iScience, Volume *23*

Supplemental Information

Manipulating the Tumor Microenvironment in Tumor Organoids Induces Phenotypic Changes and Chemoresistance

Anthony Dominijanni, Mahesh Devarasetty, and Shay Soker

Transparent Methods:

Cell culture

Human hepatic stellate cells, LX-2 cells, provided by Dr. Scott Friedman (Icahn School of Medicine at Mount Sinai, Ney York, NY), and human metastatic colon colorectal carcinoma cell line, HCT-116 (#CCL-247, ATCC, Manasses, VA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Switzerland) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Cell lines were cultured in conditions of 37°C and 5% CO_2. All cell types were cultured and expanded in plastic 15-cm tissue-treated dishes. Cells were cultured to 80%-90% confluence before being harvested for use or passage. All cells were detached from the plates with Trypsin/EDTA (Hyclone) and resuspended in media before further use in studies.

Spheroid and organoid fabrication

Spheroids of HCT-116 cells ($1.0x10^4$ cells each) were prepared by suspending cells in culture media at $1.0x10^5$ cells/mL and dispensing 100 µL of cell-media suspension into each well of an ultra-low attachment round-bottom 96-well plate (CoStar #7007; Corning, Corning, NY). Cells formed tight, spherical structures after 3 days in culture at which time they were implanted in organoids immediately.

Organoids were fabricated as described previously [71]. Type I Rat Tail Collagen (#354236; Corning) was prepared per manufacturers' protocol at a concentration of 2 mg/mL on ice. LX-2 cells were trypsinized and counted, then suspended in desired Col1 concentration at $5.0x10^6$ cells/mL. Media from round-bottom plates containing the spheroids was aspirated and 100 µL of LX-2-Col1 or collagen only solution was pipetted into each well without disturbing the spheroid. The 100 µL LX-2-Col1-spheroid mixture was slowly pipetted up to suspend the spheroid and was dispensed into a polydimethylsiloxane (PDMS; DOW Sylgard 184, Midland, MI) molds as described previously (**Fig. 1a-top**) [71]. After ensuring that the spheroid was in the center of the hydrogel solution, the plates were stored at 37ºC for 30 minutes to allow the gel to polymerize. Coculture organoids were produced using a homogenous mixture of $5.0x10^5$ LX-2 cells and/or $5.0x10^4$ HCT-116 cells in Col1. After collagen polymerization, media was added with or without

10 ng/mL TGF-β, and molds were removed (**Fig. 1a-bottom)** [46]. Organoids were cultured for 7 days total but varied depending on the experiment. Media was replenished every three days.

Contraction assay

Organoid contraction was measured through time by diameter calculation via image analysis of digital images. For each organoid, an image was taken at various time points (list time points here) with a ruler in the image for scale. The image was then opened in ImageJ and the pixel/mm was converted based on the ruler scale. The diameter of each organoid was measured in triplicate.

Rheological measurements and analysis

Organoid stiffness was determined using a Discovery HR2 Rheometer (TA Instruments), by applying a sinusoidal strain on the material. The elastic moduli of the organoids were determined through generation of a force-displacement curve through compression testing with a flat, 8mm, round geometry. Organoids were placed on the center of the rheometer stage and excess liquid was removed. The geometry was set to compress the organoid and collect force and gap distance measurements every 0.25 s. Samples were discarded after compression. Stress values were generated by dividing force measurements by sample area, determined through digital imaging. Strain values were generated by subtracting the gap distance from the sample height and dividing the total sum by the height. Stress (y-axis) and strain (x-axis) were then plotted to yield a stress-strain curve consisting of two phases: an initial amorphous phase and a subsequent crystalline phase occurring after a curve elbow. Elastic modulus was calculated using the slope of the amorphous phase.

Immunohistochemistry staining and analysis

Organoids were fixed in 4% paraformaldehyde overnight at 4°C, then washed with phosphate buffered saline (PBS), and stored in 70% ethanol before paraffin processing. Following paraffin processing and embedding, 5 µm sections were cut using a microtome (Leica Microsystems Inc., Buffalo Grove, IL) and mounted to slides. For all stains, slides were baked for 2 h at 60˚C followed by deparaffinization and rehydration. Hematoxylin & Eosin (H&E) staining was performed by core facilities at the Wake Forest Institute for Regenerative Medicine. Picrosirius Red (PS-Red)

staining was done using a commercially available staining kit (#24901; PolySciences, Warrington, PA) following the manufacturer's protocol.

For immunohistochemistry (IHC), all incubations were performed at room temperature. Antigen retrieval was performed using Proteinase K (DAKO; Carpinteria, CA). Samples were permeabilized with 0.05% Triton-X in PBS for 5 min. Non-specific antigen blocking was performed using Protein Block Solution (#ab156024; Abcam, Cambridge, MA) incubation for 30 min. Slides were then incubated with the appropriate primary antibody against β-Catenin (#71-2700; Invitrogen-ThermoFisher), E-Cadherin (#ab40772; Abcam), N-Cadherin (#ab76011; Abcam), CD44 (#ab51037; Abcam), CD133 (MAB4399-I; Millipore Sigma), Cleaved Caspase-3 (#9661; Cell Signaling Technology), focal adhesion kinase (FAK) (#ab40794, fibroblast activation protein (FAP) (#ab53066; Abcam), α-smooth muscle actin (SMA) (#ab5694; Abcam) or Ki-67 (#ab16667; Abcam) at recommended dilutions in a humidified chamber overnight at 4°C. Slides were then washed and incubated for 1 h with the appropriate secondary antibody. Slides were exposed to DAPI for 5 min and mounted with Prolong Gold (Invitrogen) before imaging. Relevant control slides were prepared for each condition and each antibody combination by excluding the primary antibody incubation. PS-Red stained slides were imaged using linearly polarized light while immunofluorescent stained slides were imaged utilizing laser excitation and were captured with an Olympus BX63 microscope (Olympus; Center Valley, PA) with an Olympus DP80 camera (Olympus).

IHC images were imported as uncompressed files into Visiopharm software (Broomfield, CO) for analysis and quantification. An application for each experiment was developed and modified using the Visiopharm software (**Fig. S3**). Briefly, a script was written to deconvolve each immunofluorescence signal, then isolate the nuclei using the DAPI stain. After each cell was segmented, a second script was written to deconvolve the fluorescence signal and quantify the cells expressing of EMT markers, Caspase-3, FAP, s-SMA and/or Ki-67 markers. These results were imported in Microsoft Excel and calculated for number significance.

Collagen fiber imaging and quantification

Organoid sections were obtained as previously described and stained using a picrosirius red stain kit (#24901; PolySciences, Warrington, PA). PS-Red imaging was performed on an Olympus BX63 (Olympus; Center Valley, PA) upright microscope under brightfield with linearly polarized

light. Once images were captured, PS-Red signal was quantified using hue analysis of collagen signal and collagen fiber geometric parameter segmentation. Hue analysis to identify different levels of collagen bundling and fibrilization was performed using a MATLAB script. Fiber parameter segmentation and quantification of regions of interest's was performed using CT-FIRE (Laboratory for Optical and Computation Instrumentation, University of Wisconsin). Settings were optimized for one data set and remained untouched for remaining images. Data was inputted into MATLAB for analysis and GraphPad prism for graphing.

Drug treatment and analysis

Organoids with embedded spheroids were cultured for 72 hours, then transferred to new well plates and incubated with media containing chemotherapeutics. Organoids were exposed to chemotherapeutics or small molecule inhibitors for a further 72 hours before analysis. FAK phosphorylation inhibition was achieved with defactinib (#S7654, Sellekchem, Houston, TX) solubilized in DMSO to produce a stock solution of 1 mM. Stock solution was added to DMEM to produce a final concentration of 100 nM. TGF-β inhibition was achieved using SB 431542 (#AB-100-NA, R&D Systems) solubilized in DMSO at 15 mM. Stock solution was added to DMEM to obtain a final concentration of 10 μM. Chemotherapeutic formulations were prepared using the following concentrations: 5-Fluorouracil 1mM, Oxaliplatin 25μM, Irinotecan 50μM, and Leucovorin 50μM. Organoids were fixed and sectioned prior to IHC staining with Ki-67 and cleaved caspase-3. Images were analyzed using VisioPharm software similar to EMT quantification (**Fig. S3**).

Statistical analysis

All experiments were performed in triplicate or greater. Quantitative results are presented as mean–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. Significance values are denoted in figure legends.

Figure Titles and Legends:

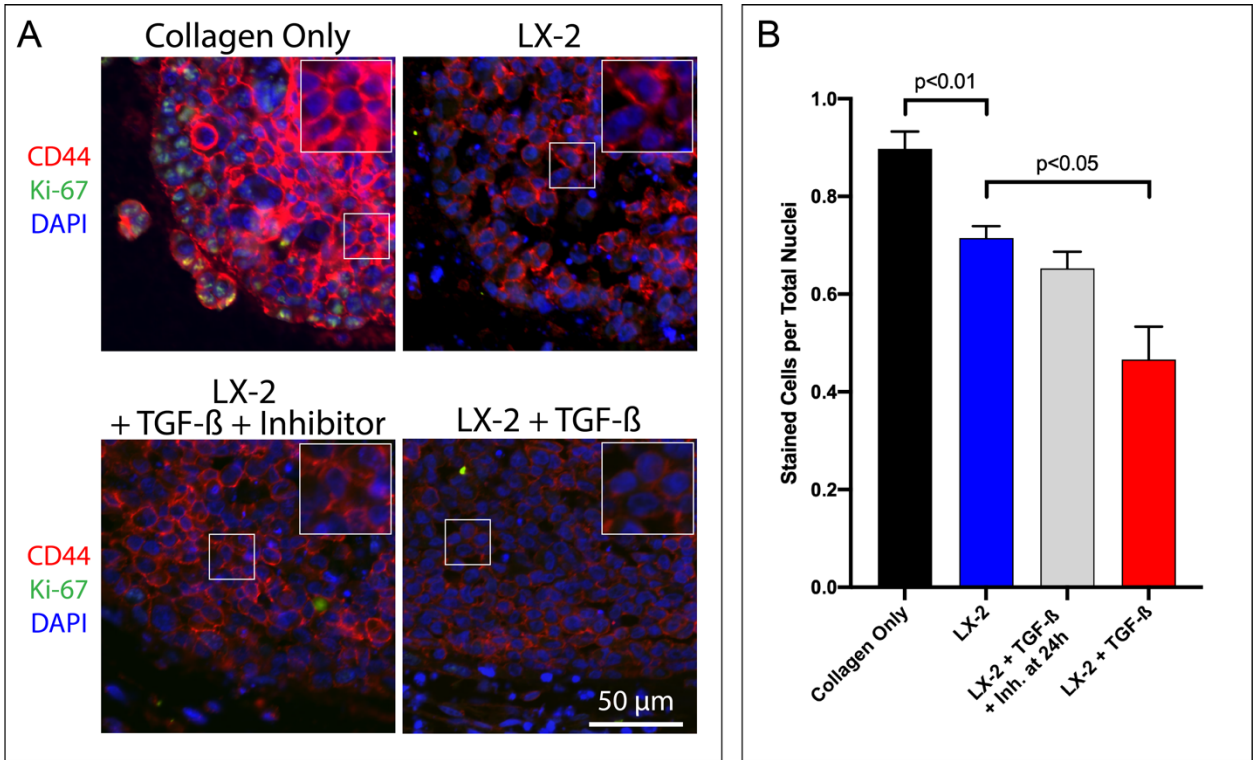

Figure S1. Organoid with dense ECM reduces CD44 expression in HCT-116, Related to Figure 4. (A) *IHC staining of organoid sections for CD44 (red), Ki-67 (green) and DAPI (blue)* at 40x magnification*. All images use the same scale bar = 50 µm.* White box on top right of each individual frame is a 2x zoom of the white box within image to show the red signal within individual cells. ***(B)*** *IHC quantification of HCT-116 cells was determined using VisioPharm software by calculating the percentage of positively expressed cells by the total nuclei present. Graphs represent mean ± s.e.m (n=5) and generated using GraphPad prism.*

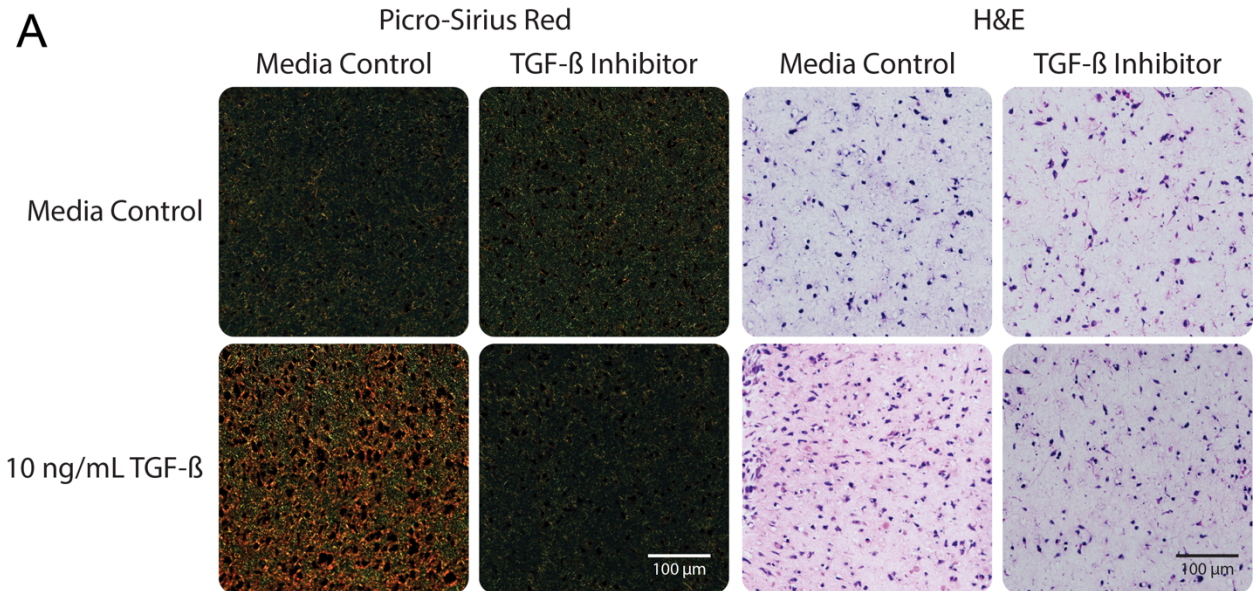

Figure S2. TGF-β inhibitor effectively prevents type I collagen remodeling by LX-2 cells, Related to Figure 7. (A) *Organoids containing LX-2s and type I collagen were sectioned and stained with PSR (left) to highlight naïve (green) and remodeled/bundled (red/orange) collagen fibers under polarized light and H&E (right)*

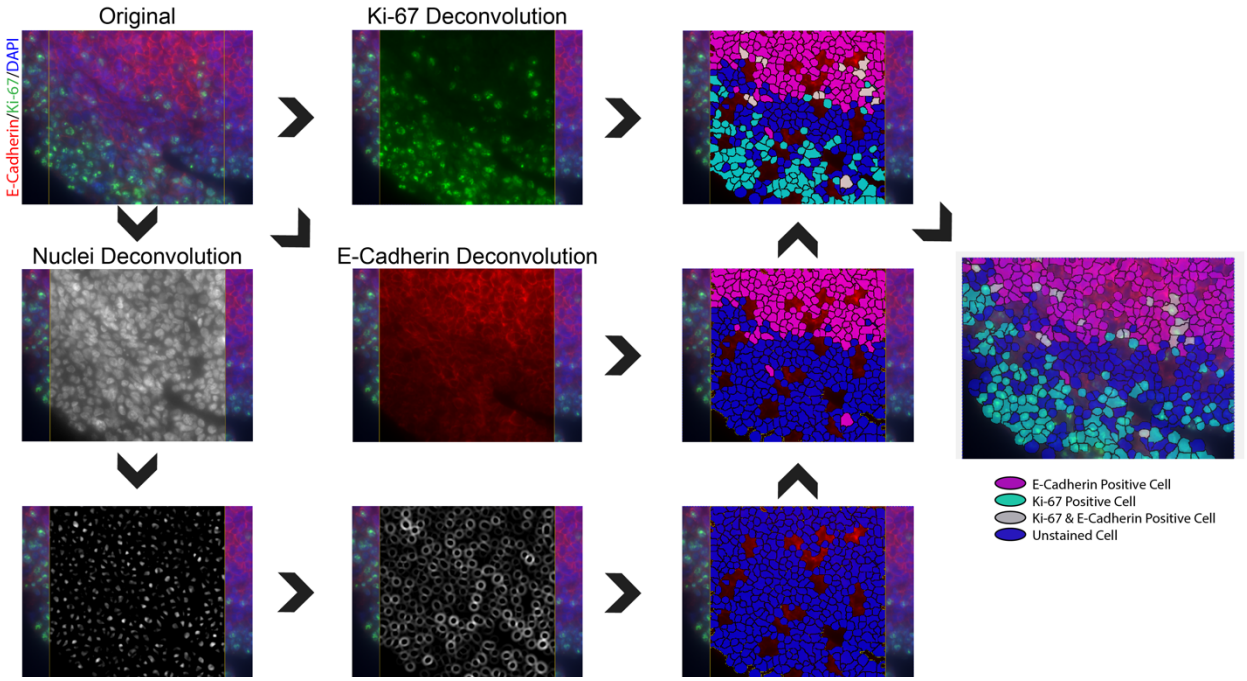

Figure S3. Image analysis using VisioPharm Software, Related to Figure 4. *IHC images were imported as uncompressed files into Visiopharm software (Broomfield, CO) for analysis and quantification. A script was written to deconvolve each immunofluorescence signal, then isolate the nuclei using the DAPI stain. After each cell was segmented, a second script was written to deconvolve the fluorescence signal and quantify the cells expressing of Ki-67 and E-Cadherin markers. These results were imported in excel and calculated for number significance.*