A pro-carcinogenic colon microbe promotes breast tumorigenesis and metastatic progression and concomitantly activates Notch and β-catenin axes

Sheetal Parida1, Shaoguang Wu2§, Sumit Siddharth1, Guannan Wang1, Nethaji Muniraj1, Arumugam Nagalingam1, Christina Hum3, Panagiotis Mistriotis3, Haiping Hao4, C. Conover Talbot Jr.4, Konstantinos Konstantopoulos1,3, Kathleen L. Gabrielson1,5, Cynthia L. Sears1,2,6, Dipali Sharma1*

1Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, 2Department of Medicine, 3Department of Chemical and Biomolecular Engineering, 4Johns Hopkins Transcriptomics and Deep Sequencing Core, 5Molecular and Comparative Pathobiology, 6Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

§ Co-second author

Running Title: ETBF promotes breast carcinogenesis

Key Words: B. fragilis, ETBF, breast cancer, Notch, β-catenin

Acknowledgements: This work was supported by NCI NIH R01CA204555 (DS), Breast Cancer Research Foundation (BCRF) 90047965 (DS), NCI NIH CA183804 (KK), Bloomberg Philanthropies (CS). We acknowledge Dr. Xinqun Wu for her technical help.

* Corresponding author:

Dipali Sharma, Department of Oncology
The Sidney Kimmel Comprehensive Cancer Center,
Johns Hopkins University School of Medicine,
1650 Orleans Street, CRB 1, Rm 145, Baltimore, MD 21231
Office: 410-455-1345, FAX: 410-614-4073, Email: dsharma7@jhmi.edu

Conflict of Interest: The authors declare no conflict of interest.
ABSTRACT

Existence of distinct breast microbiota has been recently established but their biological impact in breast cancer remains elusive. Focusing on the shift in microbial community composition in diseased breast compared to normal breast, we identified the presence of Bacteroides fragilis in cancerous breast. Mammary gland as well as gut-colonization with enterotoxigenic Bacteroides fragilis (ETBF), that secretes B. fragilis toxin (BFT), rapidly induces epithelial hyperplasia in the mammary gland. Breast cancer cells exposed to BFT exhibit ‘BFT-memory’ from the initial exposure. Intriguingly, gut or breast-duct colonization with ETBF strongly induces growth and metastatic progression of tumor cells implanted in mammary ducts in contrast to non-toxigenic Bacteroides fragilis. This work sheds light on the oncogenic impact of a pro-carcinogenic colon bacterium ETBF, on breast cancer progression, implicates βcatenin and Notch1 axis as its functional mediators, and proposes the concept of ‘BFT-memory’ that can have far-reaching biological implications after initial exposure to ETBF.

Significance: Bacteroides fragilis is an inhabitant of breast tissue and gut or mammary duct colonization with enterotoxigenic Bacteroides fragilis (ETBF) triggers epithelial hyperplasia and augments breast cancer growth and metastasis. A short-term exposure to B. fragilis toxin elicits a ‘BFT memory’ with long-term implications, functionally mediated by βcatenin and Notch1 pathways.
INTRODUCTION

The abundance of commensal microorganisms colonizing the human body can be appreciated by the fact that the number of microbial cells living within and on human body is roughly equal to the total number of human cells (1). Though accounting only for ~2-7% of biomass owing to the miniscule size of microbes, the human microbiome encodes for 100-fold more genes than the human genome indicating an important role in human health (2). Microbiota and host maintain a dynamic equilibrium referred to as eubiosis that actively influences many physiological processes and is generally beneficial to the host. However, a state of disequilibrium or dysbiosis may evolve contributing to various disease states. A major advance in the microbiome field was achieved in the last decade with the completion of the Human Microbiome Project (HMP) that identified, characterized and elucidated the role of microbes in five major sites including the nasal passages, oral cavity, skin, gastrointestinal tract and urogenital tract (3-5). More recent developments show the existence of microbiota in other body sites, initially considered ‘sterile’, such as bladder, lung, endometrium, prostate as well as breast (6-11).

Breast cancer is a heterogeneous disease with multiple subtypes and interestingly, microbial signatures may differ between the subtypes. Triple-negative breast cancer (TNBC) and triple-positive breast cancer have distinct signatures that differ from estrogen-receptor positive and Her2-positive breast cancer which share similar microbial profiles (12). Two distinct microbial signatures are proposed from a screening of ~100 TNBC samples suggesting a possibility of further segregating TNBC subtype based on associated microorganisms (13). Comparison of breast tumor tissue and paired normal adjacent tissue highlights distinct alterations in bacterial species enriched in breast tumor tissues demonstrating that the total bacterial DNA load is lower in tumor tissue in contrast to normal breast (14). Bacterial species having the ability to induce DNA double-strand breaks are more abundant in breast cancer patients compared to the breast tissue of healthy subjects suggesting a possibility of DNA-damage leading to chromosomal aberrations (15). Not only is the breast microbiota different between normal healthy tissue and tumor tissue, an enrichment of low abundant taxa including genera Hydrogenophaga, Atopobium, Fusobacterium, and Gluconacetobacter are observed in malignant disease in comparison to benign disease showing how malignancy associates with unique microbial signature (16). Furthermore, the presence of microbiota is also reported in nipple aspirate fluid (NAF) showing that unique microbes inhabit the ductal system of human breast and, interestingly, community composition differs significantly between NAF of breast cancer patients and healthy women (17). Diversity in composition of the breast microbiota (18,19) indicates that microbial dysbiosis may play an important role in breast cancer growth as well as metastatic progression.

In addition to breast microbiota, some studies have shown that gut microbiota may also influence breast cancer. Analysis of fecal microbiota shows that postmenopausal women with breast cancer harbor compositionally different gut microbiota than healthy volunteers (20) and exhibit enrichment of several...
bacterial species (21). *Bacteroides fragilis* (*B. fragilis* is a commonly found colon colonizer (22) and individuals can be asymptatically colonized by enterotoxigenic *Bacteroides fragilis* (ETBF) (23) whose virulence is attributed to a 20-kDa zinc metalloprotease toxin termed the *B. fragilis* toxin (BFT) (24). *B. fragilis* forms a small fraction of total gut bacteria estimated to about ~0.1-0.5% (22) but is regarded as an important symbiont that can function as a potent pathogen and a determinant of the structure of microbial communities based on its secretory products (25). These rogue symbionts in addition to causing diarrhea and inflammatory bowel disease (IBD), are capable of inducing oncogenic transformation in the gut mucosa leading to formation of spontaneous tumors (22,23,25,26). Owing to its unique virulence traits, ETBF has been proposed as an ‘alpha bug’ capable of direct pro-oncogenic actions, remodeling the bacterial community to enhance its own induction as well as selective ‘crowding out’ of protective microbial species (22).

We identified the presence of *B. fragilis* in breast tumor tissue using meta-analyses of breast cancer microbiome studies forming the rationale of our work that the growth and progression of breast cancer may be impacted by the pro-oncogenic actions of ETBF. Consequently, we aim to decipher i) whether gut or intraductal colonization with ETBF impacts normal breast tissue, ii) the effect of BFT exposure on breast epithelial cells and the underlying molecular networks, and iii) whether ETBF colonization of gut or breast ducts can aid breast cancer growth and metastasis. Our results demonstrate both the distant (via gut colonization) and local (via intraductal colonization) effects of ETBF on breast and involvement of βcatenin and Notch1 pathways in mediating the oncogenic effects of BFT thereby proposing ETBF as a potential pathogen in breast carcinogenesis.
RESULTS

ETBF colonization of mammary ducts or gut induces mammary hyperplasia. We started this investigation by meta-analysis of clinical data examining the microbiota of the breast specifically selecting studies that compared differences in microbial community composition between benign breast tumors and malignant breast cancer (PRJNA335375, EBI-ENA) and nipple aspirate fluids of breast cancer survivors and healthy volunteers (SRP071608, NCBI-SRA). 16S rRNA gene sequencing data were retrieved from the sequence read archives and analyzed using One Codex. We observed that Bacteroides fragilis is consistently detected in all the breast tissue samples from benign and malignant breast cancer as well as nipple aspirate fluids (Fig. 1a). Since the 1970’s, B. fragilis has been known to be the most invasive of the colon anaerobes. In other words, B. fragilis is the anaerobe most likely to enter the bloodstream. Since that time the pathogenicity of B. fragilis has expanded even further by the discovery of toxin-producing strains of B. fragilis (enterotoxigenic B. fragilis or ETBF). ETBF is known to trigger a colon mucosal cascade resulting in colitis and colon neoplasia in mouse models and has also been demonstrated to be common in human populations. In these murine models, ETBF has been shown to modulate systemic immune responses including increasing IL-17, a known contributor to multiple types of cancer. Collectively, identification of B. fragilis in the breast microbiota by our metagenomic analyses combined with our understanding of the role B. fragilis and ETBF play in gut and systemic immune responses led us to test the hypothesis that B. fragilis, and specifically ETBF, contributes to breast oncogenesis. To examine the impact of ductal dysbiosis on normal mammary tissue, we initially used an intraductal approach to colonize mammary ducts of mice with enterotoxigenic B. fragilis (ETBF) or its isogenic mutant with an in-frame deletion of the chromosomal bft gene (086Mut) (Fig. 1b). Intraductal injection of mouse teats with $10^8$ CFU of ETBF or 086Mut resulted in mammary gland colonization (Fig.1c). Mammary glands of mice harboring ETBF infection showed the presence of BFT while no BFT was detected in 086Mut-infected mice (Fig. 1d, e). Marked differences in mammary tissue architecture were observed in the ETBF-group exhibiting widespread local inflammation and tissue fibrosis with increased epithelial cell proliferation as evident by Ki67 and PCNA staining, higher T cell infiltration indicated by CD3 staining and significantly altered expression of pan-keratin in comparison to 086Mut and sham controls (Fig 1f, g).

ETBF is a gut commensal in some individuals and colon disease-associated (e.g., diarrhea, colitis, tumorigenesis) in others (22,23,25,26) but extra-colonic disease links are unknown. Hence, we queried whether gut infection with ETBF is capable of inducing distant effects on mammary gland epithelium. C57BL/6 mice orally infected with ETBF developed brief diarrhea by 2-3 days that resolved by 4-5 days after colonization with high level persistent gut colonization ($\geq 10^9$ CFU). We observed the presence of ETBF as well as BFT in mammary glands of mice harboring gut-ETBF-infection while 086Mut and sham-control mice exhibited none (Fig. 2a, b). ETBF-gut-colonized mice also showed circulating serum levels of BFT which peaked at week 1 followed by a slight decline at week 3 while no BFT was detected in sham-control mice (Fig. 2 c). Intriguingly,
mammary glands in ETBF-gut-colonized mice showed significantly enlarged terminal end buds (TEBs) with more prominent bifurcations indicating a proliferative and inflammatory response (Fig. 2d). Blinded scoring of focal hyperplasia (score range 0-3) showed that all of the mice in the ETBF group scored 2 or 3 at week 3 post-infection whereas none of the sham-control mice scored 2 or 3 (Fig. 2d). Only ETBF-gut-colonized mice showed a marked increase in thickening of the breast duct lining and hyperproliferation of breast epithelium. Blinded scoring based on the thickness of the duct lining and cellularity of the inner margins of the ducts (score range 0-3) classified most of the mice harboring gut-ETBF infection in the score 3 group and all of the 086Mut and sham-control mice showed normal ducts (score 0-1) (Fig. 2e). Histopathology of mammary glands from ETBF-gut-colonized mice confirmed increases in stromal infiltration, collagen deposition, hyperplasia and T cell infiltration as evident by Trichrome, Ki67, pan-keratin and CD3 staining compared to 086Mut and sham-control mice (Fig. 2f). Overall, these data indicate the presence of B. fragilis in cancerous breast and show that ETBF is capable of exerting pathogenic effects on mammary gland locally as well as remotely plausibly via BFT.

B. fragilis toxin (BFT) induces prominent morphological and functional alterations in normal breast epithelial cells and breast cancer cells. Virulence of ETBF is ascribed to a 20-kDa zinc metalloprotease termed the B. fragilis toxin (BFT) (24). Thus, we examined the effect of BFT exposure on normal breast epithelial cells and breast cancer cells. MCF10A and MCF7 cells were treated with varying concentration of BFT ranging from 25-150 ng/ml (~1-7 nM) and structural changes examined. Cells exhibited membrane-blebbing and increased intracellular spacing in response to 100 ng/ml (5nM) BFT, which was deemed the optimum concentration for exerting a morphological effect on breast cells (Supplementary Fig. 1a). Indeed, treatment with 100 ng/ml (5 nM) BFT exhibited a temporal decrease of the tight junction protein E-cadherin consistent with structural changes in the cells (Supplementary Fig. 1b). Immunofluorescence analysis with an antibody specific for the intracellular domain of E-cadherin showed a loss of membrane-bound E-cadherin upon BFT treatment (Supplementary Fig. 1c). Rhodamine-phalloidin staining of F-actin showed that BFT-treated cells underwent a gradual but prominent cytoskeletal organization, increased intracellular separation, membrane blebbing and increase in F-actin stress fibers while the control cells showed uniform cuboidal, smooth-edged structures (Supplementary Fig. 2a). Next, we examined the effect of BFT treatment on cell viability and clonogenicity and observed that MCF7 and MCF10A cells treated with 5 nM BFT did not show any significant alterations in cell viability or colony formation (Supplementary Fig. 2b, c). A closer examination of colonies formed from the BFT-treated cells showed spindle-shaped cells emanating from the colonies indicating a migratory phenotype, a distinctive feature not observed in control colonies (Fig. 3a). To further elucidate these phenomena, MCF7 cells were exposed to 5 nM BFT for 48 hours and subjected to RNA-sequencing (RNA-seq) analysis. A differential expression analysis was conducted to characterize global differences in RNA transcript
levels induced upon BFT-exposure. Genes associated with cytoskeletal remodeling, cell movement and migration are highlighted in a volcano plot (in green) (Fig. 3b) and genes specifically associated with cell movement of breast cancer cell lines are presented in a functional annotation graph (Fig. 3c, Supplementary Fig. 2d). These data point towards a possibility that BFT exposure might impart a migratory and invasive phenotype to breast cells.

To query if BFT exposure truly leads to a migratory/invasive phenotype, single cell migration was investigated using a microfluidic device where cells were allowed to migrate across microfluidic channels of different widths ranging from 3 μm to 50 μm towards a gradient of EGF used as a chemo-attractant (27,28). Following a brief 12 hour exposure to 5 nM BFT, MCF7 cells migrated with significantly higher velocity and persistence through the microchannels compared to the control cells (Fig. 3d-f, Supplementary Fig. 3a). MCF10A cells, owing to their larger size and tendency to move in clusters, did not migrate far along in the microchannels as single cells and their velocity and persistence could not be calculated but visually, BFT-exposed cells seemed to migrate more than control MCF10A cells (Supplementary Fig. 3a). Rapid wound closure within 48 hours was observed for both cell lines in a scratch-migration assay. Control MCF7 cells migrated at a speed of 0.212 μm/h whereas BFT-treated cells migrated at a speed of 0.657 μm/h. In the case of MCF10A, the migration rate increased from 0.709 μm/h to 1.34 μm/h in response to BFT treatment. (Supplementary Fig. 3b, c). BFT-exposed MCF7 and MCF10A cells showed increased invasion potential (Fig. 3g). Cell adhesion assay suggested a reduced adhesion to collagen I as significantly fewer BFT-exposed MCF10A and MCF7 cells attached to collagen I-coated plates (Fig. 3h,i). We further validated our observations using a 3D spheroid-migration assay. When spheroids of MCF7 and MCF10A cells were treated with 5 nM BFT, increased migration of cells from the spheroids was observed (Supplementary Fig. 3d). Cells migrated an average distance of 19.55±3.051 μm in control MCF7 spheroids (n=3) which increased to 24.81±3.009 μm in BFT-treated MCF7 spheroids (n=3) within 48 hours. In the case of MCF10A spheroids, the distance traversed increased from 24.39 μm to 44.39±1.932 μm in BFT-treated spheroids (Supplementary Fig. 3e). Multiple human breast cancer cell lines, upon exposure to BFT, showed increased invasion and migration potential (Supplementary Fig. 4a-d). BFT treatment also decreased adhesion and increased migration and invasion of 4T1 mouse mammary cancer cells (Supplementary Fig. 5a-d). These results suggest that BFT treatment induces cytoskeletal reorganization in the cells; consequently, they exhibit increased migration and invasion potential.

In addition to an enrichment of genes associated with a migratory phenotype, RNA-sequencing analysis revealed an upregulation of an embryonic stem cell pluripotency pathway in BFT-exposed MCF7 cells (Supplementary Fig. 6a). Higher expression of a set of 38 embryonic stemness-associated genes was observed in BFT-exposed MCF7 cells in comparison to control cells (Supplementary Fig. 6b). Encouraged by the RNA-seq results implicating an enhancement of stemness in BFT-exposed cells, we examined the self-renewal potential of BFT-treated MCF7 and MCF10A cells using mammosphere formation assay. BFT-treated cells
formed a higher number of mammospheres both in solid and liquid medium (Supplementary Fig. 6c,d). Additionally, the liquid mammospheres from BFT-exposed cells continued to form higher numbers of secondary and tertiary mammospheres in two subsequent generations (Supplementary Fig. 6c). Collectively, these results show that breast cells undergo distinct morphological and molecular changes supporting an invasive and migratory phenotype with enhanced stemness potential upon BFT exposure.

**B. fragilis toxin (BFT) exposure enables the formation of multifocal breast tumors with elevated stemness character.** Encouraged by our *in vitro* findings we investigated whether BFT exposure impacts tumorigenicity of breast cancer cells. MCF7 and MCF10A cells were pretreated with 5 nM BFT for 72 hours followed by mammary gland implantation in SCID-NOD mice and tumor incidence and progression was monitored for 7 weeks. More rapid tumor progression was observed in BFT-pretreated group compared to the control group (Fig. 4a). To our surprise, starting at week 3, tumors in the BFT-pretreated group extended locally forming multifocal tumors resembling local metastases (Fig. 4a). Tumors were dissociated into single cells and evaluated for their functional potential pertaining to migration, invasion, adhesion and mammosphere formation. As expected, tumor cells from BFT-pretreated group exhibited higher invasion and migration potential with reduced adhesion (Fig. 4b). Mammosphere formation potential was also elevated in tumor cells from the BFT-pretreated group, a characteristic sustained for three generations forming a higher number of primary, secondary and tertiary mammospheres (Supplementary Fig. 7a,b). Primary and tertiary mammospheres from BFT-pretreated group showed elevated expression of Oct4, Nanog and Sox2 compared to control mammospheres (Supplementary Fig. 7c). Histology of tumor sections surprisingly revealed widespread fibrosis and stromal infiltration in tumors formed by BFT-pretreated MCF7 cells. Masson trichrome staining showed regions rich in connective tissue. Immunohistochemical staining showed cMyc-positive nuclei and denser Ki67 nuclear staining in BFT-pretreated tumors indicating higher proliferation. Also, these tumors were more richly vascularized as confirmed by enhanced CD31 staining (Fig. 4c, Supplementary Fig. 7d). BFT-pretreated MCF10A cells did not form tumors since they are non-transformed cells. However, BFT-pretreated MCF10A-Kras cells formed more aggressive tumors compared to control cells (Supplementary Fig. 8a-c). Dissociated tumor cells from the BFT-pretreated MCF10A-Kras group exhibited significantly higher invasion, migration and lower adhesion potential compared to control group; qualities similar to tumor cells from BFT-pretreated MCF7 group (Supplementary Fig. 8d-f). Mammosphere formation potential was also elevated in tumor cells from the BFT-pretreated MCF10A-Kras group, as exhibited by the formation of a higher number of primary, secondary and tertiary mammospheres and elevated expression of Oct4, Nanog and Sox2 compared to mammospheres generated from control group (Supplementary Fig. 8g-i).

Formation of multifocal tumors by BFT-pretreated MCF7 cells and the fact that tumor-dissociated cells continue to form higher number of mammospheres through three generations prompted us to investigate if these
tumors possessed increased tumor-initiating cells. Tumors formed by BFT-pretreated MCF7 cells were excised and dissociated to single cells followed by secondary transplants in limiting dilution (Fig. 4d). Monitoring of tumor incidence and progression showed that BFT-pretreated MCF7 tumor group formed larger secondary tumors with a shorter tumor-free survival in comparison to the control-MCF7 tumor group (Fig. 4e,f). BFT-pretreated MCF7 tumor group exhibited significantly increased tumor-initiating frequencies when transplanted into secondary hosts at limiting dilutions. The frequency of breast tumor-initiating cells in the BFT-pretreated MCF7 tumor group was determined to be 1 in 38,481 compared to 1 in 151,950 in the control-group at week 4 and 1 in 11,644 compared to 1 in 35,573 at week 6 (Fig. 4g). Tumor cells dissociated from the secondary tumors from BFT-pretreated group retained higher matrigel-invasion, spheroid-migration and mammosphere-formation along with reduced adhesion characteristics (Supplementary Fig. 9a-c). Elevated expression of Oct4 and Nanog was also observed in primary and tertiary mammospheres formed with the tumor cells dissociated from the secondary tumors from BFT-pretreated MCF7 group (Supplementary Fig. 10a-c). To uncover the underlying molecular changes, the secondary tumors formed in the in vivo limiting dilution assay were subjected to RNA-sequencing (RNA-seq) analysis. Interestingly, an enrichment of stemness markers was observed in the BFT-pretreated group (Supplementary Fig. 10d). In addition, BFT-pretreated group showed higher expression of genes associated with cell invasion potential, cell movement of tumor cell lines, and cell movement (Supplementary Fig. 11-13). Together, these data provide the intriguing notion that a 72-hour long exposure of breast cancer cells to BFT is sufficient to induce morphological and molecular changes that impart a highly migratory, invasive and stemness-rich phenotype to MCF7 cells that is maintained through in vitro and in vivo generations lasting up to 13 weeks.

Involvement of βcatenin and Notch1 pathways in mediating the impact of B. fragilis toxin (BFT) in the breast. We next asked which signaling pathways underlie the biological effects of BFT. To address this query, we further analyzed the RNA-seq data obtained from the secondary tumors from the BFT-pretreated MCF7 group and control group. Differential expression analysis of the global differences in RNA transcript levels induced in BFT-pretreated vs. control group revealed a significant upregulation of the βcatenin and Notch1 pathways. Genes associated with βcatenin (marked in blue) and Notch1 (marked in pink) pathways are highlighted in the volcano plot (Fig. 5a) and genes specifically associated with Wnt-βcatenin pathway are presented in a functional annotation graph (Supplementary Fig. 14).

Further exploration of the direct involvement of βcatenin in BFT function showed increased expression of βcatenin in breast cancer cells treated with 5 nM BFT (Fig. 5b,c). Deposphorylation of βcatenin prevents its ubiquitination and subsequent proteasomal degradation leading to its cytoplasmic accumulation and increased nuclear localization enabling transcriptional activity (29). BFT exposure resulted in higher expression of total βcatenin protein and a reduction in phospho-βcatenin levels (Fig. 5c). Increased levels of nuclear βcatenin and a
decrease in cytoplasmic levels of β-catenin was observed in BFT-treated MCF7 cells while MCF10A cells exhibited an increase in nuclear as well as cytoplasmic β-catenin (Fig. 5d). Immunofluorescence analysis confirmed nuclear accumulation of β-catenin upon BFT treatment in MCF7 cells (Fig. 5e). Increased expression of β-catenin-responsive genes including Slug, cMyc, Jagged and Twist was noted in BFT-treated MCF7 and MCF10A cells (Fig. 5f, g). Further, immunohistochemical analysis of tumors from BFT-pretreated MCF7 group compared to control group showed a higher expression of β-catenin in the BFT group (Fig. 5h, Supplementary Fig. 15a). These results explicitly show that BFT activates the β-catenin pathway in breast cancer and breast epithelial cells (Fig. 5i). Since we observed an enrichment of Notch1 pathway genes in the RNA-seq of secondary tumors from BFT-pretreated MCF7 group, we next examined the direct effect of BFT on Notch1 in breast cells. Notch receptors are membrane-bound receptors with a cytoplasmic region or Notch intracellular domain (NICD). NICD translocates to the nucleus upon activation and mediates transcriptional activity (30). We found that BFT treatment indeed increased NICD expression in a temporal manner in MCF7 and MCF10A cells and increased its nuclear localization (Fig. 6a, b). Immunohistochemical analysis of tumors from the BFT-pretreated MCF7 group and control group showed a higher expression of NICD in the BFT group (Supplementary Fig. 15b). BFT increased the expression of NICD and β-catenin and reduced the level of phospho-β-catenin in 4T1 cells while no significant effect was observed with biologically inactive mutant BFT-H352Y (Supplementary Fig. 15c). BFT-treated cells also showed elevated expression of NICD-responsive genes including p21/Waf and Hes1 (Fig. 6c). We then evaluated the interdependence of NICD and β-catenin in BFT-treated cells. As evident in the immunofluorescence analysis, BFT-treated cells showed increased nuclear accumulation of β-catenin and NICD (Supplementary Fig. 16a, b). Co-treatment of cells with BFT and Notch inhibitor-DAPT (a γ-secretase inhibitor) resulted in lower nuclear accumulation of β-catenin while the DAPT only group showed cytoplasmic localization of β-catenin (Supplementary Fig. 16a). Breast cancer cells co-treated with BFT and the β-catenin inhibitor-ICG001 showed reduced nuclear accumulation of NICD and ICG001 alone group showed cytoplasmic localization of NICD (Supplementary Fig. 16b). Further evaluation of the functional importance of these pathways in the context of BFT treatment showed that a single treatment with DAPT and ICG001 could significantly reduce BFT-mediated elevated invasion and migration of cells while cells treated with a combination of DAPT and ICG001 further improved abrogation of BFT’s effect (Supplementary Fig. 16c-e).

Examining the in vivo relevance of our in vitro findings, MCF7 cells were exposed to BFT and treated with DAPT and/or ICG001 in vitro followed by mammary gland implantation in mice. BFT-exposed MCF7 cells formed significantly larger tumors (4-fold) than control cells. Reduced tumor growth was observed in BFT-exposed cells treated with DAPT or ICG001 alone while co-treatment with DAPT and ICG001 led to even more inhibition. DAPT and ICG001 treated control cells also showed tumor inhibition (Fig. 6d). Tumor dissociated cells, examined for the presence of stemness marker CD49f, showed higher expression (60.3%) in
BFT alone group in comparison to control (23.5%) while BFT-exposed cells with DAPT and/or ICG001 treatment showed lower CD49f expression (19.6%, 12.6% and 21%) (Fig. 6e). In vitro treatment with DAPT and/or ICG001 could inhibit BFT’s tumorigenic and stemness effect on breast cancer cells. Next, we examined whether in vivo treatment with DAPT and/or ICG001 could inhibit tumors formed by BFT-exposed breast cancer cells. Mice treated with ICG001 achieved a 58.5% regression while DAPT reduced the tumor-load by 72.2% in BFT-exposed group. Interestingly, combined treatment with DAPT and ICG001 further regressed the tumors by 86.4% in BFT group. DAPT and/or ICG001 treated control group (no BFT exposure) also showed tumor inhibition (Fig. 6f). Histopathology of tumors showed increased Ki67 (Fig. 6g) in BFT-pretreated group while DAPT and ICG001 treated groups showed reduced expression. Importantl, BFT-induced ‘memory effect’ was inhibited with in vivo DAPT/ICG001 treatment as evident from abrogated mammosphere-formation (Fig.6h), transwell-migration, matrigel-invasion and elevated adhesion potential of dissociated tumor cells (Supplementary Fig. 17a, b). Increased CD31 (Supplementary Fig. 17c) was observed in BFT-pretreated group compared to DAPT and ICG001 treated groups. Taken together, these results show that βcatenin and NICD present an important functional node in mediating the biological effects of BFT in breast cells.

**Gut or ductal colonization by ETBF accelerates breast cancer growth and metastatic progression.** To evaluate the impact of gut or ductal colonization with ETBF on breast cancer progression, we used a mammary intraductal (MIND) model in syngeneic mice. Our goal was to determine whether preexisting gut or breast duct infection with ETBF affects mammary tumorigenesis and metastatic progression. For gut colonization, Balb/c mice were given an antibiotic cocktail for 1 week followed by oral infection with 10^8 CFU of ETBF or 086Mut; high level gut colonization persisted for the duration of the experiment (≥ x 10^9 CFU). Mammary gland ducts of mice harboring gut colonization with ETBF or 086Mut were then injected (via teats) with 20,000 4T1-Luc2 cells to initiate mammary tumors in the ductal tree (Supplementary Fig.18a). We monitored the tumor progression by bioluminescent imaging. Mice harboring gut-colonization with ETBF exhibited increased tumor progression compared to 086Mut and sham-control mice (Fig. 7a). RNA-seq data obtained from the secondary tumors from BFT-pretreated MCF7 group and control group showed higher expression of genes associated with migration, homing and metastasis (Supplementary Fig. 18b) indicating that exposure to BFT owing to gut colonization with ETBF might enhance metastatic progression. Intriguingly, significantly higher levels of lung as well as liver metastases were observed in ETBF-colonized mice compared to either the sham-control group or 086Mut-colonized mice (Fig. 7b, c, Supplementary Fig.18c). Analysis of tumor sections showed sheets of tumor cells with mesenchymal-like phenotype in the ETBF group whereas no such phenotype was observed in any tumors in sham-control or 086Mut groups (Supplementary Fig. 18d). Liver and lung sections from ETBF-colonized mice exhibited higher number and increased area of metastatic lesions in comparison to sham-control
and 086Mut-colonized mice (Fig.7d-f, Supplementary Fig. 18e). Next, we queried the impact of mammary ducts colonization with ETBF on breast cancer progression. To this end, $10^8$ CFU of ETBF or 086Mut were introduced in mammary ducts via teats (Supplementary Fig.18a); high level ductal colonization persisted for the duration of the experiment (Supplementary Fig. 19a). Presence of BFT was observed in mammary glands of mice harboring ductal ETBF infection whereas no BFT was detected in 086Mut-infected mice (Supplementary Fig. 19b). 4T1-Luc2 cells injected (via teats) in mammary gland ducts showed increased tumor progression and formed ~3.9-fold larger tumors in ETBF-infected group in comparison to 086Mut or sham-control groups (Fig.7g, h). Increased lung and liver metastasis were observed in mice harboring ductal colonization of ETBF in comparison to either the sham-control group or 086Mut-colonized mice (Fig.7i-l, Supplementary Fig.19c). Tumors formed in ETBF group were more proliferative as evident from Ki67 staining, showed a mesenchymal phenotype and also exhibited markedly higher stromal infiltration evident from Trichrome staining compared to sham-control or 086Mut group (Supplementary Fig.19d). Next, we queried the impact of gut colonization with ETBF on MCF7-tumor formation. Indeed, mice harboring gut ETBF infection exhibited significantly increased (3.3-fold larger tumors) tumor progression than sham-control and 086Mut groups (Fig. 7m). MCF7-tumors formed in ETBF infected mice were more proliferative and richly vascularized as evident from Ki76 and CD31 specific IHC (Fig 7n). These results using multiple preclinical murine models unequivocally show that gut or ductal colonization with ETBF markedly enhances breast tumorigenesis and metastatic progression pointing to a novel role of ETBF.
DISCUSSION

To our knowledge, the results presented here are the first to demonstrate a direct role for enterotoxigenic *Bacteroides fragilis* (ETBF) infection and its secreted toxin, BFT, in breast carcinogenesis. We found the presence of *B. fragilis* in human breast tissues and nipple aspirates using meta-analysis of clinical data and then developed *in vivo* approaches to examine its involvement in breast tumorigenesis. This involved using a mouse-intraductal (MIND) model to introduce ETBF directly in the breast ducts or gut colonization with ETBF, both leading to the development of ductal hyperplasia as well as metastatic progression. BFT is the only identified virulence factor of ETBF and, notably, in the course of these studies we show that even a short exposure to BFT elicits a long-term oncogenic memory in breast cells. Particularly significant is the attainment of a highly migratory, invasive, stemness-rich phenotype that is supported by the drastic change in molecular machinery as expression of the genes supporting cell movement, embryonic pluripotency pathway and metastasis are induced in BFT-exposed cells. Mechanistic evaluations identify the involvement of βcatenin and Notch1 pathways whose inhibition leads to the abrogation of BFT-mediated cell migration and invasion underscoring their biological importance. Few recent studies report the presence of breast microbiota and have identified some bacterial species as selective inhabitants of breast tumors (10,14,16-19,31). *Methylobacterium radiotolerans* is abundantly present in tumor tissues while *Sphingomonas yanoikuyae* is enriched in normal breast (14). Presence of *Lactobacillus* and *Bifidobacterium*, species known for health-promoting effects, in addition to taxa generally associated with pathogenesis such as *Enterobacteriaceae*, *Pseudomonas*, and *Streptococcus agalactiae* in breast tissues have been noted (10). A higher abundance of *Escherichia coli* and *Staphylococcus epidermidis* has been observed in breast tumors in comparison to healthy breast tissue (10,15). Although these studies establish the existence of breast microbiota and some even identify differential existence of distinct microbial species in normal vs. tumor samples, their biological impact on breast cancer initiation and progression has not been investigated.

Our study implicates an oncogenic role of gut colonization with ETBF in breast cancer initiation and metastatic progression. Direct mammary ductal-colonization or indirect gut-colonization with ETBF is sufficient to initiate mammary gland hyperplasia within three weeks of ETBF infection and, unexpectedly, gut colonization with ETBF potentiates mammary tumor growth and metastatic progression. We observed the presence of ETBF in mammary ducts of mice harboring gut ETBF infection but it is unclear whether ETBF traveled internally from gut to breast or gut-infected mice acquired mammary gland infection through environment. A large body of work establishing the pathogenic role of this gut commensal in colonic inflammation and colon cancer (23,32-34) has helped form the “Alpha-bug hypothesis” proposing that a single pathogenic microbe, such as ETBF, possessing unique virulence traits can direct oncogenesis acting alone or via modulating the bacterial community in the organ and selectively “crowding out” microbes with protective roles (22,23). Interestingly, BFT is a zinc-dependent metalloprotease toxin which is structurally similar to other...
important bacterial toxins like botulinum toxin or tetanus toxin (32). While BFT has been shown to induce proliferation of human colonic epithelial cells (35), its impact on invasion, migration and stemness potential of cancer cells has never been examined. Cytoskeletal remodeling is a salient feature of epithelial to mesenchymal transition (36) and it also regulates cell migration and invasion in response to extracellular stimuli and aids in metastasis (37). Upon BFT exposure, breast cells undergo distinctive morphological changes acquiring mesenchymal-like phenotype and acquire a highly migratory and invasive phenotype. Tumors may harbor cancer stem cells (CSCs) that characteristically possess the capability of self-renewal and differentiation and are key for tumor-initiation, metastatic-progression, therapy-resistance as well as relapse (38,39). In addition to the enrichment of gene expression associated with cell movement and invasion potential, BFT-exposed breast cells also show enhanced embryonic stem cell pluripotency pathway. Importantly, increased stemness potential is observed in BFT-treated breast cells as reflected in the formation of mammospheres and demonstrated as well by the in vivo-limiting dilution assay. Of note, the non-toxigenic mutant Bacteroides fragilis (086Mut) does not secrete BFT and exhibits no oncogenic effect on breast tumor growth and metastatic progression, suggesting that BFT is central to ETBF’s actions. Our data show that ETBF acts as an “Alpha bug” triggering an oncogenic cascade resulting in breast cancer progression via its toxin.

Our results advance the understanding of the molecular mechanisms underlying ETBF-BFT and breast cancer progression. RNA-seq analyses of secondary tumors developed with BFT-pretreated cells and breast cancer cells treated with BFT show enrichment of the βcatenin pathway. Several canonical and non-canonical βcatenin responsive genes exhibit increased expression in response to BFT. Wnt-βcatenin signaling is important for human breast development as well as breast cancer progression and is elevated in multiple breast cancer subtypes. Higher expression of βcatenin associates with higher tumor grade and poor prognosis (29). These results are consistent with previous studies presenting BFT-induced activation of βcatenin via cleavage of E-cadherin in colonic epithelial cells (35). We have also discovered that BFT triggers the activation of the Notch1 pathway and the expression of several canonical and non-canonical notch-responsive genes are enriched in breast cancer cells. In addition to growth and progression of breast cancer, Notch1 is also implicated in the maintenance of mammary stem cells (40). It is interesting to note that Wnt-βcatenin and Notch signaling pathways crosstalk via GSK3β and Jagged1 (41). Inhibiting Notch and βcatenin results in abrogation of BFT-mediated migration and invasion of breast cells. Several inhibitors of Wnt-βcatenin pathway including porcupine inhibitors-LGK974 and ETC159, pathway antibodies-Vantictumab and Ipafricept, and βcatenin/TCF inhibitor-PRI-724 are under investigation (42). Preclinical and clinical studies are also examining γ-secretase inhibitors, peptide inhibitors and anti-notch monoclonal antibodies for efficient inhibition of notch pathway (43). Our results indicate a possibility of overcoming the molecular impact of ETBF infection via inhibiting the actionable key molecular nodes.
An intriguing relationship has emerged between infectious diseases and cancer pathogenesis where a chronic infection with a virus such as human papilloma virus (HPV) associates with cervical cancer, hepatitis B and C viruses associate with liver cancer and Helicobacter pylori associates with gastric cancer. B. fragilis is a common colonizer of the gut (22) and 5 to 35% of studied populations are asymptomatically colonized by ETBF (23). Although it forms a small fraction of the total gut biome (22), it is regarded as a potent pathogen and its pathogenic role is well established in colitis and colon cancer (32-34). Despite multiple established risk factors, a large number of breast cancers arise in women harboring none of the established risk factors indicating the need to look beyond. We conclude that microbial perturbations may associate with breast cancer development. Our study is a first step to show the involvement of a common, often commensal, colon microbiome member, ETBF, in breast carcinogenesis; additional studies are needed to clarify whether ETBF can be the sole driver to directly trigger the transformation of breast cells in humans and/or if other microbiota members also display pro-carcinogenic activity for the breast. Drugging or targeting a specific microbiota member is very challenging yet a very exciting goal for microbiome-breast cancer research. Combining microbiome analysis and molecular subtyping of breast tumors may help identify new contributors to the pathogenesis of breast cancer as well as patients most likely to benefit from prevention strategies.
METHODS

Cell lines and Bacterial Strains. Breast cancer cell lines MCF7, HCC1806, HCC1569, B474, HCC1937 and normal breast epithelial cell line MCF10A were procured from American Type Culture Collection (ATCC) and maintained at 37°C in 5% CO2 and 95% humidity. MCF10A-Kras cells were a gift from Dr. Ben Park. 4T1-luc cells were a gift from Dr. Saraswati Sukumar. Cells were used for experiments within 10 to 20 passages from thawing. All cells were authenticated via short tandem repeat testing. Mycoplasma detection was routinely performed using the MycoAlert Detection Kit (Lonza, LT07-218). Cultures of enterotoxigenic Bacteroides fragilis (ETBF) strain 86-5443-2 (BFT2-secreting strain) and its isogenic nontoxigenic mutant that does not secrete BFT due to an in-frame deletion of the chromosomal bft gene were maintained anaerobically at 37°C. Bacterial pellets were washed and resuspended with 1× Dulbecco’s phosphate buffered saline (1× PBS free of calcium chloride and magnesium chloride) for mouse inoculums.

Reagents. BFT was HPLC purified from culture supernatants of ETBF as previously described (44) and stored at -80°C. Briefly, BFT producing wild type B. fragilis strain or biologically inactive mutant were cultured anaerobically in BHC medium (37 g of brain heart infusion base [Difco Laboratories, Detroit, Michigan]/liter, 5 g of yeast extract [Difco Laboratories, Detroit, Michigan]/liter, and 1 μg of vitamin K/ml, 5 μg of hemin/ml, and 0.5 g of L-cysteine/ml) for 24 hours. After removing the bacterial cells by centrifugation at 4°C and sterilization by filtering through 0.22-μm-pore size filter, the supernatant containing BFT-2 protein or biologically inactive mutant BFT-H352Y were concentrated five- to six-fold at 4°C by ultrafiltration using a membrane with a molecular mass exclusion of 10 kDa (Millipore Corporation, Bedford, Massachusetts). The ultrafiltrate was chromatographically (FPLC) purified under the denaturing condition in urea. Purified BFT and BFT-H352Y were stored at -80°C. For in vitro studies, cells were treated with different concentrations of BFT. The optimum concentration of BFT is 5 nM (100 ng/ml) (45). Rabbit monoclonal BFT2 antibody was a generous gift from Dr. Saraspadee Mootien, L2 Diagnostics LLC, New Haven, Connecticut, USA (46). For western blot and immunohistochemistry, rabbit monoclonal anti-E-cadherin, anti-E-cadherin, anti-GSK3β, anti-p-GSK3β, anti-p-βCatenin, anti-Slug, anti-Snail, anti-Vimentin, anti-cMyc, anti-Notch1, anti-NICD, anti-RBPSUH, anti-MAML1, anti-Hes1, anti-cyclin D3, anti-p21/Waf1, anti-Oct4, anti-Sox2, anti-Nanog, anti-KLF4 and anti-Ki67 antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA. Anti-βCatenin, anti-CD31, anti-Jagged1, anti-Twist, anti-Snail, anti-Occludin, and anti-Lamin B were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. IHC specific rabbit monoclonal eMyc was procured from Abcam, Cambridge, MA, USA and mouse monoclonal β-Actin was procured from Sigma-Aldrich, St. Louis, MO, USA. Horseradish peroxidase conjugated goat anti-rabbit IgG, goat anti-mouse IgG and donkey anti-goat IgG were purchased from Sigma-Aldrich, St. Louis, MO, USA. DAPT and ICG001 were procured from Sigma Aldrich (St. Louis, MO, USA). Chemiluminescent peroxidase substrate and 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) were procured from Sigma Aldrich, St. Louis, MO, USA. Rhodamine Phallloidin was procured from Invitrogen Corporation, CA, USA.

**In silico analysis.** Raw data from the studies evaluating the breast microbiome in breast cancer patients was accessed from NCBI-SRA, EMBL-EBI and NCBI-GEO. Data sets PRJEB4755, SRP071608 and PRJNA335375 examining local microbiota of normal breast tissue, cancerous breast, benign breast cancer, malignant breast cancer and nipple fluid aspirate from healthy woman and breast cancer survivors were analyzed using One Codex. Abundant and rare species were identified from each cohort and further strain level classification was performed. Comparisons were made across studies as well as between cohorts. Potential pathogenic organisms were identified for further experimental studies.

**Mammary gland and gut colonization with ETBF and whole mammary gland analysis.** All animal studies were in accordance with the guidelines of Johns Hopkins University ACUC. For mammary gland colonization, twice parous Balb/C mice (obtained from Charles River and maintained in house), were given antibiotic cocktail (clindamycin 0.1g/L and streptomycin 5 g/L) in water bottles (Hospira and Amresco) for 7 days and discontinued. Mice were also injected with antibiotic cocktail intraductally to clear ductal microbiome at the same time. Mice in ETBF or 086Mut group were then injected with \(~10^8\) CFU of ETBF or 086Mut in 1X PBS via intraductal administration. For sham-control, mice were intraductally injected with 1X PBS. For gut colonization, female, 3 weeks old, C57BL/6 mice (obtained from Jackson Laboratories and maintained in-house) were given antibiotic cocktail (clindamycin 0.1 g/L and streptomycin 5 g/L) in water bottles (Hospira and Amresco) for 7 days and discontinued. Mice in ETBF or 086Mut group were given \(~10^8\) CFU of ETBF in 1X PBS via oral gavage. For sham-control, mice were gavaged with 1X PBS. We quantified fecal bacterial colonization as colony-forming units per g stool (34). Mice were euthanized and serum was collected 1 week and 3 weeks post-infection as indicated. Whole mammary glands were excised and immediately spread on a glass slide and fixed in Carnoy’s’s fixative (ethanol, chloroform and glacial acetic acid in the ratio 6:3:1) for 48 hours. After fixation, the whole mammary glands were incubated in 70% ethanol for 1 hour and then stained with carmine alum stain for 2 days. Stained mammary glands were then dehydrated in a series of graded alcohol, 1 hour each and immersed in xylene for 4 days to get rid of the immense fat deposits of the breast. Once clear of most of the fat deposits, tissue was mounted using high viscosity Cytoseal 260. Ductal structure of the mammary gland was examined microscopically.

**Immunohistochemistry and Enzyme-linked immunosorbent assay** Mammary gland tissue and tumor tissue excised from MCF7 xenografts, MCF10A-Kras xenografts and 4T1 intraductal tumors were fixed in 10% formalin, paraffin embedded and sectioned and immunohistochemical analyses were performed using anti–Ki-67, anti-Pan-keratin, anti-CD3, anti-PCNA, anti–CD31, anti–βcatenin, anti-E-Cadherin, anti-N-cadherin and anti-cMyc antibodies. Images were captured using Lieca microscope at 20X magnification. Presence of BFT in the serum of ETBF-colonized mice was determined using ELISA by antigen capture method. For tissue
*ELISA,* whole breast was excised from 3 week old mice bearing ETBF or 086Mut infection and corresponding Shams. Tissue was lysed in Sigma CellLytic™ mammalian tissue lysis buffer as per manufacturer protocol. 96 well plates were coated with lysate containing 100μg protein made up to 30μl in PBS. BFT was quantified by antigen capture ELISA. Standards were used for quantitation.

**Cell morphology, rhodamine/phalloidin staining, cell viability and clonogenicity assay.** *Cellular morphology* of BFT-treated cells was monitored using phase contrast microscopy. For *Rhodamine/Phalloidin staining,* cells were washed with PBS and stained with rhodamine conjugated phalloidin to stain the F-actin filaments for 30 minutes followed by counter staining with DAPI nuclear stain for 5 minutes. Cytoskeletal changes were examined and imaged under Lieca E800 fluorescent microscope. *Cell viability* was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. The percentages of live cells were calculated and plotted using Prism software (GraphPad Prism, CA, USA). For *clonogenicity* assay, colonies containing >50 normal-appearing cells were counted and pictures were taken using a digital camera.

**Microfluidic migration assay.** Microfluidic devices containing an array of parallel microchannels of 3-50 μm dimensions were fabricated by standard lithography and coated with 20 μg/mL collagen type I (BD Biosciences, San Jose, CA). Cells were allowed to migrate towards a gradient of EGF used as a chemo-attractant. Phase contrast time-lapse images were captured at 30-min intervals for up to 10 h on an inverted Nikon microscope (10X objective) at multiple stage positions via stage automation (Nikon Elements, Nikon, Japan). Cell speed, velocity and persistence were computed using a custom-written Matlab program (The MathWorks, Natick, MA).

**Cell adhesion and Spheroid migration assay.** Serum starved cells were treated with 5 nM BFT for 48 hours prior to the assay. For adhesion assay, wells of a microtiter plate were coated with 30 μl Collagen I (40 μg/ml in PBS) for 16 hours at 4°C followed by introduction of BFT-treated cells. Adhered cells were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet and were imaged using bright-field microscope. For Spheroid migration assay, migration of single cells from the tumor spheroids over time was monitored using phase-contrast microscopy. Distance migrated was measured using Lieca image scope software and plotted as a measure of cell movement using GraphPad Prism 5 software (CA, USA).

**Matrigel-invasion, transwell-migration and scratch-migration assay.** To assess the migratory and invasive potential of BFT treated MCF7 and MCF10A cells, we utilized the conventional Matrigel-invasion, scratch-migration and transwell-migration assays. For *Matrigel-invasion assay,* BFT (5 nM)-treated MCF7 and MCF10A cells (20,000) were seeded in Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA). For *transwell-migration assay,* BFT (5 nM)-treated MCF7 and MCF10A cells (20,000) were seeded in an upper chamber with an 8μm pore size and cells were allowed to invade or migrate through the matrigel or filter for 24-48 hours. The number of invaded/migrated cells on representative sections of each membrane were
counted under light microscope. For *scratch-migration assay*, monolayers of MCF7 and MCF10A were allowed to form; a 1-mm wide scratch was made across the cell layer using a sterile pipette tip and media was replaced with fresh serum-free media containing 5 nM BFT or vehicle control. Plates were photographed immediately after scratching and migration of cells was followed for various time intervals. Wound closure was quantified from distance between edges using Leica ImageScope software. Speed of migration was calculated and wound closure was plotted using GraphPad Prism 5 software.

**Mammosphere assay.** For *liquid mammosphere* assay, 5,000 MCF7 or MCF10A cells were seeded in 2 ml of liquid mammosphere media in 30 mm ultra-low attachment plates. Cells were treated with vehicle or 5 nM BFT and allowed to grow for 7 days. For *solid mammosphere* assay, 5,000 MCF7 or MCF10A cells were suspended in 2 ml mammosphere medium containing methylcellulose, plated on ultra-low attachment plates and incubated for 7 days. Cultures were observed under microscope and spheres (>50 µm) were counted.

**Protein isolation, sub-cellular fractionation and western blotting.** Whole cell lysates were prepared using modified RIPA buffer. For *protein lysates of tumor samples*, tumor tissues were homogenized using a tissue homogenizer in mammalian tissue lysis buffer on ice. For *sub-cellular fractionation*, nuclei were separated from the cytosolic fraction resuspended in nuclear protein extraction buffer.

**RT-PCR and immunofluorescence.** For *RT-PCR*, cells/tissue/mammosphere samples were lysed in Trizol, RNA was isolated by chloroform-isopropanol method and cDNA was synthesized using iScript cDNA synthesis kit. Reverse transcription-polymerase chain reaction (RT-PCR) was performed and imaged using Gel Doc image system (Bio-Rad). For *immunofluorescence*, fixed cells were permeabilized using 0.1% Triton-X-100 followed by overnight incubation with primary antibody at 1:100 dilution in 3% BSA. Cells were then incubated with FITC/TRITC tagged secondary antibody. Cells were examined under Lieca E800 fluorescent microscope. Images were captured at 60X magnification using oil immersion objective with Lieca Elements software.

**Detection of cancer stem cell markers by flow cytometry:** The expression profile of CD24 (*anti-human#555427, BD Biosciences*) and CD49f (*anti-human/mouse#313616, BioLegend*) in tumor-derived cells were analyzed by flow cytometry. Briefly, 10⁶ cells were stained with respective antibodies following antibody manufacturer’s specific protocol. Labeled cells were acquired by BD FACS LSR II and analyzed using FACS Diva 6.0 software.

**Orthotopic-xenograft model and limiting-dilution orthotopic-xenograft model** SCID-NOD mice (female, 6-8 weeks old) were acquired from SKCCC animal facility and maintained in house. Exponentially growing MCF7 or MCF10A-Kras cells, treated with 5 nM BFT for 48 hours (5x10⁷ cells in 100 µl matrigel), were implanted in the 4th mammary fat pad on either side. Tumor volumes were monitored. Tumors were excised and processed for further analysis. For *limiting-dilution orthotopic-xenograft model* (47), cells dissociated from primary tumors formed by BFT-treated MCF7 cells were injected at limiting dilutions (5x10⁶- 5x10³), into the
mammary fat pads of immunocompromised SCID-NOD mice. Tumor incidence was regularly monitored and stem Cell frequency is calculated based on tumor incidence.

**DAPT and ICG001 treatment animal models** For *in vitro* drug treatments, MCF7 cells were pre-treated with 5nM BFT alone or in combination with β-catenin inhibitor ICG001 or γSecretase inhibitor DAPT or both for 48 hours. 5×10⁶ pre-treated cells were then implanted into the mammary fat pads of 4 to 6 weeks old NOD-SCID mice previously implanted with E2 pellets subcutaneously. Tumor progression was monitored for 7 weeks. For *in vivo* drug treatments, MCF7 cells were pre-treated with 5nM BFT for 72 hours. 5×10⁶ MCF7 or BFT pre-treated MCF7 cells were then implanted into the mammary fat pads of 4 to 6 weeks old NOD-SCID mice previously implanted with E2 pellets subcutaneously. Tumors were allowed to reach a minimum volume (200mm³ in BFT pre-treatment group) and mice were randomized into 8 experimental groups. The animals were then treated with ICG001 (20 mg/Kg body weight, daily in 2% DMSO + 50% PEG300 + 5% Tween 80 + ddH2O) or DAPT (20 mg/kg, alternate days in corn oil) or a combination of both drugs through the course of the experiment. Tumor progression was monitored regularly. Tumors were excised at the end of the experiment and processed for further analyses.

**Mammary intraductal (MIND)-syngeneic model.** Twice parous Balb/C mice were obtained from Charles River and maintained in house. Mice were given antibiotic cocktail (clindamycin 0.1g/L and streptomycin 5 g/L) in water bottles (Hospira and Amresco) for 7 days and discontinued. Mice in ETBF or 086Mut group were then oral gavaged with ~10⁸ CFU of ETBF or 086Mut strains of *B. fragilis* in 1X PBS and allowed to colonize for 3 days. Intraductal infection groups received ~10⁸ CFU of ETBF or 086Mut strains of *B. fragilis* in 1X PBS via teat-injection. For sham-control, mice were oral gavaged or injected in teats with 1X PBS. To establish intraductal mammary tumors, 20,000 4T1-Luc2 cells were injected directly into the mammary ducts of mice on one side. Tumor progression was regularly monitored by bioluminescent imaging using IVIS spectrum at SKCCC animal resources. For bioluminescent imaging, animals were injected with 10 μl/g D-luciferin (15mg/ml in PBS) intraperitoneally and images were captured 8-10 minutes post injection. At the end of the experiment, *ex-vivo* bioluminescent images of major organs, lungs and liver were captured to investigate metastatic progression. Briefly, animals were given an intraperitoneal injection of D-luciferin and were euthanized after 10 minutes. Lungs and liver were excised and images were captured using IVIS system. Tumors were excised, weighed and preserved for subsequent studies. For MCF7 tumor model, 4-6 weeks old NOD/SCID mice with given a cocktail of antibiotics as described above for 1 week in drinking water. Mice were then infected with 10⁸ CFUs of ETBF or 086Mut via oral gavage and allowed gut colonization for 1 week followed by implantation of 5×10⁶ MCF7 cells in mammary fat pads. Tumor progression was monitored for 7 weeks. Tumors were excised, weighed and preserved for subsequent studies.

**4T1 metastasis assay.** Lungs and liver excised from 4T1 Luc2 tumor bearing Balb/c mice were harvested in DMEM F12 medium, minced into pieces and transferred into 15ml tubes containing 2.5ml of respective
digestion cocktail; RPMI containing 10 mg/ml Collagenase A for liver and RPMI containing 10mg/ml of Collagenase A+10 mg/ml of Hyaluronidase for lungs. The organs were then placed in shaking water bath at 37°C for 30 minutes to allow complete dissociation and filtered through separate 70 μm nylon cell strainer to remove large chunks of undigested tissue. Samples were collected in 50 ml tubes, centrifuged for 5 minutes/1500 rpm/RT and supernatants were discarded. Samples were washed twice by centrifugation in PBS. Pellets were resuspended in culture media containing 10 μl of 60 mM 6-thioguanine and plated onto 6-well culture plates. Plates were incubated in 37°C / 5% CO₂ to allow growth of colonies for 3-7 days (48).

**RNA sequencing and data analysis.** RNA extraction, sequencing, and sequence analysis were performed by the Johns Hopkins Transcriptomics and Deep Sequencing Core. Detailed method is provided in the supplemental section.

**Statistical analysis.** All experiments were performed thrice in triplicates. RNA sequence expression analysis was done using Partek software, and functional pathway analyses using Spotfire and Ingenuity software. Measurements of micrographs and IHC quantitation were done using Leica Aperio ImageScope, Leica Biosystems. Western blot and RT-PCR quantification were performed using GelQuant. Statistical analyses were done using GraphPad Prism 5. Results were considered to be statistically significant if P < 0.05. Results were expressed as mean ± SE between triplicate experiments performed thrice. For comparison between multiple groups, statistical significance was determined by One-way ANOVA and Bonferroni analysis. Comparison between two groups were done using Student’s t test.
References


FIGURES LEGENDS

Figure 1. Identification of B. fragilis in breast cancer patients and the effect of intraductal administration of enterotoxigenic B. fragilis (ETBF). (a) Relative abundance of B. fragilis in breast tissue or nipple aspirate fluid (NAF) of women with malignant breast cancer (n=14), benign breast cancer (n=9), breast cancer survivors (n=16) and controls (n=21) (meta-analysis of available metagenomic data using OneCodex). *** p<0.0005, ** p<0.005. (b) Cartoon showing intraductal administration of B. fragilis. (c) Detection of ETBF and 086Mut in mammary gland by qPCR (d) Detection of BFT in mammary gland by qPCR. (e) BFT is detected and quantified in mammary glands of sham-control mice and mice bearing ETBF or 086Mut ductal infection using ELISA; n=5; *** p<0.0005. (f, g) Representative H&E, Trichrome and IHC images of Ki67, PCNA, Pan-keratin, CD45 and CD3 stained mammary ductal epithelial cells in mice bearing ductal colonization of ETBF, 086Mut or sham-controls (n=5). Dot blots show corresponding quantitative analysis of Ki67, PCNA, CD45, CD3 and Pan-keratin staining. Scale bar: 200μm=260.4 pixels. *** p<0.0005.

Figure 2. ETBF colonization leads to hyperproliferation of mammary ductal epithelium. (a) Detection of B. fragilis in mammary tissue of C57BL/6 mice with ETBF or 086Mut-gut colonization and sham-control by PCR analysis. (b) Bacteroides fragilis toxin (BFT) gene detected in mammary tissue of ETBF or 086Mut-colonized and sham-control mice using PCR analysis. (c) Circulating BFT detected and quantified in serum of sham-control mice and mice bearing ETBF or 086Mut infection using ELISA; n=5; *** p<0.0005, ** p<0.005. (d) Representative images of carmine alum-stained mammary glands of ETBF-colonized and sham-control Balb/C mice and corresponding scoring of focal hyperplasia at week 1 and week 3 post-infection (e) Representative H&E images of mammary duct lining in ETBF or 086Mut-colonized mice and sham-control mice; histological scoring based on thickness of duct lining and cellularity of inner margins of the duct. Tabular representation of histological grading of breast tissue sections. Dot plot showing difference in thickness of breast ducts in ETBF-colonized groups (n=5) compared to 086Mut-colonized group and sham-control (n=5) at week 1 and week 3 post-infection. *** p<0.0005. (f) Representative IHC images of Ki67, Pan-keratin and CD3-stained mammary ductal epithelial cells in mice bearing enteric colonization of ETBF or 086Mut and sham-controls (n=5). Dot blots show corresponding quantitative analysis of Ki67, Pan-keratin and CD3 staining. Scale bar: 200μm=260.4 pixels. *** p<0.0005.

Figure 3. BFT induces morphological changes and enhances the migratory and invasive potential of MCF7 and MCF10A cells. (a) Colony morphology of MCF7 and MCF10A cells treated with 5 nM BFT for 7 days in a clonogenicity assay. All images are representative of three independent experiments. (b) RNA sequencing was performed on MCF7 cells treated with 5 nM BFT for 48 hours. Volcano plot showing
upregulation of genes associated with cytoskeletal remodeling, cell movement and migration. (c) Ingenuity functional annotation graph showing differential regulation of genes involved in movement of breast cancer cell lines upon treatment with BFT. (d) Micrographs show untreated and 5 nM BFT-treated MCF7 cells migrating through the 10 μM width microfluidic device at various time intervals as indicated. (e, f) Bar graphs show velocity (e) and persistence (f) of untreated and 5 nM BFT-treated MCF7 cells migrating through microchannels of different widths as indicated (3-50 μM). Data represents the mean ± s.e. of cells from n=4 independent experiments. *p<0.00005****, **p<0.005**p<0.05*. (g) Representative images of MCF7 and MCF10A cells invading through matrigel invasion chamber upon treatment with 5 nM BFT. Untreated cells serve as control. (h, i) Cell adhesion assay estimating the change in adhesion potential of the MCF7 and MCF10A cells to type I collagen upon treatment with 5 nM BFT. Representative images are shown. Bar graph shows the absorbance at 550 nm.

**Figure 4. Exposure to BFT increases tumor-formation potential of MCF7 cells resulting in tumors harboring features of stemness and metastatic progression.** (a) Representative images of unifocal and multifocal tumors formed in mammary fat pads of SCID/NOD mice implanted with untreated MCF7 cells or MCF7 cells exposed to BFT for 72 hours. Line graph shows tumor progression curves of control-MCF7 and BFT-exposed MCF7 cells. Dot plot shows cumulative weights of tumors from each experimental group. (number of mice/group=6). (b) Resected tumors from control and BFT-exposed group were dissociated and isolated tumor cells were subjected to *in vitro* functional assays. Representative images of dissociated tumor cells from resected tumors from control and BFT-exposed group undergoing matrigel invasion, spheroid migration, transwell migration and adhesion assay are shown. Dot plot shows quantitative presentation of adhesion assay. Data represents more than four independent experiments. (c) H&E, Trichrome and IHC images of CD31, Ki67, cMyc, E-cadherin and N-cadherin-stained tumor sections from tumors formed in mammary fat pads of SCID/NOD mice implanted with untreated MCF7 cells or MCF7 cells exposed to BFT for 72 hours are shown. (n=6). Scale bar, 100 μm. (d) Schematic outline of the *in vivo* limiting dilution assay. (e) Tumor volume of secondary tumors established with 5X 10^3 tumor-dissociated cells from tumors formed in mammary fat pads of SCID/NOD mice implanted with untreated MCF7 cells or MCF7 cells exposed to BFT for 72 hours. (f) Plots show Kaplan-Meier curves (green-control, red-BFT) for time to detect tumors in mice bearing secondary tumors. (g) Tumor incidence at week 4, 5, and 6 of secondary transplants of untreated MCF7 cells or MCF7 cells exposed to BFT for 72 hours at limiting dilutions. The tumors/numbers of mice/group are shown. The bottom row indicates the estimated breast tumor-initiating/stem cell (SC) frequencies.
**Figure 5.** BFT pre-treatment activates βcatenin and Notch 1 pathways in MCF7 xenografts. (a) Differential expression statistics comparing samples of secondary tumors established with tumor-dissociated cells from primary tumors formed with untreated MCF7 cells or MCF7 cells exposed to BFT for 48 hours were calculated and results are shown in a Volcano plot. Upregulation of βcatenin responsive genes (marked in blue) and Notch 1 responsive genes (marked in pink) as observed in RNA-seq analyses is shown. (b) Expression of βcatenin gene in MCF7 cells treated with 5 nM BFT for various time intervals as indicated using RT-PCR analysis. β-actin was included as control. (c) Immunoblot analysis total βcatenin and phospho-βcatenin expression in MCF7 cells treated with BFT for various time intervals as indicated. Expression of β-actin was included as control. (d) Expression of total βcatenin in nuclear and cytoplasmic lysates of MCF7 and MCF10A cells treated with 5 nM BFT for 3-12 hours as indicated. Lamin B expression serves as a control for nuclear lysates and β-actin was included as control for cytoplasmic lysates. (e) Immunofluorescence analyses of βcatenin in MCF7 cells treated with 5 nM BFT. Nuclei are stained with DAPI. Scale bar, 20 µm. (f) Immunoblot analyses of βcatenin-responsive genes (Slug, c-Myc, Jagged and Twist) in MCF7 and MCF10A cells treated with 5 nM BFT for various time intervals as noted. β-actin was included as control. (g) Immunofluorescence analyses of Jagged and Slug in cells treated with 5 nM BFT. Nuclei are stained with DAPI. Scale bar, 20 µm. (h) Representative IHC images of βcatenin-stained secondary tumors established with tumor-dissociated cells from primary tumors formed with untreated MCF7 cells or MCF7 cells exposed to BFT for 48 hours. Scale bar, 100 µm. (i) Schematic representation of activation of βcatenin pathway by BFT.

**Figure 6.** BFT concomitantly activates Notch 1 and βcatenin axes in MCF7 and MCF10A cells. (a) Immunoblot analysis showing temporal upregulation of NICD expression in MCF7 and MCF10A cells treated with 5 nM BFT. β-actin was included as control. (b) Immunofluorescence analyses of NICD in cells treated with 5 nM BFT. Nuclei are stained with DAPI. Scale bar, 10 µm. (c) Immunoblot analyses of Notch-responsive genes (p21/Waf and Hes1) in MCF7 and MCF10A cells treated with 5 nM BFT for various time intervals as noted. Immunoblot with β-actin was included as control. (d, e) Line graph shows tumor progression curves (d) of control-MCF7, and MCF7 cells pre-treated (for 48 hours) with 5 nM BFT alone or in combination with 25 μM DAPT (Notch 1 inhibitor) or 5 μM ICG001 (βcatenin inhibitor) or DAPT+ICG001 in vitro and implanted in mammary fat pads of SCID/NOD mice. Cells treated with inhibitors alone were also included. p<0.0005***, p<0.005**. (e) Flow cytometry analysis of dissociated tumor cells using CD49f and CD24 staining. (f-h) Line graph shows tumor progression curves (f) of MCF7 cells exposed to 5 nM BFT for 72 hours and implanted in mammary fat pads of SCID/NOD mice. Mice were treated with ICG001, DAPT and ICG001+DAPT for 6 weeks. p<0.0005***. (g) Representative sections of tumors from all the treatment groups showing Ki67 staining. (h) Representative images of primary and secondary mammosphere formed by dissociated tumor cells.
are shown. Bar graphs show number of primary and secondary mammospheres formed in each group. *p<0.0005***, *p<0.005**.

**Figure 7. Gut or ductal colonization with ETBF enhances breast tumor growth and distant organ metastasis in multiple mouse models.** (a-f) *4T1 intraductal breast tumor implant in Balb/C mice bearing enteric ETBF or 086Mut infection.* (a) Curves showing tumor progression of 4T1 cells implanted intraductally in mice harboring no infection or gut colonization with ETBF or 086Mut (n=5). *p<0.0005***, *p<0.005**. (b) Representative images of *ex vivo* bioluminescent imaging of lungs and livers of mice from the sham-control, 086Mut-gut colonized and ETBF-gut colonized groups. (c) Representative images of *ex vivo* metastasis assay of lungs and livers of mice from the sham-control, 086Mut- gut colonized and ETBF-gut colonized groups. Graph shows number of metastatic colonies in each group. (d) Representative sections showing metastatic area in liver and lungs of control, ETBF and 086Mut groups. (e, f) Graphs showing the met diameter in livers and lungs of mice from the sham-control, 086Mut- gut colonized and ETBF-gut colonized groups. *p<0.0005***, *p<0.005**. (g-l) *4T1 intraductal breast tumor implant in Balb/C mice bearing intraductal ETBF or 086Mut infection.* (g) Curves showing tumor progression of 4T1 cells implanted intraductally in mice harboring no infection or breast ductal colonization with ETBF or 086Mut (n=5). *p<0.0005***. (h) Graph shows tumor weight in sham-control, 086Mut- ductal colonized and ETBF-ductal colonized groups. *p<0.0005***, *p<0.005**. (i) Representative sections showing metastatic area in livers and lungs of control, ETBF and 086Mut groups. (j, k, l) Representative images of *ex vivo* metastasis assay of lungs and livers of mice from the sham-control, 086Mut- ductal colonized and ETBF-ductal colonized groups. Graph shows number of metastatic colonies in lungs and livers of each group. *p<0.0005***. (m-n) Curves (m) showing tumor progression of MCF7 cells implanted in mammary fat pads of mice harboring no infection or gut colonization with ETBF or 086Mut (n=5). *p<0.0005***. (n) Representative H&E, Trichrome and IHC images of Ki67 and CD31 stained tumors from mice harboring no infection or gut colonization with ETBF or 086Mut.
Figure 3

(a) MCF7 and MCF10A cells under control and BFT conditions.

(b) Heatmap showing gene expression changes with 

(c) Network diagram illustrating gene interactions.

(d) Time-lapse images of cell migration.

(e) Graphs depicting cell velocity and persistence.

(f) Persistence graph with statistical significance.

(g) Histological images of MCF7 and MCF10A cells.

(h) Electron micrographs showing cell morphology.

(i) Absorbance at 550 nm for MCF7 and MCF10A cells.

Downloaded from cancerdiscovery.aacrjournals.org on February 5, 2021. © 2021 American Association for Cancer Research.
Figure 4

(a) Primary and MCF7 Tumor Growth
- Control vs. BFT
- Time in weeks vs. Tumor volume (mm³)
- P-value: 0.017

(b) Dissociated Tumor Cells
- Matrigel Invasion
- Spheroid Migration
- Transwell Migration

(c) Tumor Weight
- Control vs. BFT
- p-value: 0.1297

(d) Adhesion Assay
- Control vs. BFT

(e) Secondary Tumor Growth
- Control vs. BFT
- Tumor Volume (mm³)

(f) Tumor Free Survival
- Control vs. BFT

(g) SC Frequency
- Week 4 vs. Week 5 vs. Week 6
- Control vs. BFT

<table>
<thead>
<tr>
<th></th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>BFT</td>
<td>Control</td>
</tr>
<tr>
<td>50000000</td>
<td>5/5</td>
<td>4/4</td>
<td>5/5</td>
</tr>
<tr>
<td>5000000</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>50000</td>
<td>1/5</td>
<td>4/5</td>
<td>2/5</td>
</tr>
<tr>
<td>5000</td>
<td>0/5</td>
<td>4/5</td>
<td>0/5</td>
</tr>
<tr>
<td>SC frequency</td>
<td>1 in 151,950</td>
<td>1 in 38,481</td>
<td>1 in 63,097</td>
</tr>
</tbody>
</table>
Figure 7

(a) Total Flux (p/s) for Control (Gut), ETBF (Gut), and 086Mut (Gut) for Intraductal 4T1.

(b) Liver and Lungs ex vivo metastasis assay showing Control, ETBF, and 086Mut.

(c) Liver and Lungs metastatic colonies showing Control, ETBF, and 086Mut.

(d) Liver and Lungs histology for Control, ETBF, and 086Mut.

(e) Liver metastatic diameter (µm) for Control, ETBF, and 086Mut.

(f) Lung metastatic diameter (µm) for Control, ETBF, and 086Mut.

(g) Total Flux (p/s) for Control (Ductal), ETBF (Ductal), and 086Mut (Ductal) for Intraductal 4T1.

(h) Tumor weight (g) for Control, ETBF, and 086Mut.

(i) Liver and Lungs histology for Control, ETBF, and 086Mut.

(j) Liver and Lungs histology for Control, ETBF, and 086Mut.

(k) No. of metastatic colonies for Control, ETBF, and 086Mut.

(l) No. of metastatic colonies in liver for Control, ETBF, and 086Mut.

(m) Tumor volume (mm³) for Control (Gut), ETBF (Gut), and 086Mut (Gut) for MCF7.

(n) H&E, Trichrome, Ki67, and CD31 staining for Control, ETBF, and 086Mut.
CANCER DISCOVERY

A pro-carcinogenic colon microbe promotes breast tumorigenesis and metastatic progression and concomitantly activates Notch and βcatenin axes

Sheetal Parida, Shaoguang Wu, Sumit Siddharth, et al.

Cancer Discov  Published OnlineFirst January 6, 2021.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-20-0537

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2021/01/05/2159-8290.CD-20-0537.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/early/2021/01/05/2159-8290.CD-20-0537.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.