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Enterococcus faecalis promotes a migratory and invasive phenotype in colon cancer cells

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Abstract

Much about the role of intestinal microbes at the site of colon cancer development and tumor progression following curative resection remains to be understood. We have recently shown that collagenolytic bacteria such as *Enterococcus faecalis* predominate within the colon postoperatively, particularly at the site of the colon reconnection (i.e. anastomosis) in the early period of post-surgical recovery. The presence of collagenolytic bacteria at this site correlates with the tumor progression in a mouse model of post-surgical tumor development. In the present study we hypothesized, that collagenolytic bacteria, such as *E. faecalis,* play an important yet to be discovered role in tumor formation and progression. Therefore the aims of this study were to assess the role of collagenolytic *E. faecalis* on the migration and invasion of a murine colon cancer cell line. Results demonstrated that both migration and invasion were induced by *E. faecalis* with collagenolytic activity being required for only invasion. Bidirectional signaling in the *E. faecalis*-cancer cell interaction was observed by the discovering that the expression of *gelE* in *E. faecalis*, the gene required for collagenase production, is expressed in response to exposure to CT26 cells. The mechanism by which migration enhancement via *E. faecalis* occurs appears to be dependent on its ability to activate pro-uPA, a key element of the urokinase-plasminogen system, a pathway that is well – known to be important in cancer cell invasion and migration. Finally, we demonstrated that collagenase producing microbes preferentially colonize human colon cancer specimens.

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Keywords: Enterococcus faecalis, gelE, uPA, uPAR, Colon cancer

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world with nearly 2-million cases diagnosed annually [1]. Originally described by Vogelstein and colleagues, the pathogenesis of CRC is reliant upon the accumulation of genetic and epigenetic mutations [2]. These genomic alterations in oncogenes or tumor suppresser genes ultimately cause the irreversible loss in the control of cell division and replication that ultimately results in malignancy. In the four decades since this initial description, the critical roles of the immune, stromal, and microbial compartments of the tumor microenvironment in both primary tumorigenesis and subsequent metastasis have come to light.

The human gastrointestinal tract harbors over 100 billion bacterial organisms, and it has become increasingly recognized that bacteria plays a role in CRC pathogenesis [3,4]. There are multiple mechanisms by which gut microbiota may influence carcinogenesis. First, certain species can be pro-carcinogenic. For example, *Fusobacterium nucleatum* can activate β-catenin signaling via a *FadA – E-cadherin* interaction, leading to NF-kB gene expression and enhanced tumor proliferation [5]. Similarly, certain species, (i.e. *Escherichia*), can generate genotoxins such as cytolethal distending toxin and colibactin that can directly cause double-strand DNA breaks [6]. Further,

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bacterial metabolism of short-chain fatty acids or bile acids can create an environment filled with pro-tumorigenic metabolites [4].

Enterococcus faecalis is a human gut commensal that has had a controversial role in CRC pathogenesis [7]. Some strains are thought of as probiotic and have protective anti-inflammatory effects via induction of interferon-gamma, IL-10 and other anti-inflammatory cytokines [8], while other strains have been shown to trigger carcinogenesis, primarily due to their ability to generate reactive oxygen species (ROS) leading to DNA damage and genomic instability [9]. These conflicting results have been exemplified in a recent report in which various strains of *E. faecalis* isolated from human stool had differential effects when co-incubated with cancer cells [10].

Our laboratory has been interested in the role of collagenolytic *E. faecalis* strains on CRC tumorigenesis. We have created a mouse model that replicates CRC tumor formation following surgery, in which mice undergo a colon resection (mimicking the surgery humans would undergo for cure) followed by exposure to cancer cells via enema (mimicking intraluminal exfoliated cancer cells known to exist in humans) [11]. We observed that extraluminal tumors form, but only when the intestine is colonized by strains of *E. faecalis* that exhibit a phenotype of enhanced bacterial collagenolytic activity (i.e., gelatinase (collagenase) production). Given that non-collagenolytic *Enterococcus* species do not associate with tumor formation, this data suggests that bacterial collagenase plays a critical and yet to be defined role in CRC tumorigenesis. We therefore hypothesized that collagenolytic *E. faecalis* could directly promote the progression of CRC cells. Finally, we sought to determine the mechanism by which *E. faecalis* might influence tumor progression in CT26 cells by focusing on the urokinase→pro-urokinase→plasminogen system as this pathway has been clinically and mechanistically linked to tumor progression.

Methods

Cell line and preparation

Luciferase/tdTomato-labeled monoclonal (L2T) CT26 cells derivative of CT26 WT (ATCC CRL-2638) colon carcinoma cell line derived from BALB/c mice were used in this study. The (L2T) CT26 cells were generated by the Ralph Weichselbaum laboratory at University of Chicago.

To create the aliquots used for experiments, 1×10^6 L2T CT26 cells were thawed and grown in RPMI 1640 media supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, dose of glutamine (2mM), and 100 mg/mL streptomycin. CT26 cells were maintained in cell culture at 5% CO_2 and 37°C in T75 cell culture flasks. Once cells reached 80% confluency, cells were removed from the plate with 0.05% trypsin, spun, washed, and resuspended in RPMI 1640 with 10% FBS.

Bacterial strains

The *E. faecalis* strain V583 and its derivative mutant, Δ*gel/sprE*, were kindly provided by Lynn Hancock [12]. *Escherichia coli* strain OP50, a commensal strain (ATCC #25922), was used as a control. All strains were stored in 10% glycerol stock at −80°C. Bacterial cells freshly plated from stock were used in all experiments. Cells from stock were plated onto tryptic soy broth plates (TSB) and grown overnight at 37°C. Liquid suspensions of bacteria from an overnight culture were dilated to an optical density of 600nm = 0.6 and then used for experimentation.

Urokinase, pro-urokinase, plasminogen, and urokinase receptor assays

All experiments using urokinase (Biodivision) and pro-urokinase (Biodivision) assays were conducted using physiologic concentrations of 4 nM following the vendor protocols. All experiments using plasminogen PLG (human glu-plasminogen, Haematologic technologies) were conducted using physiologic concentrations at 250 nM following the vendor protocol. uPAR blocking was tested using the affinity-purified polyclonal goat anti-mouse uPAR receptor antibody (R&D Systems; AF524) at 50μg/ml.

Binding of urokinase to bacterial surface

Binding of urokinase, also known as urokinase-type plasminogen activator (uPA) to the bacterial surface was measured as previously described, with minor modifications [13]. Bacteria were grown overnight in TSB and were diluted to a final density of roughly 8×10^6 CFU/mL. These cells were then incubated at 37°C with 250 nM FITC-labeled uPA (Oxford Biomedical Research). Cells were then pelleted, washed three times with PBS and resuspended in PBS. Fluorescence was analyzed using an Imagestream ISX flow cytometer. *E. faecalis* and *E. coli* were detected using log-forward and log-side scatter dot plots, and a gating region was set to exclude debris and aggregates of bacteria. Bacteria were analyzed for FITC-range fluorescence using log-scale amplification, of which the geometric mean fluorescence intensity was recorded as a measure for uPA binding. FITC-range fluorescent signal was confirmed by light and fluorescent microscopy of each event.

Cell migration and invasion assays

For migration assays, 8.0 μm pore size transwell permeable inserts (Costar, HighWycome, UK) were placed in the wells of 24-well culture plates; 500 μl of serum-free RPMI 1640 medium (ThermoFisher, Massachusetts, USA) was added to the lower chamber. CT26 cells at 80% confluence were trypsinized, washed twice with PBS (ThermoFisher, Massachusetts, USA), and resuspended in serum-free RPMI 1640 to a concentration of 1×10^6 cells/ml. 100 μl of cell suspension was added to the upper chamber. Bacterial cells from TSB plates were suspended in serum-free RPMI 1640 medium to an OD of 600nm = 0.6 for *E. faecalis* and OD of 600nm = 0.3 for *E. coli* as described above. 20 μl of bacterial suspension were added to the upper chambers. For certain experiments, 250 nM of pro-urokinase or urokinase and plasminogen at 10uL/100uL were added to both the upper and lower chambers to neutralize any chemo-attractant effect. After 24 hours of incubation at 37°C with 5% CO_2 CT26 cells that remained on the top side of the filter were removed using a cotton swab and Kimwipes (ThermoFisher, Massachusetts, USA). Cells adherent to the undersurface of the insert were fixed in 70% ethanol for 10 min, dried for 15 min, and stained with 0.025% crystal violent in 20% methanol. After 10 min of incubation, the inserts were washed thoroughly in water and dried. Migrated CT26 cells were counted using inverted microscopy and compared in fold increase ratios to control.

Invasion assays were performed using invasion assay inserts (EMD Millipore Corp, Massachusetts, US). Inserts were equilibrated to room temperature, then inserts were rehydrated in tissue culture incubator for 30 min with 300 μl of serum-free RPMI 1640 before use. Assays were then performed in an identical manor as the migration assays described above.

Effect of CT26 cells on gelE expression in E. faecalis

CT26 cells and V583 *E. faecalis* were grown as described above. To create conditioned media, 15 ml of CT26 culture media was spun at 5000 x g for 5 minutes, the supernatant was vacuum aspirated and then filtered using a 0.2μM filter. For coincubation experiments, 1 ml of bacterial culture was injected into T75 flasks with CT26 cells or conditioned media for a total of 15 ml total volume per flask. Suspensions were incubated for 2 hours at 37°C at 5% CO_2. 2 ml of RNA Protect Bacteria Reagent was injected into the T75 flask and incubated for 5 minutes; media and cells were then scraped and transferred into decanted 15 ml conical tubes and spun at 5000 x g for 5 minutes and cell pellets were lysed and digested

with lysozyme (Sigma-Aldrich; 100 µl of 15mg/ml), proteinase K (Sigma-Aldrich; 10µl of 600 mAU/ml) and TRI Reagent (Invitrogen) using FastPrep 24-5G homogenizer. The upper phase was isolated, incubated at room temperate for 10 minutes and then 0.2 ml of chloroform was added. Samples were then transferred to pre-spun Pellet Lock Gel tubes for further phase separation and the liquid phase RNA was purified with RNeasy kit (Qiagen #74004). RNA content was measured using a nanodrop (Nanodrop 1000; Thermo Scientific). RNA was further purified with TURBO DNase treatment enzymatic digestion (Invitrogen #AM2238). The absence of DNA in isolated RNA samples after DNAse treatment was confirmed by the absence of amplification of 23S rRNA gene. 1 μg of RNA was used for cDNA synthesis using BioRad iScript Reverse Transcription Supermix (BioRad, Hercules, CA, USA). The 23S rRNA housekeeping gene expression was used for normalization. The primer sequence for gelE was: forward 5'-CGGAACATACTGCCGGTTTAGA-3'; reverse 5'- TGGATTAGA TGCACCCGAAAT-3′. The primer sequence for the 23S ribosomal housekeeping gene was: forward 5'-CCTATCGGCCTCGGCTTAG-3′; reverse 5'-AGCGAAAGACAGGTGAGAATCC-3′. Each sample was run in triplicate, and mean values were used in calculations. The ΔΔCt was used to measure the fold change of gelE expression.

Effect of E. faecalis on gene expression in CT26 cells

CT26 cells and V583 *E. faecalis* were grown as described above. Bacterial cells from the TSB plate were suspended in serum-free RPMI 1640 medium at an OD of 600nm = 0.6. 20 μl of bacterial suspension was added to CT26 cells that were grown to 80% confluence on 24-well plates that had 500 μl of RPMI 1640 medium. Cells were then incubated at 37°C and 5% CO_2. At 4 or 8 hr the plates were removed and RNA was isolated in an identical fashion as for the isolation of *gelE* RNA. RNA-seq analysis was performed by the University of Chicago CRI Bioinformatics Core as follows. The quality of raw sequencing data was evaluated using FastQC v0.11.5 [Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/] and Illumina adapter/primer sequences were detected from sequencing reads. All RNA reads were first mapped to the mouse (mm10) reference genome using STAR v2.6.1a release with default parameters [14]. Picard v2.18.11 (http://broadinstitute.github.io/picard/) was utilized to collect mapping QC metrics. The resulting files from the previous alignment step in the RNA-seq analysis were taken individually as input to evaluate a transcriptional expression using Rsubread::featureCounts v1.5.3 [15]. Data were examined using Principal Component Analysis (PCA) based on normalized expression distribution. Differentially Expressed Genes (DEGs) and isoforms were detected using an ensemble approach of three tools; including edgeR v3.23.5, DESeq2 v1.21.22, and limma v3.37.7 [16-18]. The criteria of fold-change $\geq$ 1.5 and false discovery rate (FDR) $<$ 0.1 were used in pair-wise comparisons. Genes detected by all methods were collected to create a list of high-confidence DEGs. To obtain the groups with similar expression trends based on identified DEGs, several in-house scripts were implemented using R (https://www.r-project.org/) and Perl (https://www.perl.org/) languages. The master Perl script was used to submit R jobs in parallel to High Performance Computing (HPC) Clusters by considering different combinations of distance matrices and parameters. Then, the clustering results were gathered and visualization figures were generated. For heat maps, DEGs were shown as log2-transformed fold changes to the mean normalized expression of the control group. The identified DEGs were further used as input to functional analysis modules to identify enrichment of functional categories and regulatory networks using Gene Ontology (GO) terms and KEGG-enrichment analyses. Pathways significantly enriched in the genes of interest were identified using clusterProfiler (v3.6.0) at FDR-adjusted p-value $<$ 0.10 (hypergeometric test) [19]. Gene Set Enrichment Analysis (GSEA) was also performed using clusterProfiler.

Detection of collagenolytic bacteria in human samples of colon carcinoma

Swabs of resected colon carcinoma and tissue were collected under IRB 10-209. Patients 18 or older undergoing an elective colon resection for colon adenocarcinoma at the University of Chicago were enrolled between September 2017 to September 2018. An informed consent was obtained from all patients. Colonic tumor and tissue were swabbed (Puritan Opti-Tranz ® Liquid Amies Gel Swab Transport Systems, Issaquah, WA, USA) at three designated areas (tumor, 3 cm from tumor location, and 6 cm from the tumor location) once opened longitudinally by the Department of Pathology staff members at the University of Chicago. Swab samples were spun in tryptone/yeast extract (TY) media containing 10% glycerol and frozen at -80°C. 20 µl of semi-thawed solution was plated either onto skim milk containing *Enterococcus* agar prepared from (BBL™ *Enterococcus* agar, BD, Sparks, MD, USA) or Columbia CNA agar with 5% Sheep Blood (BBL TM Columbia, Sparks, MD, USA), or MaConkey agar (Difco TM MaConkey, BD, Sparks, MD, USA). Collagenolytic colonies were analyzed by the formation of halo area around the colonies as previously described [20].

Statistical analysis

Statistical analyses were performed using Graphpad Prism 8 software. All *in vitro* experiments were performed with at least 3 independent experiments. Unpaired Student's t-tests were used for comparisons between two means for continuous variables. ANOVA analysis was applied to compare slopes of regression lines in enzyme activity assays. Chi-square analysis was used for comparison of categorical variables. Cellular populations were compared in flow cytometric experiments through automated Kolmogorov-Smirnov analysis on FlowJo software. Statistical significance was defined as a p value $<$0.05.

Results

Collagenase producing microbes preferentially colonize human colon cancer specimens. Our previous research has demonstrated a role of collagenase producing bacteria in promoting tumorigenesis in a murine model of CRC. To determine the potential role of collagenolytic organisms in the pathogenesis of human CRC, we investigated if collagenolytic organisms preferentially colonize patients with CRC. Bacterial communities were recovered by swab from fourteen resected colon cancer specimens at three distinct sites: (1) on the tumor surface; (2) on normal appearing-adjacent mucosa 3 cm away from the tumor; (3) on normal appearing-adjacent mucosa 6 cm away from the tumor (Fig. 1). We found that the tumor surface (mean 15 CFU; range 6 – 56 CFU) had a significantly greater number of collagenase producing colonies compared to samples recovered from normal adjacent tissue 6 cm (mean 0.6 CFU; range 0 – 5 CFU) away from the tumor site ($p<0.05$; Ordinary one-way ANOVA and Student's t - test). Speciation of all of the collagenolytic colonies identified that *Enterococcus faecalis* and *Proteus mirabilis* were the most predominant species that colonized tumor tissue. On the day prior to surgery all fourteen patients underwent a combined mechanical bowel preparation with MiraLax plus 1gm Neomycin and 500 mg metronidazole three times per day.

Collagenolytic E. faecalis significantly alters gene expression in CT26 cells. Given that we have previously shown that colonization of *E. faecalis* associated with metastatic tumors in mice, and the current finding that collagenolytic *E. faecalis* associated with human tumors, we focused our subsequent experiments on *E. faecalis*. We first determined if collagenolytic *E. faecalis* can influence cellular pathways of cancer cells. To do this, we performed RNA sequencing (RNA-seq) to determine the transcriptome-wide response of CT26 undifferentiated carcinoma cells to *E. faecalis*. In these experiments, a human derived collagenolytic strain (V583) of *E. faecalis* was coincubated

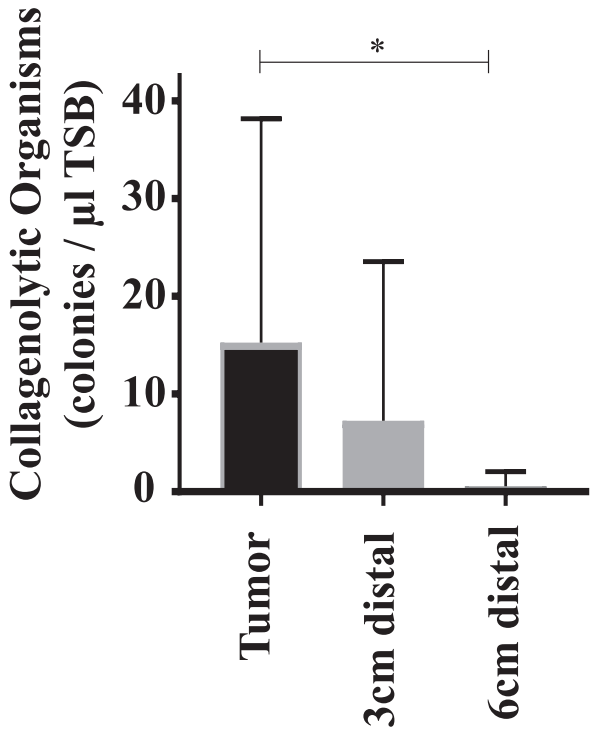


Fig. 1. Colonization of human colon cancer with collagenase producing bacteria. Tumor tissue had a significantly greater number of collagenase producing colonies (mean 15 CFU; range 6 – 56 CFU) compared to samples recovered from 6 cm (mean 0.6 CFU; range 0 – 5 CFU) away from the tumor site (error bars represent standard deviation; *p<0.05).

with CT26 cells for either 4 or 8 hours. Using a cutoff of greater than 1.5-fold change, there were 999 differentially expressed genes (DEG) when *E. faecalis* was coincubated with CT26 cells for 4 hours and 259 DEG's when incubated at 8 hours (Fig. 2A). When a false discovery rate (FDR) corrected p-value of < 0.1 was included, there were 113 DEG's at 4 hours, and 7 DEG's at 8 hours (Supplemental table S1). Gene set enrichment analysis using clusterProfile (p<.001) demonstrated that *E. faecalis* significantly influenced 4 cellular pathways: cofactor transport, regulation of microtubule cytoskeleton organization, collagen metabolic process, and integrin-mediated signaling pathway (Fig. 2B).

E. faecalis promotes the invasion of CT26 cells via a collagenase dependent mechanism and the migration of CT26 cells via a collagenase independent mechanism. Microtubule cytoskeleton regulation, collagen metabolism, and integrin signaling, the cellular pathways found to be influenced by *E. faecalis,* are well-known to be involved in tumor progression; microtubule cytoskeleton regulation and integrin signaling can be linked to migrative phenotype while collagen metabolism can promote invasiveness of cancer cells [21,22]. We therefore hypothesized that *E. faecalis* can promote enhanced invasion and migration of tumor cells. To test the influence of collagenolytic *E. faecalis* on cancer cell invasion, we coincubated bacteria with CT26 cells and analyzed invasion across a transwell chamber layered with extracellular matrix. We found that *E. faecalis* V583 significantly increased invasion, whereas neither its collagenase deficient mutant (*V583 ΔΔgel/sprE)* nor a control strain *Escherichia coli* OP50, that does not produce collagenase, showed any significant influence on invasion (Fig. 3A).

Because of our finding that bacterial collagenase was required to promote invasion, we next investigated if the interaction between CT26 cells and *E. faecalis* promotes bacterial collagenase expression. To define this relationship, we co-cultured *E. faecalis* V583 in the presence or absence of CT26 cells and used QRT-PCR to analyze expression of gelatinase *gelE*, a gene whose expression is required for collagenase production. Results demonstrated that CT26 cells induced a greater then 10-fold increase in *gelE* expression (Fig. 3B). To determine if bacteria-CT26 cell contact was required for inducing *gelE* expression, conditioned media was created by collecting the supernatant from CT26 cells grown overnight. Repeat experiments coincubating *E. faecalis* V583 with the conditioned media resulted in no increased expression of *gelE*, confirming that direct contact of *E. faecalis* to CT26 is required for cancer cell-induced *gelE* expression. Together, these data demonstrate a co-inducing loop between collagenolytic *E. faecalis* and CT26 cells in which cancer cells directly induce expression of *gelE*, which then promotes enhanced invasiveness of cancer cells.

We next assessed the effect of *E. faecalis* on migration, performing assays in an identical fashion as for invasion but without layering extracellular matrix. Similarly, we found that *E. faecalis* V583 significantly increased migration, whereas *E.coli* OP50 had no effect on the migratory potential of CT26 cells (Fig. 3C). To our surprise, V583 ΔΔ*gel/sprE* demonstrated a similar ability to promote CT26 cell migration as wild-type V583. Light microscopy at 24 and 48 hours after co-incubation of CT26 cells with both the collagenase producing *E. faecalis* V583 and collagenase deficient V583 ΔΔ*gel/sprE* strain demonstrated a significant morphological change with cellular elongation and increased pseudopodia, consistent with a migratory phenotype (Supplemental Fig. S1).

E. faecalis induces enhanced migration of CT26 cells via interaction with urokinase. Given that *E. faecalis* induction of CT26 cell migration was not dependent on bacterial collagenase, we next sought to determine an alternative mechanism by which *E. faecalis* promotes migration of cancer cells. We have previously shown that *E. faecalis* can activate plasminogen generating the production of plasmin *in vitro* [13]. uPA not only converts the zymogen plasminogen to active plasmin, but also via cell service binding to its receptor (uPAR) and downstream signaling, is well-known to promote a migratory phenotype in CRC cells. Therefore, we hypothesized that *E. faecalis* induced CT26 migration via a bacterial interaction with uPA. To test this hypothesis, we first sought to determine the potential of *E. faecalis* to bind uPA. Flow cytometric analysis found a dose dependent binding of both *E. faecalis* V583 and V583 ΔΔ*gel/sprE* to FITC-labeled pro-uPA (Kolmogorov-Smirnov analysis; p<0.05). *E. coli* OP50 demonstrated no ability to bind to urokinase (Fig. 4A).

While CT26 cells produce a basal amount of pro-uPA, to directly address the interaction between uPA and *E. faecalis* on promoting migration, we performed reiterative migration assays in the presence of exogenous pro-uPA (Fig. 4B). These experiments demonstrated that in cells pretreated with pro-uPA, both *E. faecalis* V583 and V583 ΔΔ*gel/sprE* significantly increased the migration of CT26 cells as compared to uPA treated cells without exposure to bacteria (3.12-fold V583. 2.97-fold V583 ΔΔ*gel/sprE*, 1.41-fold pro-uPA alone; p=0.008). Even in the presence of pro-uPA, *E. coli* OP50 did not significantly influence CT26 cell migration.

Blocking the uPA receptor inhibits E. faecalis induced migration of CT26 cells. To confirm that activation of uPAR by uPA was the driver of bacterial enhanced migration, migration assays were repeated in the presence of a blocking uPAR antibody. Control experiments coincubating active uPA and CT26 cells showed that in the presence of the uPAR-Ab, the known migration induction of active-uPA was prevented (Fig. 4C). Strikingly, when uPAR-Ab was present in pro-uPA treated cells, it completely prevented *E. faecalis* induced migration (no uPAR-Ab 3.12-fold vs uPAR-Ab 0.99-fold; p=0.002).

Discussion

The key influence of both commensal and pathogenic intestinal bacteria on the pathogenesis of colorectal cancer is becoming increasingly clear. The enterococci genus is comprised of 60 species, many of which reside as commensals within the gastrointestinal tract. It is estimated that there

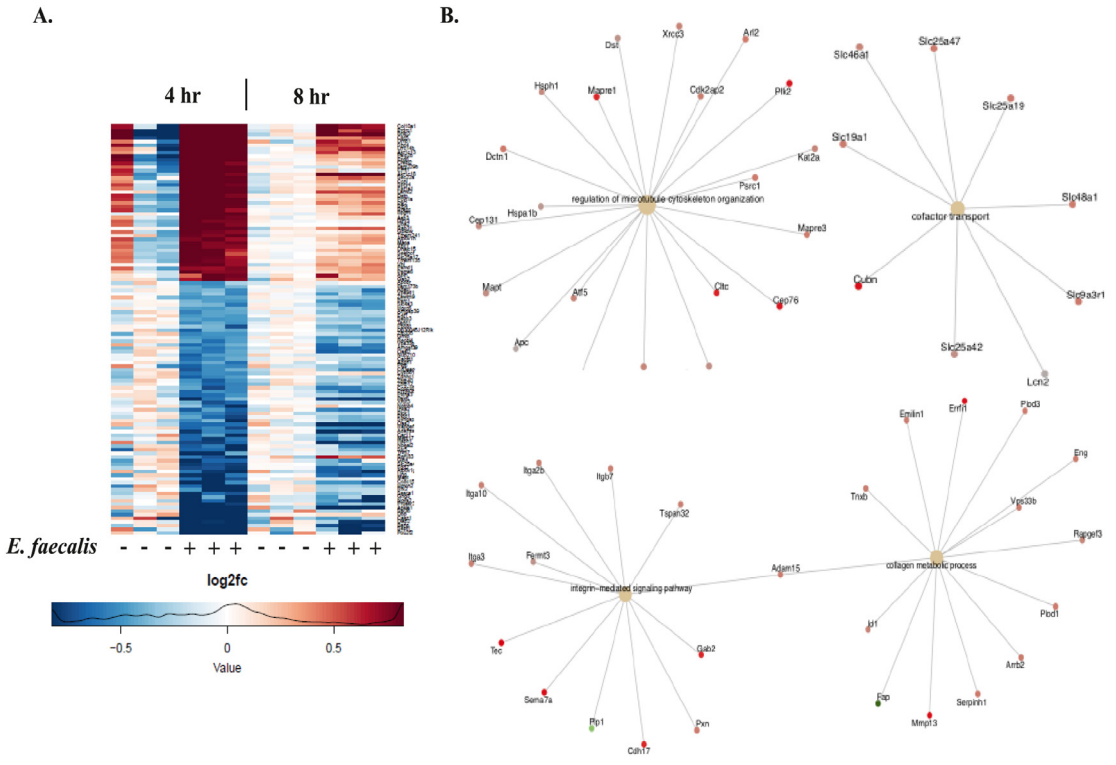


Fig. 2. Collagenolytic E. faecalis significantly alters gene expression in CT26 cells. (A) Differentially expressed genes after E. faecalis was incubated with CT26 cells for either 4 or 8 hours. (B) Gene set enrichment analysis using clusterProfile ($p<.001$) demonstrated that E. faecalis significantly influenced 4 cellular pathways: cofactor transport, regulation of microtubule cytoskeleton organization, collagen metabolic process, and integrin-mediated signaling pathway.

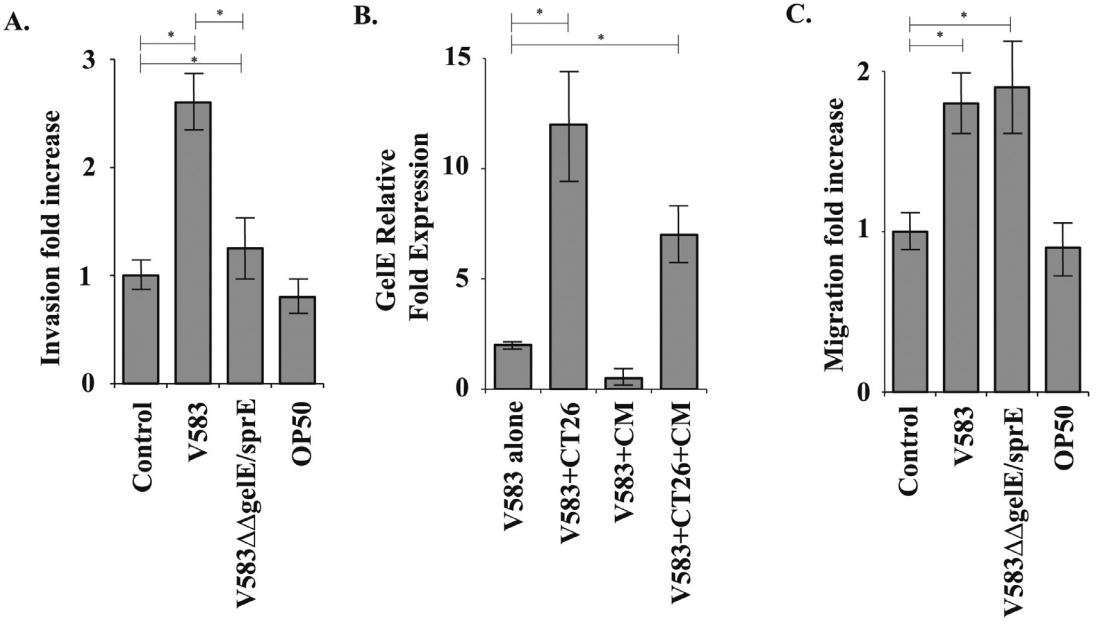
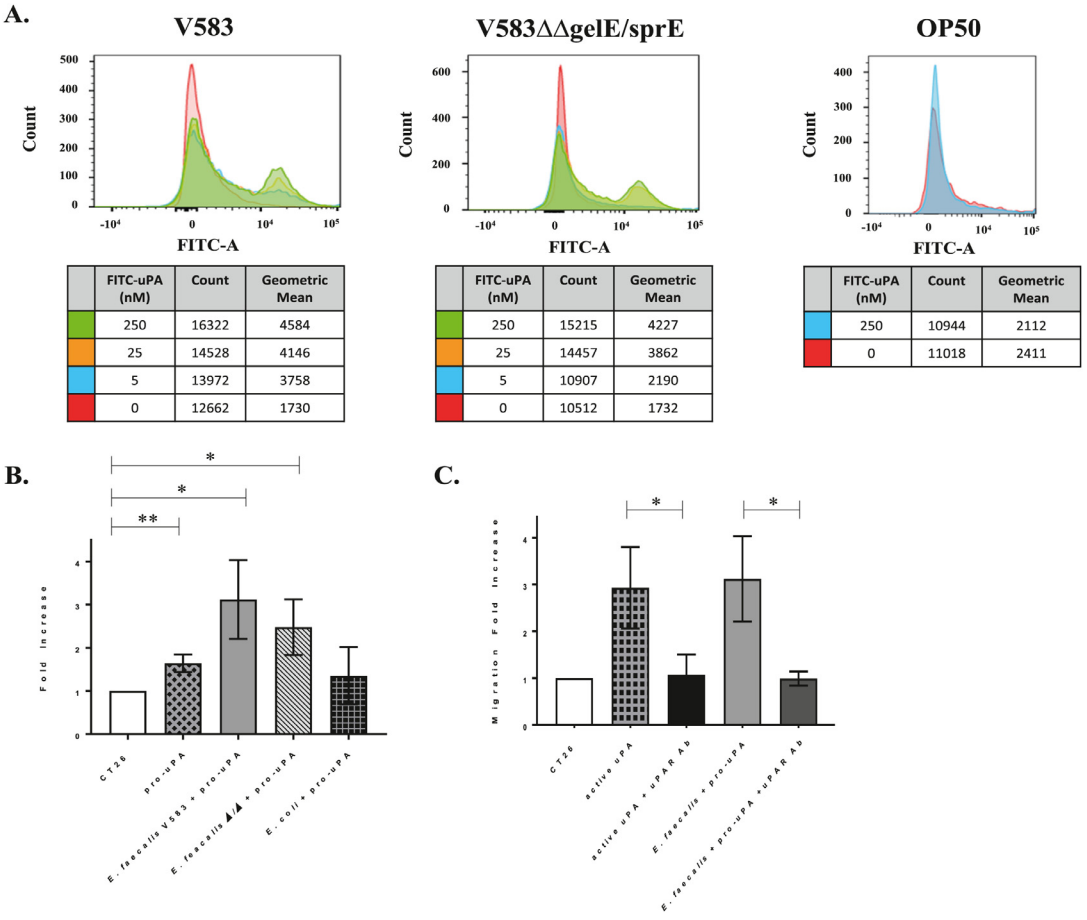


Fig. 3. E. faecalis promotes invasion of cancer cells via bacterial collagenase. (A) Invasion assay demonstrating that E. faecalis V583 significantly increased invasion, whereas its collagenase deficient mutant V583 ΔΔgel/sprE nor Escherichia coli OP50, that does not produce collagenase, showed no significant influence on invasion. (B) Coincubation of E. faecalis V583 with CT26 significantly increases E. faecalis gelE expression. Coincubation of conditioned media (CM) created from cell free supernatant after overnight culture of E. faecalis V583 with CT26 cells does not increase gelE expression demonstrating that direct contact with CT26 cells is necessary to promote expression of bacterial collagenase. (C) Migration assay demonstrating that both E. faecalis V583 and V583 ΔΔgel/sprE enhance migration of CT26 cells. E. coli OP50 demonstrated no ability to promote migration (error bars represent standard deviation; *$p<0.05$).



FITC-uPA (nM)	Count	Geometric Mean
250	16322	4584
25	14528	4146
5	13972	3758
0	12662	1730

FITC-uPA (nM)	Count	Geometric Mean
250	15215	4227
25	14457	3862
5	10907	2190
0	10512	1732

FITC-uPA (nM)	Count	Geometric Mean
250	10944	2112
0	11018	2411

Fig. 4. E. faecalis promotes migration of cancer cells by interacting with uPA-UPAR. (A) Flow cytometric analysis found a dose dependent binding of both E. faecalis V583 and V583ΔΔgel/sprE to FITC-labeled pro-uPA ($p<0.05$). E. coli OP50, demonstrated no ability to bind to urokinase. (B) In cells pretreated with pro-uPA, both E. faecalis V583 and V583ΔΔgel/sprE significantly increased the migration of CT26 cells compared to uPA treated cells without exposure to bacteria. Even in the presence of uPA, E. coli OP50 did not significantly influence CT26 cell migration. (C) Reiterative experiments in the presence of a blocking uPAR antibody (Ab). uPAR-Ab prevented E. faecalis induced migration in the presence of pro- or active uPA (error bars represent standard deviation; $^{*}p<0.05$, $^{**}p<0.005$).

are nearly 10^7 Enterococci that inhabit the gut, the majority of which are *E. faecalis* or *E. faecium* [23]. Depending upon the strain, *E. faecalis* has been found to be either a probiotic, cause infections, or promote CRC carcinogenesis. Therefore, the effect of *E. faecalis* colonization can range from promoting gut homeostasis to potentially causing cancer. Our group has demonstrated that in a murine model of CRC, tumors form in association with *E. faecalis* colonization, but only if strains demonstrated an enhanced ability to produce collagenase. How bacteria expressing a collagenolytic phenotype influence carcinogenesis has not been previously studied. We therefore investigated the mechanisms by which collagenolytic *E. faecalis* can promote the progression of colon cancer. We discovered that via both collagenase dependent and independent pathways, *E. faecalis* enhances cancer cell invasion and migration in the murine colon cancer cell line CT26, thus demonstrating a novel mechanism of *E. faecalis* induced carcinogenesis (Fig. 5).

E. faecalis is a gram-positive facultative anaerobe that has been associated with CRC. Balamurugan et al found that *E. faecalis* populations were significantly higher in CRC patients compared to healthy controls [24]. *E. faecalis* carcinogenesis has primarily been attributed to the microbes ability to produce hydroxyl radicals and other reactive oxygen species that can cause DNA breaks and DNA-cross linking leading to chromosomal instability (CIN) [9–25]. Furthermore, *in vitro* studies have shown that superoxide from *E. faecalis* induces macrophage COX-2 pathways resulting in DNA damage by the production of chromosomal-breaking factors such as clastogens [9–26]. While these studies demonstrate how *E. faecalis* induced-CIN initiating carcinogenesis, our interest has been to understand how collagenolytic strains influence exfoliated colon cancer cells to participate in their metastatic dissemination when present at the site of an intestinal wound, such as occurs following curative resection. To escape the gut lumen to form a metastasis, CRC cells must detach from the primary tumor and acquire an invasive and migratory phenotype. Rather than genetic alterations, this process is thought to be highly promoted by variations in transcriptional regulation induced by factors within the local tumor microenvironment [27,28].

Owing to an influx of immune cells, inflammatory proteins, and creation of a pre-operative anaerobic environment to become aerobic, curative resection of a primary tumor in a typical patient is associated with dramatic changes within the intraluminal microenvironment during the perioperative period. Indeed, we have previously shown a 500-fold increase in the colonization of *E. faecalis* on healing intestinal tissue with a peak colonization during postoperative days 2-5 [29,30]. In a mouse model that mimics postoperative metastasis, when exfoliated cancer cells are

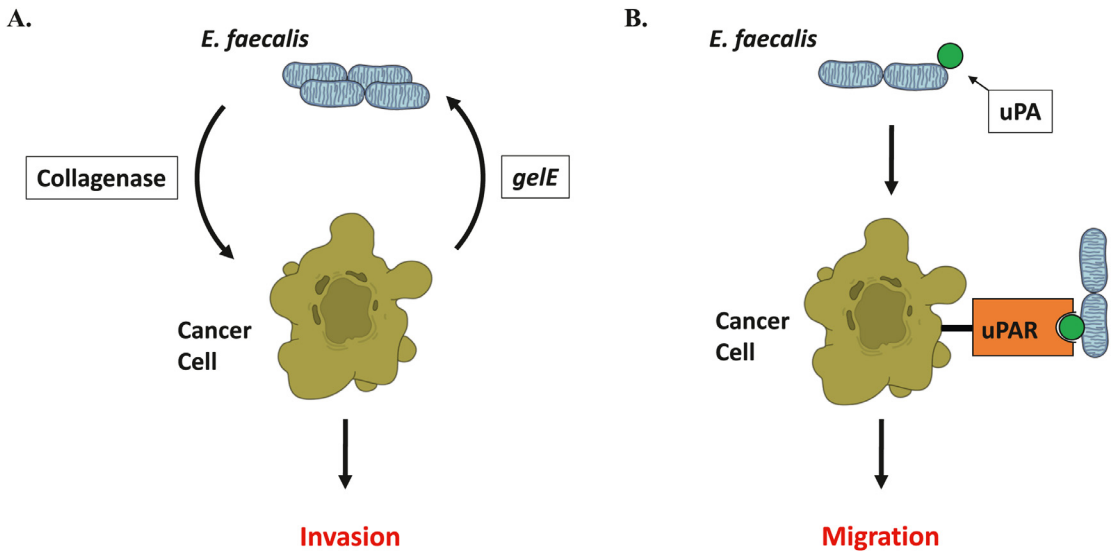


Fig. 5. Mechanism by which E. faecalis promotes the progression of CT26 cancer cells. (A) Binding of E. faecalis with CRC cells results in a bi-directional interaction that promotes gelE expression and then subsequent collagenase dependent induction of cancel cell invasion. (B) Binding of E. faecalis to uPA promotes bacterial induced induction of migration via its interaction with uPAR.

present intraluminally during this perioperative period, they are promoted to transluminally migrate across healing tissue to form extraluminal tumors, locally in adjacent lymph nodes, and micrometastatic tumor deposits in the liver [11]. Strikingly, tumors only form when the intestinal tissue is enriched with collagenolytic *E. faecalis*. Results from this current investigation demonstrate a spatial relationship of collagenolytic bacteria preferentially colonizing human CRC tumors compared to normal adjacent tissue. What precisely attracts these microbes to tumor tissue is an area of active investigation. To allow for tumor growth and metastasis, proteolytic enzymes within the tumor microenvironment promote ongoing breakdown and remodeling of the peritumor stroma exposing elements of the extracellular matrix (ECM); *E. faecalis* is particularly well-suited to hone onto tumor tissue as it has specific adhesion proteins for ECM breakdown products such as Ebp pili that binds fibrinogen, EfbA that binds fibronectin, and Ace that binds collagen [31–34].

In the present study, we discovered that collagenase producing *E. faecalis* promotes enhanced invasion of CT26 cells and further demonstrated that direct binding of CT26 cells promoted expression of *gelE* encoding *E. faecalis* gelatinase GelE, a secreted Zn-metalloproteinase with proteolytic activity against a number of substrates including gelatin, collagen, fibrin and human endothelin [35]. *E. faecalis* GelE has been shown to a be critical virulence factor that compromises the epithelial barrier allowing for bacterial translocation [36]. It therefore stands to reason that colonization with collagenolytic bacteria can create a host-microbe interaction that promotes bacterial collagenase production that subsequently degrades the peritumoral stroma promoting cancer cell invasion.

Migration of cancer cells was also enhanced by *E. faecalis*, but to our surprise was not in a bacterial collagenase dependent manor. Instead, we discovered that binding of *E. faecalis* to the urokinase plasminogen activator (uPA) significantly enhanced signaling across the urokinase plasminogen activator cell surface receptor (uPAR) leading to increased cancer cell migration. The uPA-plasminogen system is well-studied in CRC carcinogenesis; in addition to converting plasminogen to plasmin, binding of uPA to uPAR mediates integrin-dependent transmembrane signaling that activated proliferation, migration, and cell-survival pathways [37,38]. Clinically, the presence of uPA and/or other downstream proteins within the uPA-plasminogen system is associated with poor oncological outcomes and metastasis in CRC patients [39,40]. Although this is the first report describing a direct mechanism as to how a microbe promotes cancer progression via direct binding to uPA, others have shown a similar interaction within the context of infection. For example, Jacobson et al demonstrated that *E. faecalis* binds and promotes the conversion of plasminogen to plasmin, whereas Beaufort et al. found that *Pseudomonas aeruginosa* can convert pro-uPA to its active form [13–41].

Our study has several limitations. First, we performed our experiments in a single cell line (CT26) This current study was designed to further uncover the mechanism by which *E. faecalis* can promote post-surgical tumors in previously published mouse model of CRC [11]. In this previous study, CT26 cells were the only cell line that was utilized, and therefore it was logical for our current work to use CT26 cells. Reiterative studies using cell lines with different genetic backgrounds are needed to know if our results are generalizable. Second, we did not test if mutant strains of *E. faecalis* deficient in their capability to bind to extracellular matrix (Ebp, EfbA or Ace deficient mutants) lose their inducing activity to promote the migration of cancer cells. These experiments are currently underway. Finally, our RNAseq analysis used a FDR corrected cutoff of <0.1. It is possible that if a more stringent cutoff was used less DEGs would have been identified.

Taken together, this study provides mechanistic insight into how a single commensal bacteria can promote the progression of CRC via mechanisms both dependent and independent on its collagenolytic activity. Given that we previously have shown that antibiotic therapy to eliminate *E. faecalis* leads to colonization of anastomotic tissue with other collagenolytic bacteria or fungi further studies are needed to determine novel strategies to block this microbial factor to prevent or treat colorectal cancer [11].

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CRediT authorship contribution statement

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