**2017 MARY ANN LIEBERT, INC. OUTSTANDING STUDENT
AWARD OF TISSUE ENGINEERING AND REGENERATIVE
MEDICINE INTERNATIONAL SOCIETY—AMERICAS**

Bioengineered Submucosal Organoids for *In Vitro* Modeling of Colorectal Cancer

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The physical nature of the tumor microenvironment significantly impacts tumor growth, invasion, and response to drugs. Most *in vitro* tumor models are designed to study the effects of extracellular matrix (ECM) stiffness on tumor cells, while not addressing the effects of ECM's specific topography. In this study, we bioengineered submucosal organoids, using primary smooth muscle cells embedded in collagen I hydrogel, which produce aligned and parallel fiber topography similar to those found *in vivo*. The fiber organization in the submucosal organoids induced an epithelial phenotype in spheroids of colorectal carcinoma cells (HCT-116), which were embedded within the organoids. Conversely, unorganized fibers drove a mesenchymal phenotype in the tumor cells. HCT-116 cells in organoids with aligned fibers showed no WNT signaling activation, and conversely, WNT signaling activation was observed in organoids with disrupted fibers. Consequently, HCT-116 cells in the aligned condition exhibited decreased cellular proliferation and reduced sensitivity to 5-fluorouracil chemotherapeutic treatment compared to cells in the unorganized construct. Collectively, the results establish a unique colorectal tumor organoid model to study the effects of stromal topography on cancer cell phenotype, proliferation, and ultimately, chemotherapeutic susceptibility. In the future, such organoids can utilize patient-derived cells for precision medicine applications.

Keywords: colorectal cancer, tumor models, extracellular matrix, collagen organization, epithelial-to-mesenchymal transition

Introduction

FOR MANY YEARS, basic cancer research and anticancer drug development utilized primary cancer cells and cell lines cultured *in vitro* on tissue culture plastic dishes. Although the field of cancer research owes much to this style of experimentation, recent investigation has found it a poor analogue of tumor growth *in vivo*. For the most part, two-dimensional (2D) culture, such as in culture dishes, does not replicate the microenvironment of a tumor, a complex space typified by stromal cells, extracellular matrix (ECM) components, and a cocktail of signaling factors. These elements together drive cancer cells in a specific manner, and eliminating just one element might elicit a significantly different output from cancer cells. Furthermore, many *in vitro* cancer models focus on the cancer cell without its surrounding stroma despite the growing body of literature that expounds

the importance of tumor stroma-related effects.¹⁻⁴ To fully replicate a tumor system *in vitro*, models must strive to integrate as many aspects of the microenvironment as possible.

The tumor stroma can activate or inactivate cancer-related pathways, alter ECM components, thereby making migration more or less difficult, as well as secrete signaling factors that guide cancer cells in a multitude of ways. Studies have demonstrated that placing normal epithelial cells into an activated stroma can produce cancerous growth from these healthy cells; and conversely, placing malignant cancer cells in a healthy stroma can cause a reversion from a cancerous state toward a normal phenotype.^{5,6} These studies present the stroma as an important part of the cancer equation and perhaps as important as the cancer cell itself. As such, recapitulating the stroma and the complex environment therein is a crucial step in both understanding cancer and developing novel, targeted therapies.

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In colorectal cancer (CRC), epithelial cells lining the lumen of the intestine mutate and develop into cancer cells. These cells are faced with the luminal space on one side and the submucosa on the other representing two different micro-environments. Initially, cancer cells proliferate and produce a polyp that protrudes into the luminal space, but as the tumor grows and requires a more robust source of nutrients, the cells migrate toward the submucosa. The submucosa is a collagen-rich tissue populated primarily with smooth muscle cells (SMCs), which facilitate the peristalsis of the colon. In addition, the collagen found here is highly aligned in a manner conducive to smooth muscle function. It is theorized that the alignment of submucosal collagen can be altered and disorganized due to injury (such as an autoimmune disease like Crohn's); patients with such injury are often at higher risk of developing late-stage CRC, and thus, stromal topography may play a substantial role in cancer development.^{7–11} Investigation of CRC tissue has also shown a stark difference in collagen alignment when compared to healthy tissue.^{12,13} With these results in mind, we hypothesize that specific collagen architecture in the microenvironment can drive differences in cancer phenotype and response.

Herein we describe a bioengineered submucosal tissue construct, or a submucosal organoid, made with primary colonic SMCs and collagen I (Col I) that include fiber topography similar to *in vivo* ECM. The data were supplemented with image segmentation to analyze and quantify the collagen fibers in these organoids. We then embedded a tumor spheroid into the submucosal organoid and studied the tumor phenotype as well as the corresponding drug response. Our results draw a correlation between collagen fiber alignment, tumor cell phenotype, and most importantly, the response to a chemotherapeutic drug, which corroborates clinical cancer data.

Materials and Methods

Cell culture

Rabbit colon smooth muscle cells (RCSMCs) were provided by Dr. Khalil Bitar at the Wake Forest Institute for Regenerative Medicine (Winston-Salem, NC). Briefly,

RCSMCs were isolated from fresh rabbit colons, purified through attachment, and then sorted. HCT-116 cells were obtained from ATCC (#CCL-247; ATCC, Manassas, VA). CaCO2 cells were also obtained from ATCC (#HTB-37; ATCC). Some HCT-116 cells were also engineered to express the TOP-GFP.mC plasmid, which was a gift from Ramesh Shivdasani (Addgene Plasmid No. 35491). A MATLAB script was used to quantify red and green fluorescent signal generated by the TOP-GFP.mC plasmid. All cell types were cultured and expanded in 2D on tissue culture plastic using 15-cm tissue-treated dishes. Cells were cultured to 90% confluence before being harvested for use or passaged into new plates. All cell types were cultured with Dulbecco's Minimum Essential Medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal bovine serum (Hyclone, Logan, UT). Cells were detached from the substrate with Trypsin/EDTA (Hyclone) and resuspended in media before further use in studies.

Microwell insert and organoid fabrication

Overall scheme of microwell insert and organoid fabrication is outlined and illustrated in Figure 1. First, we modeled a well insert mold using SketchUp™. We then printed the model on a MakerBot Replicator 2×. After printing, we mixed polydimethylsiloxane (PDMS, Sylgard 184; Dow-Corning, Auburn, MI) in a 10:1 ratio of elastomer to curing agent. We then poured the uncured PDMS into the mold, applied vacuum to the PDMS via a desiccator chamber to remove all bubbles, and allowed the mold to cure overnight at 80°C. After curing, we sterilized the inserts with 70% ethanol by immersion for at least 24 h. These inserts were then placed into a six-well plate using sterile gloves. Inserts are then liberally washed with phosphate-buffered saline (PBS) to remove all traces of ethanol, and then, inserts are allowed to air-dry under ultraviolet light.

To produce organoids, we first prepared a solution of 0.5 mg/mL Col I (Corning, Corning, NY), using a protocol modified from the supplied protocol to produce this solution. Briefly, we mixed 600 μ L of 3.45 mg/mL Col I, 286 μ L DMEM, 100 μ L PBS 10×, and 14 μ L 1 N NaOH. This recipe produced a 2 mg/mL Col I solution, which was further diluted with DMEM to the required concentration of

FIG. 1. Schematic of organoid fabrication. (A) A CAD program was used to produce a model of a six-well insert negative mold that was subsequently printed with a 3D printer. (B) PDMS was poured into the mold and allowed to cure. (C) After curing, the mold was removed, cleaned, and trimmed of any excess of PDMS. (D) The mold was placed in a six-well plate and cell-Col I solution was deposited into the microwells. (E) Culture media were added and the construct was allowed to crosslink and contract. (F) At predetermined times, constructs were harvested for analysis. Col I, collagen I; PDMS, polydimethylsiloxane. Color images available online at www.liebertpub.com/tea

0.5 mg/mL. After mixing, we kept the collagen solution on ice until use. We then harvested RSCMCs using trypsin, after which we suspended RSCMCs in Col I at a density of 5 million cells per milliliter collagen. After fully suspending the cells, we obtained a homogenous cell–gel solution. The cell–gel solution is then pipetted into the microwells of the insert, and each microwell can accommodate ~100 μ L of solution. The well plate was then incubated at 37°C for 30 min to allow collagen to crosslink. Media was then carefully added to the wells, ensuring the hydrogels are not overly disturbed, to cover organoids fully.

Spheroid production and embedding

Spheroids were produced from HCT-116 and CaCO₂ colon carcinoma cell lines. Cells were harvested and suspended in DMEM at a density of 100,000 cells per milliliter. One hundred microliters of cell suspension was pipetted into each well of a nonadherent, round-bottomed, 96-well plate to produce spheroids (#7007; Corning), with a density of 10,000 cells per spheroid. Well plates were incubated and observed daily until spheroid formation and then immediately used in experiments. To embed spheroids into collagen hydrogel, the media were first aspirated from the spheroid well, hydrogel solution was added to the well with the spheroid, and the whole mixture was then pipetted into the microwell to prepare the submucosal organoids as described above.

Immunohistochemistry

Organoids were removed from culture at the appropriate time point. Samples were then fixed using 4% paraformaldehyde for 1 h. The samples were then processed for sectioning. They were dehydrated with graded ethanol washes followed by xylene incubation. They were then embedded in paraffin, sectioned at 5 μ m/section, and mounted to slides.

For immunohistochemistry (IHC), all incubations were carried out at room temperature unless otherwise stated. Slides were warmed at 60°C for 1 h to increase section bonding to the slides. Antigen retrieval was performed on all slides and achieved with incubation in proteinase K (Dako, Carpinteria, CA) for 5 min. Sections were permeabilized by incubation in 0.05% Triton-X in PBS for 5 min. Nonspecific antibody binding was blocked by incubation in the Protein Block Solution (#ab156024; Abcam, Cambridge, MA) for 15 min. Sections were incubated for 60 min in a humidified chamber with the appropriate antibody from the following: desmin (#ab32362; Abcam), alpha-smooth muscle actin (#ab5694; Abcam), calponin (#ab216651; Abcam), CK-18 (#ab82254; Abcam), E-cadherin (#ab40772; Abcam), N-cadherin (#ab207608; Abcam), ZO-1 (#ab59720; Abcam), MMP9 (#ab38898; Abcam), β -catenin (#712700; Invitrogen, Carlsbad, CA), or Ki-67 (#ab16667; Abcam). All antibodies are used at 1:200 dilution in antibody diluent (#ab64211; Abcam).

Following primary incubation, slides were washed three times in PBS for 5 min/wash. Samples were then incubated for 1 h with anti-rabbit Alexa Fluor 647 secondary (#ab150075; Abcam) and anti-mouse Alexa Fluor 594 antibodies (#ab150116; Abcam) as appropriate in antibody diluent (1:200 dilution). Cells were counterstained with DAPI for 5 min and washed three times with 1 $\times$ PBS before fluorescent imaging. Negative controls were performed in parallel

with the primary antibody incubations and included incubation with blocking solution in place of the primary antibody. No immunoreactivity was observed in the negative control sections. Samples were imaged with fluorescence at 647, 594, and 380 nm with a Leica DM 4000B upright microscope.

Fiber imaging and quantification

Organoid sections were obtained as previously described and stained using a picosirius red stain kit (#ab150681; Abcam). Briefly, slides are deparaffinized, hydrated to deionized water, and stained with hematoxylin to visualize nuclei. Slides were then stained with picosirius red solution for 60 min at room temperature, rinsed in acetic acid and dehydrated, and mounted with a coverslip. Picosirius red imaging was performed on a Leica DM 4000B upright microscope under brightfield with polarizers in place to visualize birefringent signal. Once images were captured, they were analyzed with the CT-FIRE program, designed by Laboratory for Optical and Computational Instrumentation, the University of Wisconsin Madison. Settings were kept at default and data were input into MATLAB for analysis and graphing.

Second-harmonic generation imaging

In vitro second-harmonic generation (SHG) and intrinsic (autofluorescent) signal acquisition: submucosal organoids with embedded HCT-116 spheroids were prepared as previously described. Organoids were sandwiched between glass slides. Samples were equilibrated in 33% TDE (thio-diethanol) Refractive Index; 1.038 in a custom printed imaging container. Organoids were imaged on a modified Olympus FV1000 using an Olympus XLSLPLN25XSVM2 objective. The sample was excited at 875 nm, the mean SHG signal was collected at 425–438 nm, and the cellular intrinsic autofluorescence was collected at 488–545 nm, respectively using GaAsP detectors. A multiarea time lapse (MATL) Z-stack of images was collected (3 μ m Z-step) encompassing the complete tumor organoids. An average organoid with an 800- μ m diameter required 20 tiles across the top by 50 Z-steps deep. These MATL Z stacks were stitched using custom software, and 3D renderings were generated using Imaris (Bitplane, London). Pseudocoloring blue represents SHG signal, whereas cellular intrinsic autofluorescence is labeled red. Each organoid was imaged three times using identical conditions (equal laser power, photomultiplier tube voltage, etc.) to ensure consistency.

Drug treatments and analysis

For chemotherapy treatment experiments, we used 5-fluorouracil (5-FU, #F6627; Sigma-Aldrich), solubilized in dimethyl sulfoxide (DMSO) and added to DMEM, as prepared for cell culture, at a concentration of 100 mM. The drug-media solution was sterilized by passing it through a syringe filter. Organoids were allowed to form for 5 days, and 5-FU, diluted to the desired concentration, was added directly to the well. Live/dead assay was performed by removing organoids and suspending them in the Live/Dead Viability/Cytotoxicity kit for mammalian cells (#L3224; Thermo Fisher, Waltham, MA). Live-dead staining was performed as instructed in the manufacturer's protocol.

Briefly, we prepared a solution of 2 μ M calcein AM and 4 μ M EthD-1 in a 1:1 solution of DMEM and PBS. Organoids were incubated in the live/dead solution for 1 h at 37°C and then imaged immediately. Imaging was performed on a Leica TCS LSI macroconfocal microscope using a 5 $\times$ objective. Confocal z-stacks were obtained with parameters of 10 μ m/stack through the entirety of the visible sample; the maximum projection of each z-stack was then generated for presentation or analysis. Quantification of live/dead signal was performed with a MATLAB script. For WNT modulation experiments, we used BIO (#B1686; Sigma-Aldrich) and XAV939 (#X3004; Sigma-Aldrich). BIO and XAV939 were solubilized in DMSO to produce stock solutions of 10 mM of each drug. Stock solutions of BIO and XAV939 were added to DMEM to produce final concentrations of 5 and 3.3 μ M, respectively. These final solutions were added directly to organoids after gelation.

Statistical analysis

All experiments were performed in triplicate or greater. Histograms were constructed from 1000 data points or more. Other quantitative results are presented as mean $\pm$ standard deviation. Significance of data values that approximate a normal distribution was evaluated using a Student's *t*-test (two tailed) with two-sample unequal variance. A Kolmogorov–Smirnov test was used to assess differences between non-normal distributions. Significance values are denoted in figure legends. *p*-Values are listed with 2, or greater, significant digits within Results text.

Results

Bioengineered submucosal microenvironmental construct

Prior studies have utilized isolated SMCs to produce functioning analogues of colonic structures such as the anal sphincter and the submucosa of the intestine.^{14–16} These engineered tissue constructs display functional characteristics of native intestinal anatomy and physiology such as cell alignment, tonicity, and contractility, and could serve as a model of the native submucosal microenvironment.¹⁷ Accordingly, we propose to use such submucosal construct (organoid), which is in direct contact with the colonic epithelium, the originating site for CRC, to create an *in vitro* CRC model. Briefly, 5 million rabbit colonic SMCs were mixed in 1 mL solution of Col I (0.5 mg/mL), and then, the hydrogel–cell solution was deposited into our microwell system (Fig. 1).

The submucosal organoids are molded as a disc-like structure that facilitates repeatable and consistent analysis. We confirmed SMC phenotypic stability in the construct using IHC for the following: α -smooth muscle actin, a component of the SMC contractile machinery¹⁸; calponin, a protein that binds to actin proteins and helps to regulate actin/myosin interactions¹⁹; and desmin, a protein found in abundance in terminally differentiated SMCs²⁰ (Fig. 2A–C). We observed positive staining of SMC markers overall, suggesting that the SMCs retained a smooth muscle phenotype throughout the study.

Construct morphology and overall SMC organization and localization were followed using hematoxylin and eosin (H&E) staining on tissue sections from constructs harvested

on days 1, 3, and 5 (Fig. 2D–F). We observed an apparent concentration of the cellular content to one side of the construct by day 3 and the construct had curled around the cellularized side, suggesting that the SMCs are generating tensile force on the collagen (Fig. 2E and F, respectively). In addition, we measured the overall construct size (diameter) over 7 days, showing that the constructs underwent significant contraction from day 1 to 4 (Fig. 2G, $p=6.41\text{e-}10$). However, by day 4, the contraction rate has slowed and there was a less significant size difference between days 4 and 7 ($p=0.052$). These results, along with the observation of bundled collagen fibers (Fig. 3A), suggest that significant collagen remodeling has occurred by day 4. Altogether, the submucosal organoids demonstrate the ability to reorganize and induce contraction similar to the native submucosa.

Submucosal organoids display significantly altered collagen alignment and structure

There is evidence that physical and structural changes associated with alignment of collagen fiber in the tumor microenvironment can alter the phenotype of tumor cells.²¹ These changes are sensed by the cells through ECM binding membranal proteins that can generate intracellular signals to up- or downregulate oncogenic pathways.^{22,23} To determine the changes in collagen fiber alignment, we performed picrosirius red staining on submucosal organoids and non-cellularized, bare collagen hydrogel (Fig. 3A and E, respectively). Picrosirius red stain highlights collagen bundles in orange or red and reticular fibers in green. In the submucosal organoid, there is an abundance of orange fibers indicating that the SMCs have extensively remodeled the collagen to create a large number of bundled collagen fibers. These fibers follow parallel patterns, demonstrating the SMC ability to align the collagen fibers during remodeling. In contrast, bare collagen showed no orange or red picrosirius staining and instead displayed exclusively green signal, indicating unbundled, reticular collagen.

We further analyzed the picrosirius red images by quantifying the collagen fiber properties using CT-FIRE, a program designed by the Laboratory for Optical and Computational Instrumentation at the University of Wisconsin, which quantifies parameters of fiber alignment.²⁴ Fibers are isolated from images by identifying edges (curvelet transform [CT]) and fiber extraction (FIRE) algorithm and connecting those edges to segment total fibers. These segmented fibers are then analyzed to generate histograms of fiber parameters, such as angle, width, length, and straightness. First, we quantified angular distribution across fields of interest in both submucosal and collagen-only constructs. Fiber angles in the submucosal constructs produce a unimodal distribution, which indicates that the majority of fibers are aligned in a single direction or, more simply, parallel fibers (Fig. 3B). Conversely, collagen-only constructs possess flat, uniform angular distributions indicating these constructs have random, unaligned fibers with no preference for direction (Fig. 3F).

We performed a Kolmogorov–Smirnov two-sample test and found a significant difference between the submucosal constructs and bare collagen ($p=5.0309\text{e-}08$). Fiber length measurements in the two constructs showed that the submucosal constructs possessed more fibers of longer length

FIG. 2. Physical and histological characterization of submucosal organoids. (A–C) Immunohistochemical staining (red) of submucosal organoids for the indicated smooth muscle markers (nuclei stained blue with DAPI) (D–F) H&E staining shows cellular organization inside the submucosal organoids at days 1 (A), 3 (B), and 5 (C). (G) Size (diameter) measurements of submucosal organoids over 7 days in culture ($^{\#}p < 0.01$, $^{*}p < 0.1$). (A–C) Scale bar = 200 μ m. (E–G) Scale bar = 50 μ m. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

than bare collagen (Fig. 3C and G, respectively, $p = 7.59 \times 10^{-24}$). Finally, fiber width measurements showed that the submucosal organoids possessed wider fibers compared with collagen-only constructs (Fig. 3D and H, respectively, $p = 4.52 \times 10^{-212}$). These CT-FIRE results indicate that SMCs remodeled the collagen to produce aligned, lengthened, and bundled fibers compared to unorganized, reticular collagen in constructs made with bare collagen.

Submucosal organoids support epithelial acini formation

Since we observed significant remodeling and alterations to the collagen topography in the submucosal organoids, we tested if these changes would alter the morphology of epithelial cells embedded within these constructs. Previous

studies have shown that collagen fiber architecture, and especially fiber orientation, has an impact on epithelial cell phenotype.^{12,24} We included CaCO2 cells, a nonaggressive colon carcinoma cell line that can differentiate and create polarized structures,²⁵ with SMCs in the submucosal construct. In parallel, we cultured CaCO2 cells in bare collagen as well as with SMCs in two separate compartments of a transwell system, to test for SMC-mediated paracrine induction of changes in epithelial morphology. H&E staining of these constructs showed acini formation in the submucosal organoids, but not in either the bare collagen or transwell systems (Fig. 4A–C), suggesting the need for direct cell–cell communication or healthy aligned submucosal matrix.

To confirm the appearance of normal epithelial morphology, we stained the constructs with anti-zonula occludens-1 (ZO-1) and anti-cytokeratin-18 (CK-18) antibodies. Both

FIG. 3. Collagen fiber properties in the submucosal organoids. Submucosal organoids (**A–D**) and bare collagen constructs (**E–H**) were stained with picrosirius red to highlight reticular (*green*) or bundled (*red/orange*) collagen (**A'** and **E'** are black and white conversions on **A** and **E** images). Images were analyzed with the CT-FIRE™ program. (**B, F**) Fiber angles (degrees vs. frequency). (**C, G**) Fiber length (pixels vs. frequency). (**D, H**) Fiber width (pixels vs. frequency). * $p < 0.01$, # $p < 0.01$, † $p < 0.01$. (**A, E**) Scale bar = 100 μ m. Color images available online at www.liebertpub.com/tea

FIG. 4. Epithelial acini formation occurs in the submucosal organoid but not in unorganized collagen. (A–C) H&E images of CaCO2 cells grown in submucosal organoids (A), bare collagen (B), and in transwell inserts (with SMCs in the bottom well). The epithelial acini structures in the submucosal constructs were immunostained (red) for ZO-1 (D) and CK-18 (E). (A–C) Scale bar = 100 μ m. (D, E) Scale bar = 25 μ m. CK-18, cytokeratin-18; SMC, smooth muscle cell; ZO-1, zonula occludens-1. Color images available online at www.liebertpub.com/tea

markers ZO-1 and CK-18 are absent in SMCs (Fig. 4D, E). Together, these results indicate that the SMC-remodeled collagen induces native-like epithelial morphology and acini formation in CaCO2 cells, suggesting that collagen fiber alignment biases epithelial cells to assume a typical, healthy morphology.

SMCs and HCT-116 colon carcinoma cells produce distinct collagen fiber topographies

ECM remodeling around tumors is performed by stromal cells, but some cancer cells are known to tailor the ECM to their needs.^{26,27} Such cancer cell-mediated remodeling can facilitate invasion into the surrounding ECM, which can lead to invasion and metastasis.^{28,29} To assess the contribution of HCT-116 cells to the overall fiber topography in the submucosal organoid, we first formed cell spheroids from HCT-116 cells, as this morphology more closely mimics foci-like structures observed in metastases,^{30–33} and embedded them into the submucosal organoids during fabrication. On inspection after 7 days, organoids had contracted similarly to those fabricated without HCT-116 spheroids. Second harmonic generation (SHG) imaging allowed whole imaging of the organoid collagen network (Fig. 5A, blue), and cells were visualized by capturing the intrinsic cellular autofluorescence (Fig. 5A, red). After identifying regions away from the spheroid (Fig. 5B) largely populated by SMCs, we observed long, aligned fibers (Fig. 5B, white arrows). This topography is similar to that found in previously performed picosirius

red staining (Fig. 3A), which also contained lengthened and aligned fibers. Conversely, regions near the tumor spheroid (Fig. 5C) were populated by comparatively short, random collagen fibers resembling those found in bare Col I networks. These areas near the HCT-116 spheroid are accessible by SMCs, but the fiber topographies do not resemble those remodeled by SMCs, as observed in Figure 3A. We then used CT-FIRE to analyze the fibers found in either location for angular distribution (Fig. 5B'', C'') and straightness (Fig. 5B''', C''').

We performed a Kolmogorov–Smirnov two-sample test and found a significant difference between the angular distributions of fibers found near and away from the HCT-116 spheroid ($p=0.0178$); the angular distribution shows no dominant angle for fiber orientation near the spheroid (Fig. 5C'') with large frequencies of fibers lying across the range. Collagen fibers away from the spheroid clustered around a similar angle (Fig. 5B'') indicating aligned, parallel topography. Fibers away from the spheroid were also significantly straighter than fibers near the spheroid ($p=1.04e-21$). The straightness of fibers is determined by the ratio of the distance from fiber end to end and the distance along the fiber path, thus a straightness value of 1 is perfectly straight and values decreasing from 1 indicate increasing waviness or curliness.³⁴ These results indicate the HCT-116 cells, and although embedded as a spheroid within an SMC organized matrix, will attempt to remodel the local ECM in the proximity. The balance between stromal cell and cancer cell remodeling may represent a crucial aspect of the progression of a tumor toward malignancy.

FIG. 5. Collagen fiber properties in submucosal organoids with embedded HCT-116 spheroids. (A) Submucosal organoids with embedded HCT-116 spheroids and Col I fibers (blue) were imaged using second-harmonic generation. Cellular autofluorescence (red) indicates cell bodies. *Inset:* a microscopic image of a 7-day organoid after fixation. A 3D reconstruction was generated in Imaris™ (A, bottom), and regions of interest, away from (B, B') and near (C, C') the tumor spheroid, were used to visualize the Col I fibers (white arrows in B denote elongated and aligned fibers). The regions of interest were analyzed with the CT-FIRE program. (B'', C'') Fiber angles (degrees vs. frequency). (B''', C''') Straightness (straightness vs. frequency). (A) Scale bar = 200 μ m. (B, C) Scale bar = 20 μ m. * p < 0.05, # p < 0.01. Color images available online at www.liebertpub.com/tea

Submucosal organoids support an epithelial phenotype of metastatic colorectal HCT-116 carcinoma cells

While CaCO2 cells are known to be nonaggressive,²⁵ HCT-116 cells were isolated from a colorectal metastasis removed from the liver, they are mesenchymal in nature, and are more aggressive than CaCO2 cells.³⁵ To assess the effects of collagen remodeling in the submucosal organoids on metastatic cells, we embedded HCT-116 spheroids in the submucosal organoids and in bare collagen. H&E staining after 7 days of culture showed a more compact structure for HCT-116 spheroids within the submucosal construct compared with those in bare collagen construct (Fig. 6A and B, respectively). In the submucosal organoid spheroid, SMCs have contracted around the dense spheroid and the HCT-116 cells migrated toward the SMCs. The spheroids in bare collagen showed a necrotic core probably due to their larger size and cell compactness compared with spheroids in the submucosal organoids. The cells of spheroids in the bare collagen had more migratory projections compared with those in the submucosal organoids, suggesting a more proliferative or motile phenotype. These results suggest that changes in matrix topography, as a result of collagen remodeling by the SMCs, affect spheroid morphology and possibly tumor cell epithelial-to-mesenchymal transition (EMT).

To verify this hypothesis, we immunostained the different constructs with known EMT markers, including N-cadherin, E-cadherin, and ZO-1 (Fig. 6C–J and Supplementary Fig. S4). N-cadherin is typically found in mesenchymal phenotype cells, while E-cadherin is more regularly located on the membranes of epithelial phenotype cells, and ZO-1 is a tight-junction protein found in epithelial cells of the colon.^{36–38} CK-18 staining was used for identification of HCT-116 cells within the constructs. We observed a higher expression of N-cadherin in HCT-116 spheroids in bare collagen (Fig. 6C, D), and conversely, a higher expression of E-cadherin in HCT-116 spheroids in the submucosal organoids (Fig. 6E, F). ZO-1 staining was abundant in HCT-116 in spheroids in the submucosal construct, but was mostly absent in spheroids in bare collagen (Fig. 6G, H). Finally, we examined the expression of matrix metalloproteinase 9 (MMP9), a proteolytic enzyme that degrades the ECM and facilitates cell migration and invasion of cancer cells to neighboring tissues.²⁹ MMP9 expression was much higher in HCT-116 in spheroids in bare collagen compared with cells in spheroids in the submucosal organoids (Fig. 6I, J). Altogether, these results clearly indicate a clear difference in phenotype between the two configurations: HCT-116 cells in remodeled and organized submucosal organoids possess a more native/benign epithelial phenotype, whereas in the unorganized bare collagen constructs, they show a more mesenchymal/tumorigenic phenotype.

WNT pathway activation in HCT-116 submucosal tumor organoids

WNT activation is a hallmark of aggressive CRC and can be associated with lower rates of patient survival.^{39,40} The canonical WNT pathway acts through the translocation of β -catenin from the cell membrane into the cell nucleus, where it forms a transcription complex to upregulate several EMT-related genes (Supplementary Fig. S1).^{22,41–43} Im-

munostaining for β -catenin on HCT-116 spheroids in submucosal organoids showed distinct membrane-localized staining patterns of β -catenin in the HCT-116 spheroids from the submucosal construct (Fig. 7A). In contrast, HCT-116 spheroids in bare collagen display nuclear expression in some cells (Fig. 7B, white arrows), suggesting activation of WNT signaling in HCT-116 cells in the bare collagen.

To validate this hypothesis, HCT-116 cells were engineered to express GFP under control of the T-cell factor/lymphoid enhancer factor (TCF/LEF) promoter cassette, activated by the translocation of β -catenin from the membrane to the nucleus, along with constitutively active nuclear mCherry fluorescence.⁴⁴ Green fluorescent signal was only observed in HCT-116 spheroids in bare collagen but not in HCT-116 spheroids in submucosal organoids (Fig. 7C, E, $p=5.04\text{e-}4$). However, when HCT-116 spheroids in submucosal organoids were incubated with a WNT agonist, BIO,^{45,46} some cells began to show GFP expression (Fig. 7F). Conversely, incubation of tumor organoids in bare collagen with a WNT antagonist, XAV939,⁴⁷ completely eliminated the green fluorescent signal (Fig. 7G). GFP fluorescence quantification indicated approximately sevenfold increase with BIO and ninefold decrease with XAV939 (Fig. 7H, $p=0.035$). Together, these results indicate that β -catenin-mediated WNT signaling is active in HCT-116 spheroids in bare collagen but not in spheroids in submucosal organoids. However, WNT signaling of spheroids is responsive to stimulus such as pharmacological activation by WNT pathway agonists/antagonists.

Chemoresistance of HCT-116 submucosal tumor organoids

The results in Figure 7 demonstrating minimal WNT signaling in HCT-116 spheroids in submucosal organoids compared with spheroids in bare collagen, suggested that there could also be a difference in HCT-116 cell proliferation between these configurations, and thus, a difference in their chemosensitivity.⁴⁸ To test these hypotheses, we first stained HCT-116 spheroids in submucosal organoids and in bare collagen for Ki-67 to identify proliferating cells (Fig. 8A and B, respectively). There were significantly more Ki-67-positive HCT-116 cells in spheroids in bare collagen compared with spheroids in submucosal organoids (Fig. 8C, $p=0.0055$). We then treated HCT-116 spheroids in submucosal organoids and bare collagen for 3 days with 10 mM 5-FU, a pyrimidine analogue targeting proliferating cells, and stained them with a Live/Dead assay kit (Fig. 8D and E, respectively). We observed marked differences between the two configurations: the majority ($\sim 260\%$ more green than red signal) of HCT-116 cells in the submucosal organoids were alive (green), whereas fewer HCT-116 cells were alive in the bare collagen constructs (approximately equivalent green and red signal) (Fig. 8F, $p=0.013$). CaCO2 spheroids cultured in these two configurations and subjected to the same 5-FU treatment showed very few dead cells in both configurations (Fig. 8G–I, $p=0.33$). Studies of CaCO2 sensitivity are inconsistent, however, our experiments confirm prior studies that indicated lower 5-FU sensitivity of CaCO2 cells in 2D compared to more aggressive lines.⁴⁹ Together, these results connect WNT signaling activation, tumor cell proliferation, and 5-FU sensitivity in HCT-116 tumor spheroids and suggest that in the remodeled and highly organized submucosal organoids, WNT signaling is less active, leading to slower cell proliferation

FIG. 6. EMT phenotype of HCT-116 submucosal tumor organoid. HCT-116 spheroids in submucosal organoids (A, C, E, G, I) and bare collagen (B, D, E, H, J) were harvested after 7 days in culture and stained for H&E (A, B) indicating cell membrane-associated proteins (C–H) and MMP (I, J). CK-18 staining was used to identify the HCT-116 cells inside the organoids. For each stain (.1), image is triple staining and (.2) image is the specific protein stain. (A, B) Scale bar = 100 μ m. (C–J) Scale bar = 25 μ m. EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase. Color images available online at www.liebertpub.com/tea

FIG. 7. The effects of collagen fiber organization on WNT pathway activation in HCT-116 submucosal tumor organoid. HCT-116 spheroids in submucosal organoids (**A**) and bare collagen (**B**) were harvested after 7 days in culture and stained for β -catenin, CK-18 (to identify HCT-116 cells), and DAPI. For each stain (**A.1**, **B.1**), images are triple staining and (**A.2**, **B.2**) images are β -catenin stain. (**C**, **D**) Spheroids of HCT-116 expressing GFP under control of WNT-activated promoter sequence (TOP-GFP) and constitutive nuclear mCherry were embedded in submucosal organoids (**C**) and bare collagen (**D**) and the ratio of signal intensity of GFP (green) and mCherry (red) was calculated after 7 days in culture (**E**, SMC—submucosal organoids; Col I—bare collagen). In parallel, these fluorescently labeled HCT-116 spheroids in submucosal organoids were incubated with WNT agonist, BIO (**F**), and WNT antagonist, XAV939 (**G**), as indicated, and signal intensity of GFP (green) and mCherry (red) was calculated after 7 days in culture (**H**). $^{\#}p < 0.01$, $^{*}p < 0.05$. (**A**, **B**) Scale bar = 25 μ m. (**C**, **D**, **F**, **G**) Scale bar = 150 μ m. Color images available online at www.liebertpub.com/tea

and higher resistance to chemotherapy, compared with spheroids from the nonremodeled bare collagen.

Discussion

Cancer research, aiming to study the mechanisms that govern tumor growth and metastasis, has been historically carried out in cancer cells in isolation, such as *in vitro* cultures of cancer cell lines in plastic dishes or suspension cultures.⁵⁰ Although many breakthroughs were discovered with these techniques, tissue culture plastic and single-cell systems are poor recapitulations of the physiologic microenvironment of cancers.^{3,4,51} In recent years, there has been a shift toward incorporating the tumor microenvironment (stroma) into the design of innovative experimental platforms to study the dynamics of cell–ECM and cell–stroma interactions *in vitro*.^{33,52,53} The microenvironmental space is complex and dynamic; there is constant ECM remodeling, interactions between the tumor and stromal cells, and a multitude of signaling factors that all drive cancer cell

phenotype in one direction or the other.^{30,54} It is now clear that the microenvironment has a profound effect on cancer cells, but it is currently difficult to precisely recapitulate its complex nature *in vitro*. To better approach this problem, one needs to develop an experimental model that will allow study of specific variables of the microenvironment, to develop an understanding of each aspect individually before adding further complexity. The current study aimed at creating an *in vitro* tumor surrogate that includes components of the tumor microenvironment such as the host tissue cells and the ECM with its unique topography, in addition to human tumor cells. To better model and study the effects of ECM fiber arrangement on cancer cell progression, we bioengineered physiologically functional submucosal tissue constructs (organoids) containing primary colonic SMCs in Col I hydrogels that represent the stromal layer of CRCs.

The submucosal organoid expressed typical SMC markers, indicating that the environment is sufficient for maintaining their differentiated phenotype, unlike some conditions that can cause SMC dedifferentiation into a fibroblast-like

FIG. 8. The effects of collagen fiber organization on 5-FU response on HCT-116 CaCO2 and submucosal tumor organoids. (A, B) HCT-116 spheroids in submucosal organoids (A) and bare collagen (B) were harvested after 7 days in culture and stained for Ki-67 for proliferating cells CK-18 to identify HCT-116 cells. (C) The proportion of Ki-67-positive cells in submucosal organoids (SMC) and bare collagen (Col I) was calculated and graphed. Spheroids of HCT-116 (D, E) and CaCO2 (G, H) were embedded in submucosal organoids (D, G) and bare collagen (E, H) and cultured for 3 days and then exposed to 10 mM 5-FU for 3 days. The organoids were stained with a Live/Dead assay kit and the green to red fluorescence signal was calculated for HCT-116 (F) and CaCO2 (I). $*p < 0.05$. (A, B) Scale bar = 50 μ m. (D, F–H) Scale bar = 150 μ m. 5-FU, 5-fluorouracil. Color images available online at www.liebertpub.com/tea

phenotype.^{55–57} Furthermore, the organoids display rapid contraction over 4–5 days, suggesting that the SMCs are actively remodeling the surrounding Col I hydrogel. Indeed, we found a significant difference in fiber alignment and bundling between the submucosal organoid and bare collagen hydrogels. Furthermore, the fiber arrangements found in the submucosal organoid are similar to the parallel, aligned patterning found in healthy, *in vivo* tissue¹²; these aligned collagen fibers are necessary to retain strength and compliance and may help maintain an epithelial phenotype in mucosal cells.⁵⁸ Conversely, the bare collagen topography was disordered and random as found in dysplastic tissue.^{12,24} Clinically, colorectal tumors are often disordered and autoimmune diseases (such as Crohn's), which cause remodeling of the submucosal stroma, are associated with low survival and poor patient prognosis

due to high rates of migratory and metastatic cells.^{7,10} Mucosal cells can sense varied stromal topography through integrin binding, which then leads to signaling cascades through focal adhesion kinase (FAK) to direct cell phenotype; these cascades can then lead to oncogenesis.⁵⁹

Since these submucosal organoids seemed to recapitulate the colonic submucosa phenotype and stromal topography, we tested if they would promote and support normal epithelial morphology. By integrating CaCO2 cells into the organoid, we observed the formation of epithelial acini. Acini formation *in vitro* is driven by the ECM and is maintained through interactions between epithelial cells and stromal ECM and cells.^{60–62} In contrast, no acini were formed in bare collagen or in a transwell culture system, indicating that Col I remodeling by the SMCs creates a native-like environment necessary for acini

formation and epithelial phenotype maintenance. Although our main interest is cancer research, the technology presented here may have implications beyond cancer: the generation of acini presents the basis for a “gut-on-a-chip” type organoid, which could be used for drug or toxicology testing.

Colon cancer usually begins with an oncogenic transformation of the colon epithelium.⁶³ After demonstrating that the submucosal organoids appeared to closely mimic the colonic submucosal layer, we proceeded to integrate colorectal tumor foci, made of HCT-116 spheroids, with the submucosal organoid, as a multitissue platform to study the impact of the ECM organization on CRC phenotype. First, we observed, through SHG imaging, the HCT-116 spheroids produced random, unaligned ECM near the spheroid boundary. Away from the spheroid, SMCs aligned and bundled collagen, as expected. Although the majority of the organoid is well organized by the SMCs, the HCT-116 cells disrupted local ECM remodeling; this might indicate that cancer cells inhibit or restrict the remodeling of nearby, native stromal cells to aid in oncogenic progression. Some studies indicate that stromal collagen alignment can increase invasiveness of cancer cells due to aligned collagen acting like “tracks” to facilitate movement.^{24,27} Although HCT-116 spheroids grew to a larger size in the collagen-only condition, HCT-116 cells exhibited propensity for invasion in the submucosal organoid (Supplementary Fig. S2; Supplementary Data are available online at www.liebertpub.com/tea), despite their phenotype, indicating alignment can increase invasion and that EMT phenotype and invasiveness may not be entirely linked.

Next, we showed that E-cadherin and N-cadherin staining mirrored one another: both were found in either condition, but E-cadherin was predominant in the submucosal organoid, while N-cadherin was predominant in the collagen-only construct. This indicates an epithelial phenotype of HCT-116 cells in the submucosal organoid and is further evidenced by the presence of ZO-1 in this condition. HCT-116 cells cultured in bare collagen also seemed more motile with projections spreading from the spheroid; together with the presence of N-cadherin, bare collagen appeared to support a mesenchymal phenotype in HCT-116 cells. We also performed transwell experiments (Supplementary Fig. S3) to determine the extent of SMC paracrine activity on the HCT-116 cancer cells; in these experiments, we saw a negligible effect from the SMCs and the cancer cells retained a mesenchymal phenotype. These data indicate that HCT-116 cells take on a more epithelial phenotype as a result of interaction with organized collagen.

In vivo CRCs are heterogeneous with a multitude of cell types, but it has been shown that many metastatic tumors have undergone EMT.^{64,65} In fact, *in vitro* systems have shown that HCT-116 cells, which are derived from a metastasis, are mesenchymal in nature.^{1,66} In addition, when these cells undergo mesenchymal-to-epithelial transition, it is associated with decreased tumor aggression.⁶⁷ We observe a similar effect through the complete loss of MMP9 expression by HCT-116 cells in remodeled submucosal organoid, but not in the bare, unorganized collagen. MMP9 is used by aggressive cancer cells to degrade ECM to facilitate migration,^{29,68} suggesting that the tumor cells in the submucosal organoids were less invasive. This result is in agreement with our previous observations that HCT-116 cells assume an epithelial phenotype in the submucosal organoids compared with a more mesenchymal phenotype in bare collagen. Furthermore, MMP9 is known

to be regulated by the WNT pathway, which is more active in HCT-116 spheroids of the collagen-only construct.^{69,70} Interestingly, high tumor stiffness is often associated with higher invasiveness of CRC cells,⁵⁹ but the stiffness of our collagen hydrogel is relatively low (464 Pa) and increases dramatically after SMC remodeling (55.4 kPa). Despite low stiffness, HCT-116 cells exhibited a mesenchymal phenotype in the collagen construct, which indicates that stiffness-related effects may be nominal in comparison to topography-based induction. In addition, there are few conclusive studies for comparing stiffness experienced by cancer cells *in vivo* to our system. These results indicate that specific fiber topography in the colon could serve as a predictive measure for oncogenic risk.

There is an unmet need to develop better model systems for screening of anticancer drugs. After observing a stark difference in HCT-116 phenotype in either aligned or unaligned collagen, we tested chemosusceptibility to the common therapeutic 5-FU. 5-FU is a nucleoside analogue and disrupts the synthesis of thymidine thereby killing rapidly proliferative cells and thus has a nominal effect on noncycling cells. *In vitro* studies have shown 5-FU has a diminished effect on normal cells compared to cancer cells.⁷¹ After treating HCT-116 spheroids in both submucosal and bare collagen constructs with 5-FU, we found corroborating evidence that cells in the submucosal organoid are reverting to a more normal, epithelial phenotype: they are less susceptible to 5-FU due to their greatly decreased proliferation rate (as evidenced by Ki-67 staining). Typical epithelial cells do not proliferate rapidly and should not be as sensitive to chemotherapeutics: this is exactly the response we observed. In fact, when we assayed CaCO2 spheroid chemosusceptibility in the same conditions, we saw little difference in 5-FU effect between submucosal organoids and bare collagen constructs, which follows as CaCO2 cells are naturally more epithelial. Conversely, HCT-116 spheroids in the bare collagen construct, which seem to be more mesenchymal and proliferative, are more susceptible to 5-FU exposure. The stark difference in chemotherapeutic response between these two conditions may also inform personalized medicine or precision oncology: the patient's unique stromal phenotype should be taken into consideration when designing the chemotherapeutic treatment.

The physical properties of the tumor microenvironment, especially ECM structure and its fiber topography, have recently been targeted for their significant contribution to cancer progression and response to therapy.⁷² However, the lack of *in vitro* models that accurately mimic how changes in ECM topography impact tumor cells significantly slows the translation of basic research data to advanced cancer therapy. Our CRC organoid platform offers a robust and replicative system to study the interactions between tumor cells and specific features of the ECM (i.e., collagen fiber organization) and how they affect the tumor cell phenotype and, importantly, response to chemotherapy. Our organoid platform is modular and tunable and can incorporate patient-derived cancer cells, stromal cells, and various features of the ECM. In addition, the basic methodology can be adapted to a variety of tissue types. Cancer organoid systems, such as ours, provide an informative and predictive platform that can be applied to better study cancer progression, drug discovery, pharmaceutical diagnostics, and ultimately, the generation of patient-specific cancer organoids for personalized medicine applications.

Acknowledgments

The authors are grateful to the TERMIS-AM Awards Committee and Council as well as Mary Ann Liebert, Inc. for the TERMIS-AM 2017 Outstanding Student Award to M.D. This research was partially supported by the NCI grants R01CA180149 (S.S.), R33CA202822 (S.S.), and by the Wake Forest Baptist Comprehensive Cancer Center's NCI Cancer Center Support Grant P30CA012197.

Authors' Contributions

M.D., A.S., and S.S. wrote the main article text. M.D. prepared Figures 1–8, and A.S. and S.S. reviewed and edited all figures. F.M. provided text and expertise on imaging analysis and methods, and reviewed and edited Figure 5. K.C. produced second-harmonic generation image. All authors reviewed the article.

Disclosure Statement

No competing financial interests exist.

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Received: September 13, 2017

Accepted: September 15, 2017

Online Publication Date: October 10, 2017