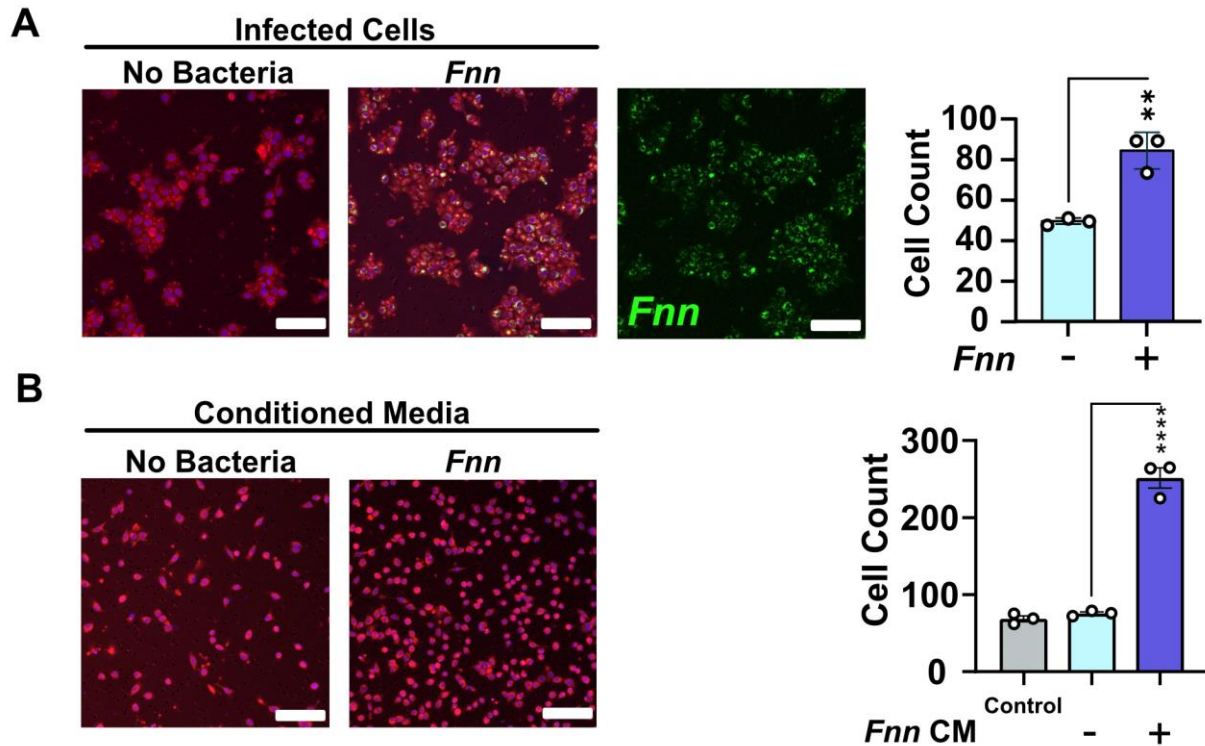


Figure 4.4 Increased cell migration observed for both *F. nucleatum* infected cells and non-infected cells in response to conditioned media obtained from *F. nucleatum* infected cells.

Representative images of Transwells with BxPC3 cells that have migrated over 16 hours in response to the control with 0.5% FBS, and conditioned media obtained from cells not infected and infected with *Fnn*. (Staining: Red: Cell Tracker Red, Blue: DAPI, Green: *Fnn* with FM 1-43X) (A) BxPC3 cells infected with *Fnn* show significantly increased migration compared to uninfected cells. (B) BxPC3 cells show significantly increased migration in response to conditioned media obtained from infected cells (Staining: Red: Cell Tracker Red, Blue: DAPI) (scale bar = 100µm). N = 3 independent experiments, compared using ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test. P-value significance denoted by ns (not significant) for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$.

4.3.5 Normal pancreatic epithelial cells secrete cytokines upon *F. nucleatum* invasion that stimulate proliferation and migration of cancer cells

We next studied the infection of a normal pancreatic epithelial cell line (which will be referred to as nPEC in this text) and compared its response with that from infection of pancreatic cancer cell lines. Flow cytometry analysis of nPECs upon infection with *Fnn* and *Fnn Δfap2* indicated that the infection occurs at the same rate for both strains (**Figure 4.5A, Figure 4.10**). Next, ELISA of conditioned media from nPECs confirmed a significant secretion of GM-CSF, CXCL1, IL-8, and MIP-3 α in response to *Fnn* and *Fnn Δfap2* infection, which was absent upon infection with *E. coli* (**Figure 4.5B**). There was also reduced secretion of GM-CSF upon infection with *Fnn Δfap2* when compared to infection with *Fnn*. Proliferation assays were subsequently performed and in contrast to the cancer cell lines, nPECs did not increase proliferation upon infection or in the presence of conditioned media obtained from infected nPECs (**Figure 4.5C**). Similarly, the nPECs did not proliferate in the presence of 200pg/mL recombinant hGM-CSF (**Figure 4.5D**). However, when conditioned media obtained from infected nPECs was added to BxPC3 cells, it stimulated BxPC3 cells to proliferate (**Figure 4.5E**). Next, we studied migratory response in a Transwell assay and observed that infection of nPECs did not impact migration in comparison to uninfected nPECs (**Figure 4.5F**). Furthermore, we observed that conditioned and concentrated media from infected nPEC cells did not impact nPEC migration, but significantly increased the cell migration of BxPC3 cells (**Figure 4.5G**).

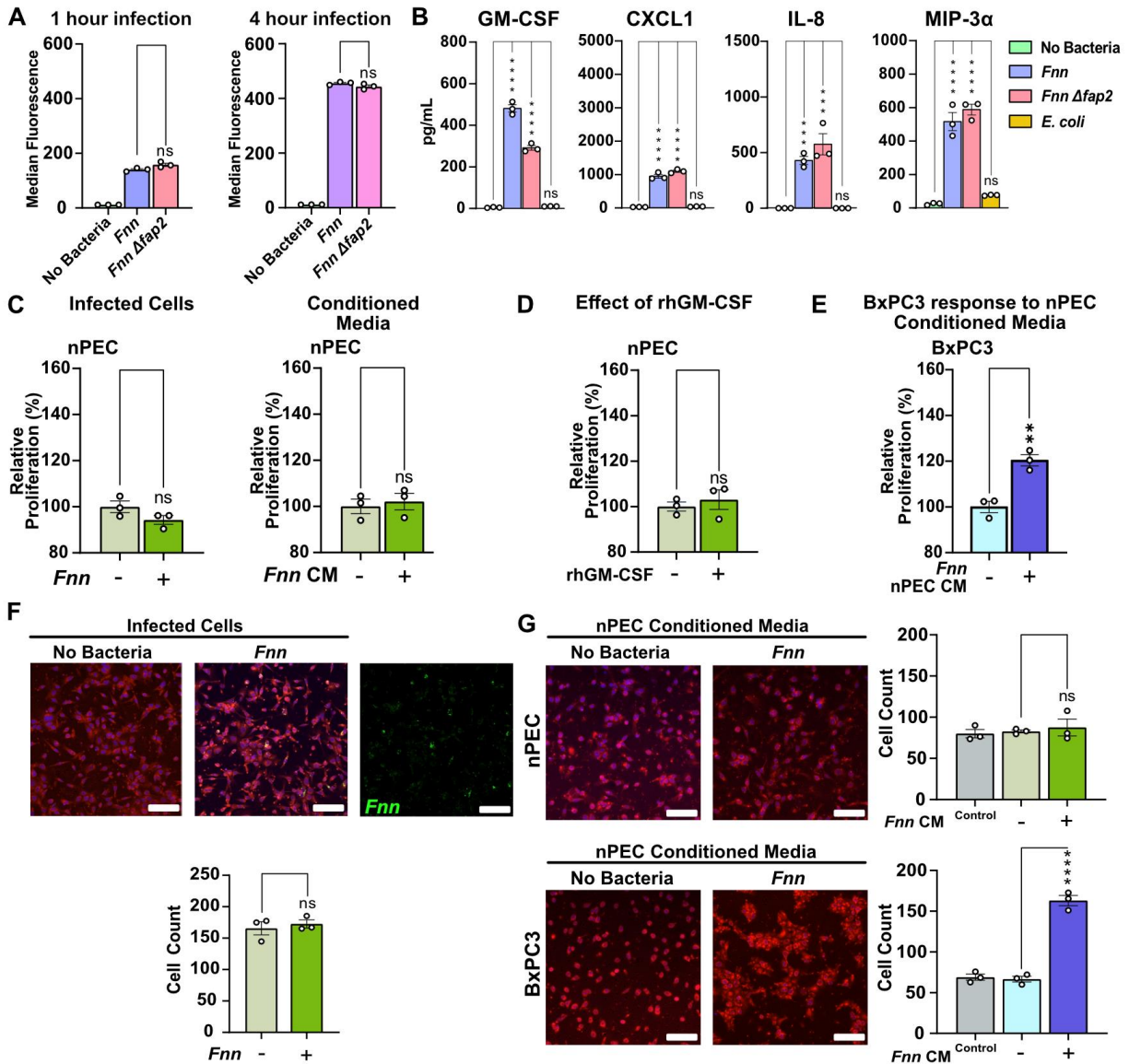


Figure 4.5 Normal Pancreatic Cells response to *F. nucleatum* infection.

(A) Flow cytometry analysis of nPEC infection with *Fnn* and *Fnn* Δ fap2 at 1 and 4 hours indicates equal rate of infection. (B) Increased secretion of GM-CSF, CXCL1, IL-8, and MIP-3 α , from nPECs upon infection with *Fnn*, *Fnn* Δ fap2, and *E. coli* compared with the uninfected condition. (C) XTT proliferation assays show no increase in the proliferation of infected nPECs, nPECs in response to conditioned media obtained from infected cells (D) nPECs do not increase their proliferation in response to rhGM-CSF. (E) BxPC3 cells show increased proliferation in response to conditioned media obtained from infected nPECs. Transwell migration assay shows that infected nPECs do not increase migration in response to infection. (F) Infected nPEC do not show an increase in migration (G) Transwell migration assays with conditioned and concentrated media obtained from infected nPEC cells do not stimulate migration of nPEC cells but stimulate BxPC3 cells to migrate at a significantly increased rate (scale bar: 100 μ m). N = 3 independent experiments, compared using ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test. P-value significance

denoted by ns (not significant) for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$.

4.4 Discussion

Motivated by our prior work on the role of *F. nucleatum* in inducing cytokine secretion and cellular migration in the CRC TME, we hypothesized that *F. nucleatum* may play analogous critical roles within the PDAC TME, potentially directly acting to enhance cancer progression. We sought in this study to leverage in vitro co-culture models to begin to define and quantify these roles that would be challenging to identify and dissect in vivo. We confirmed the intracellular invasion of this bacteria in pancreatic normal and cancer cell lines and have identified the specific secretion of cytokines that may adversely affect tumor progression, by increasing tumor cell proliferation and enhancing tumor cell migration.

Using flow cytometry, SEM, and confocal imaging, we first confirmed intracellular invasion and localization of *F. nucleatum* in pancreatic cell lines. *F. nucleatum* is well-known to bind and invade a variety of epithelial cell types^{19,44} although in most cases, exact mechanisms for organ specific cell binding and invasion need to be investigated further. Among the host of binding adhesins expressed on *F. nucleatum*, a critical adhesin and lectin involved in binding is the 390 kDa outer membrane protein Fap2. Fap2 is a member of the type 5a secreted autotransporter family of proteins which are critical virulence factors expressed by Gram-negative bacteria. This protein functions in adhesion, cell-cell aggregation, biofilm formation, and bacterial invasion. In CRC, it has been shown that Fap2 binding to human inhibitory receptor TIGIT on natural killer (NK) cells and the tumor itself can protect tumors from being targeted by NK cells and also inhibits T cell function by causing tumor-immune evasion^{45,46}. *F. nucleatum* binding to tumor cells is also mediated by Fap2, evidenced in CRC³⁰ and breast cancer³². Its target is Gal-GalNAc (D-galactose- β (1-3)-N-acetyl-D-galactosamine) that is expressed at high levels in adenocarcinomas, including pancreatic cancer^{31,33}. Furthermore, it was

identified that the Fap2 lectin domain can be inhibited by galactose and galactose derivative molecules^{47,48}. Our results reveal significantly decreased infection of BxPC3 and Panc1 cells by the Fap2 deficient *F. nucleatum* in comparison to the wild-type, indicating that Fap2 binding plays a critical role in the intracellular invasion of the bacterium in pancreatic cancer as well. Notably, this difference is not observed upon binding and invasion of normal pancreatic cells by the *fap2* deletion mutant, indicating that healthy pancreatic cell invasion is not driven by Gal-GalNAc docking, and perhaps adhesins other than Fap2 contribute to binding normal epithelial cells through undiscovered protein-protein interactions. A frequently proposed approach to inhibit *F. nucleatum* binding to tumor cells has been to use galactose-containing compounds to inhibit Fap2 lectin domain interaction. However, if normal tissue adjacent to the tumor can also be infected at the same rate, targeting Fap2 interactions would not be effective in stopping healthy tissue secretion of pro-cancerous cytokine profiles that contribute to proliferation and migration through paracrine mechanisms. Therefore, we acknowledge that further research is required to identify and characterize additional adhesins *F. nucleatum* uses to bind to and invade normal cells to block these cross-talking pathways in cancer.

The next focus of our study was the pancreatic cancer cells' response to *F. nucleatum* infection, specifically host cell secretions of cytokines. Cytokines are soluble secreted proteins usually with a molecular weight <30 kDa that mediate cell-cell communication. They bind to high-affinity receptors present on cell membranes that activate paracrine or autocrine signaling pathways to stimulate host cells in response to injury, cellular stress, inflammation, and immune cell recruitment. Cancer cells have hijacked these signaling mechanisms and exploit them to promote angiogenesis, metastasis, differentiation, immune suppression, and drug resistance. Thus, there is considerable focus on developing cancer immunotherapies utilizing or targeting cytokines⁴⁹. However, there is immense difficulty in delineating the individual roles of cytokines due to their synergistic and antagonistic mechanism of action, which additionally have complex temporal and concentration dependence. Our

findings from the cytokine arrays identified *F. nucleatum* specifically induced the secretion of GM-CSF, CXCL1, IL-8, and MIP-3 α by BxPC3, Panc1, and HPAC cells, and increased IL-6, GM-CSF, and MIP-3 α secretion in addition to the constitutive secretion of IL-8 and CXCL1 by Capan1 cells. Pancreatic cancer cells are highly secretory, and in addition to other constitutively secreted factors, these cytokines are well-known players in PDAC tumor progression and immune suppression.

The cytokines that we have identified in this study in response to *F. nucleatum* infection have been implicated in various forms of cancer⁵⁰. Notably, IL-8 is known to regulate multiple aspects of tumor progression⁵¹, CXCL1 has been implicated in chemoresistance and metastasis⁵² as well as pre-metastatic niche formation in CRC⁵³, and MIP-3 α (or CCL20) has multifaceted roles within the TME⁵⁴. MIP-3 α production has also been observed in *F. nucleatum* infection of human oral epithelial cells⁵⁵. Intriguingly, GM-CSF is known to exhibit both stimulatory and suppressive effects on tumor progression^{56,57} and has been shown to play a role in liver metastasis⁵⁸. These observations still do not reveal if this is an active or passive process (referring to increased expression versus cytokine release from cells), but the selectivity of secretion indicates the activation of specific pathways within the host cells. The ubiquitous presence of these cytokines in several cancer types suggests their potential role as cancer biomarkers⁵⁹.

Previous studies have also revealed roles for these cytokines in pancreatic cancer. It has been shown that KRAS-induced upregulation of GM-CSF can promote the development of pancreatic neoplasia⁶⁰. GM-CSF simultaneously plays a role in oncogenic activation and the evasion of antitumor immunity in PDAC. Moreover, it is an important regulator of inflammation and immunosuppression within the TME, such as its characterized role in the recruitment of immunosuppressive Gr1⁺CD11b⁺ myeloid cells⁶¹. GM-CSF secreted by mesenchymal stem cells within the PDAC stroma has been previously shown to promote tumor cell growth and metastasis⁶². These multiple roles have been tied to worsening patient outcomes. In fact, in a study of 68 patients with PDAC, GM-CSF high populations

had shown a significantly lower survival rate (a median survival of 13.9 months compared to 25.7 months for GM-CSF low population)⁶³. MIP-3 α is known to promote Panc-1 cell invasion in vitro by binding to its CCR6 receptor, complemented with the up-regulation of matrix metalloproteinases (MMP9)⁶⁴. Furthermore, Takamori et. al have shown that IL-8 and CXCL1 autocrine signaling increases the proliferation of Capan1 cells⁶⁵. These studies further corroborate our results that *F. nucleatum* induced host cell secretion of these cytokines contribute adversely to cancer progression.

Our results reveal an additional role for GM-CSF in pancreatic cancer. We studied the individual and collective impact of the identified cytokines using in vitro co-culture assays of proliferation and migration which are common features of aggressive cancers. We demonstrated that cytokines secreted from infected cancer cells can directly impact other uninfected cancer cells. We first noted increased proliferation of infected cells. To ascertain that host cell cytokine secretions could play a role to explain this observation, we isolated conditioned media from the *F. nucleatum* infected cancer cells and found that it was sufficient to increase proliferation of the pancreatic cancer cells, and this effect was partially negated when we depleted GM-CSF from the media. We also demonstrated that the addition of exogenous recombinant GM-CSF increased cell proliferation and its depletion negated the observed effect. Together, these observations define an independent role of GM-CSF in BxPC3 and Panc1 cells, to directly contribute to cancer progression by impacting proliferation.

We additionally observed increased cell migration of infected cells compared to uninfected cells, and hypothesized that secreted cytokines could account for this altered phenotype. We demonstrated that conditioned and concentrated media obtained from *F. nucleatum* infected cells was sufficient to significantly increase the cellular migration of uninfected cells. It is essential to note that this conditioned media contained a mix of other secreted cytokines such as IL-6, IL-10, TNF α , and VEGF, albeit in lower concentrations, in addition to high levels of GM-CSF, CXCL1, IL-8, and MIP-3 α , as revealed from the cytokine arrays. Nevertheless, the resulting cell migratory response arising from a

synergistic fusion of these cytokine interactions was specific to *F. nucleatum* infection. These observations indicate that infected cancer cells can impact both infected as well as uninfected cancer cells within the PDAC TME through both autocrine and paracrine signaling, an important potential mechanism whereby the impact of relatively low numbers of bacteria in vivo⁶⁶ may be amplified locally through host cell crosstalk.

While the influence of stromal cells such as tumor-associated fibroblasts and pancreatic stellate cells are well characterized in PDAC^{40,67–70}, the role of normal epithelial cells has not received as much scrutiny. Our results show that they too can regulate the fate of tumor cells. We show *F. nucleatum* infects normal pancreatic epithelial cells using a Fap2-independent mechanism, but that this infection still results in the secretion of the same cytokines released by infected PDAC cells. These cytokines, which do not elicit a proliferative response from the normal cells, are able to impact tumor proliferation and migration. Cytokines such as IL-8 and CXCL1 are well known players in formation of pre-metastatic niches^{53,71} priming organs and tissues to attract circulating tumor cells (CTC), break dormancy, and establish micrometastatic sites. Furthermore, as tumor cells increase migration in response to cytokines, infected normal cells can stimulate the expansion of the tumor, providing increased opportunity for the tumor cells to access vasculature and disseminate. This cell migration can be either individual or collective in multicellular clusters of cells as we have observed with BxPC3 cells. Collective cell migration has been considered far more detrimental due to tumor cells enhanced pro-survival adaptations and increased metastatic potential^{72,73}. In fact, many of the mechanisms that impact metastatic progression of the cancer also contribute to enhanced therapeutic resistance⁷⁴. Thus, the crosstalk between normal and tumor cells through both paracrine and autocrine signaling could lead to the evolution of potentially enhanced tumor subpopulations with increased mobility and can contribute to worse prognosis. Moreover, infection of normal cells also suggests that *F. nucleatum* infections in the vicinity of the tumor can extend to adjacent tissues and organs.

There have been hints as to the mechanisms by which *F. nucleatum* can impact cancer cell proliferation. However, these specific interactions have not been studied in pancreatic cancer. In CRC, a proposed mechanism of action is that FadA adhesin protein from *F. nucleatum*, upon binding to E-cadherin, enables host cell invasion that promotes E-cadherin phosphorylation resulting in increased β -catenin translocation to the nucleus and subsequently increased transcription of Wnt signaling genes³⁴. Furthermore, FadA upregulates the expression of annexin A1 (ANXA1), a phospholipid-binding protein, in CRC cells by binding E-cadherin, and ANXA1 can engage β -catenin to activate cyclin D1 to promote cellular proliferation⁷⁵. In mice, it was found that *F. nucleatum* increases CRC proliferation by activating TLR4 signaling, NF κ B activation, and the upregulation of miR21⁷⁶. Whether similar pathways are activated in pancreatic cancer remains to be determined. Interestingly, BxPC3 cells do not contain the driver KRAS mutation found in over 90% of pancreatic cancer cells, which is a critical nexus for multiple intracellular signaling pathways related to cell proliferation and differentiation. However, BxPC3 cells still contain chronically active effector pathways related to BRAF⁴ that are further tied to ERK and MAPK pathways that impact proliferation⁷⁷. In fact, GM-CSF is known to activate MAPK and NF κ B signaling in other cell types^{56,78,79}. In CRC, it was identified that patients with mesenchymal tumors with Fusobacteria have a worse prognosis than patients with a more epithelial subtype³⁵. Future work should therefore [also investigate](#) *F. nucleatum* impact based on the context of the genomic constitution or molecular subtype of pancreatic cancer^{80–82}.

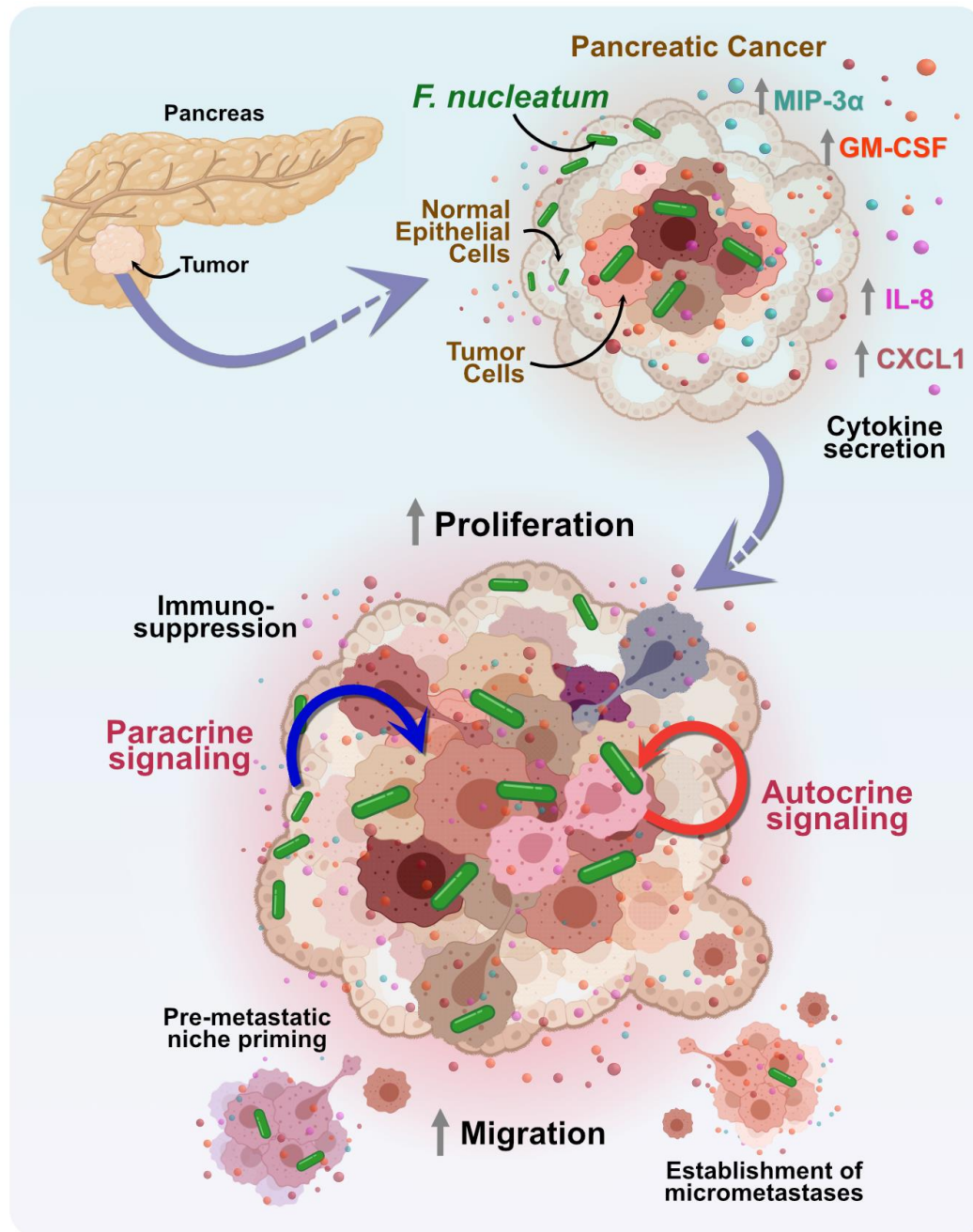


Figure 4.6 A model of pancreatic cancer response to *F. nucleatum* infection induced cytokine signaling.

F. nucleatum binds to pancreatic cancer cells in a Fap2-driven mechanism and induces the specific secretion of cytokines, GM-CSF, CXCL1, IL-8, and MIP-3α. These cytokines play a role to enhance proliferation and migration of pancreatic cancer cells through paracrine and autocrine signaling. In addition to known mechanisms of immunosuppression, these responses together adversely impact cancer progression by stimulating metastatic spread, pre-metastatic niche priming, and the establishment of micrometastases.

Tissue-resident microbes can influence cancer susceptibility in diverse and complex ways, for example by modulating inflammation and immune response, altering the metabolic profiles within tumors, and promoting DNA damage. In addition, we now show that host cell secretions upon infection can confer altered proliferative and migratory phenotypes to tumor cells to further exacerbate the cancer. A planned limitation of this study is the lack of response of immune cells to increased cytokine secretion. However, we did this to show that autocrine and paracrine signaling from the tissue cells themselves is sufficient to exacerbate tumor growth. Our work is restricted to the single bacterium *F. nucleatum*, but we note that this bacterium could synergistically act in coordination with other microbes identified within the pancreatic TME.

While PDAC remains one of the most therapy-resistant cancer types, treatments that consider the broader microenvironment of PDAC may provide a more holistic approach, and provide more beneficial outcomes for patients. Our work reveals for the first time that the PDAC-resident bacterium *F. nucleatum* plays a role in directly enhancing tumor cell proliferation and migration, two key hallmarks of PDAC as well as other cancers. The infection of normal cells surrounding the TME further extends the impact of *F. nucleatum* to surrounding tissues and organs, demonstrating that although *F. nucleatum* may not directly transform normal cells into cancer cells, it can still indirectly contribute to tumor progression through paracrine crosstalk. We believe that our data provides evidence that encourages future studies to investigate whether antibiotic-mediated therapy regimes in conjunction with chemotherapy or inhibition of cytokine and chemokine receptors may prove beneficial for treating these as well as other cancers that contain a tumor microbiome which may act to exacerbate disease.

4.5 Materials & Methods

4.5.1 Culturing Bacteria

Fusobacterium nucleatum subsp. nucleatum ATCC 23726 (referred to as *Fnn*) and its Fap2 surface adhesin deletion mutant, *F. nucleatum* Δ fap2 (referred to as *Fnn* Δ fap2), were grown as detailed previously³⁷. Bacterial colonies are initiated on solid agar plates made with Columbia Broth (Gibco) substituted with hemin (5 μ g/mL) and menadione (0.5 μ g/mL) (CBHK) and grown in anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) at 37°C for two days. Single colonies were then retrieved and added to CBHK media to initiate the liquid culture and were grown for ~16 hours at 37°C anaerobically. The optical density (OD) at 600 nm was then measured (~0.6-0.8) to obtain bacteria at the mid-exponential growth phase of the culture. Bacterial counts were obtained based on standardized curves created for the spectrophotometer. All experiments were carried out with a 50:1 multiplicity of infection (MOI) which is the bacteria to host cell ratio.

To stain the bacteria, 1 mL of the bacterial cell suspension was collected and spun down at 1500g for 3 minutes. The pellet was resuspended in 100 μ L CBHK media and then stained with FM 1-43FX lipophilic styryl dye (Invitrogen F35355) (5 μ g/mL) for 5 minutes. This stains the outer membrane of the bacteria to emit green fluorescence upon excitation. The stained cells were subsequently spun down at 1500g for 3 minutes, washed with 500 μ L media, and again resuspended in 1 mL of media and used for experiments.

Escherichia coli TOP10 was grown in the shaker incubator at 37°C in Luria Broth (LB) overnight. Bacterial culture was then accordingly diluted to OD_{600nm} of 0.6-0.8 and used for experiments.

4.5.2 Culturing Normal and Cancerous Epithelial Cells

Cancerous epithelial cells were purchased from ATCC and grown on tissue culture-treated plates and flasks in their respective cell culture media. BxPC3 (ATCC CRL-1687) cells were grown in RPMI-1640 supplemented with 10% FBS, and Panc1 (ATCC CRL-1469) cells were grown in DMEM supplemented with 10% FBS. Normal primary pancreatic epithelial cells were purchased from CellBiologics (H-6037), and grown in epithelial cell culture medium supplemented with 5% FBS (Cell Biologics H-6621). Additional cell lines HPAC (ATCC CRL-2119) and Capan1 (ATCC HTB-79) cells were grown in DMEM:F12 supplemented with 10% FBS and IMDM supplemented with 20% FBS, respectively. All cell culture media contained 1% penicillin and streptomycin, but infections were performed in serum-free and antibiotic-free media. Cells were grown at 37°C with 5% CO₂ and were passaged every 2-3 days based on their confluency with gentle trypsinization (0.25% trypsin with EDTA) and reseeding. The cells used for the experiments did not exceed passage 12. For infection experiments, cells were grown to confluency in 6, 12, 24, and 96 well plates.

4.5.3 Scanning Electron Microscopy

BxPC3 cells were seeded on coverslips and allowed to adhere overnight. The samples were then infected with *Fnn* for 2 hours at 50:1 MOI (multiplicity of infection). Preparation for scanning electron microscopy was performed as previously described⁸³. In brief, samples were fixed for 24 h with 4% paraformaldehyde and 1% glutaraldehyde in PBS, post-fixed for 45 min with 1% osmium tetroxide in distilled water, and subsequently dehydrated in serial dilutions of ethanol of 25%, 50%, 70%, 95%, 100%, and 100% before critical point drying the samples. Samples were then sputter-coated with iridium fast coating (5 nm) on a Leica EM ACE600 and imaged on LEO (Zeiss) 1550 Field Emission SEM.

4.5.4 Flow Cytometry

Fnn and *Fnn Δfap2* were obtained and first stained with FM 1-43FX as detailed above to facilitate green fluorescence detection. The epithelial cells were then infected in serum-free and antibiotic-free media with the stained bacteria at 50:1 MOI for 1 hour and 4 hours. The cells were then washed twice with PBS, trypsinized, and collected for flow cytometry and cell sorting. The population of cells containing a mix of non-infected and infected cells was then loaded onto an S3e flow cytometer (Bio-Rad). 50,000 cells were analyzed using single-cell gates to measure the median green fluorescence induced by intracellular *Fnn*. FlowJo10 was then used to determine the median fluorescence of the samples and the data was transferred to GraphPad Prism for statistical analysis.

To visualize intracellular bacterial counts based on fluorescence intensity, the cells were sorted based on gates created within the infected cell population for low, medium, and high fluorescence. The sorted cells were collected in 100% FBS, transferred to growth media, and then plated on coverslips for imaging. After adherence, the cells were stained with MemBrite 568/580 (Biotium BTM30095) and NucBlue (ThermoFisher Scientific R37605) before being fixed with 10% formalin. The cells were imaged on a Zeiss LSM 800 using a 63X oil objective and z-stacks were obtained to recreate the 3D structure of the infected cells on Zen Blue.

4.5.5 Cell Proliferation Assays

Proliferation assays were performed using XTT Assay Kit (ATCC 30-1011K) and BrdU Cell Proliferation Assay Kit (K306, BioVision) based on the protocols provided. Briefly, epithelial cells were seeded on 96 well plates at optimized cell numbers (10,000 cells/well) and allowed to adhere for 12 hours in their respective cell culture media. The media was then replaced with the treatment (conditioned media or cell culture media with added cytokines 200 pg/mL, each supplemented with 0.5% FBS) or the cells were directly infected on the 96-well plate at 50:1 MOI for 4 hours. The cancer

cells were further incubated for 48 hours, and the primary normal cells were incubated for 24 hours at 37°C, which was optimized to account for their doubling time. The recombinant cytokines that were used were Recombinant Human GM-CSF Protein (R&D Systems, 215GM), Recombinant Human CCL20/MIP-3 α Protein (R&D Systems, 360-MP), IL-8 Monocyte Recombinant Human Protein (ThermoFisher, PHC0884), and Recombinant Human CXCL1/GRO α Protein (R&D Systems, 275-GR).

For the XTT assay, following the 24/48-hour incubation with conditioned/cytokine media, 50 μ L of activated XTT reagent is added to each well and the resulting color change was monitored for 2-24 hours by measuring the absorbance at 475 nm and 660 nm. For the BrdU assay, 10X BrdU solution is added to the treated cells to a final concentration of 1X and incubated for 4 hours. The medium was then removed and the cells were fixed with the Fixing/Denaturing solution for 30 minutes. Next, BrdU Detection Antibody was added and incubated for 1 hour with shaking, followed by washing thrice with Wash Buffer. Anti-mouse HRP-linked Antibody Solution was added to the wells and it was incubated for 1 hour and washed thrice again with Wash Buffer. Finally, TMB substrate was added to the wells and the color change was monitored for 5-20 minutes and the final absorbance was measured at 450 nm.

4.5.6 Preparing Conditioned Media

The epithelial cells were grown to confluence in their respective media supplemented with 10% FBS and 1% penicillin-streptomycin in T-75 flasks or 12 well plates. One set of T-75 flasks/wells was then used for infection and another set was used as a control without infection. The flasks were washed with PBS twice and replaced with serum-free and PS-free media. For testing the impact of nPEC conditioned media on cancerous cells, serum-free and PS-free media of the cancer cell line was used. The flasks were then infected with *Fnn* at 50:1 MOI for 4 hours. The media from both uninfected and infected cells was retrieved from the flasks and filtered through a 0.22 μ m syringe filter (Millipore Sigma). For proliferation assays, conditioned media was used directly without concentration. For

migration assays, the extracted media was concentrated to 16X, as determined from preliminary trials, using a 3000 MWCO Concentrator (Amplicon Millipore Sigma) by spinning the samples at 3000g for ~1.5 hours at 4°C. The samples were immediately used for the Transwell assays and ELISA.

4.5.7 Cell Migration Assays

Cell migration assays were performed on 8µm Transwells in 24-well plates (Corning CLS3422). Cells were first trypsinized (0.25% Trypsin in EDTA) and stained using CellTracker Red CMPTX (ThermoFisher C34552) for 45 minutes in serum-free media. Matrigel (Corning CB40234A) was diluted to a concentration of 250 µg/mL in serum-free media and 100µL was added to the upper chamber of each Transwell and incubated for 25 minutes at 37°C. The stained cells were resuspended to 2 million cells/mL in media containing 0.5% FBS and 100µL of the cell suspension was added on top of the Matrigel and incubated for 1 hour. The control and chemoattractant solutions were prepared in serum-free media or media with 0.5% FBS (100 ng/mL) and 600µL of the chemoattractant solution was added to the outer chamber of the Transwell. In the case of migration in response to conditioned media, 600µL of concentrated conditioned media was added to the outer chamber of the Transwell. The Transwells were then incubated for the respective time points (12/16/24) hours at 37°C/5% CO₂. The plate was collected and the cells in the upper chamber were removed using a cotton-tip applicator. The Transwells were transferred to a plate containing 10% formalin and fixed for 20 minutes. This was washed with PBS thrice and stained with DAPI (1:5000) (Sigma Aldrich D9542) in a permeabilization buffer (10 mL PBS + 0.5% Triton-X + 200 mg Bovine Serum Albumin) overnight at 4°C. Transwells were then washed thrice with PBS and stored at 4°C. The Transwells were imaged on a Zeiss LSM 800 confocal microscope using a 10X objective. The cells were counted using a custom-developed ImageJ protocol.

4.5.8 Cytokine Arrays

Cytokine arrays were obtained using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems Inc. ARY022B) that screens for 105 cytokines. Briefly, cells were grown to confluence in 6 well plates. The cells were infected with *Fnn*, *E. coli*, and *Fnn Δfap2* at 50:1 MOI for 4 hours at 37°C. Infection with *E. coli* TOP10 was used as a non-invasive infection control. The samples were first collected and filtered using a Spin-X column (Millipore Sigma) to remove bacteria within the sample. The membranes from the kit were first blocked using the Assay buffer for 1 hour and then incubated with the samples overnight at 4°C on a rocking platform shaker. Each membrane was then washed using the Wash buffer, and incubated with the Detection antibody cocktail for 1 hour. This was followed by washing thrice with Wash Buffer again and incubating with Streptavidin-HRP for 30 minutes. After a final washing procedure, Chemo-Reagent Mix was added to each membrane and it was covered for 1 minute. Finally, the membranes were transferred to an autoradiography film cassette and exposed to X-rays for 1-10 minutes.

4.5.9 Chemokine Quantitation

ELISA was used to quantify cytokines IL-8, CXCL1, MIP-3 α , and GM-CSF. The assays were performed using Duo Kits (R&D Systems DY208, DY275, DY360, and DY215, for the 4 cytokines respectively). Epithelial cells were grown to confluence on 12 well plates and infected with *Fnn*, *E. coli*, and *Fnn Δfap2* at 50:1 MOI for 4 hours at 37°C. The ELISA plate was first coated with the capture antibody overnight and blocked the next day with the reagent diluent for 1 hour. Standards were prepared and conditioned media obtained from infected cells was filtered using a Spin-X column and then added to the wells, diluted, if necessary, with the reagent diluent. After a 2-hour incubation at room temperature, the plate was washed thrice with Wash buffer, and then incubated with the Detection Antibody for 2 hours at room temperature. The plate was again washed and incubated with Streptavidin-HRP for 30 minutes. Finally, the plate was washed and incubated with the Substrate

solution for 20-30 minutes and the reaction was stopped by adding the Stop solution. The final absorbance was measured at 450 nm on a spectrophotometer.

4.5.10 Chemokine Depletion Assays

Conditioned and concentrated media was obtained as previously described. Human GM-CSF biotinylated antibody (R&D Systems, BAM215) was added to the medium to a final concentration of 90 ng/ml and incubated at room temperature with gentle shaking for 3 hours. Next, magnetic streptavidin particles (Sigma-Aldrich, 11641778001) were first washed twice with PBS and spun down at 1500 g and resuspended in the cell media, and 25 μ L/mL was added to the sample media. This was incubated at room temperature for 1 hour. The samples were then spun down at 1500g to pellet the heavier magnetic particles, and the supernatant was collected containing the conditioned medium with reduced cytokines. The medium was used for the proliferation assays and an ELISA was used to quantify and confirm the cytokine depletion.

4.5.11 Statistical Analysis

Statistical analysis was performed on GraphPad Prism 9 using t-tests and ANOVA followed by Dunnett's multiple comparisons test, with P-value significance denoted by ns (not significant) for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$. All samples for analysis were collected in independent triplicates. Plots were created on GraphPad Prism 9 and figures were designed on Affinity Designer.

4.6 Acknowledgments

This project was supported by the NIH R21 Exploratory/Developmental Research Grant 1R21CA238630-01A1 (Verbridge, Slade), NSF Career Award CBET-1652112 (Verbridge), College of Engineering, and the Center for Engineered Health of the Institute for Critical Technologies and

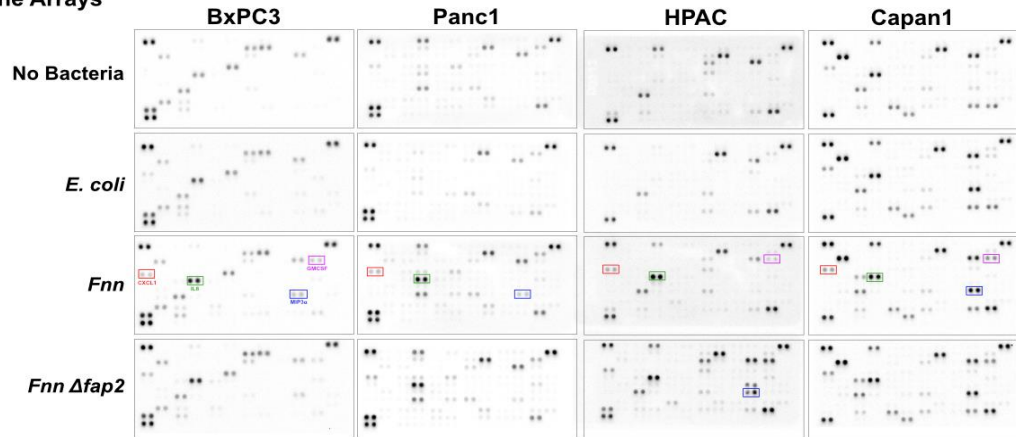
Applied Sciences at Virginia Tech. For SEM imaging experiments, we would also like to acknowledge the Nanoscale Characterization and Fabrication Laboratory, which is supported by the Virginia Tech National Center for Earth and Environmental Nanotechnology Infrastructure (NanoEarth), a member of the National Nanotechnology Coordinated Infrastructure (NNCI), supported by NSF (ECCS 1542100 and ECCS 2025151). The summary figure was designed using Biorender.com (Toronto).

Supplementary Figures

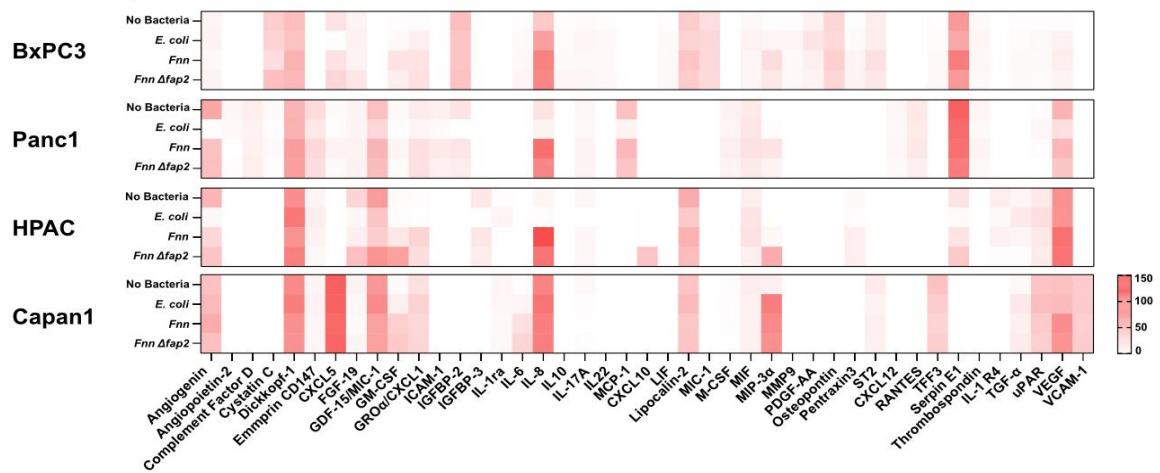
Cell Line	Age	Sex	Derivation	Metastasis	Proliferation	Differentiation	Reference
BxPC3	61	Female	Primary Tumor	No	48-60 h	Moderate to Poor	Tan et al. (1986) PubMed 3754176
Panc1	56	Male	Primary Tumor	Yes	52 h	Poor	Leiber et al. (1975) PubMed 1140870
Capan-1	40	Male	Liver metastasis	Yes	ND	Well	Kyriazis et al. (1982) PubMed 6278935
HPAC	64	Female	Primary Tumor	ND	41 h	Moderate	Gower et al. (1994)

Table 4.1: Cancer cell lines and their noted features.

A Cytokine Arrays



B Raw Intensity Values



C Percent Change in Intensity in Response to Infection

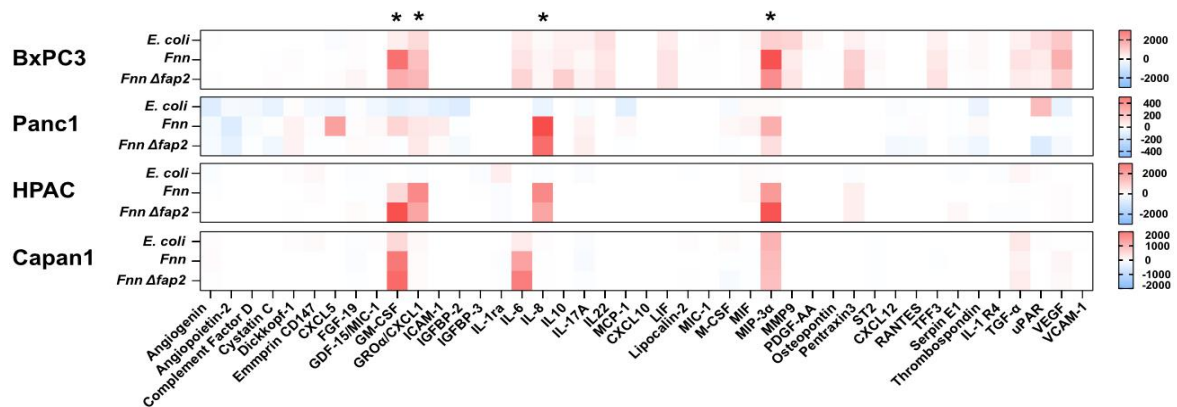


Figure 4.7 Cytokine arrays of infected host cell secretions.

(A) Cytokine secretions were tested for 4 conditions: No Bacteria, *E. coli*, *Fnn*, and *Fnn Δfap2*. using the XL Cytokine Array Kit (R&D Systems). (B) Raw intensity values based on the blots were determined on ImageJ and the intensities are plotted as a heat map. (C) Percent change in intensity relative to constitutive secretion was determined for all the infection conditions.

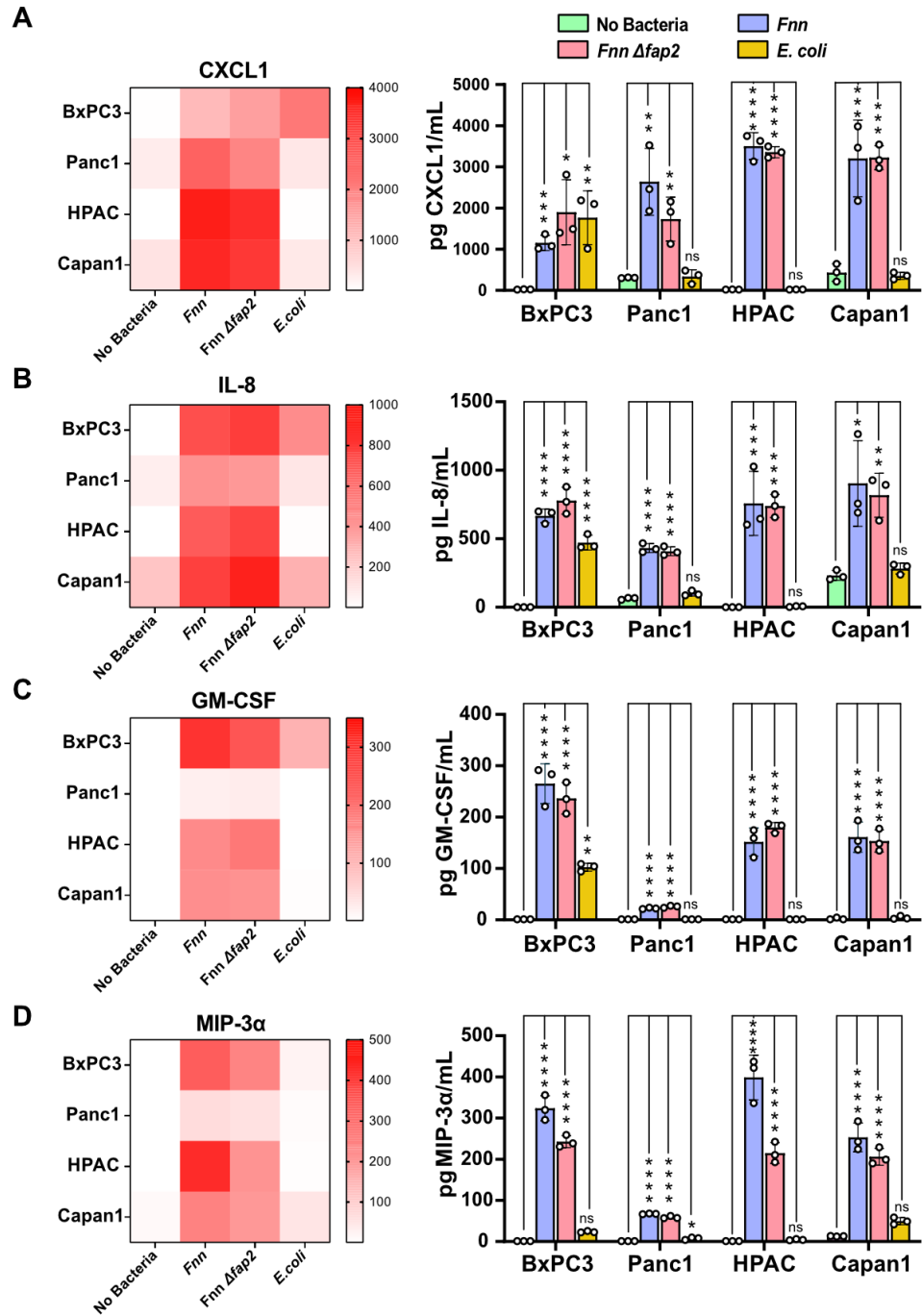


Figure 4.8 Measured values of cytokine secretion from infected host cells

ELISA was used to quantify the levels of secretion of (A) CXCL1, (B) IL-8, (C) GM-CSF, and (D) MIP-3 α for all 4 pancreatic cancer cell lines tested.

BxPC3 Cells

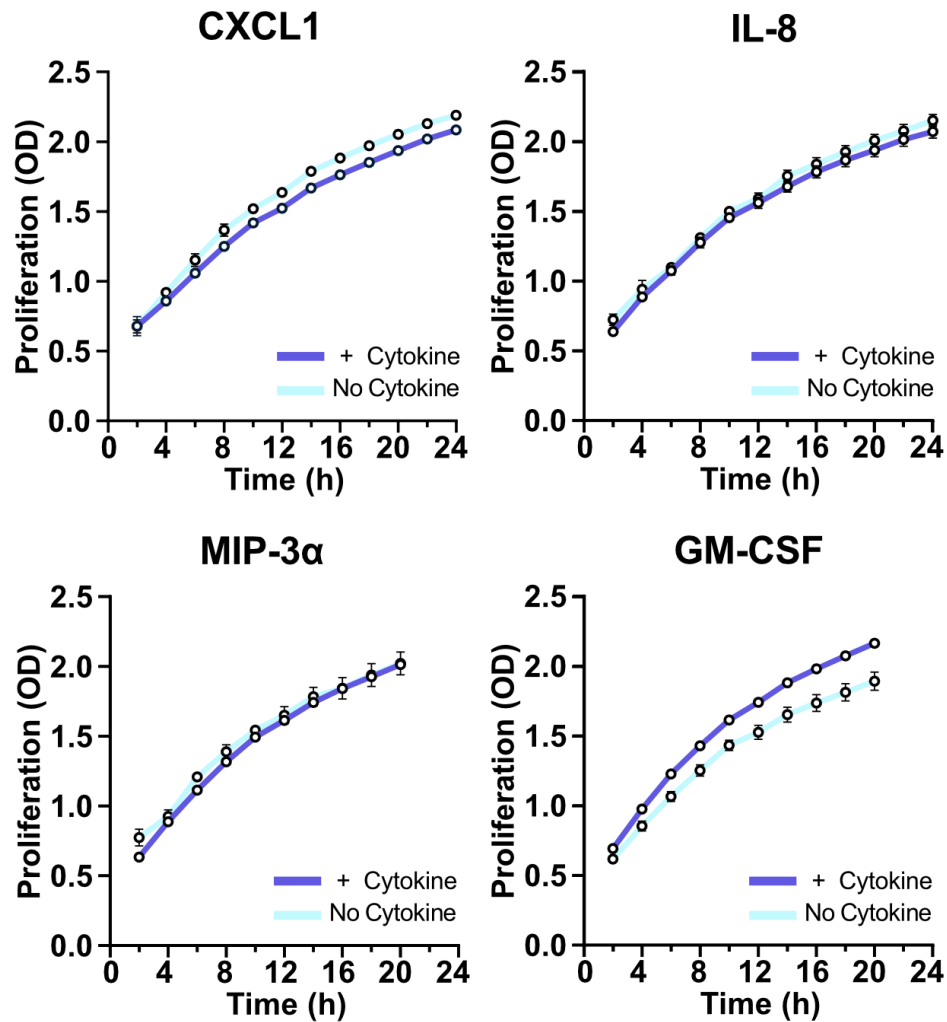


Figure 4.9 GM-CSF contributes to increased proliferation of BxPC3 cells.

Preliminary studies were conducted on BxPC3 cells to identify the impact of the 4 cytokines individually on proliferation using the XTT assay over 24 hours. GM-CSF at 200 pg/mL showed a significant increase in proliferation compared to the other cytokines.

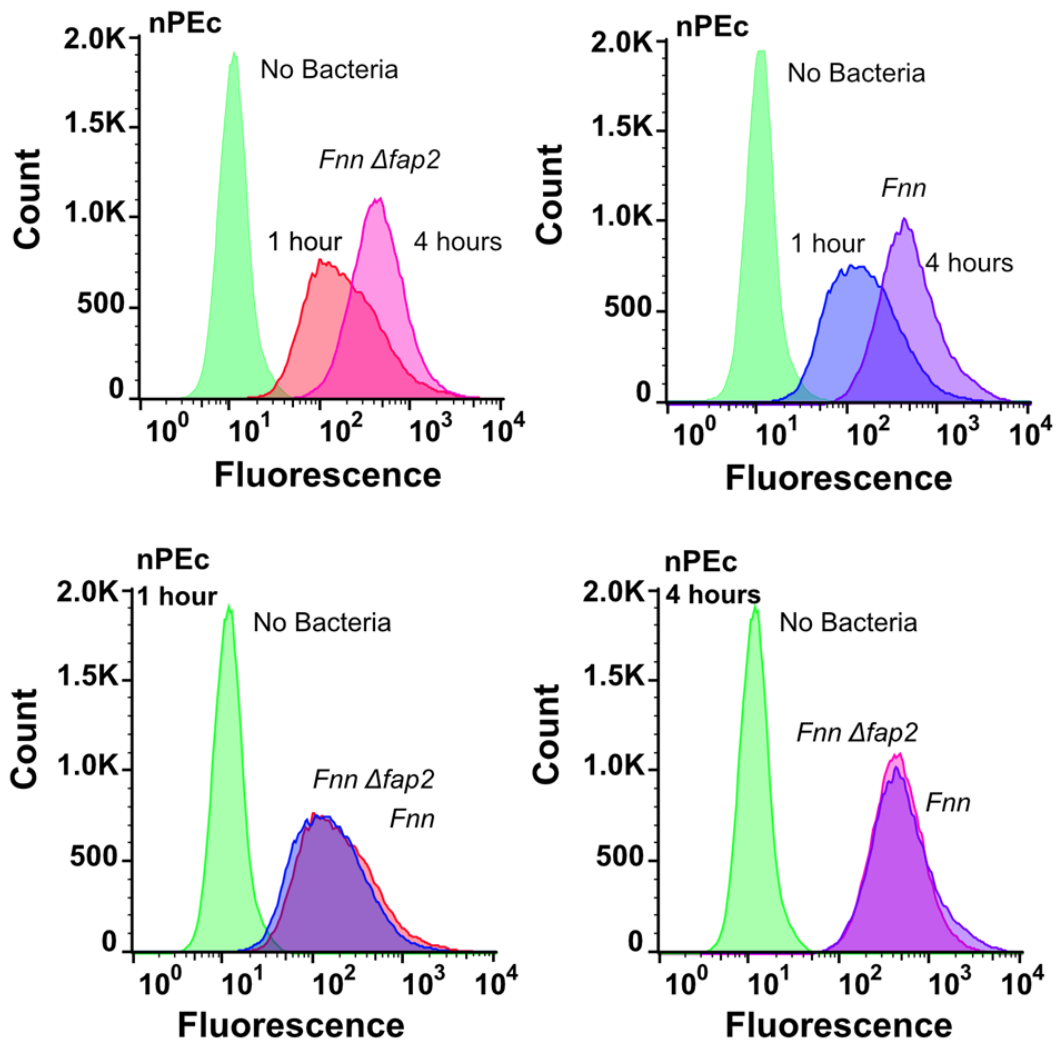


Figure 4.10 Fap2 does not impact *F. nucleatum* infection of normal pancreatic cells.

Flow cytometry data of normal pancreatic epithelial cells (nPEc) after infection with *Fnn* and *FnnΔfap2* for 1 hour and 4 hours of infection.

5 Multi-omic analysis of *F. nucleatum* infection of tumor cells in hypoxia

This chapter is adapted from

Fusobacterium nucleatum infection modulates the transcriptome and epigenome of colon cancer cells in an oxygen-dependent manner

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(In preparation for review at the time of writing)

5.1 Abstract

Fusobacterium nucleatum is an emerging oncomicrobe in colorectal cancer (CRC) and other cancers. As a Gram-negative and anaerobic commensal bacterium normally found within the oral cavity, its correlation with poor clinical outcomes in patients remains poorly understood. Furthermore, the lack of stably colonized murine models limits the power of *in vivo* studies to interrogate this host-microbe interaction. Previous *in vitro* studies have investigated interaction of *F. nucleatum* with human cells in normoxic conditions immediately after infection. However, hypoxia is known to generate highly malignant, invasive, and chemoresistant cancer phenotypes. Therefore, we sought to examine the influence of hypoxia in facilitating *F. nucleatum* invasion and host response. We first show that *F. nucleatum* infection of a CRC cell line pretreated in hypoxia for 24 hours leads to rapid bacterial invasion and significantly increased secretion of inflammatory cytokines (IL-8 and CXCL1) in comparison to infection in normoxia. We then used a multi-omics approach to investigate epigenomic alterations induced by H3K27 acetylation and global transcriptome alterations sustained within these two cell phenotypes 24 hours after initial infection with *F. nucleatum*. Using infection with non-

invasive *E. coli* as a control, analysis of H3K27ac marks which are commonly associated with enhancers, revealed fewer differential enhancers related to infection with *F. nucleatum* compared to infection in normoxia. Global RNAseq revealed 453 differentially expressed genes (DEG) in normoxic cells and 226 DEGs in hypoxic cells uniquely regulated upon *F. nucleatum* infection. The DEGs that were highly upregulated in hypoxic cells have been previously characterized to adversely impact CRC progression. We further observed that host cell response to *F. nucleatum* infection in both environments was substantially lower than host response to *E. coli*, indicating that perhaps the virulence of *F. nucleatum* can, in part, be explained through effective cloaking from host cell recognition and evasion of host defenses. Our findings reveal that a hypoxic microenvironment drastically impacts the host response to *F. nucleatum* infection that drives malignant transformation and motivates future studies to investigate host-microbe interactions in tumor tissue relevant conditions that more accurately define parameters for targeted cancer therapies.

5.2 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer related deaths among men and women in the United States ¹. Depending on the stage of the disease, its 5-year survival prognosis can range from 90% when caught early to 14% when identified after metastatic spread. CRC is further classified into four consensus molecular subtypes (CMS) based on its biological characteristics to facilitate targeted clinical intervention ^{2,3}. Early screening, surgery, neoadjuvant radiotherapy and adjuvant chemotherapy are commonly used to treat CRC ⁴. However, drug resistance continues to impede the efficacy of these therapies. Its tumor microenvironment (TME) includes a complex extracellular matrix, variable oxygen and pH gradients, and multiple tumor-infiltrating cells, including stromal and immune cells, and plays a critical role in cancer progression. Furthermore, the gut microbiome, which is in the immediate proximity of CRC, plays essential roles in infection, inflammation, and drug metabolism. Several studies have shown that the

composition of the gut microbiome varies with CRC progression and modulates the efficacy of chemotherapy ^{5,6}. It has even been shown that the local microbiome can modulate the activity of the canonical tumor suppressor gene *TP53* ⁷.

Tumor-associated bacteria, now considered a hallmark of cancer ⁸, can impact tumor progression and its response to therapy. Specific features of the TME favors the accumulation of unique species of microbes. In fact, systematic characterization of the tumor microbiome in several tumor types have revealed diverse intracellular bacteria within tumors ⁹. However, there is still limited knowledge on the role of these bacteria within tumors, their intracellular survival strategies, their long-term impact on the host cells, and if their elimination can arrest tumor progression ¹⁰. Common microbial inhabitants enriched within CRC include *Fusobacterium nucleatum*, *Streptococcus bovis*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Escherichia coli*, and *Peptostreptococcus anaerobius* ¹¹. Normally found within the oral cavity, *Fusobacterium nucleatum* is a commensal, Gram-negative, anaerobic bacilli that acts as a bridge species within the oral cavity and facilitates biofilm formation ¹². However, it behaves as an opportunistic pathogen in certain disease conditions, both oral and extraoral. It has been implicated in gingivitis, periodontitis, preterm birth complications, inflammatory bowel disease, and myocarditis ¹³⁻¹⁵. There is also considerable evidence of its role in multiple cancers including CRC, pancreatic cancer ¹⁶, oral squamous cell carcinoma ¹⁷, esophageal cancer ¹⁸, and Lauren's diffuse type gastric cancer ¹⁹.

F. nucleatum's selective enrichment in tumor tissue in comparison to adjacent healthy tissue launched investigations into the function of this microbe in cancer ^{20,21}. Clinical studies have further corroborated its role as a potential oncomicrobe. Patients harboring *F. nucleatum* within their tumors exhibited increased mortality, resistance to chemotherapy and greater risk of relapse ²²⁻²⁴. Mechanistic studies have indicated that *F. nucleatum* uses its surface lectin Fap2 to adhere to and invade tissues expressing high levels of Gal/Gal-NAc on their cell surface membrane, a feature

commonly found on tumor cells ²⁵⁻²⁷. Additionally, studies have revealed Fusobacterial FadA adhesin binds to E-cadherin on tumors and activates downstream signaling pathways, including upregulating β -catenin leading to NF κ B activation and tumor progression ²⁸. FadA has been shown to exhibit amyloid-like properties that promote CRC progression in mouse models that can be attenuated with amyloid binding molecules ²⁹. Intracellular invasion and cytoplasmic localization are unique traits of bacterial pathogens such as *F. nucleatum*. Evolutionarily, certain invasive bacterial species can exhibit adaptations that facilitate their survival, immune evasion, and dissemination ³⁰. The host cell's response to *F. nucleatum* infection can be multifactorial. We have previously shown that *F. nucleatum* binding and invasion of host cells induces the robust secretion of select cytokines that can impact tumor cell proliferation and migration even in the absence of immune cell involvement ^{31,32}. Furthermore, *F. nucleatum* can persist within the primary tumor cell for a prolonged period and even travel within the tumor cell to distant metastatic sites ³³. Concurrently, it has been shown that ICAM-1 overexpression in CRC cells can facilitate endothelial adhesion and extravasation to promote metastatic spread ³⁴. Taken together, these studies suggest that *F. nucleatum* can drive the formation of malignant phenotypes of tumor cells, but no causal relationship has yet been uncovered. Moreover, differences in human and murine biology have impeded the establishment of a stably colonized murine model to study *F. nucleatum* pathogenicity ³⁵. These findings have only begun to reveal features of this host-pathogen interaction, however there remains much to be understood on how the *F. nucleatum* adapts to the TME, modulates host cell response, and its extended impact on malignant transformation of the host.

Most *in vitro* experiments have studied *F. nucleatum* infection of cell lines in normoxic conditions (18-21% oxygen). However, physiological oxygen conditions, i.e physoxia (2-6%) and moreover, hypoxic (<1%) conditions more accurately recapitulate the TME ³⁶. Since *F. nucleatum* is an anaerobic organism, we hypothesized that hypoxic environments could be conducive to its growth and its virulence in hypoxic cells would vary from its impact on normoxic cells. Hypoxia within tumors plays

an important role in cancer progression ³⁷⁻⁴⁰. As tumors grow they develop a hypoxic core due to rapid proliferation and limited vascularization. In addition, inflammation-induced stromal and immune infiltration of the TME can increase cellular oxygen demand to result in local oxygen deficits. Hypoxia potentiates the development of more aggressive tumor cell phenotypes by altering signaling pathways, modulating cytoskeletal rearrangements, promoting epithelial-mesenchymal transition (EMT), reshaping metabolism, inducing angiogenesis, and exhibiting increased drug resistance ⁴¹. Moreover, these features can favor microbial intracellular invasion and persistence ^{42,43}. *In vitro* modeling of infection in CRC derived spheroids revealed *F. nucleatum* proliferation was significantly increased in large spheroids compared to smaller spheroids, motivating a key role of a hypoxic core to promote bacterial viability ⁴⁴. Other features of the TME that can be beneficial to microbial invaders include the immunosuppressive environment and the availability of abundant sources of nutrients. Together, these features may explain the selective enrichment of microbiomes within tumors in comparison to adjacent healthy tissue. For example, although overexpression of Gal-Gal/NAc on tumor cells can, in part, explain enhanced *F. nucleatum* adhesion to tumor cells ²⁵, a favorable TME can ensure their survival and replication.

Prior studies have investigated the epigenomic and/or transcriptomic alterations induced by *F. nucleatum* on non-cancerous cell lines including primary human colonic epithelial (HCE) cells, human carotid artery endothelial (HCAE) cells ⁴⁵, human gingival fibroblasts ^{46,47}, rat osteoblasts ⁴⁸, and human immortalized oral epithelial cells (HIOEC) ⁴⁹ in normoxia. Microarray data analysis of *F. nucleatum* infection of Caco-2 CRC cells, identified a role for CEP5 centrosomal protein that can impact cell cycle and apoptosis ⁵⁰. Other studies have focussed on the RNA landscape of multiple *F. nucleatum* strains ⁵¹ or alterations in gene expression within *F. nucleatum* upon infection of Caco-2 cells ⁵². Correlations between the methylome and bacterial abundance from CRC patient biopsies have revealed associations *F. nucleatum* to hypermethylation of tumor suppressor genes in colorectal cancer ⁵³. Another study profiled intra-tumor bacteria in 29 patients with CRC and obtained the

mutational signatures of altered genes within the tumor. The authors discovered that *Fusobacterium* was associated with several mutated genes that were involved in cell cycle related pathways ⁵⁴. However, it is not clear if the original tumor's mutational signature enabled *Fusobacterium* invasion or if after invasion, the bacterium and its metabolites promoted genomic instability thereby resulting in increased mutations at these genetic loci.

The impact of tumor microenvironmental factors such as hypoxia on the host transcriptome and epigenome in response to *F. nucleatum* infection has not been previously investigated. Although it has been documented that *F. nucleatum* enrichment in CRC derived tumor tissue correlates with worse prognosis, this effect is compounded based on the CMS subtype of the tumor, with mesenchymal subtypes exhibiting a pronounced effect ⁵⁵. Cancer cell lines derived from different CMS subtypes display distinct phenotypic and genotypic traits and possess dissimilar aberrant signaling pathways ⁵⁶. These observations underscore the need to explore the impact of host-pathogen interactions in cancer from a holistic perspective within physiologically relevant conditions such as hypoxia ⁵⁷. We hypothesized that comprehensive epigenomic and transcriptomic profiling of cancer cells in response to *F. nucleatum* infection could reveal unique genes and pathways that facilitate bacterial entry and intracellular persistence to contribute to exacerbate tumor progression in CRC. Our study used a multi-omic approach that combines RNA-seq and ChIP-seq of H3K27ac to determine the response to infection of a CRC cell line, HCT116, with *F. nucleatum* and *E. coli*, in normoxic and hypoxic environments to reveal the sustained influence of infection on host epigenome and transcriptome.

5.3 Results

5.3.1 *F. nucleatum* infects hypoxic cells at an increased rate and elicits increased IL-8 and CXCL1 secretion compared to infection of normoxic cells

To investigate whether a hypoxic microenvironment impacts *F. nucleatum* binding and invasion into host cancer cells, we first pre-treated HCT116 cells in hypoxia (<1% oxygen) and normoxia (18% oxygen) for 24 hours and subsequently infected them with *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 23726 (which will be referred to as *Fnn* in this text) for 1 and 4 hours. Flow cytometry was used to determine the fractional levels of infection and revealed an increased rate of *Fnn* infection in hypoxic cells infected in hypoxia or normoxia in comparison to infection of normoxic cells in the same conditions (**Figure 5.1A, 5.1B**). The infected cells were plated to determine the number of intracellular bacteria 1 hour and 4 hours after infection and we observed that *Fnn* within hypoxic cells had increased colony forming units (CFU/mL) 4 hours after infection in comparison to normoxic cells (**Figure 5.1C**). Since we had previously identified IL-8 and CXCL1 to be secreted by HCT116 cells upon *Fnn* infection ³¹, we quantified their secretion in response to infection in hypoxic and normoxic conditions. Normalized to cell counts, *Fnn* infection of hypoxic pre-treated cells showed a ~two-fold increase in IL-8 and CXCL1 secretion when compared to normoxic cells (**Figure 5.1D**). Using live immunofluorescence staining and live brightfield and confocal imaging, we further confirmed bacterial intracellular persistence within the host cancer cells 24 hours after the initial 4-hour infection (**Figure 5.1E**).

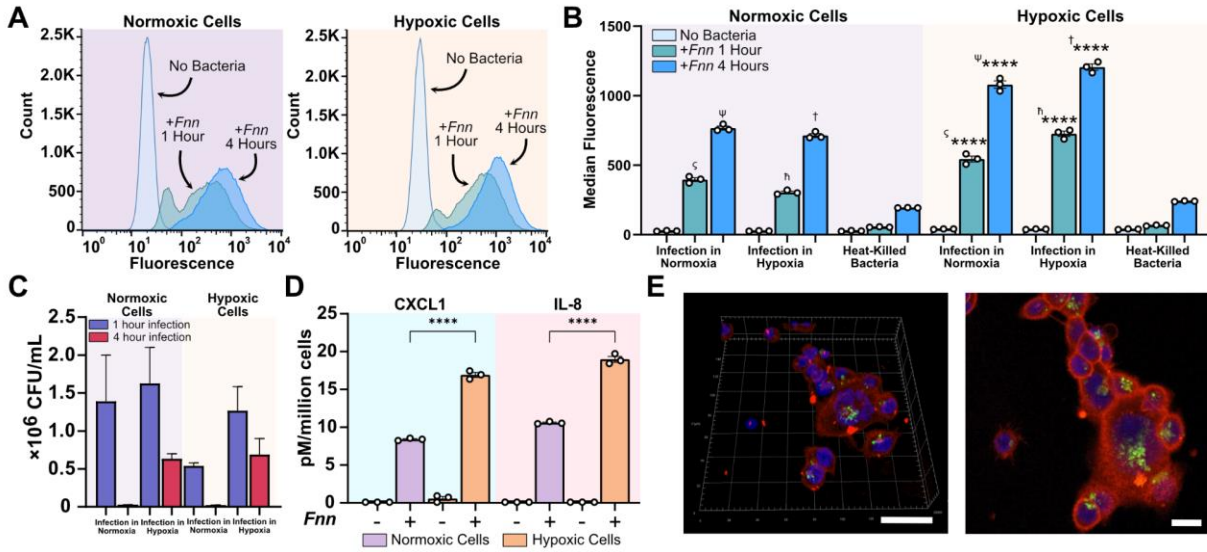


Figure 5.1: Variable rate of infection and cytokine secretion of hypoxic and normoxic cells in response to *F. nucleatum* infection.

(A). Flow cytometry analysis of infection of normoxic and hypoxic cells infected with *Fnn* for 1 and 4 hours. (B). Plot of median fluorescence obtained from flow cytometry indicates significantly increased infection of hypoxic cells in normoxia and hypoxia compared infection of normoxic cells in normoxia and hypoxic conditions. (C). Bacterial viability in hypoxia at 1 and 4 hours is greater than viability in normoxia. (D). Cytokine secretions normalized to cell counts (pM/million cells) of IL-8 and CXCL1 are significantly increased upon *Fnn* infection of hypoxic cells compared to normoxic cells. (E). Immunofluorescence confocal microscopy of *Fnn* infected HCT116 cells 24 hours after initial infection period reveals persistence of intracellular *Fnn*. (Staining: Host membrane red: MemBrite 568, Host nucleus blue: NucBlue, *Fnn* green: FM 1-43FX). Scale bar 20μm.

5.3.2 Hypoxia causes overall changes in epigenome and transcriptome

In order to quantify the epigenomic and transcriptomic alterations of host cancer cells in response to infection in both hypoxia and normoxia, we split HCT116 cells into two sets of flasks and subjected them to hypoxic and normoxic environments. One set of cells continued to proliferate in normoxic conditions (18% oxygen) while the other half was pretreated in hypoxia (<1% oxygen) for 24 hours. The time period of 24 hours was selected to capture stable and persistent changes in histone modifications and steady-state levels of mRNA transcription^{58,59}. After pretreatment, the cells were infected with *Fnn* and *E. coli* infection for 4 hours in their respective oxygen environments in pre-equilibrated media, before replacing the media. In order to study the longer term sustained response

to infection (beyond the hosts initial response to infection), the cells were further incubated for 24 hours in their respective oxygen environments with pre-equilibrated media before they were extracted for analysis. The six datasets that are described in this text are labeled as follows: (a) Normoxia - No Bacteria (NN), (b) Normoxia - infection with *Fnn* (NF), (c) Normoxia - infection with *E. coli* (NE), (d) Hypoxia - No Bacteria (HN), (e) Hypoxia - infection with *Fnn* (HF), and (f) Hypoxia - infection with *E. coli* (HE) (**Figure 5.2A**).

We applied MOWChIP-seq⁶⁰ to profile the global profiles of H3K27ac. An average Pearson correlation coefficient of 0.963 was found between ChIP-seq replicates. (**Figure 5.8A**). Between samples, the highest correlation was between NE and HE (0.961), which suggested that hypoxia caused few changes to H3K27ac signals upon infection with *E. coli*. We then compared the overall H3K27ac signal at all peak regions (**Figure 5.8B**). The H3K27ac signals of hypoxic samples (HN, HE, HF) were found to be significantly higher ($P < 0.0001$) than those of their corresponding normoxic samples (NN, NE, NF). Furthermore, the number of peaks was increased when the samples were treated in hypoxia (NN=10723, HN=22867, NE=22651, HE=31814, NF=13069, HF=21960). The H3K27ac peaks were then mapped to genomic regions (**Figure 5.8C**) and found that normoxic samples (NN, NE, NF) had a higher fraction of peaks near promoters than corresponding hypoxic samples (HN, HE, HF). Since H3K27ac indicates active promoters, this result suggests that hypoxia deactivated some genes that were active in normoxia.

We then analyzed RNA-sequencing data to further understand the effect of hypoxia on the transcriptome of the cells. The average Pearson correlation coefficient between replicates was 0.925, and the highest correlation between samples was HN and HF(0.882) (**Figure 5.8D**). Similar to the ChIP-seq data, we observed significantly increased RNA signals ($P < 0.0001$) in hypoxic samples with infection (**Figure 5.8E**). Hypoxia had a variable effect on the cells with or without infection: 3782 differentially expressed genes (DEGs) were observed between non-infected samples (NN-HN), 3223 for NE-HE and 2834 for NF-HF. Extensive changes in H3K27ac signal and genome-wide gene

expression was observed in cells cultured in hypoxia (**Figure 5.2B**). Tracks of normalized H3K27ac signal were determined for previously reported hypoxia marker genes *LDHA*⁶¹ and *SLC2A1*⁶². Both genes showed increased H3K27ac signal in hypoxia in all three comparisons (NN-HN, NE-HE, NF-HF). With respect to RNA-seq data, non-infected and *Fnn*-infected hypoxia samples showed significantly higher expression levels of those two genes.

There are limited studies that have previously investigated the impact of hypoxia on H3K27ac levels. Hypoxia can both activate and repress different gene families whose expression is correlated with multiple histone marks⁶³. H3K4me3, H3K27me3⁶⁴, and H3K9me2⁶⁵ have been shown to be increased in hypoxia. Acute hypoxia has been shown to increase HIF-A (hypoxia inducible transcription factor) binding which increases transcriptional activation⁵⁸. The authors found that transcriptional output in HCT116 cells upon 90 minutes of exposure to hypoxia was positively correlated with pre-existing levels of histone marks such as H3K27ac that relate to active genes. Another study, that subjected HepG2 cells to hypoxia for 4 hours, demonstrated increased acetylation of H3 marks in the presence of acetate⁶⁶. They reveal that hypoxia causes histone hypoacetylation by decreasing citrate and acetyl-CoA generation, which is rescued by exogenous acetate. Specifically, acetate supplementation to hypoxic cells induced significant increase of H3K9, H3K27 and H3K56 acetylation levels, but not H3K14, H3K18, H3K23 and H3K36 acetylation. In contrast, other studies suggest H3K27ac marks increase with HIF-1 binding resulting in increased enhancer activity⁶⁷. In conjunction, although HDAC (histone deacetyltransferase) activity has been correlated with transcriptional repression, it is also associated with the activation of HIF-1 related genes⁶³. Furthermore, HIF1A is negatively regulated by feedback mechanisms and can be suppressed under prolonged hypoxia⁶⁸. Together, these findings reveal a complex interconnection between hypoxia and H3K27 acetylation. Our data reveals that 52-hour total hypoxia treatment of HCT116 cells increased H3K27 acetylation under prolonged hypoxia, indicating an increase in active gene expression.

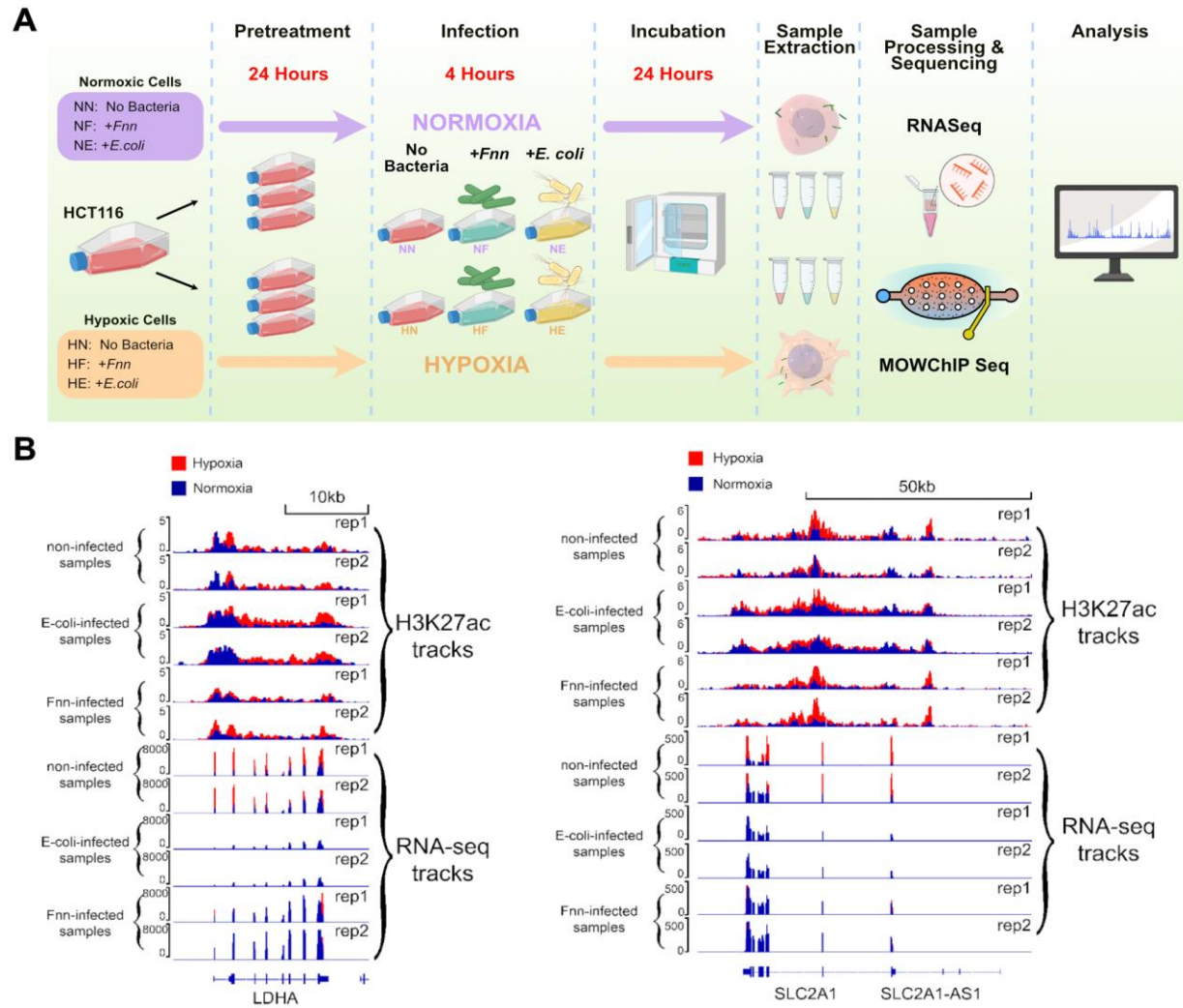


Figure 5.2 Overview of epigenomic data for HCT116 cells infected with *F. nucleatum* and *E. coli* under normoxia or hypoxia.

(A). Overview of the experimental protocol detailing the samples used and duration of hypoxia treatment and period of infection. (B). ChIPseq tracks and RNA-seq tracks for LDHA and SLC2A1 loci for normoxia and hypoxia samples.

5.3.4 Infection with invasive *F. nucleatum* leads to fewer changes to differential enhancers within host cells in hypoxia

H3K27ac plays a vital role in long-range gene regulation at enhancer regions. We, therefore, determined the locations of active enhancers, which were defined as H3K27ac high regions that do not overlap with regions near transcription start sites (TSS) (*See Methods*). We examined the total number of consensus enhancers under various treatment/infection conditions. We observed that the number of enhancers increased when cells underwent hypoxia treatment (NN = 4008, NF = 5898, NE = 9912, HN = 13081, HF = 11869, HE = 16676). In order to further explore the impact of oxygen and *Fnn* Infection on the H3K27ac profiles, differential enhancer analysis was performed with Diffbind and DEseq2. We observed significant changes in the H3K27ac profile based on the oxygen environment and the microbe. For *E. coli* infected control samples, the number of differential enhancers of the cells infected under hypoxia (HN-HE) (3097) was greater than cells infected under normoxia (NN-NE) (2781). While for *Fnn* infected samples, the number of differential enhancers of the cells infected under hypoxia condition (HN-HF) (134) was substantially less than cells infected under normoxia condition (NN-NF) (1319) (**Figure 5.3A**).

In order to investigate the unique impact of *Fnn* infection under hypoxia, we conducted overlap analysis on the genes related to differential enhancers of each culture condition (**Figure 5.3B**). *E. coli* infection samples served as a negative control and thus, the genes that overlapped with *E. coli* infection were masked. We identified 229 differential enhancer-related genes that were unique in NN-NF, and 36 genes in HN-HF. 11 differential enhancer-related genes were identified in both NN-NF and HN-HF. The majority of genes showed up in NN-NF (95%, 229 out of 240 genes as determined by dividing the number of genes in the non-overlapping regions of NN-NF comparison by the total number of genes related to *Fnn* infection under normoxia) or HN-HF (77%, 36 out of 47 genes as determined by dividing the number of genes in the non-overlapping regions of HN-HF comparison by the total number of genes related to *Fnn* infection under hypoxia) did not overlap with each other,

suggesting that *Fnn* infection under hypoxia condition presents unique epigenomic changes compared to that under normoxia.

The genes linked to the enhancers in each part mentioned above (**Figure 5.3B**) were analyzed for overrepresentation of Gene Ontology biological process gene sets. Because of the complex mechanism of activation by epigenetic modification, the differentially enhancers were not divided into up- and down-regulated enhancers for the gene ontology analysis. 18 GO terms were enriched from 229 NN-NF-specific differential enhancer-related genes (**Figure 5.3C**). Those GO terms included *regulation of cell adhesion* and *regulation of epithelial cell differentiation*, which were related to the proliferation of CRC. Terms related to transcription and cellular metabolism were also found, like *transcription from RNA polymerase II promoter* and *protein localization to nucleus*, suggesting altered transcription and translation activities in *Fnn*-infected CRC cells. In hypoxia, *Fnn* infection showed a small set of enriched genes, which limited the ability to extract GO terms. These results suggest that if hypoxic cells facilitate *Fnn* infection, then host response to the infection would be limited. Thus, for HN-HF, we focused on the roles of the 36 extracted genes as well as the 11 genes common to *Fnn* infection in both oxygen environments.

Volcano plots (**Figure 5.3D**) revealed H3K27ac signals at gene enhancers in NN-NF and HN-HF. We observed several interesting genes which may play vital roles in the regulation of cellular activities upon infection under normoxia or hypoxia. For example, the H3K27ac signal at the enhancer of *CBX7* gene significantly decreased when *Fnn* infected normoxia cells. *CBX7* (Chromobox protein homolog 7) plays a role in the proliferation inhibition of CRC cells, so the inactivation of *CBX7* enhancer provided evidence for the over-proliferation of *Fnn*-infected normoxia cells ⁶⁹. *LINC010189* expression has been correlated with larger tumor size, higher T stage, as well as lymphatic metastasis of patients with gastric cancer ⁷⁰ and has further been implicated in EMT regulation in non-small cell lung cancer ⁷¹. *SEMA6B* (Semaphorin 6b) is a protein whose increased expression was correlated with an immunosuppressive environment and poor prognosis in CRC ⁷². *MYO1C* could play a role in the

PI3K pathway and has been implicated in endometrial carcinoma ⁷³. *CARD8* (caspase recruitment domain) participates in inflammasomes which recruit Caspase-1. It's involved in pattern recognition and signals the activation of a degradation pathway for disordered and misfolded proteins ⁷⁴. A homolog, *CARD3*, has previously been implicated in increasing the migration of *Fnn* infected cells by activating autophagy signaling in response to bacterial peptidoglycan ⁷⁵. *ELN* (elastin) is a key factor in CRC development ⁷⁶ and *TMOD3* (tropomodulin 3) functions in the EGFR-PI3K-AKT pathway to drive metastasis in hepatocellular carcinoma ⁷⁷.

Cancer-related genes were observed among the genes with differential enhancers identified in *Fnn* infected hypoxic cells. *WDYHV1*, is among six genes validated to predict recurrence risk of patients with stage II/III CRC ⁷⁸. *INFGR2* is part of the interferon signaling pathway ⁷⁹. *TSPAN19* is a member of tetraspanin proteins on the cell membrane that play an important role in regulating the tumor microenvironment and cell interactions with endothelial cells to promote metastasis ⁸⁰. *CLUAP1* has been shown to be upregulated in CRC, however its role is unclear ⁸¹. *SND1* is implicated in promoting CRC progression by targeting human telomerase reverse transcriptase (hTERT) ⁸².



Figure 5.3 Analysis of differential enhancers reveals *F. nucleatum* infection specific gene regulation based on oxygen environment

(A) Number of genes related to differential enhancers, FDR = 0.05 used as cutoff. (B) The Venn diagram displays the overlap of genes between changes in oxygen level and bacteria infected. Each oval represents the number of differential enhancer-related genes obtained between each interaction (NN-NE, HN-NE, NN-NF, HN-NF) (C) GO terms enriched from 229 NN-NF specific differential enhancer-related genes (D) Volcano plots showing genes associated with differential enhancers of NN-NF and HN-HF. The differential enhancers were annotated to their nearest genes. Top 20 out of 229 significant genes uniquely found in NN-NF are labeled and top 20 out of 36 significant genes uniquely found in HN-HF are labeled. The differential enhancers were annotated to their nearest genes. The green points refer to upregulation indicated by NN(HN) < NF(HF) and purple points refer to downregulation indicated by NN(HN) > NF(HF).

5.3.5 Transcriptomic analysis reveals differential gene expression in hypoxic and normoxic cells upon *Fnn* infection

Transcriptomic analysis identified differentially expressed genes (DEGs) amongst the six culture conditions. Similar to the trend in differential enhancer analysis, for *E coli* infected samples, the number of DEGs of the cells infected under hypoxia (HN-HE) was greater than cells infected under normoxia (NN-NE). While for *Fnn* infected samples, the number of DEGs of the cells infected under hypoxia condition (HN-HF) was less than cells infected under normoxia condition (NN-NF) (**Figure 5.4A**). Overlap analysis of DEGs was performed in the same manner as enhancer analysis. (**Figure 5.4B**) We identified 453 DEGs that only showed up in NN-NF, 226 DEGs that only showed up in HN-HF and 34 DEGs that were shared by both comparisons. In either NN-NF or HN-HF specific DEGs, we found the number of DEGs up-regulated was similar to genes down-regulated. (NN-NF specific: up=260, down=193; HN-HF specific: up=120, down=106) While in *Fnn*-infection common DEGs, the majority of DEGs in HN-HF are down-regulated in HF(HN-HF: up=10, down=24). We then conducted GO and KEGG pathway enrichment analysis on the 3 gene sets marked in Figure 4B. We identified dozens of GO terms from 453 NN-NF specific DEGs (**Figure 5.4C**), with most of them being cell cycle and replication related, like *chromosome segregation*, *nuclear division* and *chromosome localization*. Those terms agreed well with the enhanced proliferation feature of *Fnn*-infected CRC that we revealed at epigenome level. Similar to differential enhancer analysis, no significant GO terms were enriched from HN-HF specific DEGs. We then identified DEGs that showed up in both normoxia and hypoxia infected cells. GO terms related to metabolism (*protein ADP-ribosylation*, *DNA recombination*), protein-DNA interaction (*positive regulation of DNA binding*) and bacteria invasion(*regulation of response to biotic stimulus*) were observed (**Figure 5.4D**). Interestingly, the NF- κ B signaling pathway was enriched from *Fnn* infection common DEGs (**Figure 5.4E**). This pathway was observed in *Fnn* infection under both normoxia and hypoxia suggesting *Fnn* infection under both conditions elicited inflammatory responses from the host cell.

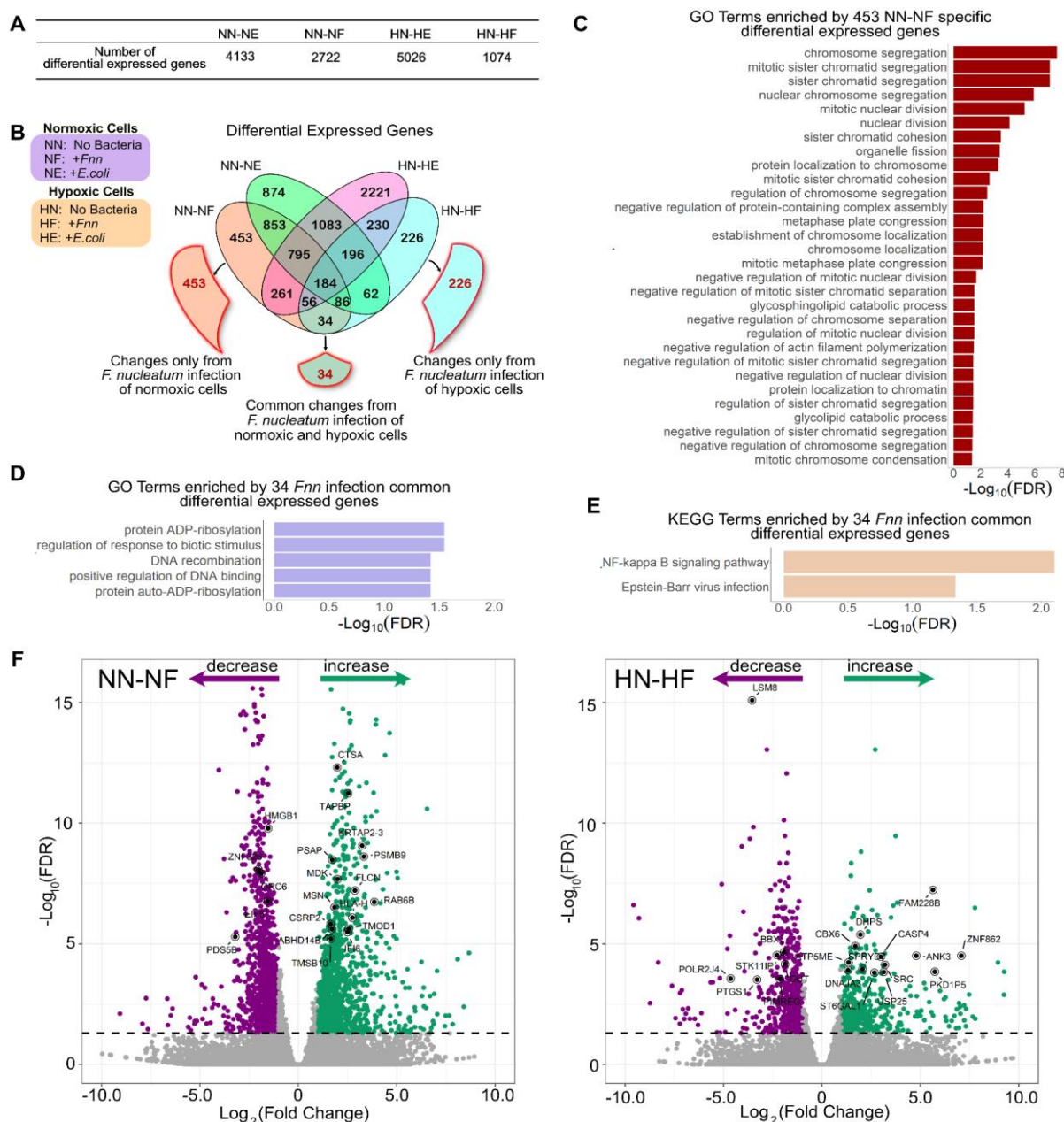


Figure 5.4 Transcriptomic analysis reveals differential gene expression in hypoxic and normoxic cells upon *F. nucleatum* infection

(A) Number of differentially expressed genes, FDR = 0.05 used as cutoff. (B) The Venn diagram displays the overlap of genes between changes in oxygen level and bacteria infected. Each oval represents the number of differentially expressed genes obtained from each interaction (NN-NE, HN-NE, NN-NF, HN-HF) (C) GO terms enriched from 453 NN-NF specific differentially expressed genes (D) GO terms enriched from 34 NN-NF and HN-HF common differentially expressed genes (E) KEGG

pathways enriched from 34 NN-NF and HN-HF common differentially expressed genes (F) Volcano plot showing genes associated with differentially expressed genes of NN-NF and HN-HF. Top 20 out of 453 significant DEGs unique to NN-NF are labeled and top 20 out of 226 significant DEGs unique to HN-HF are labeled. The green points refer to upregulation indicated by NN(HN) < NF(HF) and purple points refer to downregulation indicated by NN(HN) > NF(HF).

Volcano plots (**Figure 5.4F**) were created for NN-NF and HN-HF conditions. To identify DEGs that were altered only in *Fnn* infected hypoxic cells, we masked the DEGs induced commonly in *Fnn* infected normoxic cells as well as the DEGs that were also influenced by *E. coli* infection of hypoxic cells. The top 50 genes that exhibited the largest fold change within significance in *Fnn* infected hypoxic cells were further investigated (**Figure 5.9**). These individual genes had several functional associations with cancer progression and worse prognosis in CRC.

Upregulated DEGs in *Fnn* infected hypoxic cells. Several of the upregulated DEGs identified have been implicated in worse cancer outcomes. *TRAF1* is a TNF receptor-associated factor that regulates the activation of NFκB and JNK to regulate cell survival and apoptosis. It plays a role E3 ubiquitin-protein ligase complex and promotes ubiquitination of *MAP3K14* ⁸³. *ARHGAP31* is a Rho GTPase-activating protein that impacts cell spreading cell migration. *LZTS1* is a leucine zipper putative tumor suppressor 1 and regulates the cell cycle. *BMP7* is bone morphogenetic protein 7 that usually regulates calcium and bone homeostasis but can be expressed in cancer to influence proliferation and migration ⁸⁴. In fact, elevated *BMP7* has been clinically correlated with pathological aggressiveness of CRC including depth of tumor invasion, and liver metastasis ⁸⁵. Furthermore, *BMP7* can promote immunotherapy resistance ⁸⁶ and impact cancer progression ⁸⁷. *RUNDC3A-AS1* is a long non-coding RNA that could play a role in carcinogenesis ⁸⁸. *ANK3* has been implicated in the poor prognosis of prostate, ovarian, lung, and breast cancer ⁸⁹ and could be a potential oncogene in CRC ⁹⁰. *PIGQ* activation along with *PDGFRB* and *MYC* was found to increase cell growth ⁹¹. *TBKBP1* is a TBK-binding protein 1, which in combination with *TBK1* can respond to microbial stimuli and mediates type 1 interferon induction, can play a role in tumor-mediated immunosuppression by a mechanism

involving induction of the checkpoint molecule PD-L1 and stimulation of glycolysis ⁹². *GPR137* is a G-coupled protein receptor 137 found on the membrane and mediates signal transduction and also plays a role in intestinal pathogenesis ⁹³, gastric cancer ⁹⁴, and bladder cancer ⁹⁵. *RNF169* is a negative regulator of double-stranded DNA break repair ⁹⁶. *SRC* is involved in protein-tyrosine phosphorylation that is activated during microenvironmental stresses including hypoxia and plays a role in the evasion of apoptosis ⁹⁷. It is modulated differentially by chemotherapeutic drugs in metastatic CRC ⁹⁸. Furthermore, Src activation stimulates cytoskeletal ruffling to impact bacterial internalization ³⁰. *USP25* is a deubiquitinase and has been implicated in colonic inflammation and bacterial infection, including *F. nucleatum* colonization ⁹⁹. *ENPP1*, is a molecule that coats cancer cells and degrades cGAMP. cGAMP is created by the cGAS-STING warning system and usually signals to the immune system. However cGAMP degradation by ENPP1 results in increased immunosuppression and metastasis ^{100,101}. *TFAP4* is a transcription factor enhancer binding protein that has been correlated with poor prognosis in CRC ¹⁰². *LATS2* (large tumor suppressor 2) is another gene correlated with clinical prognosis and immune infiltration in CRC ¹⁰³. *FOXD1* promotes CRC progression via the ERK1/2 pathway ¹⁰⁴. *ARID2* is a potential tumor suppressor and driver of early onset sporadic rectal cancer ¹⁰⁵. *ST6GAL1*(β -galactoside- α 2,6-sialyltransferase 1) was found to inhibit cancer metastasis ¹⁰⁶. *TGFB1* codes for Transforming growth factor- β 1 which plays a role in CRC development and metastasis ^{107,108}. *SRR* (serine racemase) has been shown to enhance the growth of CRC cells ¹⁰⁹. *HOXA13* can promote colon cancer progression through β -catenin dependent WNT pathway ¹¹⁰. *RAB3D* expression has been implicated in poor prognosis of CRC ¹¹¹.

Downregulated DEGs in Fnn infected hypoxic cells. Several of the genes that were downregulated in hypoxic cells normally had homeostatic roles in tumor suppression and inhibiting cell invasion and metastasis. Thus, *Fnn* induced downregulation of these genes could further impact tumor progression in CRC. The most downregulated gene was a fusion *NEDD8-MDP1*. *NEDD8* codes for a ubiquitin-like protein which is added to certain substrates in a process called neddylation that

modulates many biological processes including tumorigenesis ¹¹². However, it is not clear if the gene fusion is translated to a functional protein. *NR5A2* codes for a transcription factor has been implicated in head and neck squamous cell carcinoma, and gastric cancer and plays a role in proliferation ^{113–115}. *MUC12* has been identified to be an independent marker for stage 2 and 3 colorectal cancer ¹¹⁶. *POLR2J4* is a pseudogene, and circular RNA derived from it can regulate cell proliferation ¹¹⁷. Long intergenic non-protein coding RNA (LINC00641) *PLSCR4* was also found impact proliferation ¹¹⁸. *NME1-NME2* regulation has been correlated with both pro- and anti-metastatic activities ¹¹⁹. *COX-1* is coded by *PGS1* and its downregulation could imply mitochondrial apoptosis. *EPHB6* is part of the erythropoietin-producing hepatocyte (Eph) family tyrosine kinases and EphB6 in combination with Apc mutations has been implicated in CRC tumorigenesis ¹²¹. *FADD* codes for Fas-associated death domain protein that plays a role in cellular apoptosis and has been found to impact chemoresistance to 5-fluorouracil ^{122,123}. Downregulation of *CHRNA7* was found to increase cell invasion and metastasis of a CRC cell line ¹²⁴. MAP related kinase genes have key roles in cellular proliferation and differentiation and are frequently implicated in cancer. Downregulation of *MAP3K4* is implicated in stem-cell epithelial-mesenchymal transition and has been correlated with aggressive tumor characteristics in intrahepatic cholangiocarcinoma including vascular invasion and intrahepatic or lymph node metastases ^{125,126}. Downregulation of *WWOX* has been correlated with lower patient survival in breast cancer and could play a role in CRC ^{127,128}. *WWOX* (WW domain containing oxidoreductase) acts as a tumor suppressor gene. Furthermore, HCT116 cells have complex gene deletions at the *WWOX* locus FRA16D ¹²⁹. *PPPR12B* downregulation has been found to abrogate PEAK1-mediated tumor suppression by regulating Grb2/PI3K/Akt signaling in colorectal cancer ¹³⁰.

Common DEGs regulated in normoxic and hypoxic cells upon Fnn infection. Among the common genes that exhibited differential expression in both normoxic and hypoxic cells upon infection, genes of note include *TBL1XR1*, *FUS*, *TAP1*, *RELB*, *APOBEC3C*, *BIRC5*, *CXCL8*, *SIRT2*, and *HLA-E*. *TBL1XR1* is

involved in the recruitment of the ubiquitin/19S proteasome complex to nuclear receptor-regulated transcription units for transcription activation. We observed that it is downregulated to a greater extent in normoxic cells. *HMGB1P5* is a pseudogene that can impact gene regulation through generation of siRNA or miRNA binding sites ¹³¹. The original gene *HMGB1* is an evolutionarily conserved protein with a wide spectrum of action ¹³² and plays a role in tumor progression and anti-cancer therapy. We see a reduction in its downregulation in hypoxic cells. *OPTN* codes for optineurin which is a critical node in INF and MHC signaling. The loss of this gene could impact immune evasion and intrinsic immunotherapy resistance in CRC ¹³³. *FUS* was found to be upregulated in hypoxia and downregulated in normoxia. This gene code for splicing factors and and could be involved in gene fusions, such as *FUS-DDIT3* is oncogenic in liposarcoma ¹³⁴. *FUS* RNA binding protein (FUS) functions within the heterogeneous nuclear ribonucleoprotein (hnRNP) complex which has a role in pre-mRNA splicing and the mRNA cytoplasmic export. *TAP1* is associated with antigen processing and translocation of peptide antigens from cytosol to endoplasmic reticulum (ER) for loading onto MHC class I (MHC I) molecules. It has been shown that TAP1 downregulation can elicit immune escape and poor prognosis in CRC ¹³⁵. *SIRT2* (histone deacetylase silencing information regulator two 2/sirtuin 2) plays a role in cell proliferation in CRC ¹³⁶. Moreover, SIRT2 activation has been implicated in the intracellular invasion of *L. monocytogenes* and other pathogens ¹³⁷ and can deacetylate H3K18 upon bacterial infection ¹³⁸. *HLA-E* is a non-classical MHC molecule that is involved in immune self-nonsel discrimination and its expression on tumor cells could play a role in immune evasion by preventing detection by NK cells ^{139,140}.

DEGs extracted from Fnn infection of normoxic cells. In comparison to the hypoxia dataset, we observed more DEGs regulated in normoxic cells upon *Fnn* infection compared to hypoxic cells. However, only a limited number of these have been directly implicated in cancer progression. Several could participate in host response to infection and inflammation. *NOS1* upregulation could stimulate NFkB activation in response to infection and inflammation ¹⁴¹. *WNT7A* upregulation has been tied to

proliferation of CRC cells ¹⁴². Wnt β -catenin-dependent canonical signaling pathway has been shown to impact metastatic progression of CRC cells ¹⁴³. Fatty acid 2-hydroxylase (*FA2H*) overexpression could suppress tumor growth and metastasis ¹⁴⁴. *BST-2* is involved in CRC tumor progression ¹⁴⁵. *IL-32* upregulation is associated with increased metastatic spread of CRC to lymph nodes ¹⁴⁶. *HLA-J* has been involved in breast cancer progression and immune evasion ¹⁴⁷. *HLA-DRB1* is part of a family of genes called the human leukocyte antigen (HLA) complex, and plays a critical role in immune regulation by presenting peptides derived from extracellular proteins. Its upregulation could indicate recognition of an invaded bacterium. Similarly, we also see upregulation of *IR7* that is a key regulated or type 1 interferon dependent immune response. Gene expression related to mitochondria related proteins was also found to be upregulated. Cancer related genes that were downregulated in normoxia include *SPRY3* which plays a role in the proliferation and migration of brain cancer cells ¹⁴⁸. *STAT5* (signal transducer and activator of transcription 5) in CRC is involved in cell growth, cell cycle progression and apoptosis ^{149,150}. *GAB1* codes for Grb2-associated binder 1 and participates in signaling cascades from tyrosine kinases such as Hepatocyte growth factor (Met) and can impact proliferation independent of EGFR (epidermal growth factor receptor) ^{151,152}. Downregulation of *PDS5B* has shown increased migration and invasion of non-small cell lung cancer cells ¹⁵³. Several genes involved in ubiquitin mediated proteasomal degradations were also downregulated.

The functional analysis of DEGs revealed that hypoxic cells infected with *Fnn* exhibited changes in gene expression of multiple factors directly associated with increased malignancy and worse prognosis compared to the response of normoxic cells, which notably had more DEGs. It is interesting that we do not observe Wnt signaling upregulation in hypoxia. It is likely that hypoxia conditions already increase the expression of several cancer related genes, and infection with *Fnn* uniquely compounds this response.

5.3.6 Oxygen environment and *F. nucleatum* infection modulates differential enrichment of transcription factor binding motifs

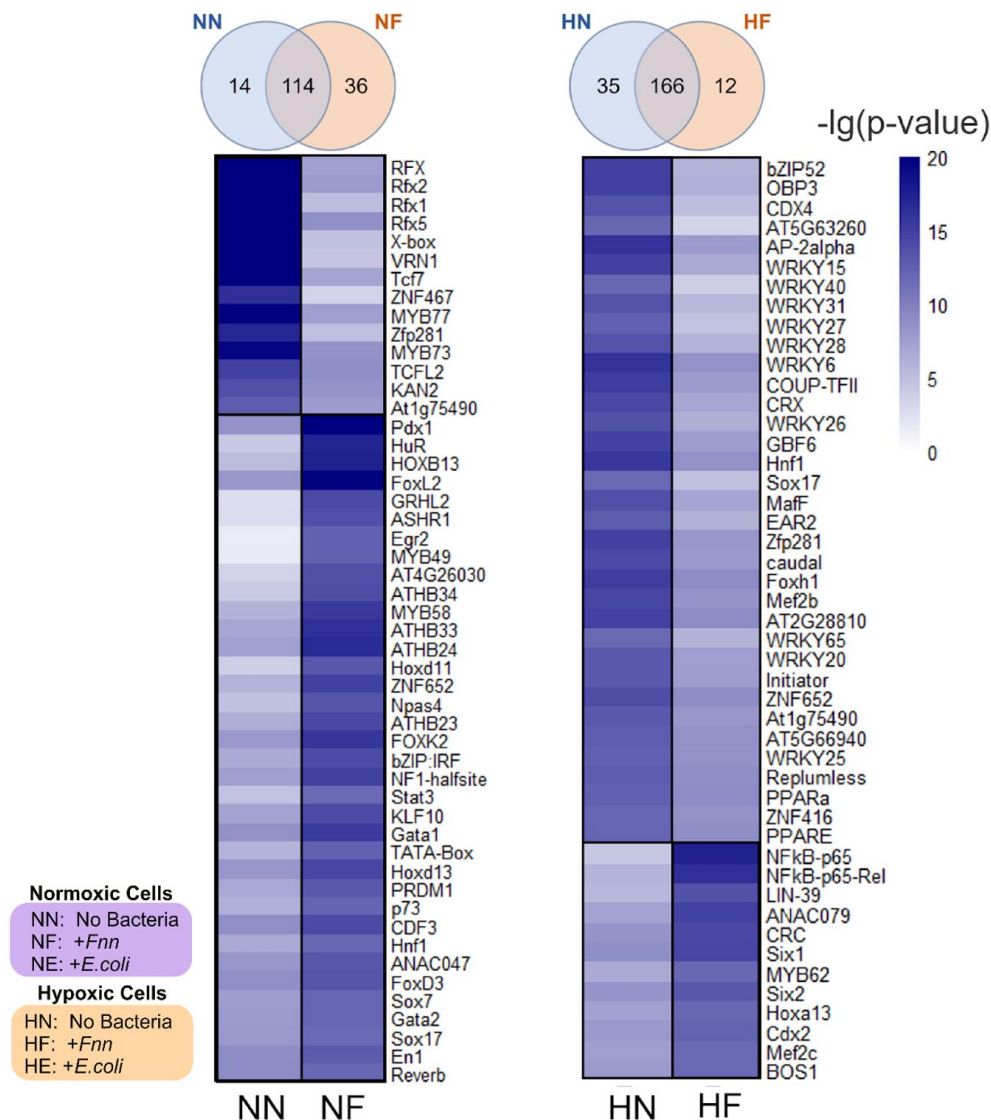


Figure 5.5 Transcription factor motif analysis in hypoxic and normoxic *F. nucleatum* infected cells reveals oxygen dependent variation in binding.

Heatmaps of motifs found to be significantly enriched in either NN (HN) or NF (HF). Color represents the level of significance. Venn diagrams of overlap between motifs enriched in NN (HN) or NF (HF) are also presented.

Enhancer regions were then scanned to determine the transcription factor (TF) binding motifs significantly enriched in each sample. **(Figure 5)** Normoxia samples (NN&NF, n=50) had a similar number of differentially enriched TF motifs as hypoxia samples (HN&HF, n=47). We defined significantly enriched TFs as those uniquely enriched in one condition (e.g., NN), and not the other (e.g., NF). In normoxia, *Fnn*-infected samples (NF, n=36) had more enriched TFs than non-infected samples (NN, n=14). On the contrary, in hypoxia, *Fnn*-infected samples (HF, n= 12) had fewer enriched TFs than non-infected sample (HN, n=35). Several transcription factors associated with CRC were found in NF. These include p73¹⁵⁴, STAT3¹⁵⁵ and GATA2¹⁵⁶. Inflammation related transcription factors NFκB-p65¹⁵⁷ and HuR¹⁵⁸ were also found to be enriched in NF. In HF, immune and development related transcription factor Mef2c¹⁵⁹ was observed. Mef2c has been implicated in negative control of angiogenesis in endothelial cells¹⁶⁰. Interestingly, we identified 5 significantly enriched TFs that appeared both in NN-NF and HN-HF. Those are: Zfp281, At1g75490, ZNF652, Hnf1 and Sox17. Hnf1 was reported as a possible tumor suppressor¹⁶¹. Hnf1 was found enriched in NF within normoxia, but was enriched in HN within hypoxia. This may suggest that hypoxia inhibited binding of Hnf1 with *Fnn* infection. In addition to NFκB-p65 enrichment in HF, we also observed the enrichment of Six1 and Six2 TFs. Transcription factors of this family (*sine oculis* (SIX)), are critical in developmental regulation and the control of cell differentiation and have been implicated in tumorigenesis, EMT, and metastasis¹⁶². Specifically, Six1 is correlated with poor prognosis in all tumor types¹⁶³. Six2 overexpression was identified in invasive breast cancer¹⁶⁴. These TFs are also connected to Wnt signaling. Together, these findings reveal variable transcriptional regulation induced by *F. nucleatum* in different oxygen environments.

5.3.7 Integrative analysis of ChIP-seq and RNA-seq data reveals genes regulated in both layers

Next, we combined ChIPseq and RNAseq data to reveal common enhancer-transcriptomic signals. Among the total enhancers and DEGs identified in NN-NF, 192 were overlapping genes. In HN-HF, a similar analysis revealed 11 overlapping genes. These overlapping genes were segregated into subsets where both were up or downregulated, or either one was up/down regulated (**Figure 6**). In hypoxia, the overlapping genes identified were *ARFGAP3*, *ARHGAP26*, *CDC42EP4*, *CLSTN1*, *DLG1*, *HSPG2*, *MAP3K4*, *RBP1*, *SRXN1*, *TNIP1*, and *UBR5*. *ARFGAP3* is part of the adenosine-diphosphate-ribosylation factor (ARF) family which is a subset of the RAS superfamily of proteins. These proteins mediate several cellular processes and have been implicated in tumorigenesis and cancer progression ¹⁶⁵. *ARHGAP26* has been shown to be implicated in ovarian cancer cell invasion and migration ¹⁶⁶. *CDC42EP4*, or Cdc42 is part of the Rho family of GTPases that can regulate cytoskeletal dynamics, cellular polarization, and aspects of proliferation, migration and invasion of tumor cells ^{167,168}. *CLSTN1* is calsynenin-1 is a secreted protein that has been previously identified as a marker for lung adenocarcinoma ¹⁶⁹. Furthermore, alternative splicing of *CLSTN1* has also been shown to impact breast cancer patient survival ¹⁷⁰. *DLG1* (discs large homolog 1) expression has been associated with poor prognosis in CRC. It is part of the Membrane Associated Guanylate Kinases (MAGUKs) and is involved in cell junction formation and cell polarity ¹⁷¹. *MAP3K4* (mitogen activated protein kinase) is a kinase that regulates multiple cellular functions including proliferation, differentiation, migration and apoptosis and can activate transcription factors and other signaling pathways in cancer ¹⁷². *RBP1* (Retinol-binding protein 1) has been shown to play a role in oral squamous cell carcinoma and other cancers ¹⁷³. *SRXN1* codes for sulfiredoxin-1 is an antioxidant enzyme and its overexpression has been correlated with poor prognosis in hepatocellular carcinoma

¹⁷⁴. Finally, UBR5 is an E3 ligase and has been shown to be overexpressed in CRC, promotes cell proliferation, and is correlated with progression and poor survival in CRC ¹⁷⁵.

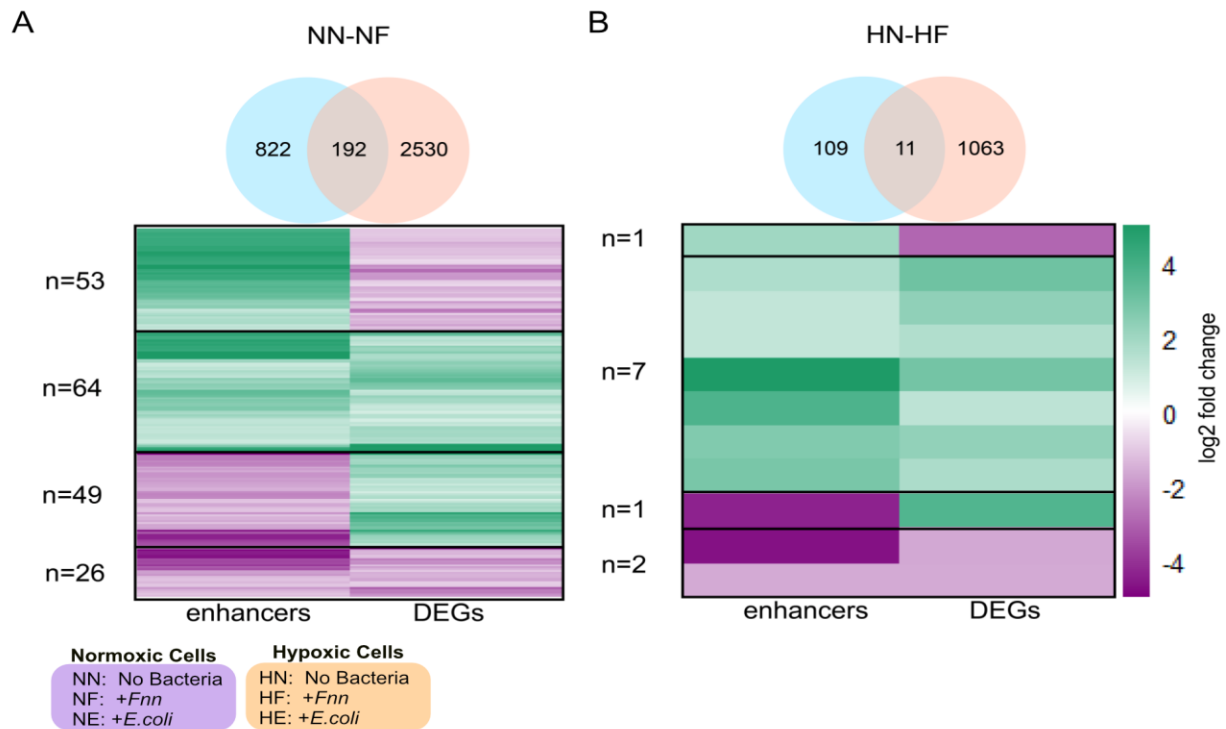


Figure 5.6 Common genes identified in both ChIPseq and RNAseq data.

Overlap of enhancer related genes and RNAseq genes and fold enrichment of genes that are changed both in H3K27ac signal at enhancers and gene expression in (A). NN-NF and (B). HN-HF. positive fold changes indicate NF<NN, and HF<HN.

5.4 Discussion

Cancer is a highly heterogeneous disease that affects the molecular machinery of the cells in myriad ways and there has been systematic documentation of the variation in a whole-genome scale ¹⁷⁶. The predominant mechanisms that regulate gene activation and gene repression are histone modifications, DNA methylation, chromatin-associated complexes, non-coding RNAs, and RNA splicing factors, which are collectively referred to as epigenetic reprogramming. Regulation of epigenetic modifications can play an important role in tumor initiation and progression ¹⁷⁷. Invasive bacteria can employ various strategies to passively or actively target these epigenetic mechanisms within the host cells which may act to subvert host defense, evade immune signaling, and allow pathogen persistence ^{100,178,179}. The composition of the TME and cellular crosstalk can also play a critical role in the host cell response to infection ¹⁸⁰. Specifically, we hypothesized that tumor hypoxia could be conducive to an anaerobic organism such as *F. nucleatum* to facilitate invasion and represents a physiologically relevant niche to study host-microbial interactions. We, therefore, investigated the sustained patho-epigenetic and transcriptomic changes upon *F. nucleatum* infection of CRC cells in high and low oxygen environments in order to extract the unique contribution of hypoxia on infection dynamics and host cell response.

DNA within cells is highly compacted within chromosomal structure. Approximately 147 bp of DNA is wound around octamers of histone proteins (H2A, H2B, H3, H4) to form nucleosomes which are arranged serially along with the linker histone H1. These complexes, in addition to non-histone proteins and RNA, form active euchromatin and inactive heterochromatin. Covalent modifications to the histones result in chromatin restructuring and alters the nucleosome structure to enable or restrict access to DNA. Histones can be modified by a variety of posttranslational modifications (PTMs) which include acetylation, methylation, phosphorylation, sumoylation, ubiquitylation, and ADP ribosylation of the histone tails. The PTMs are performed by specific enzymes including histone

acetyltransferases (HATs), histone methyltransferases (HMTs), kinases, histone deacetylases (HDACs), demethylases (HDMs) and phosphatases. The type of PTM and its location on the histones affects downstream gene expression.

The gut microbiome has an enormous impact in their contributions to CRC development via epigenetic mechanisms. Bacteria belonging to Firmicutes, Actinobacteria, Bacteroidetes, Chlamydiae, Fusobacteria, and Proteobacteria encode proteins or cell wall constituents that may interact with the epigenetic machinery of eukaryotic cells. Gut microbes produce secondary metabolites such as short chain fatty acids including acetate, propionate, and butyrate, hydrogen sulfide (H₂S), and secondary bile acids that may have both pro- and anti- carcinogenic properties. Numerous studies have definitively shown that gut microbes exert distinct impacts on DNA damage, DNA methylation, chromatin structure and non-coding RNA expression in colonic epithelial cells (CEC) ¹⁸¹. For example, *pks+* *Escherichia coli* infection of CECs have been extensively studied. Its colibactin toxin can cause DNA double-stranded breaks, chromosomal aberrations, and cell cycle arrest in vitro ¹⁸². The most common histone modifications induced by bacteria are histone acetylation/deacetylation and phosphorylation/dephosphorylation and these are usually induced by the activation of host signaling cascades such as MAPKs (ERK and p38, NFκB, PI3K) upon infection. Secreted factors can also affect the epigenetic machinery. Examples include toxins secreted by *Listeria monocytogenes* including listeriolysin O (LLO) or other virulence factors such as internalin B (InlB), and Listeria nuclear targeted protein A (LntA). LntA interacts with cMet and activates histone deacetylation via *SIRT2* inducing gene silencing ¹³⁷. It has previously been shown that treatment of gingival epithelial cells with *Prophyromonas gingivalis* and *Fusobacterium nucleatum* decreases expression of DNA methyltransferase (DNMT1) and histone deacetylase ¹⁸³. Metabolites produced by bacteria such as short chain fatty acids like butyric acid are also potent inhibitors of HDAC.

Another form of post-transcriptional control involves regulation by microRNAs (miRNAs) and lincRNA (long intergenic non-coding RNA). MicroRNAs regulate post-transcriptional gene repression by binding to the 3' UTR of mRNA molecules causing repressed translation and encouraging degradation of the target mRNA. lincRNAs can regulate protein gene expression by acting as a scaffold of histone modifying complexes, binding to transcription factors, or their DNA targets, and direct binding to RNA polymerase or sequestering miRNAs. Several miRNAs have been shown to be downregulated in CRC. In a CRC xenograft model, it has been shown that *F. nucleatum* causes resistance to oxaliplatin and 5-FU via the downregulation of miR-4802 and miR-18a*²⁴. Inhibitors to these two miRNAs caused increased drug resistance and transfection with the miRNAs resulted in drug resistance. Our data reveals the selective regulation of miRNAs (*MIR6504*), and lincRNAs (*LINC01089*, *LINC00173*, *LINC02343*, *LINC01589*, *LINC02101*, *LINC02352*, *LINC01564*, *LINC02321*, *LINC02273*, *LINC01011*) upon *F. nucleatum* infection, whose roles have not been previously characterized in CRC.

The response observed due to infection with *F. nucleatum*, is highly dependent upon the genotype and phenotype of the host cell as well as the specific strain of Fusobacterium used. A previous study investigated *F. nucleatum* infection of immortalized primary human colonic epithelial (HCE) cells and human carotid artery endothelial (HCAE) cells⁴⁵. The authors compared infection of these cell lines with 3 strains of Fusobacterium and identified upregulated genes related to inflammation and downregulated genes related to histone modifications and significantly remodeled chromatin states. Fusobacterium genes are also modified upon infection including genes related to hematogenous spread and the generation of a tumor permissive hypoxic environment⁵². Our work used a single strain, *F. nucleatum* subsp. *nucleatum* ATCC 23726, and revealed differences within a cell line subjected to varying oxygen environments. The single parameter of oxygen impacted the overall response to *F. nucleatum* infection by drastically altering the epigenomic and RNA landscape within the cell.

We have previously shown that the invasion of *F. nucleatum* into host cancer cells, leads to signaling changes. These have included the secretion of the potent chemokines, IL-8 and CXCL1 ³¹ in addition to a host of other signaling cascades including metabolic changes that affect cell behavior, epithelial-mesenchymal transition, and cellular migration and seeding ^{44,75,184}. We observed increased secretion of IL-8 and CXCL1 in hypoxia indicating a more robust response by hypoxic cells compared to normoxic cells. Furthermore, we observed rapid and higher intake of *F. nucleatum* in hypoxic cells as well as increased survival after infection in hypoxia. Hypoxia within tumors leads to a host of genotypic and phenotypic changes that can facilitate bacterial invasion and modulate its subsequent response. In response to low oxygen environments, the transcription factor HIF-1 is activated and increases expression of genes promoting erythropoiesis, angiogenesis, glucose transport and metabolism ^{58,185}. Infection in hypoxia can lead to signaling alterations that impact membrane alterations, cytoskeletal rearrangement, and endoplasmic reticulum stress during infection ⁴³. There is selective remodeling of lipid rafts on the membrane on exposure to hypoxia, redistributing membrane proteins, and increasing phosphatidylinositol that can be used by microbes to internalize into host cells. There is also an induction of selectin ligands on the cell surface that impacts cancer cell adhesion to endothelial cells to facilitate metastasis ¹⁸⁶. Hypoxia disrupts the cytoskeleton and modulates activity of actin binding proteins. These in turn can impact endocytosis and cellular permeability. The endoplasmic reticulum usually responsible for protein folding/translocation/and post translational modifications can result in misfolded proteins under hypoxia that can lead to lower protein biosynthesis, increased expression of chaperones, degradation of proteins, and eventual apoptosis. Hypoxia has been shown to promote intracellular bacterial persistence in *Staphylococcus aureus* of neutrophils ⁴². Our findings parallel these typical responses including upregulation of the ubiquitination pathway, spliceosome activity, and tumor evasion via the STING-cGAS pathway and interferon signaling in normoxia.

Epigenetic data particularly on the impact of hypoxia within CRC is limited. It has been shown that H3K27 acetylation was significantly increased in CRC samples compared with normal tissue ¹⁸⁷. A recent study showed that acetate can function as an epigenetic metabolite to promote cancer cell survival under hypoxic stress by increasing H3K9, H3K27, and H3K56 acetylation at specific promoters ⁶⁶. There is also an increase in H3K4me3, and a decrease in H3K27me3 ¹⁸⁸. However, it has also been shown that acute and chronic hypoxia can induce differential response within cells ⁵⁸. H3K27ac have been found to be associated with super-enhancer regions. Super-enhancers are regions of the DNA that exhibit a high transcription factor density and strong binding of the transcriptional machinery ¹⁸⁹. They have a role to play in tumorigenesis and it has been found that they function in the dysregulation of signaling pathways and promote cancer cell growth. In fact, these regions are identified by high levels of H3K4me1, H3K27ac, p300 or master TFs. Our data reveals fewer alterations within the H3K27ac related epigenome of host HCT116 cells in response to *F. nucleatum* infection in comparison to *E. coli* infection. These changes are almost 10-fold lower in hypoxia when compared to normoxia for *F. nucleatum*, while the numbers are comparable for *E. coli* in both environments. Qualitatively, with respect to the transcriptomic response, we observed a 63% reduction of DEGs in hypoxia for *F. nucleatum* infection, but a 21% increase in DEGs in hypoxia for *E. coli* infection. It is critical to note that the *E. coli* strain used in these experiments was non-invasive compared to the highly invasive *F. nucleatum* strain. The reduction in host transcriptomic response to *F. nucleatum* infection could indicate that intracellular invasion somehow subverts host recognition in hypoxia. Genotypic and phenotypic variations within hypoxic cells appear to favor this intracellular accumulation. It is still an open question as to how *F. nucleatum* is able to evade host recognition and subvert host defense within the cell. Biological GO terms extracted for normoxic cells indicate activity in DNA damage repair, DNA replication, spliceosome activity, metabolic changes, and antigen presentation. We also observed that certain genes that are involved in establishing metastases at new locations. Interestingly, we did not observe increased expression of

ICAM, which has previously been shown to impact HCT116 adhesion to HUVECs and extravasation³⁴. In addition, we observed *F. nucleatum* induced downregulation of METTL3, which has been previously shown to mediate metastasis via m6A epi-transcriptional modification¹⁹⁰. Furthermore, the top upregulated and downregulated genes upon *F. nucleatum* infection contained several cancer-related genes that could be uniquely activated upon *F. nucleatum* binding. Further research is needed to delineate the implications of these expressional changes.

Metabolic alterations are also common in intracellular infections as pathogens replicate within host cells. Host defenses may include generation of oxygen or nitrogen reactive species. These metabolic pathways are intrinsically tied to pathways including P13K/AKT, LKB1/AMPK, HIF-1, mTORC, MYC, p53¹⁹¹. Intracellular infection of normal cells leads cells to adopt a Warburg-like metabolism (aerobic glycolysis)¹⁹². Further studies are needed to understand how *Fusobacterium* secreted factors such as FadA, can contribute to epigenetic responses¹⁹³. *F. nucleatum* infection of oral cancer cells has previously revealed the presence of double stranded DNA breaks and recombination defects¹⁹⁴. Upregulation of DNA repair related genes could indicate similar occurrences in CRC.

There are multiple layers of regulation within the cell. Epigenomic alterations due to H3K27ac and the transcriptome contribute only a subset of information. Furthermore, we must account for the phenotypic variability of the cells in their signaling responses¹⁹⁵. Other histone modifications, post-transcriptional and post-translational modifications, intracellular and extracellular proteomic profiles, splicing variants, and protein trafficking can contribute to the complexity of these interactions to manifest differential outcomes within the tissue. The distinct metabolome of infected cells can also impact host cell response⁴⁴. Moreover, there are challenges associated with unbiased analysis of the staggering amount of data obtained with these methodologies. Nevertheless, these investigations can provide clues on fundamental mechanisms that can inform and direct future studies. Heterogeneity within tumors is another significant consideration and thus other cell lines

and responses within other CMS subtypes of CRC must be investigated. Technologies with increased sensitivity such as MOWChIP (sensitivity of <100 cells) ¹⁹⁶ can be harnessed to explore the impact of *F. nucleatum* infection *in vivo*, especially to extract the impact of tumor microbiome, normally found in low abundance ¹⁹⁷.

The intratumoral heterogeneity of the epigenome underscores the profound impact of the TME in influencing cell responses ¹⁹⁸. Epigenetic reprogramming may also result in the appearance of cancer stem-like phenotype ^{177,199,200}. The observed bacterium specific changes between infection with *F. nucleatum* and *E. coli*, indicates that the host interactions with bacteria are unique. In most cancers, there are multiple distinct species of bacteria cohabiting the tumor environment ⁹. *F. nucleatum* is known to co-aggregate with other microorganisms such as *Porphyromonas gingivalis* and facilitate their invasion in CRC cells ²⁰¹. Thus, a coordination of individual effects and concerted effects from multiple microbial species can orchestrate the ultimate response of the host cell. Microorganisms that have synergistically adapted to the ecological niche can result in the production of new metabolites that may have pathogenic effects on the host cell ^{202,203}.

In conclusion, our data reveals how features of the TME such as hypoxia can alter the epigenomic and transcriptomic landscape of HCT116 cells. We show that *F. nucleatum* infection in both normoxia and under physiologically relevant hypoxia can induce the expression of select cancer-related genes that, in part, can explain poor patient outcomes observed in CRC. The impact of the tumor microbiome on cancer progression has coalesced two normally disparate fields of study (cancer biology and infectious disease) that offers new opportunities to identify targets for therapy. We need to interrogate host-microbial interactions from a holistic perspective by integrating multiple layers of data in a TME and tissue relevant context to gain a nuanced understanding of the role of these microbes in cancer progression.

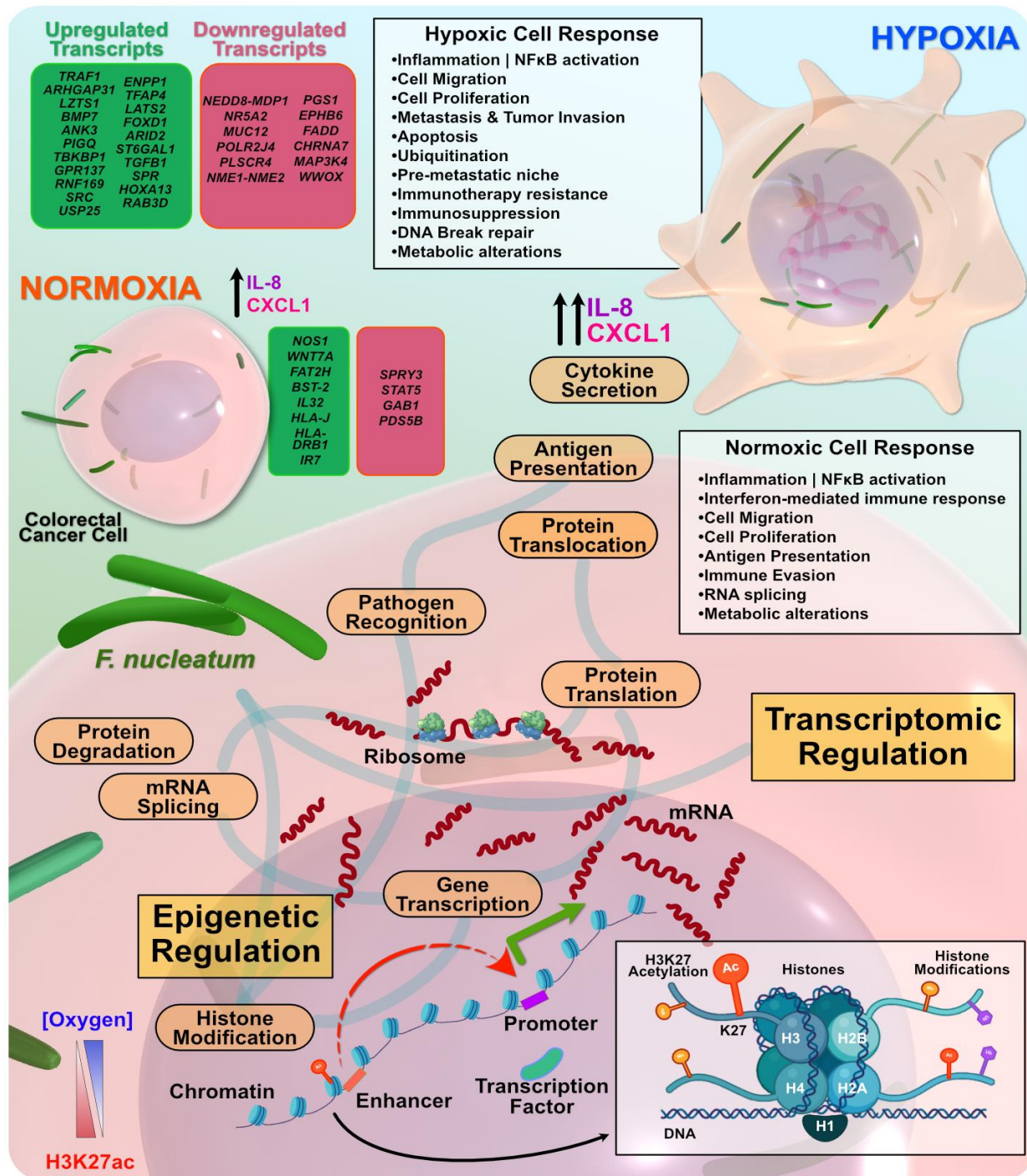


Figure 5.7 Schematic summarizing the oxygen-dependent epigenetic and transcriptomic response to *F. nucleatum* infection.

5.5 Materials and Methods

5.5.1 Culturing Bacteria

Fusobacterium nucleatum subsp. *nucleatum* ATCC23726 was grown as detailed previously³¹. Frozen cultures of bacteria were streaked on solid agar plates made with Columbia broth (Gibco) substituted with hemin (5 µg/mL) and menadione (0.5 µg/mL) (CBHK) and grown in an anaerobic chamber (90% N₂, 10% H₂, and 10% CO₂) at 37°C. Single colonies were then retrieved and inoculated in liquid cultures of CBHK media and grown for ~16 hours anaerobically to reach a mid-exponential growth phase. The optical density at 600 nm was measured to be 0.5 and the bacteria were resuspended in PBS and used for the experiments.

Escherichia coli TOP 10 was grown in Luria Broth (LB) in a shaker incubated at 37°C overnight. Bacterial culture was diluted to an optical density (600 nm) of 0.5 before being used for experiments.

5.5.2 Epithelial Cell Culture

HCT116 cells were purchased from ATCC (ATCC CCL-247) and grown in McCoy's 5A media supplemented with 10% FBS and 1% Penicillin and Streptomycin. Cells were grown in a normoxia incubator at 37 °C with 5% CO₂. Cells were passaged with gentle trypsinization (0.25% trypsin with EDTA) and re-seeding at 1:10 ratio. Cells used in the experiments did not exceed passage 12. Cells were re-seeded in 12-well plates or T25 flasks for experiments. For epigenomic and transcriptomic experiments, cells were first seeded at a 1:12 ratio in T-25 flasks and allowed to adhere overnight in normoxia. One set of cells continued to grow in normoxic conditions and another set was transferred to a hypoxic incubator for pre-treatment.

5.5.3 Hypoxia Incubator

The hypoxic environment was generated using a custom-built hypoxic chamber in the form of a modified incubator (ThermoFisher, 3578), an oxygen profiling microsensor (PreSens, PM-PSt7) connected to an oxygen meter (PreSens, OXY-1 ST), and a gas flow controller supplying 100% nitrogen

gas, controlled using a custom Python code and Arduino circuit. The chamber reduced oxygen levels to <1% within an hour of initiation and desired oxygen levels were maintained through the course of the experiment. PreSens oxygen sensor calibrations were routinely run per manufacturer's instructions. Details on the construction of the hypoxia incubator can be found at the following link:

Instructables: <https://www.instructables.com/Automated-Variable-Oxygen-Chamber/>

5.5.4 Flow Cytometry and Cell Sorting

Bacteria were first stained with FM 1-43FX lipophilic styryl dye (Invitrogen F35355) (5 µg/mL) for 5 minutes to stain the outer membrane of the bacteria. The stained cells were spun down at 1000g for 3 minutes, washed with media and resuspended in its original volume to be used for experiments.

Normoxic and hypoxic pre-treated epithelial cells were infected with stained Fnn at 50:1 multiplicity of infection (MOI, Bacteria:Epithelial) for 1 or 4 hours in their respective oxygen environments. Following infection, cells were washed twice with PBS, trypsinized, and collected for flow cytometry and cell sorting experiments. Cells were then loaded into a S3e flow cytometer (Bio-rad) and gated for single cells. 50,000 cells per sample were analyzed for green fluorescence due to intracellular *F. nucleatum*. Median fluorescence was determined using FlowJo10 before transferring data to GraphPad Prism for statistical analysis.

5.5.5 Immunofluorescence Imaging

Live immunofluorescence visualization was achieved by staining epithelial cells according to manufacturer's instructions with NucBlue (ThermoFisher Scientific 451 R37605) and MemBrite 568/580 (Biotium BTM30095) for the cell's nucleus and membrane respectively. *F. nucleatum* cell membrane was stained as previously described. Adhered epithelial cells were infected with *F. nucleatum* at 50:1 MOI for 4 hours. Z-stack images were then taken with an LSM800 confocal microscope (Zeiss) to confirm intracellular invasion of bacteria. To confirm persistence of

intracellular bacteria, z-stack images were again taken at 24 hours and 3D structures were recreated using Zen Blue.

5.5.6 Chemokine Quantitation

HCT116 cells were infected with *F. nucleatum* in normoxia and hypoxia after pre-treatment for 4 hours. The supernatant was collected and filtered using a Spin-X column to remove cell debris and bacteria. ELISA was used to quantify cytokines IL-8 and CXCL1 using Duo Kits (R&D Systems DY208, DY275). The ELISA plate was prepared by coating with Capture antibody overnight and was blocked with the reagent diluent the following day for 1 hour. Samples and standards were added to the plate and incubated at room temperature for 2 hours. The plate was then washed thrice with a Washing Buffer and the detection antibody was added. The plate was incubated for another 2 hours. The plate was again washed thrice and incubated with Streptavidin-HRP for 30 minutes. The plate was washed once again before adding the Substrate solution and the color change was monitored for 20 minutes. The reaction was stopped using the Stop solution and the absorbance at 450nm was collected using a spectrophotometer.

5.5.7 Chromatin preparation

~22,000 HCT116 cells were used for each ChIP-seq sample, which included two technical replicates (~10,000 cells per replicate) and one input assay (~2,000 cells). Cell culture medium was removed by centrifuging the cell suspension at 300g for 5 minutes at 4°C. Cells were washed twice with 1 mL cold PBS and suspended in 60 µL PBS supplemented with 0.6 µL PIC and 0.6 µL PMSF solution. 60 µL of 2X lysis buffer (4% (vol/vol) Triton X-100, 100 mM Tris-HCl [pH 8.0], 100 mM NaCl and 30 mM MgCl₂ in Milli-Q water) was added to the tube. The mixture was mixed well and incubated at room temperature for 10 minutes. 6 µL of 100 mM CaCl₂ solution and 12µL of 10 U/µL MNase (in PBS) was added to the tube and vortexed for 5 seconds. The mixture was incubated at room temperature for 10 minutes. 13.2 µL of 0.5 M EDTA was added to the tube to quench the MNase digestion. The mixture

was incubated on ice for 10 minutes, followed by centrifugation at 16,100g for 10 minutes at 4°C. Cellular debris was precipitated to the bottom of the tube, and ~120uL of supernatant containing the fragmented chromatin was collected for each ChIP-seq sample.

5.5.8 Beads preparation

~5 µL of protein A-coated Dynabeads (Life Technologies) were transferred to a microcentrifuge tube and washed twice with IP buffer (20 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100 in Milli-Q water). The beads were resuspended in 150 µL of IP buffer with 0.5 µg of H3K27ac (Active Motif Inc, cat: 39133) antibody, then rotated overnight at 4°C. The beads were washed with IP buffer three times and resuspended in 5 µL of IP buffer and placed on ice.

5.5.9 MOWChIP-seq

MNase-digested chromatin samples of ~10,000 cells per replicate were used for MOWChIP-seq to profile H3K27ac histone mark. The protocol of MOWChIP-seq has been detailed previously⁶⁰. Briefly, the sieve valve of the microfluidic device was first filled with water by setting the pressure to 30 psi for the solenoid valve. After opening the sieve valve by stopping the pressure of the solenoid valve, the chamber of device was filled with an IP buffer at a rate of 200 µL/min. The sieve valve was then closed, and the H3K27ac-coated beads were packed against the sieve valve. Extracted chromatin was then flowed through the packed bed at a rate of 1.5 µL/min. The beads then underwent oscillatory washing. ~60 µL of Low Salt washing buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 0.1% (wt/vol) SDS and 1% (vol/vol) Triton X-100 in Milli-Q water) was first used to wash the beads for 5 minutes at ~0.5 psi with opened sieve valve. This was followed with another wash with High Salt buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 2 mM EDTA, 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100 in Milli-Q water) for 5 minutes. Washed beads were retrieved in IP buffer. Finally, the ChIP DNA bound to the beads was collected by phenol-chloroform extraction. The Accel-NGS 2S Plus

DNA Library Kit (Swift-Bio) was used to prepare sequencing libraries. Libraries were sequenced with Illumina HiSeq 4000 platform. Single-end 100 nt reads were generated and trimmed to 50 nt to maintain consistency with previous data.

5.5.10 RNA-seq

Each RNA-seq library was constructed from 25,000 cells. Each experimental condition has two technical replicates. Total RNA was extracted using the RNeasy Mini Kit (74104, Qiagen) and RNase-Free DNase Set (79254, Qiagen). The extracted total RNA was suspended in 30 μ L RNase-free water and concentrated by ethanol precipitation. Total RNA was resuspended in 4.6 μ L of RNase-free water supplemented with 5% RNase inhibitor (40 U/ μ L). The SMART-seq2 protocol with minor alterations was then used for reverse transcription to produce cDNA. 2 μ L of oligo-dT primer (10 μ M), 2 μ L of dNTP mix (10 mM) and 4.3 μ L of total RNA solution were first mixed well. This mixture (8.3 μ L) was denatured at 72°C for 3 minutes and immediately stored on ice. Next, 11.7 μ L of reverse transcription mix (1 μ L of SuperScript II reverse transcriptase (200 U/ μ L), 0.5 μ L of RNase inhibitor (40 U/ μ L), 4 μ L of Superscript II first-strand buffer, 1 μ L of DTT (100 mM), 4 μ L of 5 M Betaine, 0.12 μ L of 1 M MgCl₂, 0.2 μ L of TSO (100 μ M), 0.88 μ L of nuclease-free water) was added to the 8.3 μ L mixture. The total reverse transcription reaction mix was incubated in a thermal cycler at 42°C for 90 minutes, followed by 10 cycles of 50°C for 2 minutes, 42°C for 2 minutes, and finally inactivated at 70°C for 15 minutes. The reverse transcription reaction mix was then mixed with 30 μ L of PCR mix (25 μ L KAPA HiFi HotStart ReadyMix, 0.5 μ L IS PCR primers (10 μ M), 2.5 μ L Evagreen dye, and 2 μ L nuclease-free water) and amplified with the program: 98°C for 1 minute, 9–11 cycles of 98 °C 15 seconds, 67 °C 30 seconds, 72 °C 6 minutes. Finally, the cDNA was purified using 40 μ L of SPRIselect beads and resuspended in ~7 μ L of low-EDTA TE buffer.

The RNA-seq library was made using Tn5-tagmentation. Briefly, P5/P7 transposon stock solution was first prepared by annealing 1.5 μ L 100 μ M single-strand P5/P7 transposon (IDT) with 1.5 μ L of 100 μ M pMENTS, a 5'-phosphorylated 19-bp mosaic end complementary oligonucleotide. The P5/P7 annealing mix was incubated at 95 °C for 5 minutes followed by a slow ramp to 25 °C at -0.1°C/sec and another 5 minutes at 25 °C. 3 μ L of Tn5 was mixed with 3 μ L of 50 μ M annealed P5/P7 transposon and incubated at 37°C for 1 hour. Assembled P5 and P7 transposons (6 μ L each) were mixed well to form 12 μ L assembled Tn5 and stored on ice. 8 μ L assembled Tn5 was mixed with 1 μ L cDNA (~600 pg) and 1 μ L of tagmentation buffer (TNP92110, Lucigen). The reaction mixture was incubated at 37 °C for 1 hour. 1 μ L of 10X stop buffer (TNP92110, Lucigen) was then added to the mixture to stop the tagmentation. ~12 μ L SPRIselect beads were used to purify tagmented cDNA and remove all primer dimmers. The cDNA was then resuspended in 10 μ L of low-EDTA TE buffer. 25 μ L of KAPA HiFi HotStart ReadyMix was activated by incubation at 98 °C for 30 seconds. The activated polymerase was mixed with 10 μ L tagmented cDNA and incubated at 72 °C for 5 minutes. 1.5 μ L of 25 μ M P5 primer, 1.5 μ L of 25 μ M P7 primer, 10 μ L nuclease-free water and 2.5 μ L Evagreen were added to form a 50 μ L amplification mix. The amplification mix was incubated with the following cycle: 98°C for 30 seconds; 10-12 cycles of 98°C for 10 seconds, 63°C for 30 seconds; and 72°C for 30 seconds. Amplified cDNA libraries were purified with ~40 μ L SPRIselect beads and resuspended in 7 μ L of low-EDTA TE buffer. RNA-seq libraries were sequenced with Illumina HiSeq 4000 platform. Single-end 100 nt reads were generated and trimmed to 50nt to keep consistent with our previous data.

5.5.11 ChIP-seq reads alignment and normalization

Sequencing reads were trimmed by Trim Galore! (Babraham Institute) with default settings. Trimmed reads were aligned to the hg38 genome with Bowtie2. Peaks were called using MACS2 ($q < 0.05$). Blacklisted regions in hg38 defined by ENCODE were removed to improve data quality. Mapped reads from ChIP and input samples were extended by 100 bp on either side (250bp total)

and a normalized signal was calculated using Eq. (1). The signal was calculated in 100bp windows over the entire genome and output as a bigwig file to be visualized in IGV (Broad Institute).

$$\text{Normalized Signal} = \left(\frac{\text{ChIP Signal}}{\text{Number of ChIP Reads}} - \frac{\text{Input Signal}}{\text{Number of Input Reads}} \right) \times 10^6 \quad (1)$$

5.5.12 Enhancer analysis

We predicted enhancer regions by identifying H3K27ac high regions that did not intersect with promoter regions (TSS $\pm$ 2000 bp). First, consensus H3K27ac peak sets were generated for each of the experimental conditions (NN, NE, NF, HN, HE, HF) with Diffbind. Peaks were expanded 500bp on each side around the summit, forming a 1000 bp long peak width. Any H3K27ac 1 kb regions that intersected with a promoter region was removed and the remaining regions were designated as enhancers. Differential enhancers between any two experimental conditions were identified using the bioconductor DESeq2 package (FDR < 0.05) with Benjamini-Hochberg method. Gene ontology analysis of differential enhancers was performed using GREAT with default settings.

5.5.13 RNA-seq Analysis

RNA-seq data was quantified using Salmon against the hg38 transcriptome using a full decoy. Differential gene expression analysis was performed using DESeq2, where genes with a fold change ≥ 2 and FDR < 0.05 were considered as significantly differentially expressed. Genes were analyzed for gene ontologies with clusterProfiler.

5.5.14 Statistical analysis

Statistical analysis was performed on GraphPad Prism 9 using t-tests and ANOVA followed by Dunnett's multiple comparisons test, with P-value significance denoted by ns (not significant) for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$. All samples for analysis were collected in independent triplicates. Plots were created on GraphPad Prism 9 and figures were designed on Affinity Designer.

5.6 Acknowledgements

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Supplementary Figures

Because NN was from the last batch, which was sequenced SE50bp, other samples were trimmed from 100bp to 50bp to stay consistent with NN. Furthermore, because some samples had much more reads than the others, we randomly subsampled the reads of samples with more than 25 million reads to 25 million. This can reduce bias when conducting analysis.

ChIP-seq Data Quality Control

Experimental group	Replicate	Mark	Total reads	Trimmed reads	Aligned reads	% duplicate reads	Peaks	FRip
NN	1	H3K27ac	13425098	12270795	8840811	70.9443	17833	7.50%
	2	H3K27ac	18071088	17329893	12641308	74.3666	22160	10.03%
NE	1	H3K27ac	25000000	24898398	17677948	28.8138	28590	16.99%
	2	H3K27ac	14974664	14919732	10605132	39.684	32849	19.35%
NF	1	H3K27ac	17655910	17592292	11305709	43.4656	18455	9.54%
	2	H3K27ac	25000000	24792337	15934219	42.898	19219	11.97%
HN	1	H3K27ac	17293043	17020373	9321476	62.063	37446	22.49%
	2	H3K27ac	23185060	22736499	13778059	50.8794	33606	15.72%
HE	1	H3K27ac	18319962	18210613	12641747	42.3342	44723	22.93%
	2	H3K27ac	25000000	24922168	17188659	35.3334	47621	23.55%
HF	1	H3K27ac	25000000	24624744	14807660	50.8853	34301	19.15%
	2	H3K27ac	19375219	18943094	11091715	53.3603	29804	17.94%

RNA-seq Data Quality Control

Experimental group	Replicate	Total reads	Aligned reads	% uniquely mapped reads
NN	1	9002005	8920009	76.51
	2	13399894	13282658	73.00
NE	1	14727006	14529738	56.10
	2	15474426	15254993	55.58
NF	1	13549539	13408193	63.61
	2	17936962	17761651	67.95
HN	1	13668761	13495696	46.18
	2	15085986	14883882	41.75
HE	1	14751367	14240998	53.18
	2	16620283	16008288	53.00
HF	1	14961090	14794994	33.66
	2	10913784	10783782	34.03

Table 5.1 Data Quality Controls of ChIPseq and RNAseq data.

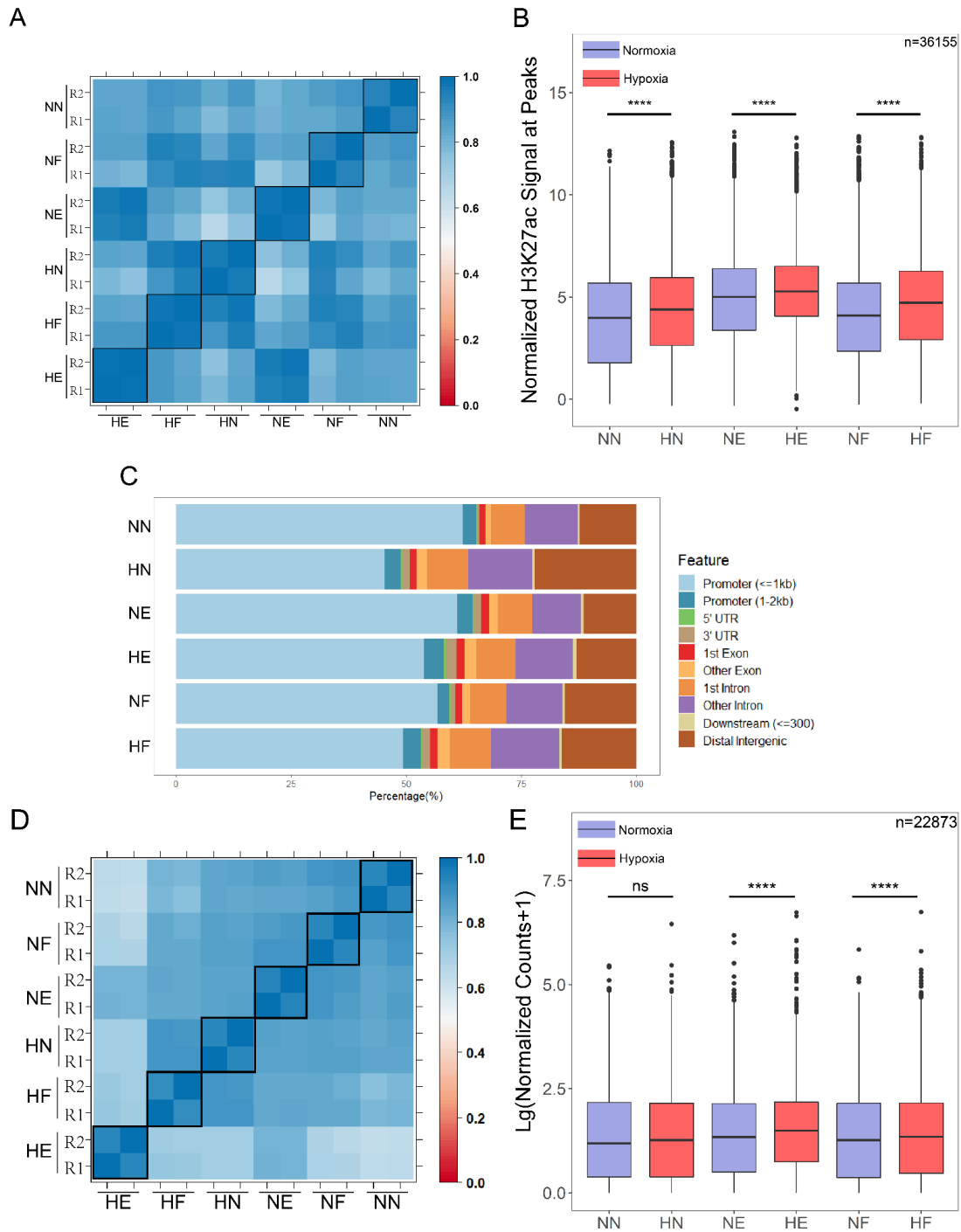


Figure 5.8 Global analysis of ChIPseq and RNAseq data.

(A) Pearson correlation of normalized H3K27ac signal around promoter regions (TSS +/- 2 kb). (B) Distribution of normalized H3K27ac signal at peaks. (C) Genomic locations of the H3K27ac peaks in normoxia and hypoxia samples. (D) Pearson correlation of normalized RNA-seq counts at genes (E) Boxplots of RNA-seq counts at genes

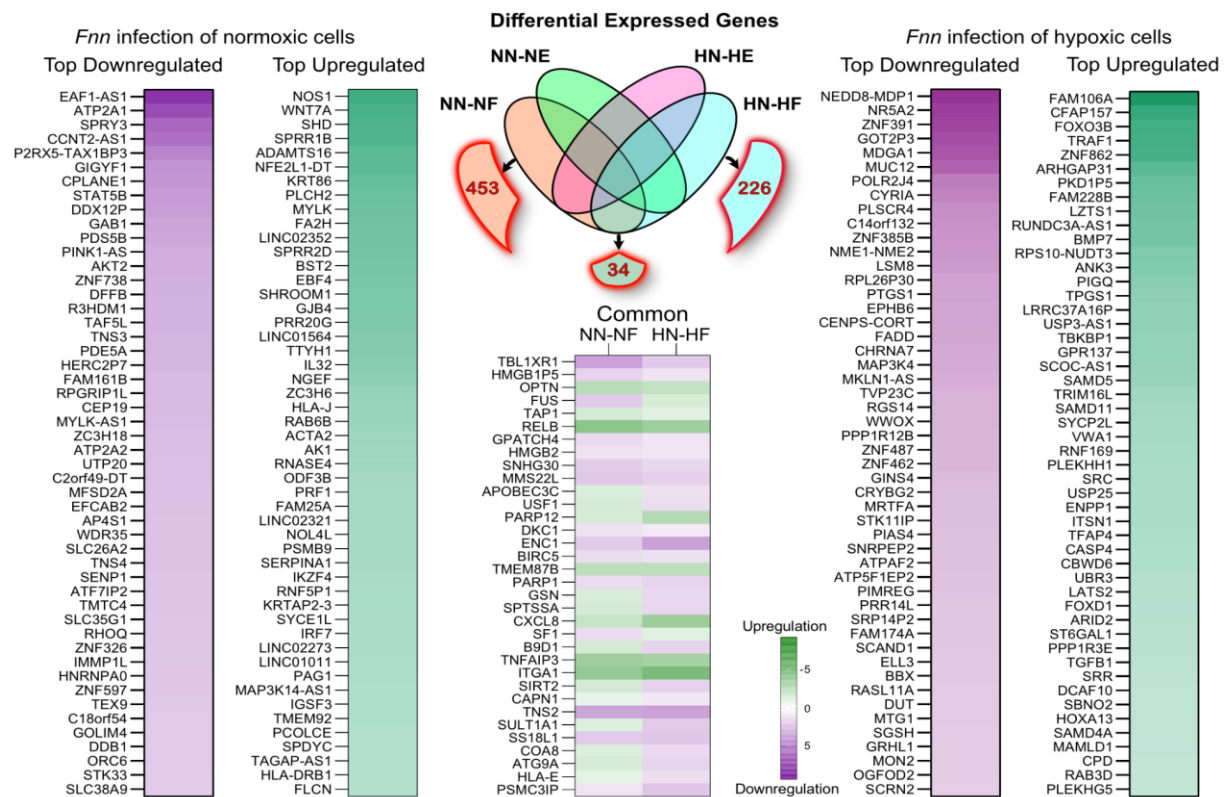


Figure 5.9 Differentially expressed genes in normoxic and hypoxic cells upon infection with *F. nucleatum*.

Top 50 upregulated and downregulated DEGs in normoxic and hypoxic cells infected with *Fnn* not including the expression changes induced by *E. coli*.

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6 Future Work

This chapter describes related studies that further explore the impact of *F. nucleatum* in a 3D culture format and in revealing bioelectrical signatures of infected cells.

6.1 3D Model Systems with Spheroids

6.1.1 Introduction

3D culture formats have significant advantages over 2D culture systems¹. Not only do cells in 3D exhibit altered signaling patterns, they respond differently to environmental stimuli and drugs. Effects observed in 2D conditions do not translate well in 3D conditions, which have a better ability to recapitulate the *in vivo* observations². A vast array of 3D models has been developed, from simple spheroid-based cultures to complex multi-organ microfluidic platforms³⁻⁶. There is limited data on *Fusobacterium* infection in 3D models such as spheroids. A simple spheroid model was published by Kasper et al. studied *F. nucleatum* infection of HT29 tumor spheroids⁷. In their study, the authors developed a co-culture model with *Fusobacterium* strains ATCC 23726 and ATCC 25586 and varying sizes of spheroids made of human colorectal adenocarcinoma cell line HT29. With a starting MOI of 1.8:1, they were able to consistently retrieve viable bacteria from with spheroids of 40,000 cells after 48-hour infections, but not from spheroids of 5,000 and 20,000 cells. Their model also indicated increased cytotoxicity to the tumor spheroids as their bacterial biofilms proliferated. Some of their more significant findings include differential regulation of cancer-related gene expression, and varying metabolite levels in the tumor spheroid microenvironment. They identified upregulation of selected gene enrichment pathways including colorectal cancer metastasis signaling, IL8 signaling, Tec kinase signaling, LPS-stimulated MAPK signaling, and host of pathways related to the molecular mechanisms of cancer. They also studied alterations in secreted metabolites. Using targeted triple quadruple LC-MS analysis, they showed a decrease in amino acids (histidine, tryptophan, glutamine, serine, methionine, threonine) over 24 hours. Moreover, using principal component analysis (PCA)

they further revealed that metabolite levels varied significantly between control (only spheroid) and co-culture with the strains 23726 and 25586 and their heat-killed counterparts. Their study emphasized the importance of studying *Fusobacterium* infection dynamics in a 3D context and further substantiates our hypothesis of bacterial localization within the hypoxic regions of the spheroid core.

We have shown that cell migration is increased in response to conditioned media from infected cells in CRC and PDAC cell lines^{8,9} in a 2.5D Transwell system. We wanted to see if this response could be recapitulated in a 3D spheroid cell culture system. This present study focused on investigating cell migration from BxPC3 and Panc1 spheroids.

6.1.2 Results

Spheroids were grown in a custom-designed 3D printed platform using the hanging-drop technique. This platform contained 4 wells cut out from PDMS attached to a coverslip to facilitate imaging (**Figure 6.1**). Images of spheroids were obtained at 0 hours and 24 hours for Panc1 spheroids (**Figure 6.2**), and BxPC3 spheroids (**Figure 6.3**). Qualitatively, infected spheroids did not exhibit increased cell migration in comparison to uninfected spheroids. However, conditioned media from both Panc1 and BxPC3 cells appeared to increase cell migration from both Panc1 and BxPC3 spheroids. Interestingly, 10ng/mL GM-CSF suppressed migration while 10ng/mL MIP-3 α stimulated migration. Quantitative analysis of BxPC3 spheroids revealed the same observations (**Figure 6.4**).

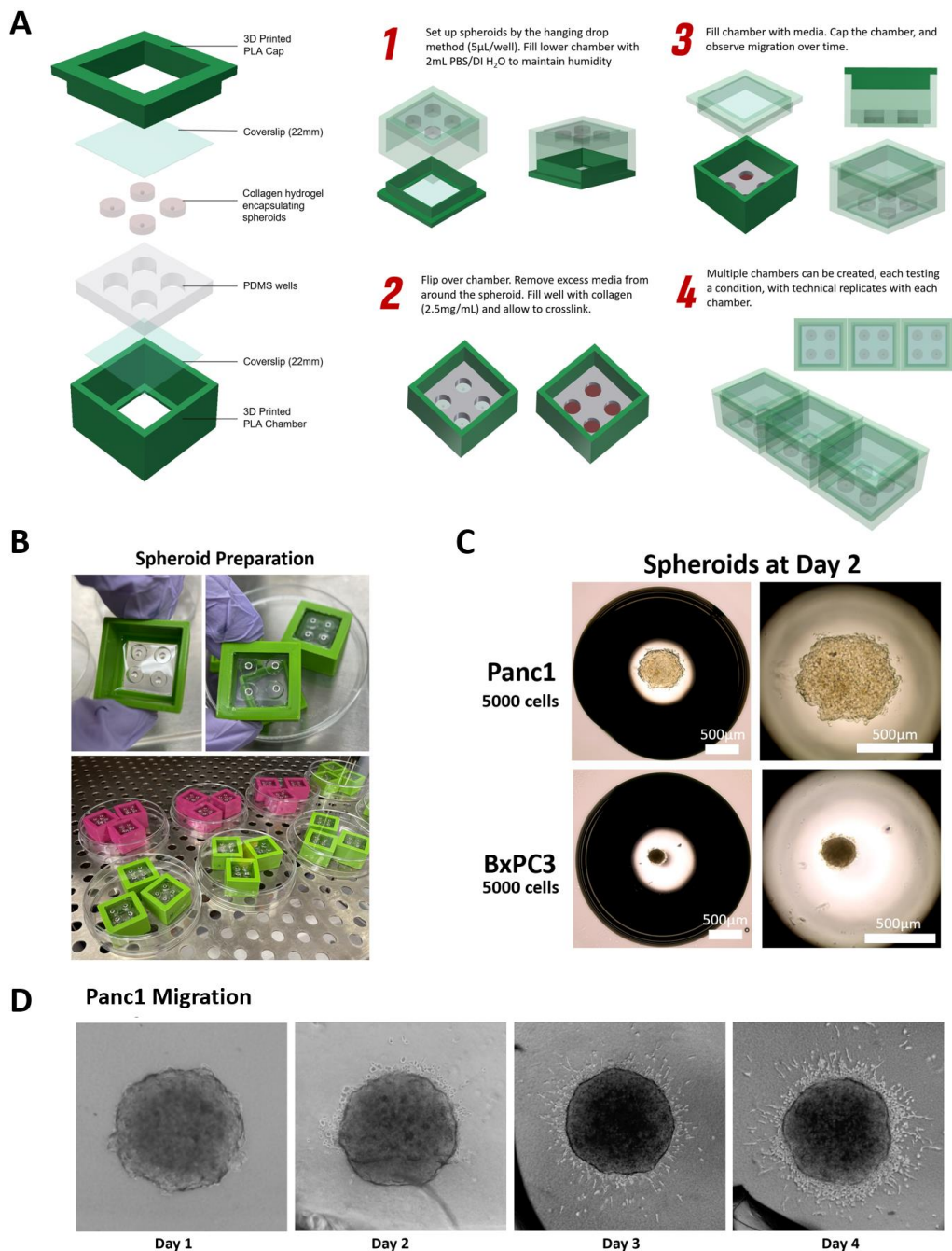


Figure 6.1 Panc1 and BxPC3 spheroids were prepared in custom designed cell culture chambers.

(A) Schematic of the spheroid culture platform consisting of a chamber, PDMS wells, coverslip and a lid. The steps to form the spheroids within each chamber is shown. (B) Photographs of the spheroid droplet at Day 1. (C) Images of spheroid formation at Day 2 (D) Observation of Panc1 cell migration over 4 days. Scale bar 500µm.

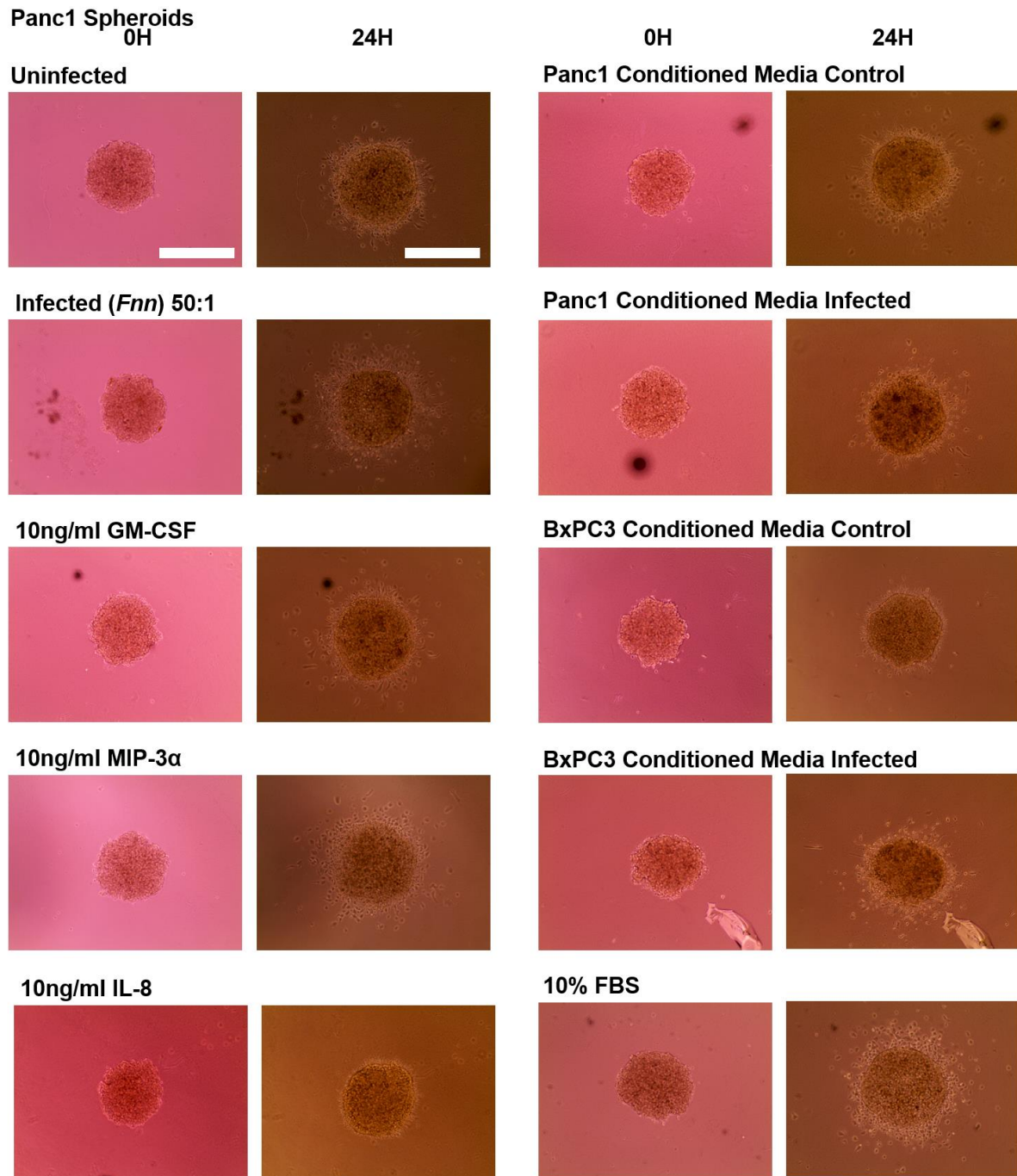


Figure 6.2 Cell migration out of Panc1 spheroids

5000 cell Panc1 spheroids were grown and imaged at 0 hours and 24 hours after infection, or treatment with cytokines or conditioned media from uninfected cells (control) and infected cells of both Panc1 and BxPC3 cells, in DMEM media. All media used was supplemented with 0.5% FBS. Scale bar 500 μ m.

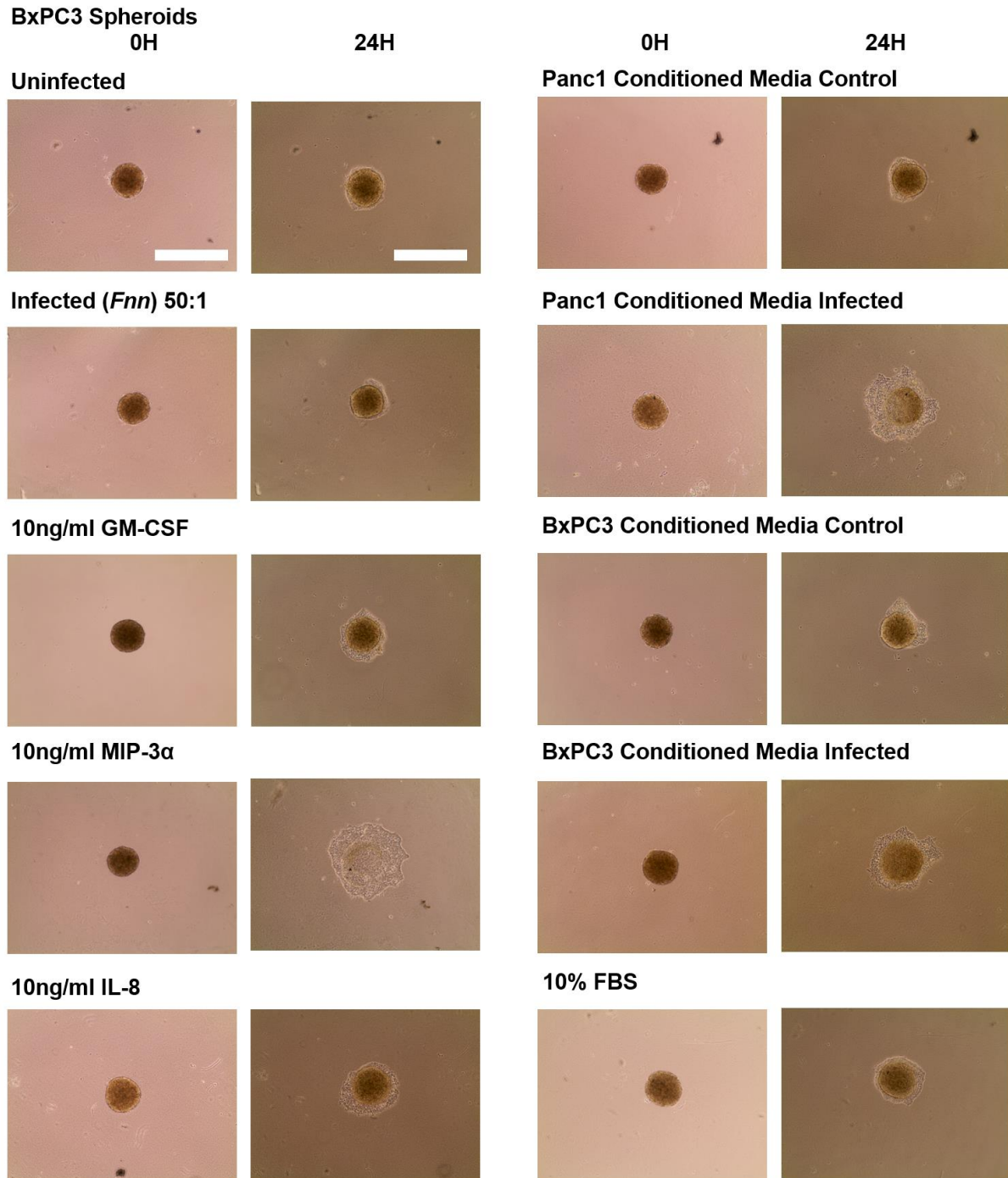


Figure 6.3 Cell migration out of BxPC3 spheroids

5000 cell BxPC3 spheroids were grown and imaged at 0 hours and 24 hours after infection, or treatment with cytokines or conditioned media from uninfected cells (control) and infected cells of both Panc1 and BxPC3 cells, in BxPC3 media. All media used was supplemented with 0.5% FBS. Scale bar 500 μ m.

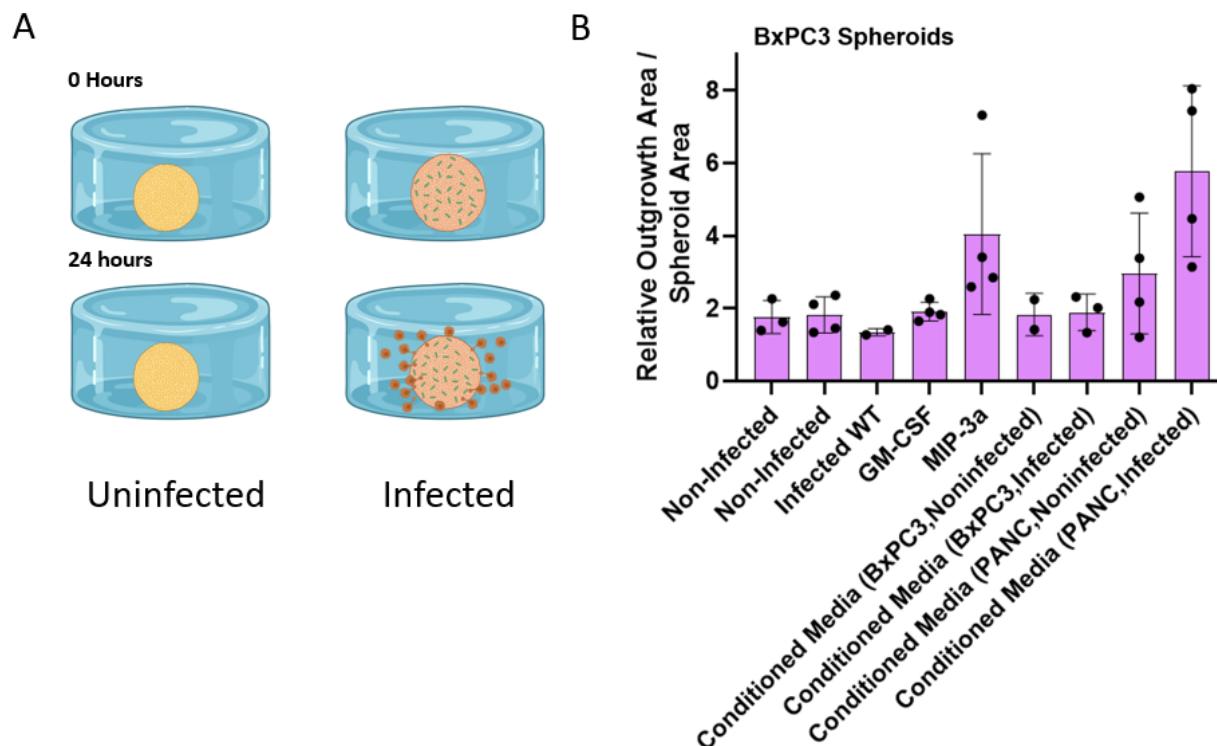


Figure 6.4 Quantification of cell migration out of BxPC3 spheroids

(A) Schematic of outward migrating cells from an embedded spheroid (B) Quantifying relative cell migration from BxPC3 spheroids calculated by dividing outgrowth area/area of spheroid.

6.1.3 Discussion

Panc1 and BxPC3 spheroids of 5000 cells were consistently grown in custom-designed spheroid chambers and cell migration was observed upon treatment with cytokines and conditioned media from infected cells. Interestingly, while infected cells show increased cell migration in a Transwell system, they do not increase migration in these spheroids. There could be general limitations with respect to the effective MOI of infection, bacterial viability, and size of the spheroids which require further optimization. These preliminary studies demonstrate the feasibility of this 3D printed chamber in quantifying cell migration. It is important to note that spheroids exhibit variable cytokine profiles in comparison to 2D models and furthermore show differential gene expression in the

varying culture formats. In addition, hypoxia, as found within tumors, can be recapitulated effectively in spheroid cultures and thus host-microbial studies can be more relevant in a 3D context.

6.1.4 Materials and Methods

6.1.4.1 Culturing *Fusobacterium nucleatum*

Fusobacterium nucleatum subsp. nucleatum ATCC 23726 is grown in CBHK media. Bacteria are first cultured on solid agar plates made with Columbia Broth (Gibco) substituted with hemin (5 µg/mL) and menadione (0.5 µg/mL) (CBHK) under anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) at 37 °C. Liquid cultures started from single colonies were grown in CBHK media under the same conditions. Colonies are retrieved from the plate and inoculated in a closed glass inoculation tube. It is incubated overnight at 37. The OD is checked the next day and the bacteria is inoculated into a fresh tube at 0.1 OD. Within 4 hours, the bacteria grow to ~0.5 OD indicating the mid-exponential growth phase of the culture. The O.D. is again checked to make sure it's within the required 0.4-0.6 range. 1 mL of the bacterial cell suspension is collected and spun down at 1000g for 3 minutes. Bacteria can be stained in advance if required with FM-143-FX (500µg/mL) for 5 minutes. The cells are spun down again after staining and washed once with media, spun down, and resuspended in 1 mL of media.

6.1.4.2 Epithelial CRC cells

BxPC3 and Panc1 cells were purchased from ATCC and grown on tissue culture treated plates and flasks in RPMI 1640 supplemented with 10% FBS, and DMEM supplemented with 10% FBS, respectively. Cells were grown to no more than passage 15 at 37 °C with 5% CO₂. For maintenance between infections, cells were passaged by gentle trypsinization and reseeding. For infection experiments, cells were grown to >90% confluency in 6- or 24-well plates.

6.1.4.3 Spheroid Culture Platform

Spheroid hanging drop chambers with lids was modeled on Autodesk Inventor and 3D printed using PLA. The chamber contained enough space to fill 2 mL of media, and the lid was designed to hold 2 mL of PBS. A coverslip was attached using PDMS to the bottom of the chamber to enable microscopy. Another coverslip was attached to the lid. The chamber was filled with PDMS to a height of 4mm and was allowed to crosslink. Then, four 6mm diameter wells were punched out using a biopsy punch to making sure to not break the coverslip. The chamber was tested for leaks using PBS and sterilized by immersion in 70% Ethanol for 15 minutes followed by drying and UV treatment for 4 hours.

6.1.4.4 Spheroid Development

Methylcellulose is an inert and non-toxic compound that was used to enhance cell-cell adhesion which is critical for spheroid formation. Methylcellulose is prepared according to¹⁰. 50mL of ultrapure water is heated to 80°C and 10g of methyl cellulose (Viscosity 15cP Sigma Aldrich M7027) is added to it. The solution is agitated until the particles were evenly dispersed. The solution was brought to a final volume of 100mL using cold ultrapure water and was stirred at 4°C until the solution became clear, and straw colored, with no visible solids. The solution was filtered through a 0.45µm filter to remove undissolved solids.

The spheroid culture medium was prepared with cell culture media containing 10% FBS and 1-5mg/mL of methylcellulose. Cells were first trypsinized and counted and resuspended to appropriated cell concentrations. To form spheroids of 5000 cells, 5µL of the cell suspension (100,000 cells/mL) was placed as individual droplets in the center of each well of the custom-printed chamber making sure the droplet did not touch the sides of the well. The chamber was flipped over gently to not disturb the droplets. The lid was filled with 2 mL PBS to maintain humidity during spheroid formation. The chamber was placed in a Petri dish and incubated at 37 °C with 5% CO₂. Spheroids take 2-3 days to form and are monitored each day under a DIC microscope. Optimal

conditions to form BxPC3 spheroids are 5mg/mL methylcellulose and 5% serum, incubated for 3-5 days. Optimal conditions to form Panc1 spheroids are 5mg/mL methylcellulose and 5% serum, incubated for 5-7 days.

Once the spheroids had formed, the lid was removed and the PBS was aspirated. The chamber was flipped over and excess media around the spheroid was carefully removed using a pipette. Next 2.5mg/mL collagen was added over the spheroid and the spheroid was centered in the collagen. 600 μ L of 5mg/mL collagen stock was first diluted with 120 μ L 10X DMEM, 450.8 μ L of 1X DMEM, and ~20 μ L of NaOH on ice. NaOH was added in small increments to make sure the pH did not exceed 7.4. After thorough mixing, 28.27 μ L collagen was added to each well containing the spheroid. This volume was calculated for a 1mm height of collagen within the 6 mm diameter well. The collagen was allowed to crosslink for 20 minutes at 37 °C with 5% CO₂. After crosslinking, the chamber was filled with media, media containing cytokines, or conditioned media as required. The migration of cells out of the spheroid was monitored over 24 hours.

6.1.4.5 Collagen Preparation

Type I collagen stock was prepared as published previously¹¹. Briefly, rat tendons were excised from Sprague Dawley rat tails. The tendons were sterilized in a beaker of 70% ethanol. Next, the tendons were dissolved in 200mL of 0.1% acetic acid/gram of tendon and refrigerated at 4°C for 48 hours with continuous magnetic stirring. Next, the solution was centrifuged for 45 minutes at 30,000g at 4°C in an Avanti J-25 centrifuge (Beckman Coulter, Brea, CA). The pelleted debris was discarded and the supernatant was lyophilized to form thin collagen fibers which were stored at -20°C. For use, the lyophilized collagen was weighed and dissolved in 0.1% acetic acid and vortexed and stored in 4°C for 48 hours for complete dissolution.

6.2 Dielectrophoresis of Infected Cells

Dielectrophoresis studies were performed in collaboration with Josie Duncan and Dr. Rafael Davalos.

6.2.1 Introduction

High-frequency dielectrophoresis (DEP) is a cell separation technique based the bioelectric properties of the cell within a non-uniform electric field. Differences in plasma membrane composition and permeability modulates the intracellular conductivity of the cell, which in turn influences the force experienced by the cells when subjected to a non-uniform electric field. The electric field gradient polarizes the cell based on cell composition and surrounding media causing them to move^{12,13}. Contactless dielectrophoresis (cDEP) developed in the Davalos Lab can harness this property to characterize the bioelectric signature of cells and exploit it for cell separation. The cDEP chip is a multi-layer microfluidic device that consist of a cell chamber positioned under an electrode layer separated by a thin layer or polydimethylsiloxane (PDMS). The chamber further consists of multiple posts to trap cells. Voltage is applied through the electrode layer which is filled with 10X concentrated phosphate buffered saline. Consequently, the cells within the chamber only experience the electric field and are not impacted by the buffer constitution. Cells are flowed through the cDEP chip at a constant flow rate and the cells are subjected to varying high frequency fields. The differences in bioelectric properties of the cells can be extracted by balancing the DEP force with the drag force within the chip (**Figure 6.5**).

Cells become polarized upon applying an AC electric field across the chip. A factor called the Clausius-Mossotti factor defines the direction of the perceived force of the cells as they are pushed parallel or antiparallel to the electric field gradient. The Clausius-Mossotti factor is defined by the following equation:

$$K(\omega) = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*}$$

where,

ϵ_p^* is the complex permittivity of the particle or cell

ϵ_m^* is the complex permittivity of the medium.

These terms are further represented by

$$\epsilon_X^* = \epsilon - i\sigma/\omega.$$

where,

ϵ is the permittivity

σ is the conductivity

ω is the angular frequency

The DEP equation for a dipole is then written as:

$$\vec{F}_{DEP} = 2\pi r^3 \epsilon_m \text{Re}\{K(\omega)\} \nabla |\vec{E}_{rms}|^2$$

where,

r is the cell radius

ϵ_m is the conductivity of the medium

$\text{Re}\{K(\omega)\}$ is the real part of the Clausius-Mossotti factor as a function of angular frequency (ω)

$\vec{E}_{rms}$ is the root mean squared of the electric field

We can then separate the DEP equation into two components: $\vec{F}_{DEP}$ as a function of only cellular properties and $r^3 \epsilon_m \text{Re}\{K(\omega)\}$ as a function of medium composition.

Dielectrophoretic techniques have previously been used to distinguish between bacterial types¹⁴ and quantify mitochondrial dynamics within cells¹⁵.

Intracellular localization of multiple bacteria within cells can modify the conductivity of the cells, and thus alter the observed bioelectric signature of the cell within cDEP. This would enable efficient separation of infected cells from uninfected cells from a mixed population. In order to achieve this, we sought to examine the differences in bioelectric signature due to *F. nucleatum* infection in PDAC cancer cell lines.

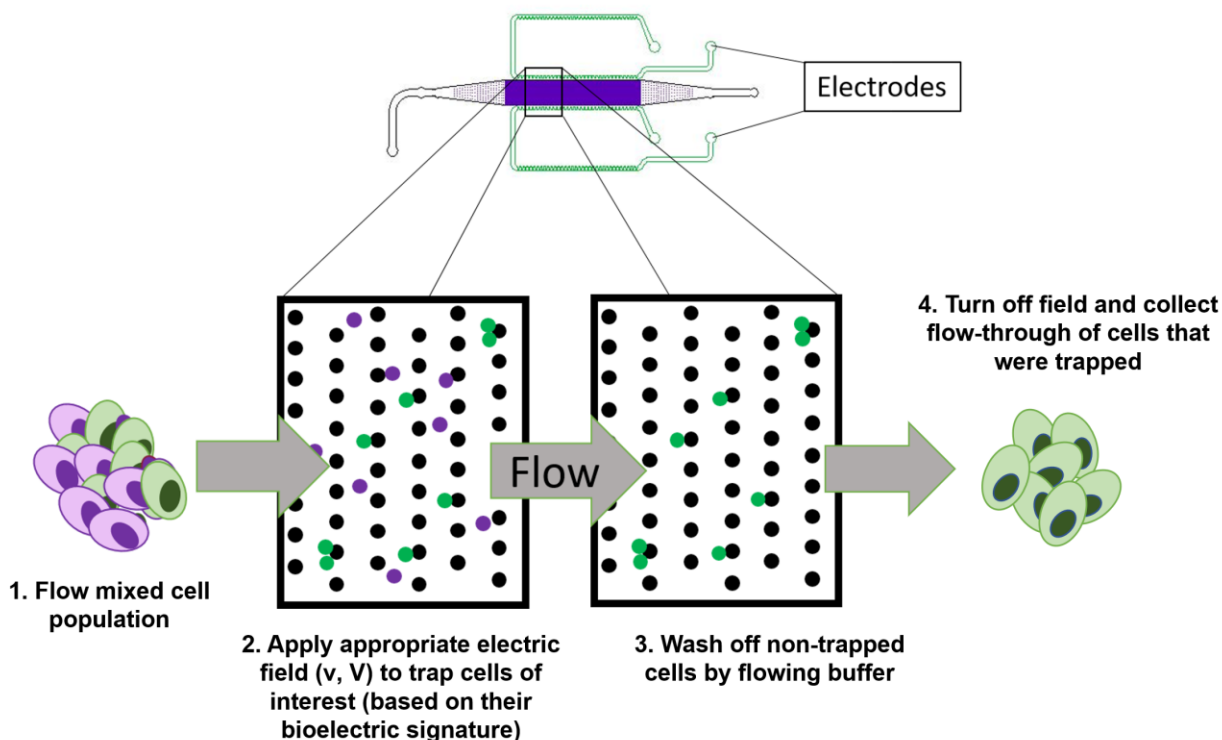


Figure 6.5 Schematic of the cDEP chip.

A mixed cell population consisting of uninfected and infected cells stained red and green is flowed through the cDEP chip. Electrodes connected to the chip enable the application of an electric field at different frequencies. Cells that respond to specific frequencies can trap when the field is on and is released when the field is off. These cells can be collected for downstream analysis.

6.2.2 Results

Computational modeling was performed using the myDEP software. Using the two-shell model of DEP, the CM over a frequency sweep revealed differences at the kHz and MHz range (**Figure 6.6**). Thus, we selected the 0-100kHz range to perform our initial trials to identify if we observe differences between infected and uninfected cells. We infected BxPC3 and Panc1 cells with *F. nucleatum* at 50:1 MOI for 4 hours and mixed them with their respective uninfected cells. We then studied their behavior in the cDEP chip at 3 flow rates (1, 2, 4 $\mu\text{L}/\text{min}$) across a range of frequencies (0-100kHz) (**Figure 6.7**).

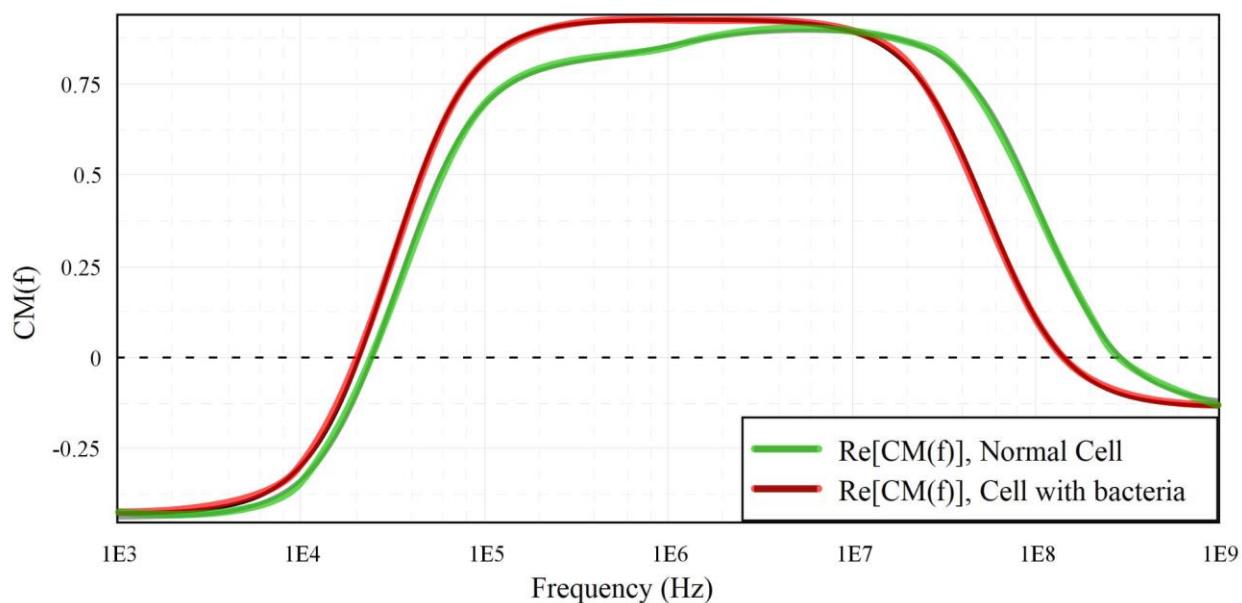


Figure 6.6 Modeling CM at different frequencies using myDEP

The Clausius-Mossotti factor defines the direction of a polarized cell in a non-uniform electric field. A frequency sweep in the myDEP software containing parameters for a normal cell and a cell with bacteria indicates shifts in the CM factor at particular frequencies which can be exploited for cell separation.

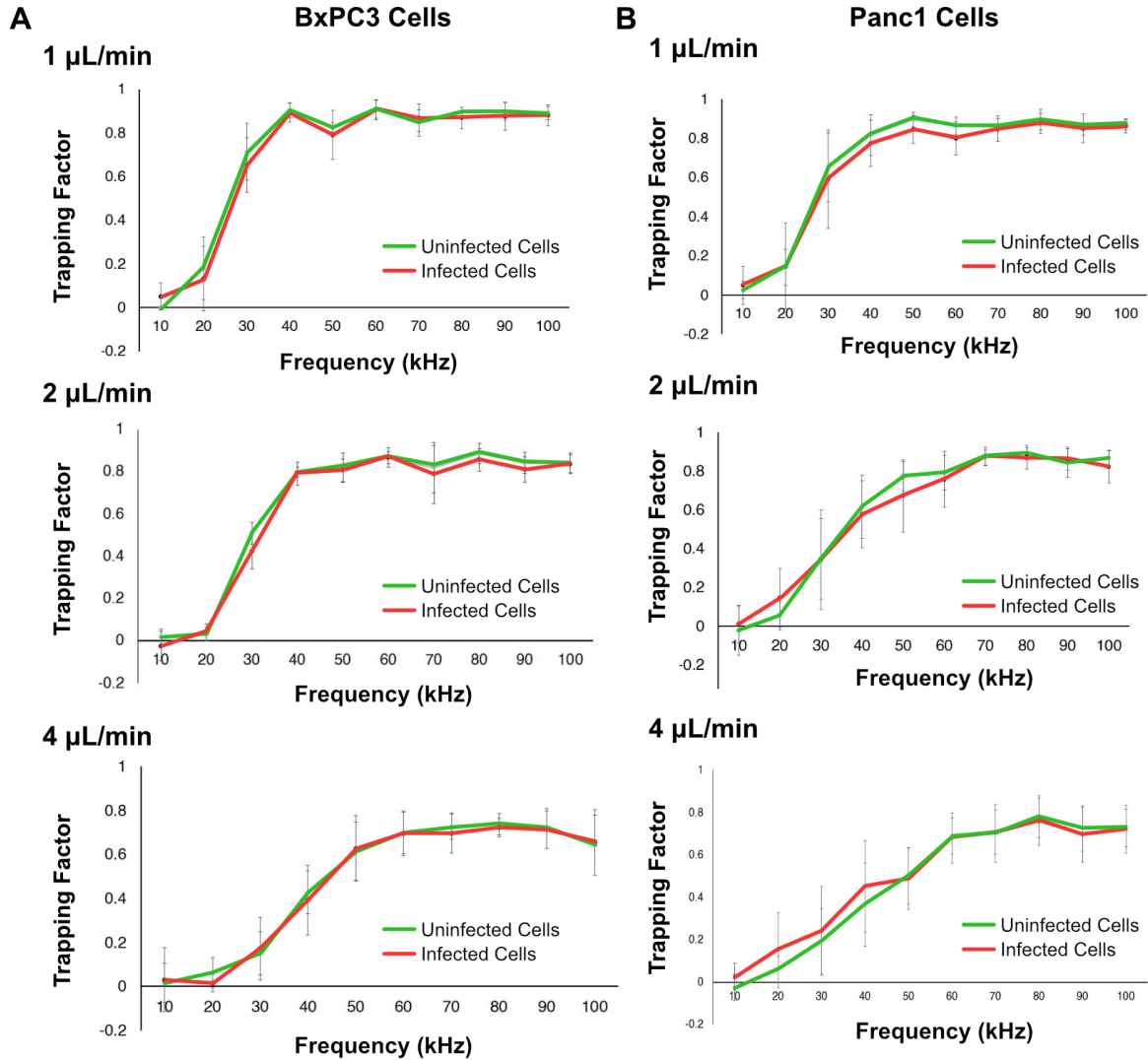


Figure 6.7 Trapping Factor vs Frequency plot for uninfected and infected BxPC3 and Panc1 cells.

Plots of trapping factor vs frequency for 3 different flow rates (1, 2, and 4 $\mu\text{L}/\text{min}$) are shown for (A) BxPC3 cells and (B) Panc1 cells. Between the 10-100kHz range, the trapping factors appear to overlap, and thus higher frequencies need to be explored. (n=3 independent experiments).

6.2.3 Methods

6.2.3.1 DEP Chip Design and Construction

The DEP chip is a multi-layer microfluidic device that is constructed using PDMS (polydimethylsiloxane). Its basic construction incorporates a central channel with an inlet and outlet

aligned over a fluidic electrode channel. These two layers are separated from each other by a plasma bonded 14 μm PDMS membrane that was created by being spun at 4000rpm on a silanized silicon wafer (University Wafers). Multiple 20 μm diameter insulating posts are found within the central channel. Non-uniform electric field gradients are formed around these posts when voltage is applied across the chip. Wires from the signal generator (Agilent 33500B Series) and amplifier (Trek Model 2205/ Tektronix) are connected to the DEP chip with alligator clips to wire leads in contact with a conductive liquid medium, 10X phosphate buffered saline (PBS). Pipette tips are connected to either end of the fluidic electrode and filled with 10X PBS. The generator is also connected to an oscilloscope for monitoring.

6.2.3.2 Cell Collection

Pancreatic cancer cell lines (BxPC3 and Panc1 (ATCC)) were passaged into two flasks and grown overnight. One flask was infected with *F. nucleatum* as previously described at 50:1 ratio for 4 hours. The other flask was kept sterile. The cells were then trypsinized and spun down at 135g for 6 mins. Infected cells and uninfected cells were stained Calcein Green or Calcein Red-Orange respectively for 15mins. The cells were then transferred into a low conductivity DEP buffer containing an isotonic solution of 8.5% sucrose (w/v) (Sigma), 0.3% glucose (w/v) (Wako), and 0.725% RPMI (v/v) (ATCC). This is performed by spinning the cells down three times 10mL of DEP buffer. The cells are then counted and resuspended to 2 million/mL. The conductivity of this solution was measured using a handheld conductivity probe (Horiba). Next, equal volumes of infected and uninfected cells are mixed before the experiment.

6.2.3.3 DEP Experiment and Analysis

The mixed cell solution is collected in a syringe which is then connected to a syringe pump that maintains constant low flow rates within the DEP Chip. A separate syringe was filled with DEP buffer and connected to another syringe pump which is used for washing. Flow rates of 1 $\mu\text{L}/\text{min}$, 2 $\mu\text{L}/\text{min}$,

and 4uL/min are used. Frequencies between 10 and 100kHz in 10 kHz increments were cycled through randomly. The field was toggled on and off three times for each frequency leaving ample time between toggles for cell trapping and release. If necessary, manual washes of DEP buffer are performed between frequencies to remove cells that have adhered to the electrodes. The entire experiment was recorded using OBS Studio (). The recorded experiment was then processed in VLC and analyzed using a custom developed ImageJ macro. The macro distinguishes stationary and moving cells in each frame. These results were then used to determine the reversible trapping factor i.e., percentage of cell released from the electrodes when the field was off.

6.2.4 Discussion

While there were no observable differences experimentally, the larger deviations at frequencies below 30kHz indicate the frequency range used in the experiments could encapsulate the first crossover frequency of each cell type. It is possible that these cell subtypes are distinguishable between 20-30kHz using dielectrophoresis as the force experienced by the cell is moving from positive to negative DEP. The sensitivity of the device, however, should be optimized by considering design alternatives or highly-controlled flow to maintain adequate balance of the drag force and extremely low DEP forces. Furthermore, prior cell sorting of infected cells to obtain a highly infected population could help increase the sensitivity of the system.

6.3 Limitations

To study host-microbial interaction, we amplify their effects in a controlled environment, which in many cases, though they seek to be physiologically relevant, are far from it. Studies in spheroids and organoids and microfluidic devices can supplement this area. Animal models such as murine models do not accurately recapitulate human immune modulation and may lack key signaling molecules. There are some inherent limitations of the proposed projects including the study of a single species of bacterium in an isolated environment. Another limitation is a lack of immune cells which play a major role in signaling and adding evolutionary pressure for strain selection and intracellular localization to evade capture. However, this work was designed as foundational studies to interrogate specific host-microbiota interactions and future studies are expected to expand on this research.

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7 Conclusions

The research presented in this dissertation adds to a rapidly growing body of literature on the profound role that microbes can play in cancer. Decades of cancer research have consistently overlooked the role of microbial inhabitants. In retrospect, however, it is clear that several observations noted throughout history have suggested their potential involvement. The tumor microbiome is now considered a hallmark of cancer^{1,2}. Its appreciation has been greatly fueled by substantial developments in next-generation sequencing and bioinformatics over the past decade that uncovered population-based statistics on microbial compositions. Advanced *in vivo* and *in vitro* models further validated and revealed mechanistic insights into the role of individual bacteria in the tumor microenvironment (TME). We now have a better understanding of how tumor-associated microbes and their metabolites can modulate some, if not all, of the expanded hallmarks of cancer. These include potentiating genomic instability, sustaining proliferative signaling, evading growth suppressors, deregulating cell metabolism, regulating immunomodulation, evading immune recognition, promoting angiogenesis, amplifying inflammation, activating metastatic programs, unlocking phenotypic plasticity, and reprogramming non-mutational epigenetics.

My research specifically illuminated the role of a single microbe in simultaneously influencing several of the aforementioned hallmarks. We discovered that *F. nucleatum* binding and invasion induces the selective secretion the cytokines, IL-8 and CXCL, in colorectal cancer (CRC) that can drive cell migration by activating metastatic pathways within the host cell. Both these cytokines have been previously implicated in pre-metastatic niche formation and in the recruitment of granulocytic myeloid cells that function to suppress natural killer cells, thereby avoiding immune destruction. Since the publication of that initial study, research in other labs have validated our findings^{3,4}. Using chromatin immunoprecipitation (ChIP), we revealed how non-mutational epigenetic reprogramming can activate dormant cancer-related pathways. Complementing ChIPseq, RNAseq studies further

revealed the tremendous phenotypic plasticity of these cancer cells in upregulating and downregulating genes associated with host defense and cancer progression, based on the oxygen environment surrounding tumor cells. The differences observed between hypoxic and normoxic cells encourages further investigations on how the TME and host cell phenotypic plasticity can impact infection dynamics and concomitant host responses. My work on exploring *F. nucleatum* infection of pancreatic ductal adenocarcinoma (PDAC), uncovered the role of additional cytokines, GM-CSF and MIP-3 α , in regulating host cell response to infection. We revealed a new role for GM-CSF in directly inducing the proliferation of pancreatic cancer cells while not impacting the proliferation of normal pancreatic epithelial cells. These findings are indicative of well-regulated pathways within normal cells that have been perturbed in cancer cells. These observations motivate future studies on the contribution of the infection of normal cell types including stromal, epithelial, and immune cells within the TME to tumor progression.

Together, these findings paint a multifarious picture of *F. nucleatum* in cancer (**Figure 7.1**). Infection can directly stimulate cancer cell migration and proliferation through paracrine and autocrine signaling. The signaling pathways we had identified have been implicated in immunosuppression and immune evasion of the host cancer cell. Furthermore, we have shown that microbial binding, invasion, and response is intrinsically tied to host cell biology, which includes the expression of cell surface receptors and modulation of the host glycocalyx. The external tumor microenvironment, specifically hypoxia, can modulate host cell phenotype and genotype which, in turn, determines the observed response to *F. nucleatum* invasion. The unique feature of intracellular localization of *F. nucleatum* may be remnant of adaptations that originated from co-evolutionary relationships with the host over millennia. To confirm these theories, future studies will require a nuanced understanding of cancer-host microbial evolution. The survival advantages conferred by intracellular localization of *F. nucleatum* is manifold. Intracellular invasion aids evasion from immune detection, and the TME creates a favorable hypoxic niche that allows anaerobic *F. nucleatum* to thrive. In

addition, the TME provides an abundant source of nutrients for microbial growth in the form of amino acids and proteins. There are many fascinating questions that remain to be explored. Is there any benefit to the host cell to continue to harbor this bacterium? It is still unclear if infection is an active or passive process. Is infection a common enemy of both the cancer and normal cells, whereby host cells secrete cytokines to recruit immune cells in order to rid the tumor of infection?

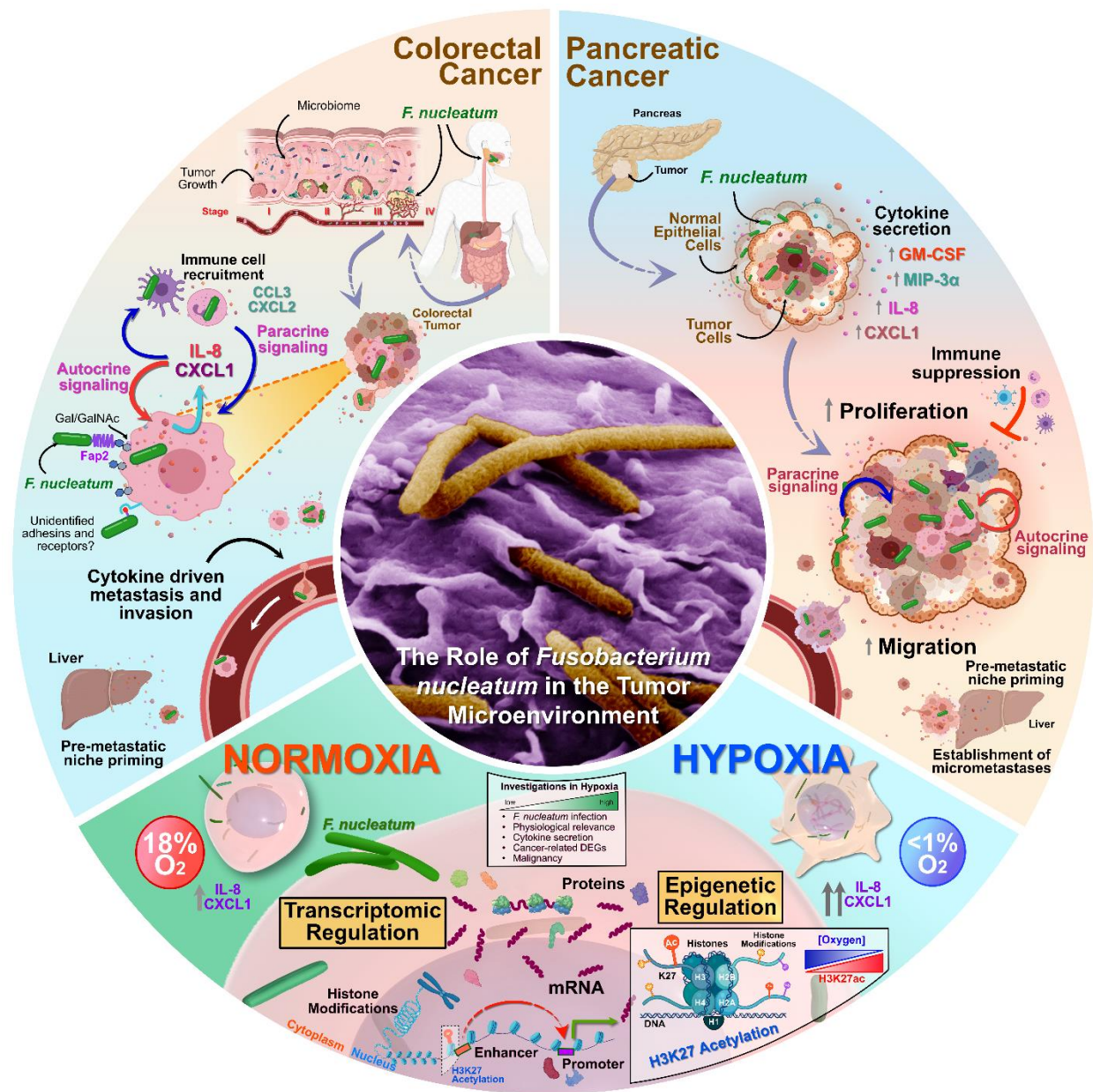


Figure 7.1 Summary of findings described within this dissertation

There are several species of intratumoral bacteria whose roles are currently unknown. Nejman et al. had revealed multiple distinct species of microbes within tumors⁵. Their behavior within the tumor can either complement or antagonize one another. Their combined influence may reveal additional features impacting cancer progression. The presence of these microbes within tumors exerts a significant effect on patient prognosis despite their relatively low abundance within tumors⁶. However, further validation of their abundance and distribution is required from clinical contexts through higher resolution imaging, incorporating tissue clearing techniques and advanced microscopy techniques, especially in 3D models and organ-on-chips. Many of the causative roles are yet to be identified, but nevertheless, we have sufficient evidence to ascertain that these bacteria are complicit in enhancing tumor progression. In addition to the microbiome, the mycobiome⁷⁻⁹, the virome and associated bacteriophages^{10,11} have also been identified to participate in tumor progression. The metabolic crosstalk between these multiple species and with the host cells within tumors needs to be investigated.

Progress in studies of the tumor microbiome will illuminate fundamental mechanisms underpinning tumorigenesis and progression. Knowledge of these mechanisms can then aid the development of diagnostic and prognostic tools to detect and eliminate cancer early. DNA and RNA derived from microbes that are highly specific to tumors based on the stage of cancer can be used as potential biomarkers¹². Compositional alterations within the gut microbiome (dysbiosis) can signal the development of cancer. Additionally, metabolic alterations can be extracted and cancer associated markers found in tumor biopsies, stool, saliva, blood samples can facilitate early detection of cancer. This research can additionally complement preventative therapy. Information on the particular microbial composition of the individual can be used to implement dietary modifications to ensure host-microbial eubiosis¹³. Since microbes are known to impact chemotherapy¹⁴, antibiotic treatment can supplement chemo-treatment to increase effectiveness. New forms of therapy such as Fecal

Microbial Transplants (FMTs) are already gaining traction in resolving dysbiosis arising due to *Clostridium difficile* infection¹⁵.

To complement these studies, advancements in physiologically relevant tumor models are required. Updates to gut-on-a-chip and other organ-on-chip devices may help create more accurate representations of biophysical conditions within the TME¹⁶. These chips can incorporate spheroids and organoids in their construction to examine interactions in 3D contexts. Furthermore, immune cells can be incorporated in these systems as they play a crucial role in inflammation. Innervation within tumors is also a less studied field where the impact of microbes needs to be determined¹⁷. Understanding cell signaling and communication may provide future insights into immunotherapy and treatments targeting these signaling cascades. Previous studies have revealed microbial peptides being presented by melanoma cells¹⁸, which raise the possibility of harnessing bacteria to activate in immune recognition, likely in combination with existing CAR-T therapies. Furthermore, *F. nucleatum* contains potential CRISPR-Cas9 systems that can be used to build new genome editing and transcriptional tuning tools¹⁹.

Can intracellular bacteria be targeted for therapy? Will elimination of intratumoral bacteria have an effect on reducing relapse? Another potential strategy in cancer therapy is to harness the tumor-homing capabilities of the bacteria. Bacteria can be loaded with drugs or nanoparticles to seek and destroy cancer cells within the body²⁰⁻²². Furthermore, synthetic biological techniques can be used to engineer bacteria to be less harmful in order to execute their desired function²³⁻²⁶. There are already initial studies on exploiting the specificity of Fap2-dependent infection in bacteriotherapy to home to tumors¹⁴. These technologies would further equip our armamentarium against cancer.

Not too far in the future, we can envision a personalized treatment approach. Patient biopsies would be tested for mutational drivers, genotypic composition, identification of heterogenous tumor subpopulations, the level of immune infiltration, cytokine composition, and microbial composition.

Artificial intelligence and machine learning technologies will aid in cumulating and analyzing this voluminous dataset to recreate a holistic picture of the patient's tumor. Modelling programs would then access these datasets and simulate various permutations of and combinations of the progression of cancer to predict the effectiveness of possible courses of treatment. These models would then enable doctors to prescribe informed treatment strategies. Results obtained from the treatment can again be fed into a global network that updates and reanalyzes the data to improve predictions.

The following key observations: (1) The high-level evidence of the widespread prevalence of microbes in multiple cancer types, (2) how the tumor microbiome can impact most of the hallmarks of cancer, and (3) the observed upregulation of cancer-related programs uniquely upon infection, raises not-so-unconventional hypotheses with far-reaching implications. Is infection prerequisite for enhanced malignancy? Does cancer without microbial influence exist in a naïve state, where proliferation and metastatic programs are activated at a slower pace? If so, can cancer be restrained to less aggressive phenotypes if we directly target the tumor microbiome?

We are at a significant crossroads in cancer research as we combine two usually disparate fields of study: Cancer Biology and Infection Biology. A fundamental understanding of the tumor microbiome and its role in cancer progression is expected to redefine the way we approach the treatment and cure of cancer.

Published Work

1. **B. Udayasuryan**, Z. Zhou, R. N. Ahmad, P. Sobol, C. Deng, T. T. D. Nguyen, R. Morrison, I. Goswami, D.J. Slade, C. Lu, S. S. Verbridge. *Fusobacterium nucleatum* infection modulates the transcriptome and epigenome of colon cancer cells in an oxygen-dependent manner. (*In preparation*)
2. **B. Udayasuryan**, T. T. D. Nguyen, A. Umaña, M. Robérts, R. Ahmad, P. Sobol, S. Jones, J.M. Munson, D. J. Slade, S. S. Verbridge. *Fusobacterium nucleatum* infection induces pancreatic cancer cell proliferation and migration through regulation of host cytokine signaling. *BioRxiv* 2022. (*Under review*)
3. J. Queen, J.C. Domingue, J. R. White, C. Stevens, **B. Udayasuryan**, T. T. D. Nguyen, S. Wu, H. Ding, H. Fan, M. McMann, A. Corona, T. Larman, F. Housseau, S. Bullman, S.S. Verbridge, D. Slade, J. L. Drewes, C. L. Sears. Comparative analysis of colon cancer-derived *Fusobacterium nucleatum* subspecies: inflammation and colon tumorigenesis in murine models. *ASM mBio*. (PubMed ID: 35130731) 2022.
4. A. J. Huntington, **B. Udayasuryan**, P. Du, S.S. Verbridge, S. D. Abramowitch, R. De Vita. Smooth muscle and nerves in the rat vagina: a first look using tissue clearing and immunolabeling. *Annals of Biomedical Engineering*. (PubMed ID: 3518228) 2022.
5. **B. Udayasuryan**, T. Nguyen, D. Slade, S. Verbridge. Harnessing Tissue Engineering Tools to Interrogate Host-Microbe Crosstalk in Cancer. *iScience Cell Press* (PubMed ID: 33344921) 2020.
6. **B. Udayasuryan***, M. A. Casasanta*, C. C. Yoo*, B. E. Sanders, A. Umaña, Y. Zhang, A.J. Duncan, Y. Wang, L. Li, S.S. Verbridge, and D.J. Slade. *Fusobacterium nucleatum* host cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. *Science Signaling*. (PubMed ID: 32694172) 2020.
7. **B. Udayasuryan**, D. Slade, S. Verbridge. "Microfluidics in Microbiome and Cancer Research." Book Chapter. *Nanotechnology and Microfluidics*. *Wiley Publications*. 2019.
8. A. Umaña, B. E. Sanders, C. C. Yoo, M. A. Casasanta, **B. Udayasuryan**, S. S. Verbridge, D.J. Slade. "Utilizing Whole *Fusobacterium* Genomes to Identify, Correct, and Characterize Potential Virulence Protein Families". *American Society for Microbiology*. (PubMed ID: 31501282) 2019.

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References

Chapter 1

1. Liubakka, A. & Vaughn, B. P. Clostridium difficile Infection and Fecal Microbiota Transplant. *AACN Adv. Crit. Care* 27, 324–337 (2016).
2. Biancheri, P., Divekar, D. & Watson, A. J. M. Could Fecal Transplantation Become Part of PD-1-Based Immunotherapy, Due to Effects of the Intestinal Microbiome? *Gastroenterology* 154, 1845–1847 (2018).
3. Griffiths, J. A. & Mazmanian, S. K. Emerging evidence linking the gut microbiome to neurologic disorders. *Genome Med.* 10, 98 (2018).
4. Martin, C. R., Osadchiy, V., Kalani, A. & Mayer, E. A. The Brain-Gut-Microbiome Axis. *Cell. Mol. Gastroenterol. Hepatol.* 6, 133–148 (2018).
5. Stefanaki, C., Peppas, M., Mastorakos, G. & Chrousos, G. P. Examining the gut bacteriome, virome, and mycobiome in glucose metabolism disorders: Are we on the right track? *Metabolism.* 73, 52–66 (2017).
6. Scarpellini, E. *et al.* The human gut microbiota and virome: Potential therapeutic implications. *Dig. Liver Dis. Off. J. Ital. Soc. Gastroenterol. Ital. Assoc. Study Liver* 47, 1007–1012 (2015).
7. Siegel, R. L., Miller, K. D., Fuchs, H. E. & Jemal, A. Cancer statistics, 2022. *CA. Cancer J. Clin.* 72, 7–33 (2022).
8. Siegel, R. L. *et al.* Colorectal cancer statistics, 2020. *CA. Cancer J. Clin.* 70, 145–164 (2020).
9. Rawla, P., Sunkara, T. & Gaduputi, V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J. Oncol.* 10, 10–27 (2019).
10. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biol.* 14, e1002533 (2016).
11. Althani, A. A. *et al.* Human Microbiome and its Association With Health and Diseases. *J. Cell. Physiol.* 231, 1688–1694 (2016).
12. Wang, Z.-K. & Yang, Y.-S. Upper gastrointestinal microbiota and digestive diseases. *World J. Gastroenterol.* 19, 1541–1550 (2013).
13. Donaldson, G. P., Lee, S. M. & Mazmanian, S. K. Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.* 14, 20–32 (2016).
14. Mima, K. *et al.* The role of intestinal bacteria in the development and progression of gastrointestinal tract neoplasms. *Surg. Oncol.* 26, 368–376 (2017).
15. Rea, D. *et al.* Microbiota effects on cancer: from risks to therapies. *Oncotarget* 9, 17915–17927 (2018).
16. Gopalakrishnan, V., Helmink, B. A., Spencer, C. N., Reuben, A. & Wargo, J. A. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. *Cancer Cell* 33, 570–580 (2018).
17. Sheflin, A. M., Whitney, A. K. & Weir, T. L. Cancer-Promoting Effects of Microbial Dysbiosis. *Curr. Oncol. Rep.* 16, 406 (2014).
18. Cummins, J. & Tangney, M. Bacteria and tumours: causative agents or opportunistic inhabitants? *Infect. Agent. Cancer* 8, 11 (2013).
19. Song, S., Vuai, M. S. & Zhong, M. The role of bacteria in cancer therapy – enemies in the past, but allies at present. *Infect. Agent. Cancer* 13, (2018).
20. Mima, K. *et al.* Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* 65, 1973–1980 (2016).
21. Mitsuhashi, K. *et al.* Association of Fusobacterium species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget* 6, 7209–7220 (2015).
22. Ternes, D. *et al.* Microbiome in Colorectal Cancer: How to Get from Meta-omics to Mechanism? *Trends Microbiol.* 28, 401–423 (2020).
23. Kostic, A. D. *et al.* Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 14, 207–215 (2013).

24. Wu, S. *et al.* A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat. Med.* 15, 1016–1022 (2009).
25. Abed, J. *et al.* Fap2 Mediates *Fusobacterium nucleatum* Colorectal Adenocarcinoma Enrichment by Binding to Tumor-Expressed Gal-GalNAc. *Cell Host Microbe* 20, 215–225 (2016).
26. Casasanta, M. A. *et al.* A chemical and biological toolbox for Type Vd secretion: Characterization of the phospholipase A1 autotransporter FplA from *Fusobacterium nucleatum*. *J. Biol. Chem.* 292, 20240–20254 (2017).
27. Bullman, S. *et al.* Analysis of *Fusobacterium* persistence and antibiotic response in colorectal cancer. *Science* 358, 1443–1448 (2017).
28. Umaña, A. *et al.* Utilizing Whole *Fusobacterium* Genomes To Identify, Correct, and Characterize Potential Virulence Protein Families. *J. Bacteriol.* 201, (2019).
29. Zambirinis, C. P., Pushalkar, S., Saxena, D. & Miller, G. Pancreatic Cancer, Inflammation and Microbiome. *Cancer J. Sudbury Mass* 20, 195–202 (2014).
30. Archibugi, L., Signoretti, M. & Capurso, G. The Microbiome and Pancreatic Cancer: An Evidence-based Association? *J. Clin. Gastroenterol.* 52 Suppl 1, Proceedings from the 9th Probiotics, Prebiotics and New Foods, Nutraceuticals and Botanicals for Nutrition&Human and Microbiota Health Meeting, held in Rome, Italy from September 10 to 12, 2017, S82–S85 (2018).
31. Yu, L.-X. & Schwabe, R. F. The gut microbiome and liver cancer: mechanisms and clinical translation. *Nat. Rev. Gastroenterol. Hepatol.* 14, 527–539 (2017).
32. Golombos, D. M. *et al.* The Role of Gut Microbiome in the Pathogenesis of Prostate Cancer: A Prospective, Pilot Study. *Urology* 111, 122–128 (2018).
33. Fernández, M. F. *et al.* Breast Cancer and Its Relationship with the Microbiota. *Int. J. Environ. Res. Public Health* 15, (2018).
34. Parhi, L. *et al.* Breast cancer colonization by *Fusobacterium nucleatum* accelerates tumor growth and metastatic progression. *Nat. Commun.* 11, 3259 (2020).
35. J, Y., S, J., Y, Z. & J, W. *Helicobacter hepaticus* infection in primary hepatocellular carcinoma tissue. *Singapore Med. J.* 54, 451–457 (2013).
36. Fan, X. *et al.* Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. *Gut* 67, 120–127 (2018).
37. Gaiser, R. A. *et al.* Enrichment of oral microbiota in early cystic precursors to invasive pancreatic cancer. *Gut* 68, 2186–2194 (2019).
38. Dominy, S. S. *et al.* *Porphyromonas gingivalis* in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Sci. Adv.* 5, eaau3333 (2019).
39. Casasanta, M. A. *et al.* *Fusobacterium nucleatum* host-cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. *Sci. Signal.* 13, (2020).
40. Fischbach, M. A. Microbiome: Focus on Causation and Mechanism. *Cell* 174, 785–790 (2018).
41. Schmidt, T. S. B., Raes, J. & Bork, P. The Human Gut Microbiome: From Association to Modulation. *Cell* 172, 1198–1215 (2018).
42. Kurilshikov, A., Wijmenga, C., Fu, J. & Zhernakova, A. Host Genetics and Gut Microbiome: Challenges and Perspectives. *Trends Immunol.* 38, 633–647 (2017).
43. Heintz-Buschart, A. & Wilmes, P. Human Gut Microbiome: Function Matters. *Trends Microbiol.* 26, 563–574 (2018).

Chapter 2

1. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* 144, 646–674 (2011).
2. Fulbright, L. E., Ellermann, M. & Arthur, J. C. The microbiome and the hallmarks of cancer. *PLoS Pathog.* 13, (2017).
3. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biol.* 14, e1002533 (2016).

4. Brestoff, J. R. & Artis, D. Commensal bacteria at the interface of host metabolism and the immune system. *Nat. Immunol.* 14, 676–684 (2013).
5. Fan, Y. & Pedersen, O. Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 1–17 (2020) doi:10.1038/s41579-020-0433-9.
6. Udayasuryan, B., Slade, D. J. & Verbridge, S. S. Microfluidics in Microbiome and Cancer Research. *Wiley Online Library* 281–317 <https://onlinelibrary.wiley.com/doi/abs/10.1002/9783527818341.ch9> (2019).
7. Xavier, J. B. *et al.* The Cancer Microbiome: Distinguishing Direct and Indirect Effects Requires a Systemic View. *Trends Cancer* 6, 192–204 (2020).
8. Durack, J. & Lynch, S. V. The gut microbiome: Relationships with disease and opportunities for therapy. *J. Exp. Med.* 216, 20–40 (2019).
9. Kadosh, E. *et al.* The gut microbiome switches mutant p53 from tumour-suppressive to oncogenic. *Nature* 1–6 (2020) doi:10.1038/s41586-020-2541-0.
10. Nejman, D. *et al.* The human tumor microbiome is composed of tumor type-specific intracellular bacteria. *Science* 368, 973–980 (2020).
11. Balhouse, B. N., Patterson, L., Schmelz, E. M., Slade, D. J. & Verbridge, S. S. N-(3-oxododecanoyl)-L-homoserine lactone interactions in the breast tumor microenvironment: Implications for breast cancer viability and proliferation in vitro. *PLOS ONE* 12, e0180372 (2017).
12. Correa, P. & Piazuelo, M. B. *Helicobacter pylori* Infection and Gastric Adenocarcinoma. *US Gastroenterol. Hepatol. Rev.* 7, 59–64 (2011).
13. Uemura, N. *et al.* *Helicobacter pylori* infection and the development of gastric cancer. *N. Engl. J. Med.* 345, 784–789 (2001).
14. Farinha, P. & Gascoyne, R. D. *Helicobacter pylori* and MALT lymphoma. *Gastroenterology* 128, 1579–1605 (2005).
15. Ferreccio, C. *Salmonella typhi* and Gallbladder Cancer. in *Bacteria and Cancer* (ed. Khan, A. A.) 117–137 (Springer Netherlands, 2012). doi:10.1007/978-94-007-2585-0_5.
16. Boleij, A., Schaeps, R. M. J. & Tjalsma, H. Association between *Streptococcus bovis* and Colon Cancer. *J. Clin. Microbiol.* 47, 516 (2009).
17. Zhan, P. *et al.* Chlamydia pneumoniae infection and lung cancer risk: a meta-analysis. *Eur. J. Cancer Oxf. Engl.* 1990 47, 742–747 (2011).
18. Kostic, A. D. *et al.* Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma. *Genome Res.* 22, 292–298 (2012).
19. Greathouse, K. L. *et al.* Interaction between the microbiome and TP53 in human lung cancer. *Genome Biol.* 19, 123 (2018).
20. Gopalakrishnan, V. *et al.* Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 359, 97–103 (2018).
21. Geller, L. T. *et al.* Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. *Science* 357, 1156–1160 (2017).
22. van Elsland, D. & Neefjes, J. Bacterial infections and cancer. *EMBO Rep.* 19, e46632 (2018).
23. Fares, J., Fares, M. Y., Khachfe, H. H., Salhab, H. A. & Fares, Y. Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct. Target. Ther.* 5, 1–17 (2020).
24. Niller, H. H. & Minarovits, J. Patho-epigenetics of Infectious Diseases Caused by Intracellular Bacteria. in *Patho-Epigenetics of Infectious Disease* (eds. Minarovits, J. & Niller, H. H.) 107–130 (Springer International Publishing, 2016). doi:10.1007/978-3-319-24738-0_6.
25. Kunzmann, A. T. *et al.* *Fusobacterium nucleatum* tumor DNA levels are associated with survival in colorectal cancer patients. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 1891–1899 (2019).
26. Mima, K. *et al.* *Fusobacterium nucleatum* in colorectal carcinoma tissue and patient prognosis. *Gut* 65, 1973–1980 (2016).
27. Mitsuhashi, K. *et al.* Association of *Fusobacterium* species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget* 6, 7209–7220 (2015).
28. Abed, J. *et al.* Fap2 Mediates *Fusobacterium nucleatum* Colorectal Adenocarcinoma Enrichment by Binding to Tumor-Expressed Gal-GalNAc. *Cell Host Microbe* 20, 215–225 (2016).
29. Parhi, L. *et al.* Breast cancer colonization by *Fusobacterium nucleatum* accelerates tumor growth and metastatic progression. *Nat. Commun.* 11, 3259 (2020).

30. Shamsuddin, A. M., Tyner, G. T. & Yang, G. Y. Common expression of the tumor marker D-galactose-beta-[1-->3]-N-acetyl-D-galactosamine by different adenocarcinomas: evidence of field effect phenomenon. *Cancer Res.* 55, 149–152 (1995).
31. Bullman, S. *et al.* Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. *Science* 358, 1443–1448 (2017).
32. Casasanta, M. A. *et al.* Fusobacterium nucleatum host cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. *bioRxiv* 2020.01.15.907931 (2020) doi:10.1101/2020.01.15.907931.
33. Xia, X. *et al.* Bacteria pathogens drive host colonic epithelial cell promoter hypermethylation of tumor suppressor genes in colorectal cancer. *Microbiome* 8, 108 (2020).
34. Kasper, S. H. *et al.* Colorectal cancer-associated anaerobic bacteria proliferate in tumor spheroids and alter the microenvironment. *Sci. Rep.* 10, 5321 (2020).
35. Fischbach, M. A. Microbiome: Focus on Causation and Mechanism. *Cell* 174, 785–790 (2018).
36. Jovel, J. *et al.* Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. *Front. Microbiol.* 7, 459 (2016).
37. Bertrand, S. *et al.* Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. *Biotechnol. Adv.* 32, 1180–1204 (2014).
38. Hay, M., Thomas, D. W., Craighead, J. L., Economides, C. & Rosenthal, J. Clinical development success rates for investigational drugs. *Nat. Biotechnol.* 32, 40–51 (2014).
39. Mestas, J. & Hughes, C. C. W. Of Mice and Not Men: Differences between Mouse and Human Immunology. *J. Immunol.* 172, 2731–2738 (2004).
40. Schroeder, B. O. Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterol. Rep.* 7, 3–12 (2019).
41. Barkal, L. J., Berthier, E., Theberge, A. B., Keller, N. P. & Beebe, D. J. Multikingdom microscale models. *PLOS Pathog.* 13, e1006424 (2017).
42. Bahcecioglu, G., Basara, G., Ellis, B. W., Ren, X. & Zorlutuna, P. Breast cancer models: Engineering the tumor microenvironment. *Acta Biomater.* 106, 1–21 (2020).
43. Ho, W. J., Jaffee, E. M. & Zheng, L. The tumour microenvironment in pancreatic cancer - clinical challenges and opportunities. *Nat. Rev. Clin. Oncol.* (2020) doi:10.1038/s41571-020-0363-5.
44. Mittal, V. *et al.* The Microenvironment of Lung Cancer and Therapeutic Implications. in *Lung Cancer and Personalized Medicine: Novel Therapies and Clinical Management* (eds. Ahmad, A. & Gadgil, S. M.) 75–110 (Springer International Publishing, 2016). doi:10.1007/978-3-319-24932-2_5.
45. Millhouse, E. *et al.* Development of an in vitro periodontal biofilm model for assessing antimicrobial and host modulatory effects of bioactive molecules. *BMC Oral Health* 14, 80 (2014).
46. Sadaghian Sadabad, M. *et al.* A simple coculture system shows mutualism between anaerobic faecalibacteria and epithelial Caco-2 cells. *Sci. Rep.* 5, (2015).
47. Park, G.-S. *et al.* Emulating Host-Microbiome Ecosystem of Human Gastrointestinal Tract in Vitro. *Stem Cell Rev. Rep.* 13, 321–334 (2017).
48. Li, L. *et al.* An in vitro model maintaining taxon-specific functional activities of the gut microbiome. *Nat. Commun.* 10, 4146 (2019).
49. Cox, M. C., Reese, L. M., Bickford, L. R. & Verbridge, S. S. Toward the Broad Adoption of 3D Tumor Models in the Cancer Drug Pipeline. *ACS Biomater. Sci. Eng.* 1, 877–894 (2015).
50. Barrila, J. *et al.* Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions. *Nat. Rev. Microbiol.* 8, 791–801 (2010).
51. Radtke, A. L., Wilson, J. W., Sarker, S. & Nickerson, C. A. Analysis of Interactions of Salmonella Type Three Secretion Mutants with 3-D Intestinal Epithelial Cells. *PLoS ONE* 5, (2010).
52. Ilhan, Z. E., Łaniewski, P., Tonachio, A. & Herbst-Kralovetz, M. M. Members of Prevotella genus distinctively modulate innate immune and barrier functions in a human three-dimensional endometrial epithelial cell model. *J. Infect. Dis.* (2020) doi:10.1093/infdis/jiaa324.
53. Chen, Y. *et al.* Robust bioengineered 3D functional human intestinal epithelium. *Sci. Rep.* 5, 13708 (2015).
54. Fujii, M. *et al.* A Colorectal Tumor Organoid Library Demonstrates Progressive Loss of Niche Factor Requirements during Tumorigenesis. *Cell Stem Cell* 18, 827–838 (2016).
55. Shamir, E. R. & Ewald, A. J. Three-dimensional organotypic culture: experimental models of mammalian biology and disease. *Nat. Rev. Mol. Cell Biol.* 15, 647–664 (2014).

56. Yao, Y. *et al.* Patient-Derived Organoids Predict Chemoradiation Responses of Locally Advanced Rectal Cancer. *Cell Stem Cell* 26, 17-26.e6 (2020).
57. Bar-Ephraim, Y. E., Kretzschmar, K. & Clevers, H. Organoids in immunological research. *Nat. Rev. Immunol.* 20, 279–293 (2020).
58. Bartfeld, S. *et al.* In Vitro Expansion of Human Gastric Epithelial Stem Cells and Their Responses to Bacterial Infection. *Gastroenterology* 148, 126-136.e6 (2015).
59. Schlaermann, P. *et al.* A novel human gastric primary cell culture system for modelling *Helicobacter pylori* infection in vitro. *Gut* 65, 202–213 (2016).
60. Pleguezuelos-Manzano, C. *et al.* Mutational signature in colorectal cancer caused by genotoxic pks + *E. coli*. *Nature* 580, 269–273 (2020).
61. Barrila, J. *et al.* Three-dimensional organotypic co-culture model of intestinal epithelial cells and macrophages to study *Salmonella enterica* colonization patterns. *NPJ Microgravity* 3, (2017).
62. Costa, E. C., Gaspar, V. M., Coutinho, P. & Correia, I. J. Optimization of liquid overlay technique to formulate heterogenic 3D co-cultures models. *Biotechnol. Bioeng.* 111, 1672–1685 (2014).
63. Nunes, A. S., Barros, A. S., Costa, E. C., Moreira, A. F. & Correia, I. J. 3D tumor spheroids as in vitro models to mimic in vivo human solid tumors resistance to therapeutic drugs. *Biotechnol. Bioeng.* 116, 206–226 (2019).
64. Shah, P. *et al.* A microfluidics-based in vitro model of the gastrointestinal human–microbe interface. *Nat. Commun.* 7, 11535 (2016).
65. Kim, H. J., Li, H., Collins, J. J. & Ingber, D. E. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc. Natl. Acad. Sci.* 113, E7–E15 (2016).
66. Kim, H. J., Lee, J., Choi, J.-H., Bahinski, A. & Ingber, D. E. Co-culture of Living Microbiome with Microengineered Human Intestinal Villi in a Gut-on-a-Chip Microfluidic Device. *J. Vis. Exp. JoVE* (2016) doi:10.3791/54344.
67. Grassart, A. *et al.* Bioengineered Human Organ-on-Chip Reveals Intestinal Microenvironment and Mechanical Forces Impacting *Shigella* Infection. *Cell Host Microbe* 26, 435-444.e4 (2019).
68. Jalili-Firoozinezhad, S. *et al.* A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. *Nat. Biomed. Eng.* 3, 520–531 (2019).
69. Shin, W. *et al.* A Robust Longitudinal Co-culture of Obligate Anaerobic Gut Microbiome With Human Intestinal Epithelium in an Anoxic-Oxic Interface-on-a-Chip. *Front. Bioeng. Biotechnol.* 7, 13 (2019).
70. Sontheimer-Phelps, A. *et al.* Human Colon-on-a-Chip Enables Continuous In Vitro Analysis of Colon Mucus Layer Accumulation and Physiology. *Cell. Mol. Gastroenterol. Hepatol.* 9, 507–526 (2020).
71. Kasendra, M. *et al.* Duodenum Intestine-Chip for preclinical drug assessment in a human relevant model. *eLife* 9, e50135 (2020).
72. Edington, C. D. *et al.* Interconnected Microphysiological Systems for Quantitative Biology and Pharmacology Studies. *Sci. Rep.* 8, 4530 (2018).
73. Trietsch, S. J. *et al.* Membrane-free culture and real-time barrier integrity assessment of perfused intestinal epithelium tubes. *Nat. Commun.* 8, 262 (2017).
74. Lanz, H. L. *et al.* Therapy response testing of breast cancer in a 3D high-throughput perfused microfluidic platform. *BMC Cancer* 17, (2017).
75. Kramer, B. *et al.* Interstitial Flow Recapitulates Gemcitabine Chemoresistance in A 3D Microfluidic Pancreatic Ductal Adenocarcinoma Model by Induction of Multidrug Resistance Proteins. *Int. J. Mol. Sci.* 20, (2019).
76. Trujillo-de Santiago, G. *et al.* The Tumor-on-Chip: Recent Advances in the Development of Microfluidic Systems to Recapitulate the Physiology of Solid Tumors. *Materials* 12, (2019).
77. Ma, Y.-H. V., Middleton, K., You, L. & Sun, Y. A review of microfluidic approaches for investigating cancer extravasation during metastasis. *Microsyst. Nanoeng.* 4, 1–13 (2018).
78. Shang, M., Soon, R. H., Lim, C. T., Khoo, B. L. & Han, J. Microfluidic modelling of the tumor microenvironment for anti-cancer drug development. *Lab. Chip* 19, 369–386 (2019).
79. Tsai, H.-F., Trubelja, A., Shen, A. Q. & Bao, G. Tumour-on-a-chip: microfluidic models of tumour morphology, growth and microenvironment. *J. R. Soc. Interface* 14, 20170137 (2017).
80. Huh, D. *et al.* Reconstituting Organ-Level Lung Functions on a Chip. *Science* 328, 1662–1668 (2010).

81. Hsu, Y.-H., Moya, M. L., Hughes, C. C. W., George, S. C. & Lee, A. P. A microfluidic platform for generating large-scale nearly identical human microphysiological vascularized tissue arrays. *Lab. Chip* 13, 2990–2998 (2013).
82. Nguyen, D.-H. T. *et al.* Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. *Proc. Natl. Acad. Sci.* 110, 6712–6717 (2013).
83. Sobrino, A. *et al.* 3D microtumors in vitro supported by perfused vascular networks. *Sci. Rep.* 6, 31589 (2016).
84. Morgan, J. P. *et al.* Formation of microvascular networks in vitro. *Nat. Protoc.* 8, 1820–1836 (2013).
85. Aung, A., Kumar, V., Theprungsirikul, J., Davey, S. K. & Varghese, S. An Engineered Tumor-on-a-Chip Device with Breast Cancer–Immune Cell Interactions for Assessing T-cell Recruitment. *Cancer Res.* 80, 263–275 (2020).
86. Boussommier-Calleja, A., Li, R., Chen, M. B., Wong, S. C. & Kamm, R. D. Microfluidics: A New Tool for Modeling Cancer–Immune Interactions. *Trends Cancer* 2, 6–19 (2016).
87. Choi, Y. *et al.* A microengineered pathophysiological model of early-stage breast cancer. *Lab. Chip* 15, 3350–3357 (2015).
88. Hassell, B. A. *et al.* Human Organ Chip Models Recapitulate Orthotopic Lung Cancer Growth, Therapeutic Responses, and Tumor Dormancy In Vitro. *Cell Rep.* 21, 508–516 (2017).
89. Beer, M. *et al.* A novel microfluidic 3D platform for culturing pancreatic ductal adenocarcinoma cells: comparison with in vitro cultures and in vivo xenografts. *Sci. Rep.* 7, (2017).
90. Ayuso, J. M. *et al.* Tumor-on-a-chip: a microfluidic model to study cell response to environmental gradients. *Lab. Chip* 19, 3461–3471 (2019).
91. Carvalho, M. R. *et al.* Colorectal tumor-on-a-chip system: A 3D tool for precision onco-nanomedicine. *Sci. Adv.* 5, eaaw1317 (2019).
92. Coughlin, M. F. & Kamm, R. D. The Use of Microfluidic Platforms to Probe the Mechanism of Cancer Cell Extravasation. *Adv. Healthc. Mater.* 9, 1901410 (2020).
93. Jeon, J. S., Zervantonakis, I. K., Chung, S., Kamm, R. D. & Charest, J. L. In vitro model of tumor cell extravasation. *PloS One* 8, e56910 (2013).
94. Xu, Z. *et al.* Application of a microfluidic chip-based 3D co-culture to test drug sensitivity for individualized treatment of lung cancer. *Biomaterials* 34, 4109–4117 (2013).
95. Datta, P., Dey, M., Ataie, Z., Unutmaz, D. & Ozbolat, I. T. 3D bioprinting for reconstituting the cancer microenvironment. *Npj Precis. Oncol.* 4, 1–13 (2020).
96. Murphy, S. V., De Coppi, P. & Atala, A. Opportunities and challenges of translational 3D bioprinting. *Nat. Biomed. Eng.* 4, 370–380 (2020).
97. Ramadan, Q. & Zourob, M. Organ-on-a-chip engineering: Toward bridging the gap between lab and industry. *Biomicrofluidics* 14, 041501 (2020).
98. Cox, M. C., Deng, C., Naler, L. B., Lu, C. & Verbridge, S. S. Effects of Culture Condition on Epigenomic Profiles of Brain Tumor Cells. *ACS Biomater. Sci. Eng.* 5, 1544–1552 (2019).
99. Zhu, B. *et al.* MOWChIP-seq for low-input and multiplexed profiling of genome-wide histone modifications. *Nat. Protoc.* 14, 3366–3394 (2019).
100. Munson, J. M. & Shieh, A. C. Interstitial fluid flow in cancer: implications for disease progression and treatment. *Cancer Manag. Res.* 6, 317–328 (2014).
101. Sontheimer-Phelps, A., Hassell, B. A. & Ingber, D. E. Modelling cancer in microfluidic human organs-on-chips. *Nat. Rev. Cancer* 19, 65–81 (2019).
102. Fetah, K. L. *et al.* Cancer Modeling-on-a-Chip with Future Artificial Intelligence Integration. *Small Wein. Bergstr. Ger.* 15, e1901985 (2019).
103. Cunningham, J. J. A call for integrated metastatic management. *Nat. Ecol. Evol.* 3, 996–998 (2019).
104. Suh, S. *et al.* Nanoscale Bacteria-Enabled Autonomous Drug Delivery System (NanoBEADS) Enhances Intratumoral Transport of Nanomedicine. *Adv. Sci.* 6, 1801309 (2019).

Chapter 3

1. D. van Elsland, J. Neefjes, Bacterial infections and cancer. *EMBO Rep.* 19, e46632 (2018), doi:10.15252/embr.201846632.

2. A. Gagnaire, B. Nadel, D. Raoult, J. Neefjes, J.-P. Gorvel, Collateral damage: insights into bacterial mechanisms that predispose host cells to cancer. *Nat. Rev. Microbiol.* 15, 109–128 (2017).
3. H. L. Kaufman, F. J. Kohlhapp, A. Zloza, Oncolytic viruses: a new class of immunotherapy drugs. *Nat. Rev. Drug Discov.* 14, 642–662 (2015).
4. C. A. Brennan, W. S. Garrett, *Fusobacterium nucleatum* — symbiont, opportunist and oncobacterium. *Nat. Rev. Microbiol.* (2018), doi:10.1038/s41579-018-0129-6.
5. Y. W. Han, *Fusobacterium nucleatum*: a commensal-turned pathogen. *Curr. Opin. Microbiol.* 23, 141–147 (2015).
6. M. R. Rubinstein, X. Wang, W. Liu, Y. Hao, G. Cai, Y. W. Han, *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/ β -catenin signaling via its FadA adhesin. *Cell Host Microbe.* 14, 195–206 (2013).
7. J. Abed, J. E. M. Emgård, G. Zamir, M. Faroja, G. Almogy, A. Grenov, A. Sol, R. Naor, E. Pikarsky, K. A. Atlan, A. Mellul, S. Chaushu, A. L. Manson, A. M. Earl, N. Ou, C. A. Brennan, W. S. Garrett, G. Bachrach, Fap2 Mediates *Fusobacterium nucleatum* Colorectal Adenocarcinoma Enrichment by Binding to Tumor-Expressed Gal-GalNAc. *Cell Host Microbe.* 20, 215–225 (2016).
8. M. Castellarin, R. L. Warren, J. D. Freeman, L. Dreolini, M. Krzywinski, J. Strauss, R. Barnes, P. Watson, E. Allen-Vercoe, R. A. Moore, R. A. Holt, *Fusobacterium nucleatum* infection is prevalent in human colorectal carcinoma. *Genome Res.* 22, 299–306 (2012).
9. A. D. Kostic, D. Gevers, C. S. Pedomallu, M. Michaud, F. Duke, A. M. Earl, A. I. Ojesina, J. Jung, A. J. Bass, J. Tabernero, J. Baselga, C. Liu, R. A. Shivdasani, S. Ogino, B. W. Birren, C. Huttenhower, W. S. Garrett, M. Meyerson, Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma. *Genome Res.* 22, 292–298 (2012).
10. S. Bullman, C. S. Pedomallu, E. Sicinska, T. E. Clancy, X. Zhang, D. Cai, D. Neuberg, K. Huang, F. Guevara, T. Nelson, O. Chipashvili, T. Hagan, M. Walker, A. Ramachandran, B. Diosdado, G. Serna, N. Mulet, S. Landolfi, S. Ramon Y Cajal, R. Fasani, A. J. Aguirre, K. Ng, E. Élez, S. Ogino, J. Tabernero, C. S. Fuchs, W. C. Hahn, P. Nuciforo, M. Meyerson, Analysis of *Fusobacterium* persistence and antibiotic response in colorectal cancer. *Science.* 358, 1443–1448 (2017).
11. S. Chen, T. Su, Y. Zhang, A. Lee, J. He, Q. Ge, L. Wang, J. Si, W. Zhuo, L. Wang, *Fusobacterium nucleatum* promotes colorectal cancer metastasis by modulating KRT7-AS/KRT7. *Gut Microbes*, 1–15 (2020).
12. Y. Chen, Y. Chen, J. Zhang, P. Cao, W. Su, Y. Deng, N. Zhan, X. Fu, Y. Huang, W. Dong, *Fusobacterium nucleatum* Promotes Metastasis in Colorectal Cancer by Activating Autophagy Signaling via the Upregulation of CARD3 Expression. *Theranostics.* 10, 323–339 (2020).
13. H. Verbeke, S. Struyf, G. Laureys, J. Van Damme, The expression and role of CXC chemokines in colorectal cancer. *Cytokine Growth Factor Rev.* 22, 345–358 (2011).
14. C. Zhuo, X. Wu, J. Li, D. Hu, J. Jian, C. Chen, X. Zheng, C. Yang, Chemokine (C-X-C motif) ligand 1 is associated with tumor progression and poor prognosis in patients with colorectal cancer. *Biosci. Rep.* 38 (2018), doi:10.1042/BSR20180580.
15. A. Li, M. L. Varney, R. K. Singh, Constitutive expression of growth regulated oncogene (gro) in human colon carcinoma cells with different metastatic potential and its role in regulating their metastatic phenotype. *Clin. Exp. Metastasis.* 21, 571–579 (2004).
16. A. Li, M. L. Varney, J. Valasek, M. Godfrey, B. J. Dave, R. K. Singh, Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis.* 8, 63–71 (2005).
17. H. Ogata, A. Sekikawa, H. Yamagishi, K. Ichikawa, S. Tomita, J. Imura, Y. Ito, M. Fujita, M. Tsubaki, H. Kato, T. Fujimori, H. Fukui, GRO α promotes invasion of colorectal cancer cells. *Oncol. Rep.* 24, 1479–1486 (2010).
18. D. Wang, H. Sun, J. Wei, B. Cen, R. N. DuBois, CXCL1 Is Critical for Premetastatic Niche Formation and Metastasis in Colorectal Cancer. *Cancer Res.* 77, 3655–3665 (2017).
19. C. Rubie, V. O. Frick, S. Pfeil, M. Wagner, O. Kollmar, B. Kopp, S. Graber, B. M. Rau, M. K. Schilling, Correlation of IL-8 with induction, progression and metastatic potential of colorectal cancer. *World J. Gastroenterol.* 13, 4996–5002 (2007).
20. L.-C. Chen, C.-Y. Hao, Y. S. Y. Chiu, P. Wong, J. S. Melnick, M. Brotman, J. Moretto, F. Mendes, A. P. Smith, J. L. Bennington, D. Moore, N. M. Lee, Alteration of gene expression in normal-appearing colon mucosa of APC(min) mice and human cancer patients. *Cancer Res.* 64, 3694–3700 (2004).
21. Y. S. Lee, I. Choi, Y. Ning, N. Y. Kim, V. Khatchadourian, D. Yang, H. K. Chung, D. Choi, M. J. LaBonte, R. D.

- Ladner, K. C. Nagulapalli Venkata, D. O. Rosenberg, N. A. Petasis, H.-J. Lenz, Y.-K. Hong, Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis. *Br. J. Cancer*. 106, 1833–1841 (2012).
22. Q. Liu, A. Li, Y. Tian, J. D. Wu, Y. Liu, T. Li, Y. Chen, X. Han, K. Wu, The CXCL8-CXCR1/2 pathways in cancer. *Cytokine Growth Factor Rev*. 31, 61–71 (2016).
 23. A. Umaña, B. E. Sanders, C. C. Yoo, M. A. Casasanta, B. Udayasuryan, S. S. Verbridge, D. J. Slade, Utilizing Whole *Fusobacterium* Genomes To Identify, Correct, and Characterize Potential Virulence Protein Families. *J. Bacteriol.* 201 (2019), doi:10.1128/JB.00273-19.
 24. M. Desvaux, A. Khan, S. A. Beatson, A. Scott-Tucker, I. R. Henderson, Protein secretion systems in *Fusobacterium nucleatum*: genomic identification of Type 4 piliation and complete Type V pathways brings new insight into mechanisms of pathogenesis. *Biochim. Biophys. Acta*. 1713, 92–112 (2005).
 25. N. Dautin, H. D. Bernstein, Protein secretion in gram-negative bacteria via the autotransporter pathway. *Annu. Rev. Microbiol.* 61, 89–112 (2007).
 26. C. Gur, Y. Ibrahim, B. Isaacson, R. Yamin, J. Abed, M. Gamliel, J. Enk, Y. Bar-On, N. Stanitsky-Kaynan, S. Coppenhagen-Glazer, N. Shussman, G. Almog, A. Cuapio, E. Hofer, D. Mevorach, A. Tabib, R. Ortenberg, G. Markel, K. Miklić, S. Jonjic, C. A. Brennan, W. S. Garrett, G. Bachrach, O. Mandelboim, Binding of the Fap2 protein of *Fusobacterium nucleatum* to human inhibitory receptor TIGIT protects tumors from immune cell attack. *Immunity*. 42, 344–355 (2015).
 27. M. L. Brewer, D. Dymock, R. L. Brady, B. B. Singer, M. Virji, D. J. Hill, *Fusobacterium* spp. Target human CEACAM1 via the trimeric autotransporter adhesin CbpF. *J. Oral Microbiol.* 11, 1565043 (2019).
 28. J. Bassler, B. Hernandez Alvarez, M. D. Hartmann, A. N. Lupas, A domain dictionary of trimeric autotransporter adhesins. *Int. J. Med. Microbiol.* 305, 265–275 (2015).
 29. J. Strauss, G. G. Kaplan, P. L. Beck, K. Rioux, R. Panaccione, R. Devinney, T. Lynch, E. Allen-Vercoe, Invasive potential of gut mucosa-derived *Fusobacterium nucleatum* positively correlates with IBD status of the host. *Inflamm. Bowel Dis*. 17, 1971–1978 (2011).
 30. Y. Yang, W. Weng, J. Peng, L. Hong, L. Yang, Y. Toiyama, R. Gao, M. Liu, M. Yin, C. Pan, H. Li, B. Guo, Q. Zhu, Q. Wei, M.-P. Moyer, P. Wang, S. Cai, A. Goel, H. Qin, Y. Ma, *Fusobacterium nucleatum* Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor- κ , Up-Regulating Expression of MicroRNA-21. *Gastroenterology* (2016), doi:10.1053/j.gastro.2016.11.018.
 31. M. Xu, M. Yamada, M. Li, H. Liu, S. G. Chen, Y. W. Han, FadA from *Fusobacterium nucleatum* utilizes both secreted and nonsecreted forms for functional oligomerization for attachment and invasion of host cells. *J. Biol. Chem.* 282, 25000–25009 (2007).
 32. U. K. Gursoy, E. K  n  nen, V.-J. Uitto, Intracellular replication of fusobacteria requires new actin filament formation of epithelial cells. *APMIS*. 116, 1063–1070 (2008).
 33. M. K. H. Schindler, M. S. Sch  tz, M. C. M  hlenkamp, S. H. M. Rooijakkers, T. Hallstr  m, P. F. Zipfel, I. B. Autenrieth, *Yersinia enterocolitica* YadA mediates complement evasion by recruitment and inactivation of C3 products. *J. Immunol.* 189, 4900–4908 (2012).
 34. J. Eitel, P. Dersch, The YadA Protein of *Yersinia pseudotuberculosis* Mediates High-Efficiency Uptake into Human Cells under Environmental Conditions in Which Invasin Is Repressed. *Infect. Immun.* 70, 4880–4891 (2002).
 35. C. Wu, A. A. M. Al Mamun, T. T. Luong, B. Hu, J. Gu, J. H. Lee, M. D’Amore, A. Das, H. Ton-That, Forward Genetic Dissection of Biofilm Development by *Fusobacterium nucleatum*: Novel Functions of Cell Division Proteins FtsX and EnvC. *Mbio*. 9 (2018), doi:10.1128/mBio.00360-18.
 36. H. Nariya, S. Miyata, M. Suzuki, E. Tamai, A. Okabe, Development and application of a method for counterselectable in-frame deletion in *Clostridium perfringens*. *Appl. Environ. Microbiol.* 77, 1375–1382 (2011).
 37. A. Ikegami, P. Chung, Y. W. Han, Complementation of the fadA mutation in *Fusobacterium nucleatum* demonstrates that the surface-exposed adhesin promotes cellular invasion and placental colonization. *Infect. Immun.* 77, 3075–3079 (2009).
 38. A. Manson McGuire, K. Cochrane, A. D. Griggs, B. J. Haas, T. Abeel, Q. Zeng, J. B. Nice, H. MacDonald, B. W. Birren, B. W. Berger, E. Allen-Vercoe, A. M. Earl, Evolution of invasion in a diverse set of *Fusobacterium* species. *Mbio*. 5, e01864 (2014).
 39. I. D. Bobanga, F. Allen, N. R. Teich, A. Y. Huang, Chemokines CCL3 and CCL4 Differentially Recruit Lymphocytes in a Murine Model of Early Metastatic Colon Cancer. *J. Surg. Res.* 186, 515–516 (2014).

40. Y. Itatani, K. Kawada, S. Inamoto, T. Yamamoto, R. Ogawa, M. M. Taketo, Y. Sakai, The Role of Chemokines in Promoting Colorectal Cancer Invasion/Metastasis. *Int. J. Mol. Sci.* 17 (2016), doi:10.3390/ijms17050643.
41. V. Papayannopoulos, Neutrophil extracellular traps in immunity and disease. *Nat. Rev. Immunol.* 18, 134–147 (2018).
42. M. Doke, H. Fukamachi, H. Morisaki, T. Arimoto, H. Kataoka, H. Kuwata, Nucleases from *Prevotella intermedia* can degrade neutrophil extracellular traps. *Mol. Oral Microbiol.* 32, 288–300 (2017).
43. B. P. Lima, W. Shi, R. Lux, Identification and characterization of a novel *Fusobacterium nucleatum* adhesin involved in physical interaction and biofilm formation with *Streptococcus gordonii*. *Microbiologyopen*. 6 (2017), doi:10.1002/mbo3.444.
44. C. W. Kaplan, R. Lux, S. K. Haake, W. Shi, The *Fusobacterium nucleatum* outer membrane protein RadD is an arginine-inhibitable adhesin required for inter-species adherence and the structured architecture of multispecies biofilm. *Mol. Microbiol.* 71, 35–47 (2009).
45. C. W. Kaplan, X. Ma, A. Paranjpe, A. Jewett, R. Lux, S. Kinder-Haake, W. Shi, *Fusobacterium nucleatum* Outer Membrane Proteins Fap2 and RadD Induce Cell Death in Human Lymphocytes, , doi:10.1128/IAI.00567-10.
46. Y. Ning, P. C. Manegold, Y. K. Hong, W. Zhang, A. Pohl, G. Lurje, T. Winder, D. Yang, M. J. LaBonte, P. M. Wilson, R. D. Ladner, H.-J. Lenz, Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. *Int. J. Cancer.* 128, 2038–2049 (2011).
47. E. Cohen-Hillel, I. Yron, T. Meshel, A. Ben-Baruch, Interleukin 8 and cell migration to inflammatory sites: the regulation of focal adhesion kinase under conditions of migratory desensitization. *Isr. Med. Assoc. J.* 9, 579–583 (2007).
48. P.-L. Kuo, K.-H. Shen, S.-H. Hung, Y.-L. Hsu, CXCL1/GRO α increases cell migration and invasion of prostate cancer by decreasing fibulin-1 expression through NF- κ B/HDAC1 epigenetic regulation. *Carcinogenesis*. 33, 2477–2487 (2012).
49. K. Mima, R. Nishihara, Z. R. Qian, Y. Cao, Y. Sukawa, J. A. Nowak, J. Yang, R. Dou, Y. Masugi, M. Song, A. D. Kostic, M. Giannakis, S. Bullman, D. A. Milner, H. Baba, E. L. Giovannucci, L. A. Garraway, G. J. Freeman, G. Dranoff, W. S. Garrett, C. Huttenhower, M. Meyerson, J. A. Meyerhardt, A. T. Chan, C. S. Fuchs, S. Ogino, *Fusobacterium nucleatum* in colorectal carcinoma tissue and patient prognosis. *Gut* (2015), doi:10.1136/gutjnl-2015-310101.
50. K. Noshio, Y. Sukawa, Y. Adachi, M. Ito, K. Mitsuhashi, H. Kurihara, S. Kanno, I. Yamamoto, K. Ishigami, H. Igarashi, R. Maruyama, K. Imai, H. Yamamoto, Y. Shinomura, Association of *Fusobacterium nucleatum* with immunity and molecular alterations in colorectal cancer. *World J. Gastroenterol.* 22, 557–566 (2016).
51. T. Yu, F. Guo, Y. Yu, T. Sun, D. Ma, J. Han, Y. Qian, I. Kryczek, D. Sun, N. Nagarsheth, Y. Chen, H. Chen, J. Hong, W. Zou, J.-Y. Fang, *Fusobacterium nucleatum* Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. *Cell.* 170, 548–563.e16 (2017).
52. C. A. Brennan, W. S. Garrett, Gut Microbiota, Inflammation, and Colorectal Cancer. *Annu. Rev. Microbiol.* 70, 395–411 (2016).
53. A. D. Kostic, E. Chun, L. Robertson, J. N. Glickman, C. A. Gallini, M. Michaud, T. E. Clancy, D. C. Chung, P. Lochhead, G. L. Hold, E. M. El-Omar, D. Brenner, C. S. Fuchs, M. Meyerson, W. S. Garrett, *Fusobacterium nucleatum* potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe.* 14, 207–215 (2013).
54. W. S. Garrett, The gut microbiota and colon cancer. *Science.* 364, 1133–1135 (2019).
55. R. Mizuno, K. Kawada, Y. Itatani, R. Ogawa, Y. Kiyasu, Y. Sakai, The Role of Tumor-Associated Neutrophils in Colorectal Cancer. *Int. J. Mol. Sci.* 20 (2019), doi:10.3390/ijms20030529.
56. J. Cools-Lartigue, J. Spicer, B. McDonald, S. Gowing, S. Chow, B. Giannias, F. Bourdeau, P. Kubes, L. Ferri, Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J. Clin. Invest.* (2013), doi:10.1172/JCI67484.
57. J. Park, R. W. Wysocki, Z. Amoozgar, L. Maiorino, M. R. Fein, J. Jorns, A. F. Schott, Y. Kinugasa-Katayama, Y. Lee, N. H. Won, E. S. Nakasone, S. A. Hearn, V. Küttner, J. Qiu, A. S. Almeida, N. Perurena, K. Kessenbrock, M. S. Goldberg, M. Egeblad, Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci. Transl. Med.* 8, 361ra138 (2016).
58. K. Mima, Y. Sukawa, R. Nishihara, Z. R. Qian, M. Yamauchi, K. Inamura, S. A. Kim, A. Masuda, J. A. Nowak,

- K. Noshio, A. D. Kostic, M. Giannakis, H. Watanabe, S. Bullman, D. A. Milner, C. C. Harris, E. Giovannucci, L. A. Garraway, G. J. Freeman, G. Dranoff, A. T. Chan, W. S. Garrett, C. Huttenhower, C. S. Fuchs, S. Ogino, *Fusobacterium nucleatum* and T Cells in Colorectal Carcinoma. *JAMA Oncol.* 1, 653–661 (2015).
59. R. Kalluri, The biology and function of fibroblasts in cancer. *Nat. Rev. Cancer.* 16, 582–598 (2016).
60. Y. Lin, J. Xu, H. Lan, Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J. Hematol. Oncol.* 12, 76 (2019).
61. A. S. Abdulmir, R. R. Hafidh, L. K. Mahdi, T. Al-jeboori, F. Abubaker, Investigation into the controversial association of *Streptococcus gallolyticus* with colorectal cancer and adenoma. *BMC Cancer.* 9, 403 (2009).
62. J. H. Abed, J. Emgård, S. Chaushu, W. Garrett, G. Bachrach, Abstract 3300: Gal-GalNAc overexpressed in colorectal carcinoma mediates attachment and colonization of *Fusobacterium nucleatum* utilizing the Fap2 lectin. *Cancer Res.* 76, 3300–3300 (2016).
63. C. N. Spaulding, R. D. Klein, S. Ruer, A. L. Kau, H. L. Schreiber, Z. T. Cusumano, K. W. Dodson, J. S. Pinkner, D. H. Fremont, J. W. Janetka, H. Remaut, J. I. Gordon, S. J. Hultgren, Selective depletion of uropathogenic *E. coli* from the gut by a FimH antagonist. *Nature.* 546, 528–532 (2017).
64. L. Harbaum, M. J. Pollheimer, P. Kornprat, R. A. Lindtner, A. Schlemmer, P. Rehak, C. Langner, Keratin 7 expression in colorectal cancer—freak of nature or significant finding? *Histopathology.* 59, 225–234 (2011).
65. B. Huang, J. H. Song, Y. Cheng, J. M. Abraham, S. Ibrahim, Z. Sun, X. Ke, S. J. Meltzer, Long non-coding antisense RNA KRT7-AS is activated in gastric cancers and supports cancer cell progression by increasing KRT7 expression. *Oncogene.* 35, 4927–4936 (2016).
66. S. M. Singel, K. Batten, C. Cornelius, G. Jia, G. Fasciani, S. L. Barron, W. E. Wright, J. W. Shay, Receptor-interacting protein kinase 2 promotes triple-negative breast cancer cell migration and invasion via activation of nuclear factor-kappaB and c-Jun N-terminal kinase pathways. *Breast Cancer Res.* 16, R28 (2014).
67. J. Zheng, J. Meng, S. Zhao, R. Singh, W. Song, *Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB. *Infect. Immun.* 76, 4498–4508 (2008).
68. A. Kuznik, M. Bencina, U. Svajger, M. Jeras, B. Rozman, R. Jerala, Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J. Immunol.* 186, 4794–4804 (2011).
69. W. S. Rasband, ImageJ. Bethesda, MD: US National Institutes of Health. Ht tp://rsb. Info. Nih. Gov/ij, 1997–2007 (1997).
70. C. Yanisch-Perron, J. Vieira, J. Messing, Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene.* 33, 103–119 (1985).
71. T.L. Bannam, J.I. Rood, *Clostridium perfringens*-*Escherichia coli* shuttle vectors that carry single antibiotic resistance determinants. *Plasmid.* 29, 233–235 (1993).
72. M. Knorr, Über die fusospirilläre symbiose, die Gattung *Fusobacterium* (KB Lehmann) und *Spirillum sputigenum*. Zugleich ein Beitrag zur Bakteriologie der Mundhöhle. II. Mitteilung Die Gattung *Fusobacterium* I Abt Orig Zentralbl Bakteriöl Parasitenkd Infektionskr Hyg. 89, 4–22 (1922).
73. M. Knorr, Über die fusospirilläre symbiose, die Gattung *Fusobacterium* (KB Lehmann) und *Spirillum sputigenum*. II Mitteilung. Die Gattung *Fusobacterium*. Zentbl Bakteriöl Parasitenkd Infekt Hyg Abt.1, 4–22 (1923).
74. J.L. Dzink, M.T. Sheenan, S.S. Socransky, Proposal of three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. *nucleatum* subsp. nov., comb. nov.; *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. Rev., comb. nov.; and *Fusobacterium nucleatum* subsp. *vincentii* subsp. nov., nom. rev., comb. nov. *Int J Syst Evol Microbiol. Microbiology Society* 40, 74–78 (1990).
75. A. Manson McGuire, K. Cochrane, A.D. Griggs, B.J. Haas, T. Abeel, Q. Zeng, J.B. Nice, H. MacDonald, B.W. Birren, B.W. Berger, E. Allen-Vercoe, A.M. Earl, Evolution of invasion in a diverse set of *Fusobacterium* species. *mBio.*;5: e01864-14 (2014).
76. S. Yamaguchi, S. Yokoyama, M. Ueno, S. Hayami, Y. Mitani, A. Takeuchi, J.E. Shively, H. Yamaue, H. CEACAM1 is associated with recurrence after hepatectomy for colorectal liver metastasis. *J. Surg. Res.* 220, 353–362 (2017).

Chapter 4

1. Adamska, A., Domenichini, A. & Falasca, M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. *Int. J. Mol. Sci.* 18, 1338 (2017).
2. Buscail, L., Bournet, B. & Cordelier, P. Role of oncogenic KRAS in the diagnosis, prognosis and treatment of pancreatic cancer. *Nat. Rev. Gastroenterol. Hepatol.* 17, 153–168 (2020).
3. Singh, K. et al. Kras mutation rate precisely orchestrates ductal derived pancreatic intraepithelial neoplasia and pancreatic cancer. *Lab. Invest.* 101, 177–192 (2021).
4. Waters, A. M. & Der, C. J. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb. Perspect. Med.* 8, a031435 (2018).
5. Ho, W. J., Jaffee, E. M. & Zheng, L. The tumour microenvironment in pancreatic cancer - clinical challenges and opportunities. *Nat. Rev. Clin. Oncol.* (2020) doi:10.1038/s41571-020-0363-5.
6. Liu, Q., Liao, Q. & Zhao, Y. Chemotherapy and tumor microenvironment of pancreatic cancer. *Cancer Cell Int.* 17, 68 (2017).
7. Du, W., Pasca di Magliano, M. & Zhang, Y. Therapeutic Potential of Targeting Stromal Crosstalk-Mediated Immune Suppression in Pancreatic Cancer. *Front. Oncol.* 11, 2606 (2021).
8. Kemp, S. B., Magliano, M. P. di & Crawford, H. C. Myeloid Cell Mediated Immune Suppression in Pancreatic Cancer. *Cell. Mol. Gastroenterol. Hepatol.* 12, 1531–1542 (2021).
9. Guo, W. et al. Tumor microbiome contributes to an aggressive phenotype in the basal-like subtype of pancreatic cancer. *Commun. Biol.* 4, 1–13 (2021).
10. Pushalkar, S. et al. The Pancreatic Cancer Microbiome Promotes Oncogenesis by Induction of Innate and Adaptive Immune Suppression. *Cancer Discov.* 8, 403–416 (2018).
11. Wei, M.-Y. et al. The microbiota and microbiome in pancreatic cancer: more influential than expected. *Mol. Cancer* 18, 97 (2019).
12. Nejman, D. et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. *Science* 368, 973–980 (2020).
13. Udayasuryan, B., Nguyen, T. T. D., Slade, D. J. & Verbridge, S. S. Harnessing Tissue Engineering Tools to Interrogate Host-Microbiota Crosstalk in Cancer. *iScience* 101878 (2020) doi:10.1016/j.isci.2020.101878.
14. Xavier, J. B. et al. The Cancer Microbiome: Distinguishing Direct and Indirect Effects Requires a Systemic View. *Trends Cancer* 6, 192–204 (2020).
15. Farrell, J. J. et al. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut* 61, 582–588 (2012).
16. Otieno, S. et al. Positive association between *Helicobacter pylori* infection and pancreatic cancer: A case-control study. *J. Clin. Oncol.* 39, e16243–e16243 (2021).
17. Riquelme, E. et al. Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. *Cell* 178, 795–806.e12 (2019).
18. Geller, L. T. et al. Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. *Science* 357, 1156–1160 (2017).
19. Brennan, C. A. & Garrett, W. S. *Fusobacterium nucleatum* - symbiont, opportunist and oncobacterium. *Nat. Rev. Microbiol.* 17, 156–166 (2019).
20. Han, Y. W. et al. *Fusobacterium nucleatum* Induces Premature and Term Stillbirths in Pregnant Mice: Implication of Oral Bacteria in Preterm Birth. *Infect. Immun.* 72, 2272–2279 (2004).
21. Swidsinski, A. et al. Acute appendicitis is characterised by local invasion with *Fusobacterium nucleatum/necrophorum*. *Gut* 60, 34–40 (2011).
22. Shammass, N. W. et al. Infective endocarditis due to *Fusobacterium nucleatum*: case report and review of the literature. *Clin. Cardiol.* 16, 72–75 (1993).
23. Li, Q. et al. Oral Pathogen *Fusobacterium nucleatum* Coaggregates With *Pseudomonas aeruginosa* to Modulate the Inflammatory Cytotoxicity of Pulmonary Epithelial Cells. *Front. Cell. Infect. Microbiol.* 11, 643913 (2021).
24. Kostic, A. D. et al. Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma. *Genome Res.* 22, 292–298 (2012).
25. Mima, K. et al. *Fusobacterium nucleatum* in colorectal carcinoma tissue and patient prognosis. *Gut* 65, 1973–1980 (2016).

26. Yamamura, K. et al. Human Microbiome *Fusobacterium Nucleatum* in Esophageal Cancer Tissue Is Associated with Prognosis. *Clin. Cancer Res.* 22, 5574–5581 (2016).
27. Gaiser, R. A. et al. Enrichment of oral microbiota in early cystic precursors to invasive pancreatic cancer. *Gut* 68, 2186–2194 (2019).
28. Mitsuhashi, K. et al. Association of *Fusobacterium* species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget* 6, 7209–7220 (2015).
29. Boehm, E. T. et al. *Fusobacterium nucleatum* is associated with worse prognosis in Lauren's diffuse type gastric cancer patients. *Sci. Rep.* 10, 16240 (2020).
30. Abed, J. et al. Fap2 Mediates *Fusobacterium nucleatum* Colorectal Adenocarcinoma Enrichment by Binding to Tumor-Expressed Gal-GalNAc. *Cell Host Microbe* 20, 215–225 (2016).
31. Abed, J. et al. Tumor Targeting by *Fusobacterium nucleatum*: A Pilot Study and Future Perspectives. *Front. Cell. Infect. Microbiol.* 7, (2017).
32. Parhi, L. et al. Breast cancer colonization by *Fusobacterium nucleatum* accelerates tumor growth and metastatic progression. *Nat. Commun.* 11, 3259 (2020).
33. Shamsuddin, A. M., Tyner, G. T. & Yang, G. Y. Common expression of the tumor marker D-galactose-beta-[1-->3]-N-acetyl-D-galactosamine by different adenocarcinomas: evidence of field effect phenomenon. *Cancer Res.* 55, 149–152 (1995).
34. Rubinstein, M. R. et al. *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/ β -catenin signaling via its FadA adhesin. *Cell Host Microbe* 14, 195–206 (2013).
35. Salvucci, M. et al. Patients with mesenchymal tumours and high *Fusobacteriales* prevalence have worse prognosis in colorectal cancer (CRC). *Gut* (2021) doi:10.1136/gutjnl-2021-325193.
36. Bullman, S. et al. Analysis of *Fusobacterium* persistence and antibiotic response in colorectal cancer. *Science* 358, 1443–1448 (2017).
37. Casasanta, M. A. et al. *Fusobacterium nucleatum* host-cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. *Sci. Signal.* 13, (2020).
38. Alkharaan, H. et al. Circulating and Salivary Antibodies to *Fusobacterium nucleatum* Are Associated With Cystic Pancreatic Neoplasm Malignancy. *Front. Immunol.* 11, (2020).
39. Murphy, K. J., Chambers, C. R., Herrmann, D., Timpson, P. & Pereira, B. A. Dynamic Stromal Alterations Influence Tumor-Stroma Crosstalk to Promote Pancreatic Cancer and Treatment Resistance. *Cancers* 13, 3481 (2021).
40. Bulle, A. & Lim, K.-H. Beyond just a tight fortress: contribution of stroma to epithelial-mesenchymal transition in pancreatic cancer. *Signal Transduct. Target. Ther.* 5, 1–12 (2020).
41. Thomas, D. & Radhakrishnan, P. Tumor-stromal crosstalk in pancreatic cancer and tissue fibrosis. *Mol. Cancer* 18, 14 (2019).
42. Deer, E. L. et al. Phenotype and Genotype of Pancreatic Cancer Cell Lines. *Pancreas* 39, 425–435 (2010).
43. Umaña, A. et al. Utilizing Whole *Fusobacterium* Genomes To Identify, Correct, and Characterize Potential Virulence Protein Families. *J. Bacteriol.* 201, (2019).
44. Han, Y. W. et al. Interactions between Periodontal Bacteria and Human Oral Epithelial Cells: *Fusobacterium nucleatum* Adheres to and Invades Epithelial Cells. *Infect. Immun.* 68, 3140–3146 (2000).
45. Gur, C. et al. Binding of the Fap2 protein of *Fusobacterium nucleatum* to human inhibitory receptor TIGIT protects tumors from immune cell attack. *Immunity* 42, 344–355 (2015).
46. Gur, C. et al. *Fusobacterium nucleatum* suppresses anti-tumor immunity by activating CEACAM1. *Oncoimmunology* 8, e1581531 (2019).
47. Copenhagen-Glazer, S. et al. Fap2 of *Fusobacterium nucleatum* is a galactose-inhibitable adhesin involved in coaggregation, cell adhesion, and preterm birth. *Infect. Immun.* 83, 1104–1113 (2015).
48. Park, J., Shokeen, B., Haake, S. K. & Lux, R. Characterization of *Fusobacterium nucleatum* ATCC 23726 adhesins involved in strain-specific attachment to *Porphyromonas gingivalis*. *Int. J. Oral Sci.* 8, 138–144 (2016).
49. Berraondo, P. et al. Cytokines in clinical cancer immunotherapy. *Br. J. Cancer* 120, 6–15 (2019).
50. Dranoff, G. Cytokines in cancer pathogenesis and cancer therapy. *Nat. Rev. Cancer* 4, 11–22 (2004).
51. Yuan, A., Chen, J. J. W., Yao, P.-L. & Yang, P.-C. The role of interleukin-8 in cancer cells and microenvironment interaction. *Front. Biosci. J. Virtual Libr.* 10, 853–865 (2005).
52. Acharyya, S. et al. A CXCL1 Paracrine Network Links Cancer Chemoresistance and Metastasis. *Cell* 150, 165–178 (2012).

53. Wang, D., Sun, H., Wei, J., Cen, B. & DuBois, R. N. CXCL1 Is Critical for Premetastatic Niche Formation and Metastasis in Colorectal Cancer. *Cancer Res.* 77, 3655–3665 (2017).
54. Korbecki, J., Grochans, S., Gutowska, I., Barczak, K. & Baranowska-Bosiacka, I. CC Chemokines in a Tumor: A Review of Pro-Cancer and Anti-Cancer Properties of Receptors CCR5, CCR6, CCR7, CCR8, CCR9, and CCR10 Ligands. *Int. J. Mol. Sci.* 21, E7619 (2020).
55. Ghosh, S. K., Gupta, S., Jiang, B. & Weinberg, A. *Fusobacterium nucleatum* and human beta-defensins modulate the release of antimicrobial chemokine CCL20/macrophage inflammatory protein 3 α . *Infect. Immun.* 79, 4578–4587 (2011).
56. Hong, I.-S. Stimulatory versus suppressive effects of GM-CSF on tumor progression in multiple cancer types. *Exp. Mol. Med.* 48, e242–e242 (2016).
57. Zhan, Y., Lew, A. M. & Chopin, M. The Pleiotropic Effects of the GM-CSF Rheostat on Myeloid Cell Differentiation and Function: More Than a Numbers Game. *Front. Immunol.* 10, 2679 (2019).
58. Thorn, M. et al. Tumor-associated GM-CSF overexpression induces immunoinhibitory molecules via STAT3 in myeloid-suppressor cells infiltrating liver metastases. *Cancer Gene Ther.* 23, 188–198 (2016).
59. Yako, Y. Y., Kruger, D., Smith, M. & Brand, M. Cytokines as Biomarkers of Pancreatic Ductal Adenocarcinoma: A Systematic Review. *PLOS ONE* 11, e0154016 (2016).
60. Pylayeva-Gupta, Y., Lee, K. E., Hajdu, C. H., Miller, G. & Bar-Sagi, D. Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. *Cancer Cell* 21, 836–847 (2012).
61. Bayne, L. J. et al. Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer Cell* 21, 822–835 (2012).
62. Waghray, M. et al. GM-CSF Mediates Mesenchymal-Epithelial Cross-talk in Pancreatic Cancer. *Cancer Discov.* 6, 886–899 (2016).
63. Takeuchi, S. et al. Chemotherapy-Derived Inflammatory Responses Accelerate the Formation of Immunosuppressive Myeloid Cells in the Tissue Microenvironment of Human Pancreatic Cancer. *Cancer Res.* 75, 2629–2640 (2015).
64. Campbell, A. S., Albo, D., Kimsey, T. F., White, S. L. & Wang, T. N. Macrophage inflammatory protein-3 α promotes pancreatic cancer cell invasion. *J. Surg. Res.* 123, 96–101 (2005).
65. Takamori, H., Oades, Z. G., Hoch, O. C., Burger, M. & Schraufstatter, I. U. Autocrine growth effect of IL-8 and GRO α on a human pancreatic cancer cell line, Capan-1. *Pancreas* 21, 52–56 (2000).
66. Sepich-Poore, G. D. et al. The microbiome and human cancer. *Science* 371, eabc4552 (2021).
67. Liot, S. et al. Stroma Involvement in Pancreatic Ductal Adenocarcinoma: An Overview Focusing on Extracellular Matrix Proteins. *Front. Immunol.* 12, (2021).
68. Jiang, B. et al. Stroma-Targeting Therapy in Pancreatic Cancer: One Coin With Two Sides? *Front. Oncol.* 10, (2020).
69. Datta, R. et al. Interactions with stromal cells promote a more oxidized cancer cell redox state in pancreatic tumors. *Sci. Adv.* 8, eabg6383.
70. Sherman, M. H. et al. Stromal cues regulate the pancreatic cancer epigenome and metabolome. *Proc. Natl. Acad. Sci. U. S. A.* 114, 1129–1134 (2017).
71. Li, R., Wen, A. & Lin, J. Pro-Inflammatory Cytokines in the Formation of the Pre-Metastatic Niche. *Cancers* 12, 3752 (2020).
72. Cheung, K. J. & Ewald, A. J. A collective route to metastasis: Seeding by tumor cell clusters. *Science* (2016) doi:10.1126/science.aaf6546.
73. Wrenn, E. D. et al. Regulation of Collective Metastasis by Nanolumenal Signaling. *Cell* 183, 395–410.e19 (2020).
74. Weiss, F., Lauffenburger, D. & Friedl, P. Towards targeting of shared mechanisms of cancer metastasis and therapy resistance. *Nat. Rev. Cancer* (2022) doi:10.1038/s41568-021-00427-0.
75. Rubinstein, M. R. et al. *Fusobacterium nucleatum* promotes colorectal cancer by inducing Wnt/ β -catenin modulator Annexin A1. *EMBO Rep.* 20, (2019).
76. Yang, Y. et al. *Fusobacterium nucleatum* Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor- κ B, and Up-regulating Expression of MicroRNA-21. *Gastroenterology* 152, 851–866.e24 (2017).
77. Zhang, W. & Liu, H. T. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res.* 12, 9–18 (2002).
78. Al-Rashed, F., Thomas, R., Al-Roub, A., Al-Mulla, F. & Ahmad, R. LPS Induces GM-CSF Production by

- Breast Cancer MDA-MB-231 Cells via Long-Chain Acyl-CoA Synthetase 1. *Mol. Basel Switz.* 25, E4709 (2020).
79. Kolonics, A. et al. Activation of Raf/ERK1/2 MAP kinase pathway is involved in GM-CSF-induced proliferation and survival but not in erythropoietin-induced differentiation of TF-1 cells. *Cell. Signal.* 13, 743–754 (2001).
 80. Brunton, H., Caligiuri, G., Inman, G. J. & Bailey, P. PDAC Subtypes/Stratification. in *Translational Pancreatic Cancer Research: From Understanding of Mechanisms to Novel Clinical Trials* (eds. Michalski, C. W., Rosendahl, J., Michl, P. & Kleeff, J.) 117–128 (Springer International Publishing, 2020). doi:10.1007/978-3-030-49476-6_8.
 81. Collisson, E. A., Bailey, P., Chang, D. K. & Biankin, A. V. Molecular subtypes of pancreatic cancer. *Nat. Rev. Gastroenterol. Hepatol.* 16, 207–220 (2019).
 82. Flowers, B. M. et al. Cell of Origin Influences Pancreatic Cancer Subtype. *Cancer Discov.* 11, 660–677 (2021).
 83. Shurer, C. R. et al. Physical Principles of Membrane Shape Regulation by the Glycocalyx. *Cell* 177, 1757–1770.e21 (2019).

Chapter 5

1. Luo Z, Li Y, Wang H, Fleming J, Li M, Kang Y, Zhang R, Li D. Hepatocyte nuclear factor 1A (HNF1A) as a possible tumor suppressor in pancreatic cancer. *PLoS One.* 2015 Mar 20;10(3):e0121082. doi: 10.1371/journal.pone.0121082. PMID: 25793983; PMCID: PMC4368635.
2. Cui XG, Han ZT, He SH, et al. HIF1/2 α mediates hypoxia-induced LDHA expression in human pancreatic cancer cells. *Oncotarget.* 2017;8(15):24840–24852. doi:10.18632/oncotarget.15266
3. Park HS, Kim JH, Sun BK, Song SU, Suh W and Sung JH: Hypoxia induces glucose uptake and metabolism of adipose-derived stem cells. *Mol Med Rep* 14: 4706–4714, 2016
4. Siegel, R. L., Miller, K. D., Fuchs, H. E. & Jemal, A. Cancer statistics, 2022. *CA. Cancer J. Clin.* 72, 7–33 (2022).
5. Guinney, J. et al. The consensus molecular subtypes of colorectal cancer. *Nat. Med.* 21, 1350–1356 (2015).
6. Menter, D. G. et al. Back to the Colorectal Cancer Consensus Molecular Subtype Future. *Curr. Gastroenterol. Rep.* 21, 5 (2019).
7. Van der Jeught, K., Xu, H.-C., Li, Y.-J., Lu, X.-B. & Ji, G. Drug resistance and new therapies in colorectal cancer. *World J. Gastroenterol.* 24, 3834–3848 (2018).
8. Saus, E., Iraola-Guzmán, S., Willis, J. R., Brunet-Vega, A. & Gabaldón, T. Microbiome and colorectal cancer: Roles in carcinogenesis and clinical potential. *Mol. Aspects Med.* 69, 93–106 (2019).
9. Ternes, D. et al. Microbiome in Colorectal Cancer: How to Get from Meta-omics to Mechanism? *Trends Microbiol.* 28, 401–423 (2020).
10. Kadosh, E. et al. The gut microbiome switches mutant p53 from tumour-suppressive to oncogenic. *Nature* 1–6 (2020) doi:10.1038/s41586-020-2541-0.
9. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 12, 31–46 (2022).
10. Nejman, D. et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. *Science* 368, 973–980 (2020).
11. Udayasuryan, B., Nguyen, T. T. D., Slade, D. J. & Verbridge, S. S. Harnessing Tissue Engineering Tools to Interrogate Host-Microbiota Crosstalk in Cancer. *iScience* 101878 (2020) doi:10.1016/j.isci.2020.101878.
12. Cheng, Y., Ling, Z. & Li, L. The Intestinal Microbiota and Colorectal Cancer. *Front. Immunol.* 11, (2020).
13. Brennan, C. A. & Garrett, W. S. *Fusobacterium nucleatum* - symbiont, opportunist and oncobacterium. *Nat. Rev. Microbiol.* 17, 156–166 (2019).
14. Han, Y. W. *Fusobacterium nucleatum*: a commensal-turned pathogen. *Curr. Opin. Microbiol.* 0, 141–147 (2015).
15. Storm, J. C., Ford, B. A. & Streit, J. A. Myocardial infection due to *Fusobacterium nucleatum*. *Diagn. Microbiol. Infect. Dis.* 77, 373–375 (2013).
16. Vander Haar, E. L., So, J., Gyamfi-Bannerman, C. & Han, Y. W. *Fusobacterium nucleatum* and adverse

- pregnancy outcomes: Epidemiological and mechanistic evidence. *Anaerobe* 50, 55–59 (2018).
17. Mitsuhashi, K. *et al.* Association of Fusobacterium species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget* 6, 7209–7220 (2015).
 18. Yamamura, K. *et al.* Intratumoral Fusobacterium Nucleatum Levels Predict Therapeutic Response to Neoadjuvant Chemotherapy in Esophageal Squamous Cell Carcinoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 25, 6170–6179 (2019).
 19. Yamamura, K. *et al.* Human Microbiome Fusobacterium Nucleatum in Esophageal Cancer Tissue Is Associated with Prognosis. *Clin. Cancer Res.* 22, 5574–5581 (2016).
 20. Boehm, E. T. *et al.* Fusobacterium nucleatum is associated with worse prognosis in Lauren’s diffuse type gastric cancer patients. *Sci. Rep.* 10, 16240 (2020).
 21. Kostic, A. D. *et al.* Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res.* 22, 292–298 (2012).
 22. Kostic, A. D. *et al.* Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 14, 207–215 (2013).
 23. Mima, K. *et al.* Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* 65, 1973–1980 (2016).
 24. Serna, G. *et al.* Fusobacterium nucleatum persistence and risk of recurrence after preoperative treatment in locally advanced rectal cancer. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* 31, 1366–1375 (2020).
 25. Yu, T. *et al.* Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. *Cell* 170, 548–563.e16 (2017).
 26. Abed, J. *et al.* Fap2 Mediates Fusobacterium nucleatum Colorectal Adenocarcinoma Enrichment by Binding to Tumor-Expressed Gal-GalNAc. *Cell Host Microbe* 20, 215–225 (2016).
 27. Parhi, L. *et al.* Breast cancer colonization by Fusobacterium nucleatum accelerates tumor growth and metastatic progression. *Nat. Commun.* 11, 3259 (2020).
 28. Parhi, L. *et al.* Placental colonization by Fusobacterium nucleatum is mediated by binding of the Fap2 lectin to placentally displayed Gal-GalNAc. *Cell Rep.* 38, 110537 (2022).
 29. Rubinstein, M. R. *et al.* Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/ β -catenin signaling via its FadA adhesin. *Cell Host Microbe* 14, 195–206 (2013).
 30. Meng, Q. *et al.* Fusobacterium nucleatum secretes amyloid-like FadA to enhance pathogenicity. *EMBO Rep.* 22, e52891 (2021).
 31. Colonne, P. M., Winchell, C. G. & Voth, D. E. Hijacking Host Cell Highways: Manipulation of the Host Actin Cytoskeleton by Obligate Intracellular Bacterial Pathogens. *Front. Cell. Infect. Microbiol.* 6, (2016).
 32. Casasanta, M. A. *et al.* Fusobacterium nucleatum host-cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. *Sci. Signal.* 13, (2020).
 33. Udayasuryan, B. *et al.* Fusobacterium nucleatum induces pancreatic cancer cell proliferation and migration by regulating host autocrine and paracrine signaling. 2021.11.19.469245 (2022) doi:10.1101/2021.11.19.469245.
 34. Bullman, S. *et al.* Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. *Science* 358, 1443–1448 (2017).
 35. Zhang, Y. *et al.* Fusobacterium nucleatum promotes colorectal cancer cells adhesion to endothelial cells and facilitates extravasation and metastasis by inducing ALPK1/NF- κ B/ICAM1 axis. *Gut Microbes* 14, 2038852 (2022).
 36. Queen, J. *et al.* Comparative Analysis of Colon Cancer-Derived Fusobacterium nucleatum Subspecies: Inflammation and Colon Tumorigenesis in Murine Models. *mBio* e0299121 (2022) doi:10.1128/mbio.02991-21.
 37. McKeown, S. R. Defining normoxia, physoxia and hypoxia in tumours—implications for treatment response. *Br. J. Radiol.* 87, 20130676 (2014).
 38. Mi, Y. *et al.* Hypoxic colorectal cancer cells promote metastasis of normoxic cancer cells depending on IL-8/p65 signaling pathway. *Cell Death Dis.* 11, 1–11 (2020).
 39. Qi, L., Chen, J., Yang, Y. & Hu, W. Hypoxia Correlates With Poor Survival and M2 Macrophage Infiltration in Colorectal Cancer. *Front. Oncol.* 10, (2020).
 40. Tímár, J., Sebestyén, A., Kopper, L. & Dankó, T. Hypoxia Signaling in Cancer: From Basics to Clinical Practice. *Pathol. Oncol. Res.* 0, (2021).
 41. Yu, S. *et al.* Hypoxia promotes colorectal cancer cell migration and invasion in a SIRT1-dependent

- manner. *Cancer Cell Int.* 19, 116 (2019).
42. Xu, K. *et al.* Hypoxia Induces Drug Resistance in Colorectal Cancer through the HIF-1 α /miR-338-5p/IL-6 Feedback Loop. *Mol. Ther.* 27, 1810–1824 (2019).
 43. Hajdamowicz, N. H., Hull, R. C., Foster, S. J. & Condliffe, A. M. The Impact of Hypoxia on the Host-Pathogen Interaction between Neutrophils and *Staphylococcus aureus*. *Int. J. Mol. Sci.* 20, 5561 (2019).
 44. Zeitouni, N. E., Chotikatum, S., von Köckritz-Blickwede, M. & Naim, H. Y. The impact of hypoxia on intestinal epithelial cell functions: consequences for invasion by bacterial pathogens. *Mol. Cell. Pediatr.* 3, 14 (2016).
 45. Kasper, S. H. *et al.* Colorectal cancer-associated anaerobic bacteria proliferate in tumor spheroids and alter the microenvironment. *Sci. Rep.* 10, 5321 (2020).
 46. Despins, C. A. *et al.* Modulation of the Host Cell Transcriptome and Epigenome by *Fusobacterium nucleatum*. *mBio* 12, e0206221 (2021).
 47. Ahn, S.-H. *et al.* Transcriptome profiling analysis of senescent gingival fibroblasts in response to *Fusobacterium nucleatum* infection. *PLOS ONE* 12, e0188755 (2017).
 48. Kang, W. *et al.* Time-Course Transcriptome Analysis for Drug Repositioning in *Fusobacterium nucleatum*-Infected Human Gingival Fibroblasts. *Front. Cell Dev. Biol.* 7, (2019).
 49. Gao, H. *et al.* The Pathogenic Effects of *Fusobacterium nucleatum* on the Proliferation, Osteogenic Differentiation, and Transcriptome of Osteoblasts. *Front. Cell Dev. Biol.* 8, 807 (2020).
 50. Zhang, S. *et al.* Analysis of differentially expressed genes in oral epithelial cells infected with *Fusobacterium nucleatum* for revealing genes associated with oral cancer. *J. Cell. Mol. Med.* 25, 892–904 (2021).
 51. Zhang, J., Wang, Z., Lv, H. & Li, G. Identification and Validation of Potential Candidate Genes of Colorectal Cancer in Response to *Fusobacterium nucleatum* Infection. *Front. Genet.* 12, (2021).
 52. Ponath, F. *et al.* RNA landscape of the emerging cancer-associated microbe *Fusobacterium nucleatum*. *Nat. Microbiol.* 6, 1007–1020 (2021).
 53. Cochrane, K., Robinson, A. V., Holt, R. A. & Allen-Vercoe, E. A survey of *Fusobacterium nucleatum* genes modulated by host cell infection. *Microb. Genomics* 6, (2020).
 54. Xia, X. *et al.* Bacteria pathogens drive host colonic epithelial cell promoter hypermethylation of tumor suppressor genes in colorectal cancer. *Microbiome* 8, 108 (2020).
 55. Okuda, S. *et al.* Profiling of host genetic alterations and intra-tumor microbiomes in colorectal cancer. *Comput. Struct. Biotechnol. J.* 19, 3330–3338 (2021).
 56. Salvucci, M. *et al.* Patients with mesenchymal tumours and high *Fusobacteriales* prevalence have worse prognosis in colorectal cancer (CRC). *Gut* (2021) doi:10.1136/gutjnl-2021-325193.
 57. Berg, K. C. G. *et al.* Multi-omics of 34 colorectal cancer cell lines - a resource for biomedical studies. *Mol. Cancer* 16, 116 (2017).
 58. Westermann, A. J., Gorski, S. A. & Vogel, J. Dual RNA-seq of pathogen and host. *Nat. Rev. Microbiol.* 10, 618–630 (2012).
 59. Andrysik, Z., Bender, H., Galbraith, M. D. & Espinosa, J. M. Multi-omics analysis reveals contextual tumor suppressive and oncogenic gene modules within the acute hypoxic response. *Nat. Commun.* 12, 1375 (2021).
 60. Smythies, J. A. *et al.* Inherent DNA-binding specificities of the HIF-1 α and HIF-2 α transcription factors in chromatin. *EMBO Rep.* 20, e46401 (2019).
 61. Zhu, B. *et al.* MOWChIP-seq for low-input and multiplexed profiling of genome-wide histone modifications. *Nat. Protoc.* 14, 3366–3394 (2019).
 62. Cui, X.-G. *et al.* HIF1/2 α mediates hypoxia-induced LDHA expression in human pancreatic cancer cells. *Oncotarget* 8, 24840–24852 (2017).
 63. Park, H. S. *et al.* Hypoxia induces glucose uptake and metabolism of adipose-derived stem cells. *Mol. Med. Rep.* 14, 4706–4714 (2016).
 64. Johnson, A. B., Denko, N. & Barton, M. C. Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. *Mutat. Res.* 640, 174–179 (2008).
 65. Prickaerts, P. *et al.* Hypoxia increases genome-wide bivalent epigenetic marking by specific gain of H3K27me3. *Epigenetics Chromatin* 9, 46 (2016).
 66. Chen, H., Yan, Y., Davidson, T. L., Shinkai, Y. & Costa, M. Hypoxic stress induces dimethylated histone H3 lysine 9 through histone methyltransferase G9a in mammalian cells. *Cancer Res.* 66, 9009–9016 (2006).

67. Gao, X. *et al.* Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nat. Commun.* 7, 11960 (2016).
68. Kindrick, J. D. & Mole, D. R. Hypoxic Regulation of Gene Transcription and Chromatin: Cause and Effect. *Int. J. Mol. Sci.* 21, E8320 (2020).
69. Cavadas, M. A. S. *et al.* REST mediates resolution of HIF-dependent gene expression in prolonged hypoxia. *Sci. Rep.* 5, 17851 (2015).
70. Pallante, P. *et al.* The loss of the CBX7 gene expression represents an adverse prognostic marker for survival of colon carcinoma patients. *Eur. J. Cancer Oxf. Engl.* 1990 46, 2304–2313 (2010).
71. Guo, X. & Li, M. LINC01089 is a tumor-suppressive lncRNA in gastric cancer and it regulates miR-27a-3p/TET1 axis. *Cancer Cell Int.* 20, 507 (2020).
72. Li, X. *et al.* LINC01089 Inhibits Tumorigenesis and Epithelial–Mesenchymal Transition of Non-small Cell Lung Cancer via the miR-27a/SFRP1/Wnt/ β -catenin Axis. *Front. Oncol.* 10, (2020).
73. Li, T. *et al.* SEMA6B Overexpression Predicts Poor Prognosis and Correlates With the Tumor Immunosuppressive Microenvironment in Colorectal Cancer. *Front. Mol. Biosci.* 8, 687319 (2021).
74. Visuttijai, K. *et al.* Lowered Expression of Tumor Suppressor Candidate MYO1C Stimulates Cell Proliferation, Suppresses Cell Adhesion and Activates AKT. *PLoS ONE* 11, e0164063 (2016).
75. Chui, A. J. *et al.* Activation of the CARD8 Inflammasome Requires a Disordered Region. *Cell Rep.* 33, 108264 (2020).
76. Chen, Y. *et al.* Fusobacterium nucleatum Promotes Metastasis in Colorectal Cancer by Activating Autophagy Signaling via the Upregulation of CARD3 Expression. *Theranostics* 10, 323–339 (2020).
77. Li, J. *et al.* Elastin is a key factor of tumor development in colorectal cancer. *BMC Cancer* 20, 217 (2020).
78. Zheng, H. *et al.* Tropomodulin 3 modulates EGFR-PI3K-AKT signaling to drive hepatocellular carcinoma metastasis. *Mol. Carcinog.* 58, 1897–1907 (2019).
79. Liu, Z. *et al.* Development and clinical validation of a novel six-gene signature for accurately predicting the recurrence risk of patients with stage II/III colorectal cancer. *Cancer Cell Int.* 21, 359 (2021).
80. Slattery, M. L., Lundgreen, A., Bondurant, K. L. & Wolff, R. K. Interferon-signaling pathway: associations with colon and rectal cancer risk and subsequent survival. *Carcinogenesis* 32, 1660–1667 (2011).
81. Detchokul, S., Williams, E. D., Parker, M. W. & Frauman, A. G. Tetraspanins as regulators of the tumour microenvironment: implications for metastasis and therapeutic strategies. *Br. J. Pharmacol.* 171, 5462–5490 (2014).
82. Takahashi, M., Lin, Y.-M., Nakamura, Y. & Furukawa, Y. Isolation and characterization of a novel gene CLUAP1 whose expression is frequently upregulated in colon cancer. *Oncogene* 23, 9289–9294 (2004).
83. Diao, C. *et al.* SPT6 recruits SND1 to co-activate human telomerase reverse transcriptase to promote colon cancer progression. *Mol. Oncol.* 15, 1180–1202 (2021).
84. Zhu, S. *et al.* Genetic Alterations of TRAF Proteins in Human Cancers. *Front. Immunol.* 9, (2018).
85. Alarmo, E.-L. *et al.* BMP7 influences proliferation, migration, and invasion of breast cancer cells. *Cancer Lett.* 275, 35–43 (2009).
86. Motoyama, K. *et al.* Clinical significance of BMP7 in human colorectal cancer. *Ann. Surg. Oncol.* 15, 1530–1537 (2008).
87. Cortez, M. A. *et al.* Bone morphogenetic protein 7 promotes resistance to immunotherapy. *Nat. Commun.* 11, 4840 (2020).
88. Bach, D.-H., Park, H. J. & Lee, S. K. The Dual Role of Bone Morphogenetic Proteins in Cancer. *Mol. Ther. - Oncolytics* 8, 1–13 (2018).
89. Li, H., Ma, S.-Q., Huang, J., Chen, X.-P. & Zhou, H.-H. Roles of long noncoding RNAs in colorectal cancer metastasis. *Oncotarget* 8, 39859–39876 (2017).
90. Glinsky, G. V., Berezovska, O. & Glinskii, A. B. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J. Clin. Invest.* 115, 1503–1521 (2005).
91. Menyhart, O. *et al.* Uncovering Potential Therapeutic Targets in Colorectal Cancer by Deciphering Mutational Status and Expression of Druggable Oncogenes. *Cancers* 11, 983 (2019).
92. Palin, K. *et al.* Contribution of allelic imbalance to colorectal cancer. *Nat. Commun.* 9, 3664 (2018).
93. Zhu, L. *et al.* TBKBP1 and TBK1 form a growth factor signaling axis mediating immunosuppression and tumorigenesis. *Nat. Cell Biol.* 21, 1604–1614 (2019).
94. Mager, L. F. *et al.* The ESRP1-GPR137 axis contributes to intestinal pathogenesis. *eLife* 6, e28366 (2017).

95. Wang, Z., Zhang, H., Wang, J., Yang, Y. & Wu, Q. RNA interference-mediated silencing of G protein-coupled receptor 137 inhibits human gastric cancer cell growth. *Mol. Med. Rep.* 11, 2578–2584 (2015).
96. Lu, J., Zhong, F., Sun, B. & Wang, C. GPR137 is a promising novel bio-marker for the prognosis of bladder cancer patients. *Medicine (Baltimore)* 98, e16576 (2019).
97. Poulsen, M., Lukas, C., Lukas, J., Bekker-Jensen, S. & Møllgaard, N. Human RNF169 is a negative regulator of the ubiquitin-dependent response to DNA double-strand breaks. *J. Cell Biol.* 197, 189–199 (2012).
98. Mahbub Hasan, A. K. M., Ijiri, T. & Sato, K. Involvement of Src in the Adaptation of Cancer Cells under Microenvironmental Stresses. *J. Signal Transduct.* 2012, 483796 (2012).
99. Kopetz, S. *et al.* Src activity is modulated by oxaliplatin and correlates with outcomes after hepatectomy for metastatic colorectal cancer. *BMC Cancer* 14, 660 (2014).
100. Wang, X.-M. *et al.* The deubiquitinase USP25 supports colonic inflammation and bacterial infection and promotes colorectal cancer. *Nat. Cancer* 1, 811–825 (2020).
101. Cornejo, E., Schlaermann, P. & Mukherjee, S. How to rewire the host cell: A home improvement guide for intracellular bacteria. *J. Cell Biol.* 216, 3931–3948 (2017).
102. Li, J. *et al.* Metastasis and Immune Evasion from Extracellular cGAMP Hydrolysis. *Cancer Discov.* 11, 1212–1227 (2020).
103. Wei, J. *et al.* Overexpression of transcription factor activating enhancer binding protein 4 (TFAP4) predicts poor prognosis for colorectal cancer patients. *Exp. Ther. Med.* 14, 3057–3061 (2017).
104. Zhao, C. *et al.* Large tumor suppressor 2 is a prognostic biomarker and correlated with immune infiltrates in colorectal cancer. *Bioengineered* 12, 11648–11661 (2021).
105. Pan, F., Li, M. & Chen, W. FOXD1 predicts prognosis of colorectal cancer patients and promotes colorectal cancer progression via the ERK 1/2 pathway. *Am. J. Transl. Res.* 10, 1522–1530 (2018).
106. Bala, P. *et al.* Exome sequencing identifies ARID2 as a novel tumor suppressor in early-onset sporadic rectal cancer. *Oncogene* 40, 863–874 (2021).
107. Zhou, L. *et al.* The β -galactoside α 2,6-sialyltransferase 1 (ST6GAL1) inhibits the colorectal cancer metastasis by stabilizing intercellular adhesion molecule-1 via sialylation. *Cancer Manag. Res.* 11, 6185–6199 (2019).
108. Stanilova, S., Stanilov, N., Julianov, A., Manolova, I. & Miteva, L. Transforming growth factor- β 1 gene promoter -509C/T polymorphism in association with expression affects colorectal cancer development and depends on gender. *PLOS ONE* 13, e0201775 (2018).
109. Villalba, M., Evans, S. R., Vidal-Vanaclocha, F. & Calvo, A. Role of TGF- β in metastatic colon cancer: it is finally time for targeted therapy. *Cell Tissue Res.* 370, 29–39 (2017).
110. Ohshima, K. *et al.* Serine racemase enhances growth of colorectal cancer by producing pyruvate from serine. *Nat. Metab.* 2, 81–96 (2020).
111. Gu, Y. *et al.* HOXA13 promotes colon cancer progression through β -catenin-dependent WNT pathway. *Exp. Cell Res.* 395, 112238 (2020).
112. Luo, Y. *et al.* High expression of Rab3D predicts poor prognosis and associates with tumor progression in colorectal cancer. *Int. J. Biochem. Cell Biol.* 75, 53–62 (2016).
113. Zhou, L., Jiang, Y., Luo, Q., Li, L. & Jia, L. Neddylation: a novel modulator of the tumor microenvironment. *Mol. Cancer* 18, 77 (2019).
114. Bayrer, J. R., Mukkamala, S., Sablin, E. P., Webb, P. & Fletterick, R. J. Silencing LRH-1 in colon cancer cell lines impairs proliferation and alters gene expression programs. *Proc. Natl. Acad. Sci. U. S. A.* 112, 2467–2472 (2015).
115. Liu, L. *et al.* Nr5a2 promotes tumor growth and metastasis of gastric cancer AGS cells by Wnt/ β -catenin signaling. *Oncotargets Ther.* 12, 2891–2902 (2019).
116. Zhang, K., Xiao, M., Jin, X. & Jiang, H. NR5A2 Is One of 12 Transcription Factors Predicting Prognosis in HNSCC and Regulates Cancer Cell Proliferation in a p53-Dependent Manner. *Front. Oncol.* 11, (2021).
117. Matsuyama, T. *et al.* MUC12 mRNA expression is an independent marker of prognosis in stage II and stage III colorectal cancer. *Int. J. Cancer* 127, 2292–2299 (2010).
118. Lu, X., Yu, Y., Liao, F. & Tan, S. Homo Sapiens Circular RNA 0079993 (hsa_circ_0079993) of the POLR2J4 Gene Acts as an Oncogene in Colorectal Cancer Through the microRNA-203a-3p.1 and CREB1 Axis. *Med. Sci. Monit. Int. Med. J. Exp. Clin. Res.* 25, 6872–6883 (2019).
119. Xue, D. *et al.* LINC00641 induces the malignant progression of colorectal carcinoma through the miRNA-424-5p/PLSCR4 feedback loop. *Eur. Rev. Med. Pharmacol. Sci.* 25, 749–757 (2021).
120. Romani, P., Ignesti, M., Gargiulo, G., Hsu, T. & Cavaliere, V. Extracellular NME proteins: a player or a

- bystander? *Lab. Invest.* 98, 248–257 (2018).
121. Ding, L. *et al.* Downregulation of cyclooxygenase-1 stimulates mitochondrial apoptosis through the NF- κ B signaling pathway in colorectal cancer cells. *Oncol. Rep.* 41, 559–569 (2019).
122. Xu, D. *et al.* EphB6 overexpression and Apc mutation together promote colorectal cancer. *Oncotarget* 7, 31111–31121 (2016).
123. He, X. *et al.* Adenovirus-mediated overexpression FADD induces a significant antitumor effect on human colorectal cancer cells both in vitro and in vivo. *Cell. Mol. Biol. Noisy--Gd. Fr.* 64, 31–35 (2018).
124. Yin, A., Jiang, Y., Zhang, X. & Luo, H. Overexpression of FADD enhances 5-fluorouracil-induced apoptosis in colorectal adenocarcinoma cells. *Med. Oncol. Northwood Lond. Engl.* 27, 397–405 (2010).
125. Xiang, T., Yu, F., Fei, R., Qian, J. & Chen, W. CHRNA7 inhibits cell invasion and metastasis of LoVo human colorectal cancer cells through PI3K/Akt signaling. *Oncol. Rep.* 35, 999–1005 (2016).
126. Abell, A. N. *et al.* MAP3K4/CBP-Regulated H2B Acetylation Controls Epithelial-Mesenchymal Transition in Trophoblast Stem Cells. *Cell Stem Cell* 8, 525–537 (2011).
127. Yang, L.-X. *et al.* Mitogen-activated protein kinase kinase 4 deficiency in intrahepatic cholangiocarcinoma leads to invasive growth and epithelial-mesenchymal transition. *Hepatology* 62, 1804–1816 (2015).
128. Pluciennik, E. *et al.* WWOX--the FRA16D cancer gene: expression correlation with breast cancer progression and prognosis. *Eur. J. Surg. Oncol. J. Eur. Soc. Surg. Oncol. Br. Assoc. Surg. Oncol.* 32, 153–157 (2006).
129. Żelazowski, M. J. *et al.* WWOX expression in colorectal cancer—a real-time quantitative RT-PCR study. *Tumour Biol.* 32, 551–560 (2011).
130. Alsop, A. E. *et al.* Homozygous deletions may be markers of nearby heterozygous mutations: The complex deletion at FRA16D in the HCT116 colon cancer cell line removes exons of WWOX. *Genes. Chromosomes Cancer* 47, 437–447 (2008).
131. Ding, C. *et al.* The PEA3-PPP1R12B axis inhibits tumor growth and metastasis by regulating Grb2/PI3K/Akt signalling in colorectal cancer. *Cancer Lett.* 442, 383–395 (2019).
132. Kalyana-Sundaram, S. *et al.* Expressed pseudogenes in the transcriptional landscape of human cancers. *Cell* 149, 1622–1634 (2012).
133. Smolarczyk, R., Cichoń, T., Jarosz, M. & Szala, S. [HMGB1--its role in tumor progression and anticancer therapy]. *Postepy Hig. Med. Doswiadczalnej Online* 66, 913–920 (2012).
134. Du, W. *et al.* Loss of optineurin drives cancer immune evasion via palmitoylation-dependent IFNGR1 lysosomal sorting and degradation. *Cancer Discov.* 11, 1826–1843 (2021).
135. Göransson, M. *et al.* The myxoid liposarcoma FUS-DDIT3 fusion oncoprotein deregulates NF-kappaB target genes by interaction with NFKBIZ. *Oncogene* 28, 270–278 (2009).
136. Ling, A. *et al.* TAP1 down-regulation elicits immune escape and poor prognosis in colorectal cancer. *Oncoimmunology* 6, e1356143 (2017).
137. Li, C. *et al.* Regulation of SIRT2 by Wnt/ β -catenin signaling pathway in colorectal cancer cells. *Biochim. Biophys. Acta Mol. Cell Res.* 1868, 118966 (2021).
138. Bierne, H. & Hamon, M. Targeting host epigenetic machinery: The Listeria paradigm. *Cell. Microbiol.* 22, e13169 (2020).
139. Eskandarian, H. A. *et al.* A role for SIRT2-dependent histone H3K18 deacetylation in bacterial infection. *Science* 341, 1238858 (2013).
140. Levy, E. *et al.* HLA-E over-expression in colorectal cancer patients decreases disease free survival. *Cancer Res.* 67, 2751 (2007).
141. Zeestraten, E. C. M. *et al.* Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. *Br. J. Cancer* 110, 459–468 (2014).
142. Baig, M. S. *et al.* NOS1-derived nitric oxide promotes NF- κ B transcriptional activity through inhibition of suppressor of cytokine signaling-1. *J. Exp. Med.* 212, 1725–1738 (2015).
143. Li, C. *et al.* Wnt7a Promotes the Occurrence and Development of Colorectal Adenocarcinoma. *Front. Oncol.* 11, 522899 (2021).
144. Nie, X., Liu, H., Liu, L., Wang, Y.-D. & Chen, W.-D. Emerging Roles of Wnt Ligands in Human Colorectal Cancer. *Front. Oncol.* 10, (2020).
145. Sun, L. *et al.* 2-Hydroxylation of Fatty Acids Represses Colorectal Tumorigenesis and Metastasis via the YAP Transcriptional Axis. *Cancer Res.* 81, 289–302 (2021).
146. Mukai, S. *et al.* Overexpression of Transmembrane Protein BST2 is Associated with Poor Survival of

- Patients with Esophageal, Gastric, or Colorectal Cancer. *Ann. Surg. Oncol.* 24, 594–602 (2017).
147. Yang, Y. *et al.* Dysregulation of over-expressed IL-32 in colorectal cancer induces metastasis. *World J. Surg. Oncol.* 13, 146 (2015).
148. Würfel, F. M. *et al.* HLA-J, a Non-Pseudogene as a New Prognostic Marker for Therapy Response and Survival in Breast Cancer. *Geburtshilfe Frauenheilkd.* 80, 1123–1133 (2020).
149. Celik-Selvi, B. E. *et al.* Sprouty3 and Sprouty4, Two Members of a Family Known to Inhibit FGF-Mediated Signaling, Exert Opposing Roles on Proliferation and Migration of Glioblastoma-Derived Cells. *Cells* 8, 808 (2019).
150. Du, W. *et al.* STAT5 isoforms regulate colorectal cancer cell apoptosis via reduction of mitochondrial membrane potential and generation of reactive oxygen species. *J. Cell. Physiol.* 227, 2421–2429 (2012).
151. Xiong, H. *et al.* Inhibition of STAT5 induces G1 cell cycle arrest and reduces tumor cell invasion in human colorectal cancer cells. *Lab. Investig. J. Tech. Methods Pathol.* 89, 717–725 (2009).
152. Ortiz-Padilla, C. *et al.* Functional characterization of cancer-associated Gab1 mutations. *Oncogene* 32, 2696–2702 (2013).
153. Seiden-Long, I. *et al.* Gab1 but not Grb2 mediates tumor progression in Met overexpressing colorectal cancer cells. *Carcinogenesis* 29, 647–655 (2008).
154. Xu, H. *et al.* PDS5B inhibits cell proliferation, migration, and invasion via upregulation of LATS1 in lung cancer cells. *Cell Death Discov.* 7, 1–8 (2021).
155. Kotulak, A. *et al.* Decreased expression of p73 in colorectal cancer. *Folia Histochem. Cytobiol.* 54, 166–170 (2016).
156. Corvinus, F. M. *et al.* Persistent STAT3 activation in colon cancer is associated with enhanced cell proliferation and tumor growth. *Neoplasia N. Y. N* 7, 545–555 (2005).
157. Liu, X. *et al.* GATA2 rs2335052 Polymorphism Predicts the Survival of Patients with Colorectal Cancer. *PloS One* 10, e0136020 (2015).
158. Lee, S. A., Liu, F., Riordan, S. M., Lee, C. S. & Zhang, L. Global Investigations of *Fusobacterium nucleatum* in Human Colorectal Cancer. *Front. Oncol.* 9, (2019).
159. Srikantan, S. & Gorospe, M. HuR function in disease. *Front. Biosci. Landmark Ed.* 17, 189–205 (2012).
160. Canté-Barrett, K., Pieters, R. & Meijerink, J. P. P. Myocyte enhancer factor 2C in hematopoiesis and leukemia. *Oncogene* 33, 403–410 (2014).
161. Sturtzel, C., Testori, J., Schweighofer, B., Bilban, M. & Hofer, E. The Transcription Factor MEF2C Negatively Controls Angiogenic Sprouting of Endothelial Cells Depending on Oxygen. *PLOS ONE* 9, e101521 (2014).
162. Luo, Z. *et al.* Hepatocyte Nuclear Factor 1A (HNF1A) as a Possible Tumor Suppressor in Pancreatic Cancer. *PLoS ONE* 10, (2015).
163. Meurer, L., Ferdman, L., Belcher, B. & Camarata, T. The SIX Family of Transcription Factors: Common Themes Integrating Developmental and Cancer Biology. *Front. Cell Dev. Biol.* 9, (2021).
164. Blevins, M. A., Towers, C. G., Patrick, A. N., Zhao, R. & Ford, H. L. The SIX1-EYA transcriptional complex as a therapeutic target in cancer. *Expert Opin. Ther. Targets* 19, 213–225 (2015).
165. Wang, C.-A. *et al.* Homeoprotein Six2 promotes breast cancer metastasis via transcriptional and epigenetic control of E-cadherin expression. *Cancer Res.* 74, 7357–7370 (2014).
166. Casalou, C., Ferreira, A. & Barral, D. C. The Role of ARF Family Proteins and Their Regulators and Effectors in Cancer Progression: A Therapeutic Perspective. *Front. Cell Dev. Biol.* 8, 217 (2020).
167. Chen, X. *et al.* SMURF1-mediated ubiquitination of ARHGAP26 promotes ovarian cancer cell invasion and migration. *Exp. Mol. Med.* 51, 1–12 (2019).
168. Farrugia, A. J. & Calvo, F. Cdc42 regulates Cdc42EP3 function in cancer-associated fibroblasts. *Small GTPases* 8, 49–57 (2016).
169. Feng, Q. *et al.* CDC42EP3 promotes colorectal cancer through regulating cell proliferation, cell apoptosis and cell migration. *Cancer Cell Int.* 21, 169 (2021).
170. Chu, Y. *et al.* Calsyntenin-1, clusterin and neutrophil gelatinase-associated lipocalin are candidate serological biomarkers for lung adenocarcinoma. *Oncotarget* 8, 107964–107976 (2017).
171. Hu, X. *et al.* The RNA-binding protein AKAP8 suppresses tumor metastasis by antagonizing EMT-associated alternative splicing. *Nat. Commun.* 11, 486 (2020).
172. Zhu, G.-D. *et al.* Elevated Expression of DLG1 Is Associated with Poor Prognosis in Patients with Colorectal Cancer. *Ann. Clin. Lab. Sci.* 47, 657–662 (2017).
173. Slattery, M. L., Lundgreen, A. & Wolff, R. K. MAP kinase genes and colon and rectal cancer.

- Carcinogenesis* 33, 2398–2408 (2012).
174. Gao, L. *et al.* The RBP1–CKAP4 axis activates oncogenic autophagy and promotes cancer progression in oral squamous cell carcinoma. *Cell Death Dis.* 11, 1–16 (2020).
 175. Rao, Q. *et al.* Sulfiredoxin-1 is a promising novel prognostic biomarker for hepatocellular carcinoma. *Cancer Med.* 9, 8318–8332 (2020).
 176. Wang, J., Zhao, X., Jin, L., Wu, G. & Yang, Y. UBR5 Contributes to Colorectal Cancer Progression by Destabilizing the Tumor Suppressor ECRG4. *Dig. Dis. Sci.* 62, 2781–2789 (2017).
 177. Campbell, P. J. *et al.* Pan-cancer analysis of whole genomes. *Nature* 578, 82–93 (2020).
 178. Poli, V., Fagnocchi, L. & Zippo, A. Tumorigenic Cell Reprogramming and Cancer Plasticity: Interplay between Signaling, Microenvironment, and Epigenetics. *Stem Cells International* vol. 2018 e4598195 <https://www.hindawi.com/journals/sci/2018/4598195/> (2018).
 179. Bierne, H., Hamon, M. & Cossart, P. Epigenetics and Bacterial Infections. *Cold Spring Harb. Perspect. Med.* 2, a010272 (2012).
 180. Niller, H. H. & Minarovits, J. Patho-epigenetics of Infectious Diseases Caused by Intracellular Bacteria. in *Patho-Epigenetics of Infectious Disease* (eds. Minarovits, J. & Niller, H. H.) 107–130 (Springer International Publishing, 2016). doi:10.1007/978-3-319-24738-0_6.
 181. Andrews, M. C., Reuben, A., Gopalakrishnan, V. & Wargo, J. A. Concepts Collide: Genomic, Immune, and Microbial Influences on the Tumor Microenvironment and Response to Cancer Therapy. *Front. Immunol.* 9, (2018).
 182. Allen, J. & Sears, C. L. Impact of the gut microbiome on the genome and epigenome of colon epithelial cells: contributions to colorectal cancer development. *Genome Med.* 11, 11 (2019).
 183. Pleguezuelos-Manzano, C. *et al.* Mutational signature in colorectal cancer caused by genotoxic pks + E. coli. *Nature* 580, 269–273 (2020).
 184. Yin, L. & Chung, W. O. Epigenetic regulation of human β -defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria. *Mucosal Immunol.* 4, 409–419 (2011).
 185. Chen, S. *et al.* *Fusobacterium nucleatum* promotes colorectal cancer metastasis by modulating KRT7-AS/KRT7. *Gut Microbes* 11, 511–525 (2020).
 186. Daly, L. A. *et al.* Oxygen-dependent changes in binding partners and post-translational modifications regulate the abundance and activity of HIF-1 α /2 α . *Sci. Signal.* 14, eabf6685 (2021).
 187. Koike, T. *et al.* Hypoxia induces adhesion molecules on cancer cells: A missing link between Warburg effect and induction of selectin-ligand carbohydrates. *Proc. Natl. Acad. Sci. U. S. A.* 101, 8132–8137 (2004).
 188. Karczmarski, J. *et al.* Histone H3 lysine 27 acetylation is altered in colon cancer. *Clin. Proteomics* 11, 24 (2014).
 189. Tsai, Y.-P. & Wu, K.-J. Epigenetic regulation of hypoxia-responsive gene expression: focusing on chromatin and DNA modifications. *Int. J. Cancer* 134, 249–256 (2014).
 190. Tang, F., Yang, Z., Tan, Y. & Li, Y. Super-enhancer function and its application in cancer targeted therapy. *Npj Precis. Oncol.* 4, 1–7 (2020).
 191. Chen *et al.* *Fusobacterium nucleatum* reduces METTL3-mediated m6A modification and contributes to colorectal cancer metastasis. *Nat. Commun.* 13, 1248 (2022).
 192. Eisenreich, W., Heesemann, J., Rudel, T. & Goebel, W. Metabolic host responses to infection by intracellular bacterial pathogens. *Front. Cell. Infect. Microbiol.* 3, 24 (2013).
 193. Escoll, P. & Buchrieser, C. Metabolic reprogramming of host cells upon bacterial infection: Why shift to a Warburg-like metabolism? *FEBS J.* 285, 2146–2160 (2018).
 194. Umaña, A. *et al.* Utilizing Whole *Fusobacterium* Genomes To Identify, Correct, and Characterize Potential Virulence Protein Families. *J. Bacteriol.* 201, (2019).
 195. Geng, F., Zhang, Y., Lu, Z., Zhang, S. & Pan, Y. *Fusobacterium nucleatum* Caused DNA Damage and Promoted Cell Proliferation by the Ku70/p53 Pathway in Oral Cancer Cells. *DNA Cell Biol.* 39, 144–151 (2020).
 196. Topolewski, P. *et al.* Phenotypic variability, not noise, accounts for most of the cell-to-cell heterogeneity in IFN- γ and oncostatin M signaling responses. *Sci. Signal.* 15, eabd9303.
 197. Cao, Z., Chen, C., He, B., Tan, K. & Lu, C. A microfluidic device for epigenomic profiling using 100 cells. *Nat. Methods* 12, 959–962 (2015).
 198. Sepich-Poore, G. D. *et al.* The microbiome and human cancer. *Science* 371, eabc4552 (2021).

199. T, M., A, P., Js, S. & Jf, C. Intratumoral Heterogeneity of the Epigenome. *Cancer cell* vol. 29 <https://pubmed.ncbi.nlm.nih.gov/27070699/> (2016).
200. Skrypek, N., Goossens, S., De Smedt, E., Vandamme, N. & Berx, G. Epithelial-to-Mesenchymal Transition: Epigenetic Reprogramming Driving Cellular Plasticity. *Trends Genet.* 33, 943–959 (2017).
201. Wainwright, E. N. & Scaffidi, P. Epigenetics and Cancer Stem Cells: Unleashing, Hijacking, and Restricting Cellular Plasticity. *Trends Cancer* 3, 372–386 (2017).
202. Saito, A. *et al.* *Fusobacterium nucleatum* enhances invasion of human gingival epithelial and aortic endothelial cells by *Porphyromonas gingivalis*. *FEMS Immunol. Med. Microbiol.* 54, 349–355 (2008).
203. Zhang, W. *et al.* Gut Microbiota-Derived Metabolites in Colorectal Cancer: The Bad and the Challenges. *Front. Oncol.* 11, (2021).
204. Zhang, Z., Tang, H., Chen, P., Xie, H. & Tao, Y. Demystifying the manipulation of host immunity, metabolism, and extraintestinal tumors by the gut microbiome. *Signal Transduct. Target. Ther.* 4, 1–34 (2019).

Chapter 6

1. Cox, M. C., Reese, L. M., Bickford, L. R. & Verbridge, S. S. Toward the Broad Adoption of 3D Tumor Models in the Cancer Drug Pipeline. *ACS Biomater. Sci. Eng.* 1, 877–894 (2015).
2. Duval, K. *et al.* Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology* 32, 266–277 (2017).
3. Ashammakhi, N. *et al.* Gut-on-a-chip: Current progress and future opportunities. *Biomaterials* 120196 (2020) doi:10.1016/j.biomaterials.2020.120196.
4. Jalili-Firoozinezhad, S. *et al.* A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. *Nat. Biomed. Eng.* 3, 520–531 (2019).
5. Kim, H. J., Li, H., Collins, J. J. & Ingber, D. E. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc. Natl. Acad. Sci.* 113, E7–E15 (2016).
6. Shah, P. *et al.* A microfluidics-based in vitro model of the gastrointestinal human–microbe interface. *Nat. Commun.* 7, 11535 (2016).
7. Kasper, S. H. *et al.* Colorectal cancer-associated anaerobic bacteria proliferate in tumor spheroids and alter the microenvironment. *Sci. Rep.* 10, 5321 (2020).
8. Casasanta, M. A. *et al.* *Fusobacterium nucleatum* host-cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. *Sci. Signal.* 13, (2020).
9. Udayasuryan, B. *et al.* *Fusobacterium nucleatum* induces pancreatic cancer cell proliferation and migration by regulating host autocrine and paracrine signaling. 2021.11.19.469245 (2022) doi:10.1101/2021.11.19.469245.
10. Maritan, S. M., Lian, E. Y. & Mulligan, L. M. An Efficient and Flexible Cell Aggregation Method for 3D Spheroid Production. *J. Vis. Exp. JoVE* (2017) doi:10.3791/55544.
11. Cox, M. C., Deng, C., Naler, L. B., Lu, C. & Verbridge, S. S. Effects of Culture Condition on Epigenomic Profiles of Brain Tumor Cells. *ACS Biomater. Sci. Eng.* 5, 1544–1552 (2019).
12. Douglas, T. A., Alinezhadbalalami, N., Balani, N., Schmelz, E. M. & Davalos, R. V. Separation of Macrophages and Fibroblasts Using Contactless Dielectrophoresis and a Novel ImageJ Macro. *Bioelectricity* 1, 49–55 (2019).
13. Alinezhadbalalami, N., Douglas, T. A., Balani, N., Verbridge, S. S. & Davalos, R. V. The feasibility of using dielectrophoresis for isolation of glioblastoma subpopulations with increased stemness. *Electrophoresis* 40, 2592–2600 (2019).
14. Fernandez, R. E., Rohani, A., Farmehini, V. & Swami, N. S. Review: Microbial Analysis in Dielectrophoretic Microfluidic Systems. *Anal. Chim. Acta* 966, 11–33 (2017).
15. Rohani, A. *et al.* Label-Free Quantification of Intracellular Mitochondrial Dynamics Using Dielectrophoresis. *Anal. Chem.* 89, 5757–5764 (2017).

Chapter 7

1. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 12, 31–46 (2022).
2. Fulbright, L. E., Ellermann, M. & Arthur, J. C. The microbiome and the hallmarks of cancer. *PLoS Pathog.* 13, (2017).
3. Yin, H. et al. *Fusobacterium nucleatum* promotes liver metastasis in colorectal cancer by regulating the hepatic immune niche and altering gut microbiota. *Aging* 14, (2022).
4. Zhang, Y. et al. *Fusobacterium nucleatum* promotes colorectal cancer cells adhesion to endothelial cells and facilitates extravasation and metastasis by inducing ALPK1/NF- κ B/ICAM1 axis. *Gut Microbes* 14, 2038852 (2022).
5. Nejman, D. et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. *Science* 368, 973–980 (2020).
6. Sepich-Poore, G. D. et al. The microbiome and human cancer. *Science* 371, eabc4552 (2021).
7. Li, X. & Saxena, D. The mycobiome-immune axis: The next frontier in pancreatic cancer. *Cancer Cell* 40, 120–122 (2022).
8. Vallianou, N. et al. Mycobiome and Cancer: What Is the Evidence? *Cancers* 13, 3149 (2021).
9. Elaskandrany, M. et al. Fungi, host immune response, and tumorigenesis. *Am. J. Physiol.-Gastrointest. Liver Physiol.* 321, G213–G222 (2021).
10. Broecker, F. & Moelling, K. The Roles of the Virome in Cancer. *Microorganisms* 9, 2538 (2021).
11. Massimino, L., Lovisa, S., Antonio Lamparelli, L., Danese, S. & Ungaro, F. Gut eukaryotic virome in colorectal carcinogenesis: Is that a trigger? *Comput. Struct. Biotechnol. J.* 19, 16–28 (2021).
12. Poore, G. D. et al. Microbiome analyses of blood and tissues suggest cancer diagnostic approach. *Nature* 579, 567–574 (2020).
13. Rinninella, E. et al. Nutritional Interventions Targeting Gut Microbiota during Cancer Therapies. *Microorganisms* 9, 1469 (2021).
14. Geller, L. T. et al. Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. *Science* 357, 1156–1160 (2017).
15. Dodin, M. & Katz, D. E. Faecal microbiota transplantation for *Clostridium difficile* infection. *Int. J. Clin. Pract.* 68, 363–368 (2014).
16. Udayasuryan, B., Nguyen, T. T. D., Slade, D. J. & Verbridge, S. S. Harnessing Tissue Engineering Tools to Interrogate Host-Microbiota Crosstalk in Cancer. *iScience* 101878 (2020) doi:10.1016/j.isci.2020.101878.
17. Frontiers | Nerves in the Tumor Microenvironment: Origin and Effects | Cell and Developmental Biology. <https://www.frontiersin.org/articles/10.3389/fcell.2020.601738/full>.
18. Kalaora, S. et al. Identification of bacteria-derived HLA-bound peptides in melanoma. *Nature* 592, 138–143 (2021).
19. Umaña, A. & Slade, D. J. CRISPR-Cas systems in *Fusobacterium*: An untapped genetic frontier. 2022.01.21.477265 (2022) doi:10.1101/2022.01.21.477265.
20. Yeh, Y.-C., Huang, T.-H., Yang, S.-C., Chen, C.-C. & Fang, J.-Y. Nano-Based Drug Delivery or Targeting to Eradicate Bacteria for Infection Mitigation: A Review of Recent Advances. *Front. Chem.* 0, (2020).
21. Zheng, D.-W. et al. Biomaterial-mediated modulation of oral microbiota synergizes with PD-1 blockade in mice with oral squamous cell carcinoma. *Nat. Biomed. Eng.* 6, 32–43 (2022).
22. Zheng, D.-W. et al. Phage-guided modulation of the gut microbiota of mouse models of colorectal cancer augments their responses to chemotherapy. *Nat. Biomed. Eng.* 3, 717–728 (2019).
23. Harimoto, T. & Danino, T. Engineering bacteria for cancer therapy. *Emerg. Top. Life Sci.* 3, 623–629 (2019).
24. Harimoto, T. et al. Rapid screening of engineered microbial therapies in a 3D multicellular model. *Proc. Natl. Acad. Sci.* 116, 9002–9007 (2019).
25. Leventhal, D. S. et al. Immunotherapy with engineered bacteria by targeting the STING pathway for anti-tumor immunity. *Nat. Commun.* 11, 2739 (2020).
26. Abedi, M. H. et al. Ultrasound-controllable engineered bacteria for cancer immunotherapy. *Nat. Commun.* 13, 1585 (2022).
27. Ganesan, K., Guo, S., Fayyaz, S., Zhang, G. & Xu, B. Targeting Programmed *Fusobacterium nucleatum* Fap2 for Colorectal Cancer Therapy. *Cancers* 11, (2019).

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