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Supplemental Information

Prostate Stroma Increases the Viability and Maintains the Branching Phenotype of Human Prostate Organoids

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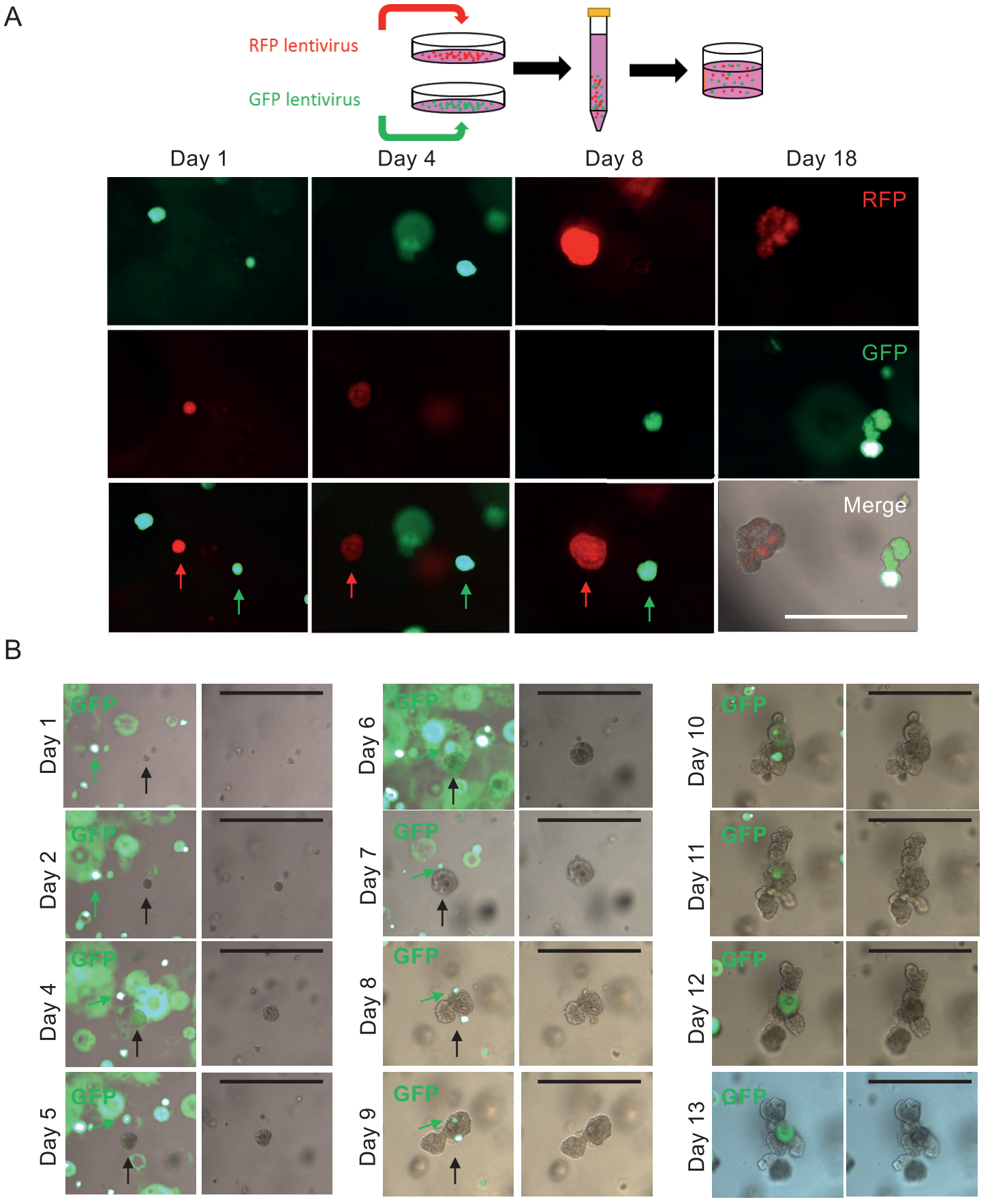
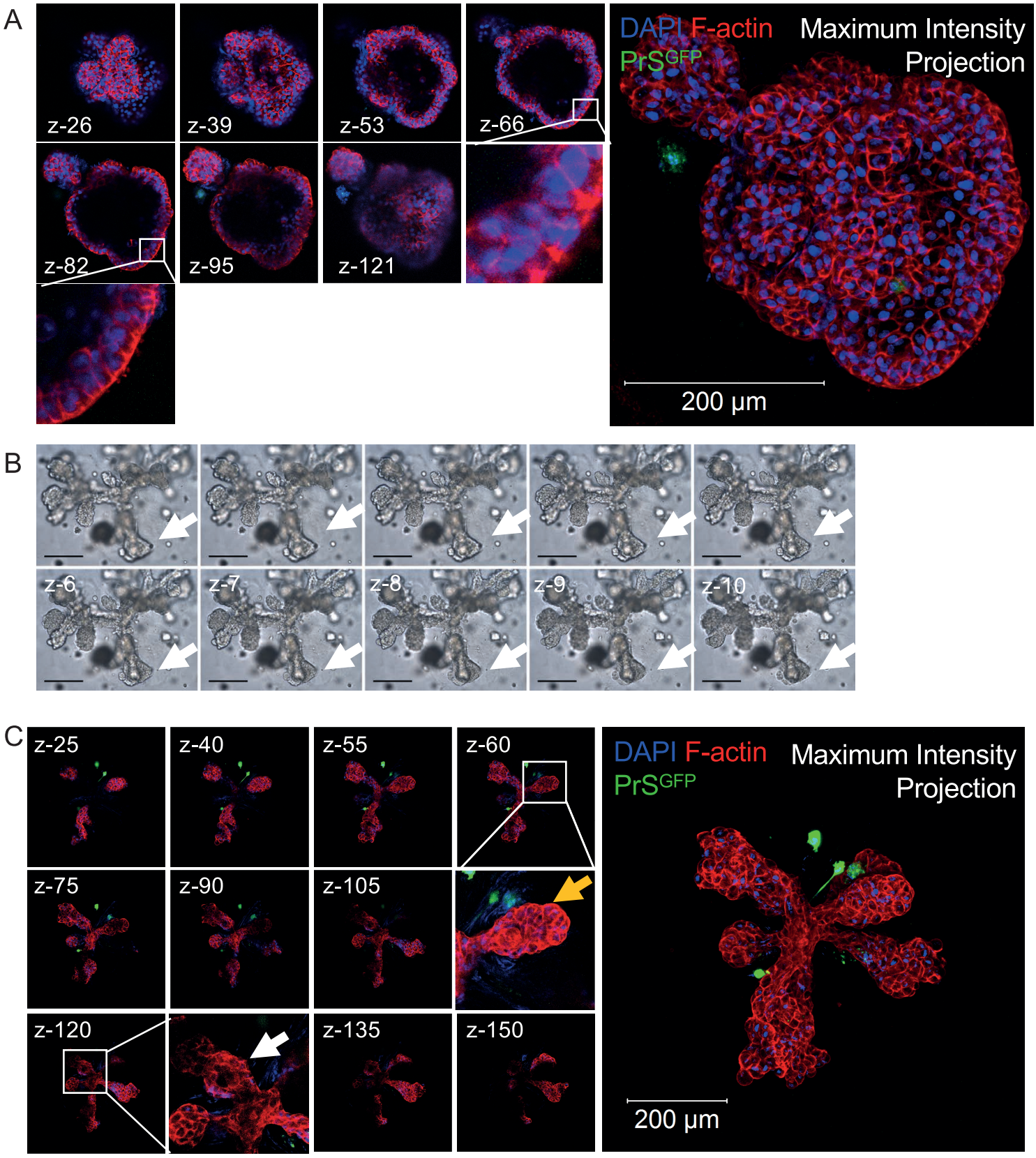
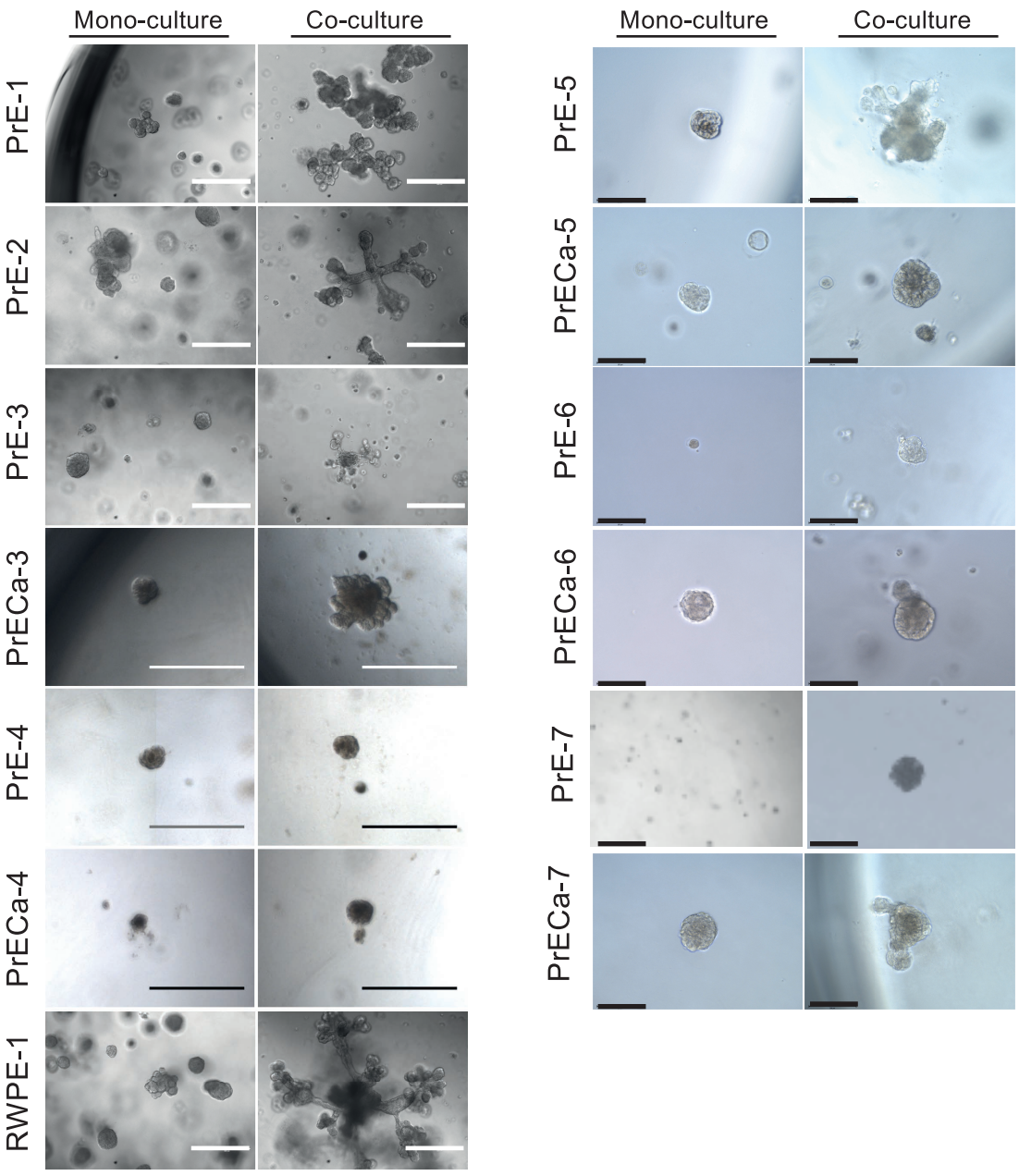


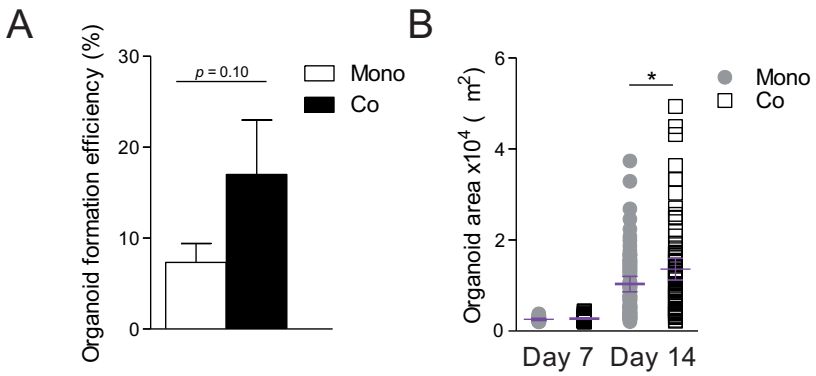
Supplemental Figure 1. Organoids are derived from single cells, related to Figure 1.



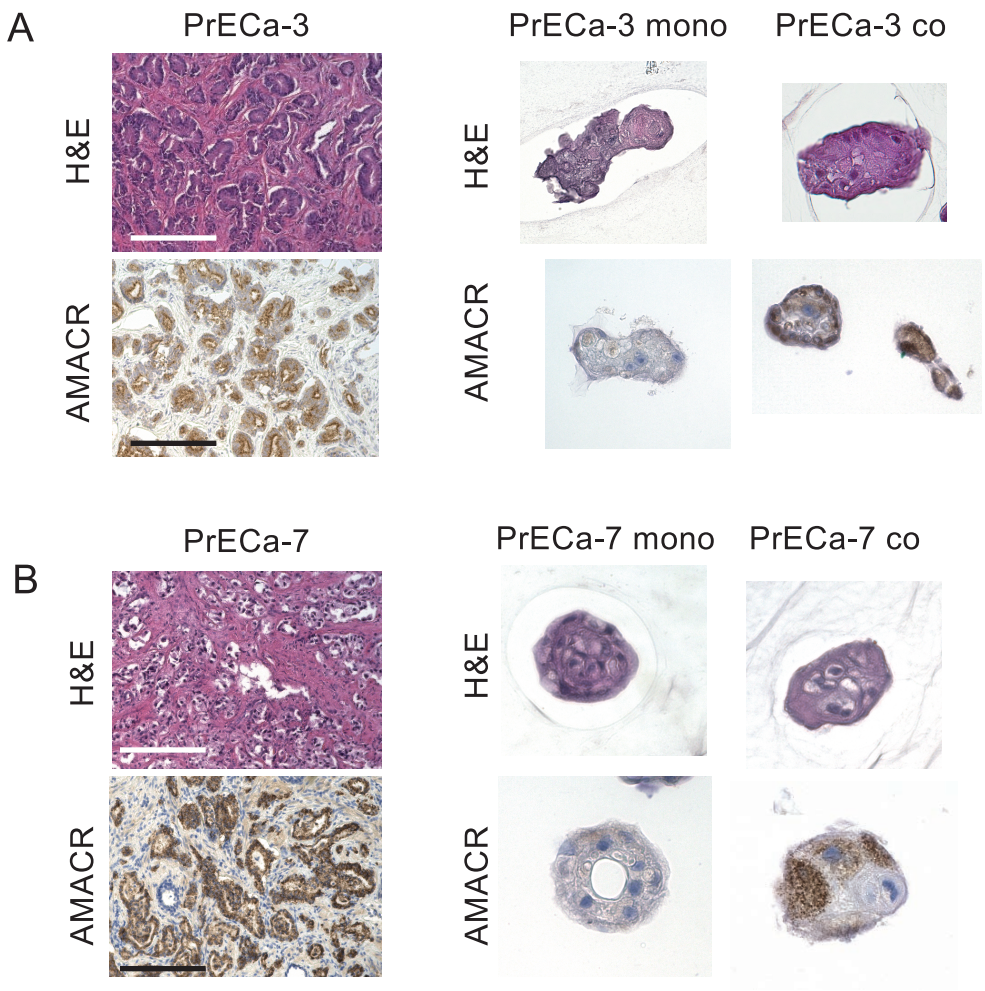
Supplemental Figure 2. Organoids in co-culture contain lumens, related to Figure 2



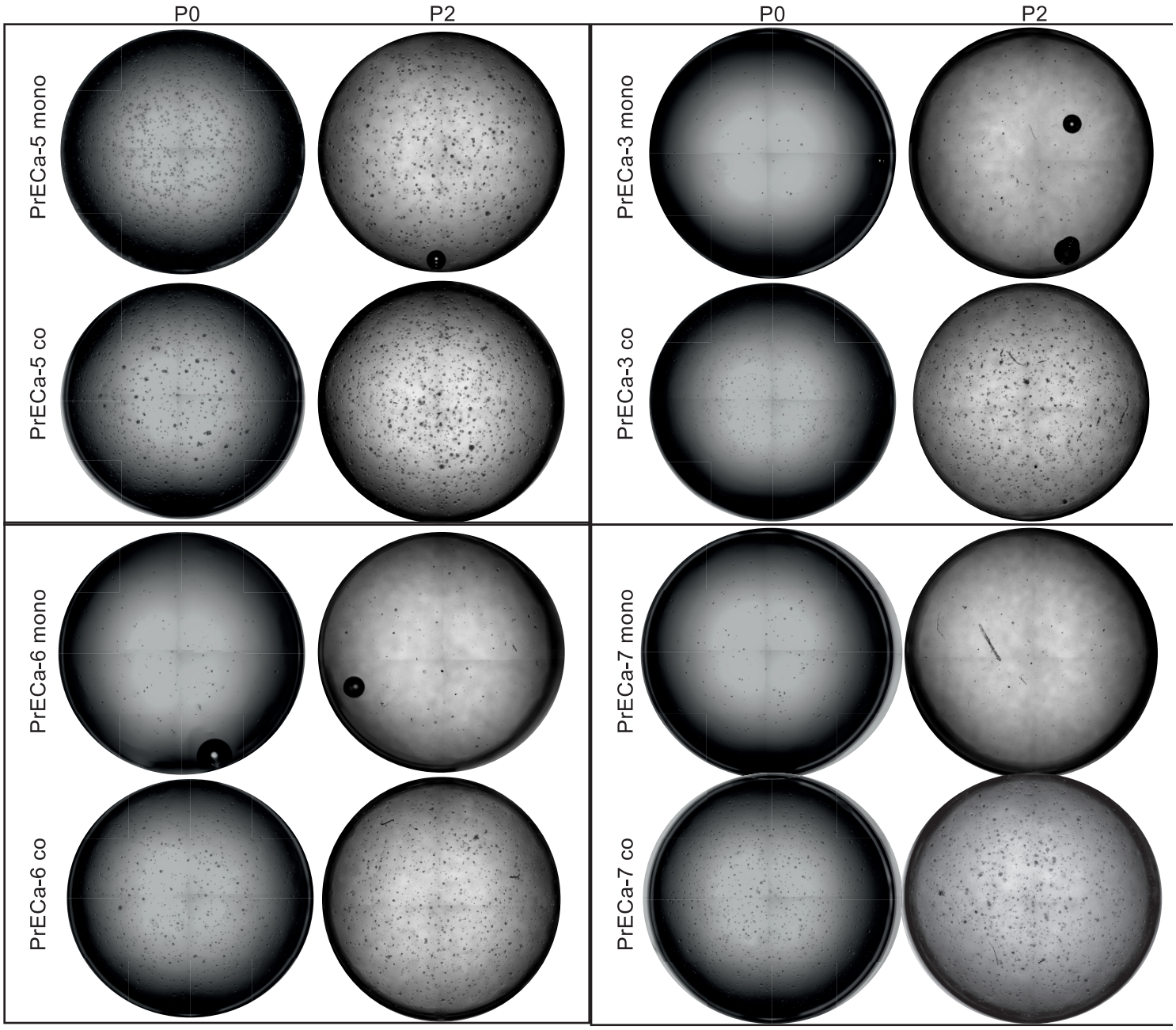
Supplemental Figure 3. Organoids exhibit inter-patient heterogeneity, related to Figure 2 and Table 1

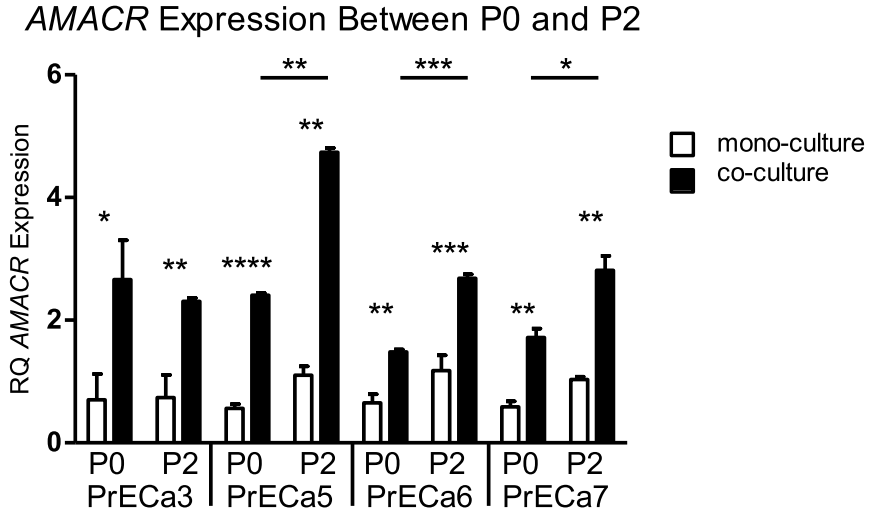


Supplemental Figure 4. Formation efficiency and area of organoids in co-culture with NIH-3T3 fibroblasts, related to Figure 4



Supplemental Figure 5. Images and AMACR staining in co-cultured organoids derived from cancer regions, related to Figure 5.





Supplemental Figure 6. Images and *AMACR* expression of co-cultured organoids derived from cancer regions at passage 0 and passage 2, related to Figure 5

Supplemental Video, Data and Excel Table Title and Legends

Video Titles

Movie 1. Organoid at day 7 in co-culture exhibiting branching morphogenesis. Cells used, PrE-1 and PrS mix 1.

Movie 2. Organoid at day 7 in mono-culture exhibiting branching morphogenesis. Cells used, PrE-1.

Movie 3. Stromal cell (right) taxis towards a branching organoid (left) in co-culture. Cells used, PrE-1 and PrS mix 1.

Movie 4. Stromal cell (bottom left) taxis towards an organoid and subsequent influence on organoid budding and branching distal to stroma-organoid contact. Cells used, PrE-1 and PrS mix 1.

Movie 5. Stromal cell pseudopodia extension (bottom right) and direct contact with an organoid. Subsequent bud formation and elongation occurred distal to stroma-organoid contact. Cells used, PrE-1 and PrS mix 1.

Movie 6. Stromal cells (top right) in direct contact and migrating with a branched organoid. Complex stalk elongation occurred distal to stroma-organoid contact. Cells used, PrE-1 and PrS mix 1.

Movie 7. Organoid in co-culture exhibiting complex branching morphogenesis. Bud detachment occurred after 29 hours (top of organoid), and stalk detachment at 31 hours (top of frame). Cells used, PrE-1 and PrS mix 1.

Movie 8. Organoid co-cultured with GFP-labeled PrS cells integrated with more complex organoid structures. Cells used, PrE-1 and PrS mix 3.

Movie 9. PrS cell at day 7 in co-culture exhibiting pseudopodia expansion and contraction, and remodeling of the surrounding matrix. Cells used, PrE-1 and PrS mix 1.

Movie 10. Organoid co-cultured with GFP-labeled mouse embryonic fibroblasts (NIH-3T3 cells). Extensive cell-cell contact and encapsulation of organoids inhibited branching. Cells used, PrE-1.

Movies are composed of time-lapse images taken every 5 minutes. Time format, hour:min.

Supplemental Data Titles and Legends

Supplemental Figure 1. Organoids are derived from single cells, related to Figure 1

(A) PrE-2 cells were separately transduced with a lentivirus RFP or GFP reporter, mixed and plated into 3D culture. Resulting organoids were imaged at day 1, 4, 8, and 18 and show clonal origin of each organoid. Scale bar 500μm.

(B) PrE-1 cells were plated in co-culture with GFP-labeled PrS mix 5, epithelial cells were identified as GFP(-) and their locations were recorded, followed, and imaged over days 0-13 to capture expansion into organoid. Figure shows outgrowth of a single cell to a branched organoid. GFP+ stromal cells (green arrow) are visible throughout, moving around day to day and touching the organoid (black arrow). Scale Bar 500μm.

Supplemental Figure 2. Organoids in co-culture contain lumens, related to Figure 2

(A) Whole-mounted fluorescent z-stack images and maximum intensity projected image (right) of PrE-1 organoid co-cultured with PrS^{GFP}, stained with phalloidin and DAPI; lumen in zoom panels at z-66 and z-82.

(B) Sequential z-stack images of a day 14 organoid grown from PrE-1 cells. White arrow shows a branch containing hollow luminal space. Scale bar 200μm

(C) Whole-mounted fluorescent z-stack images and maximum intensity projected image (right) of PrE-1 organoid co-cultured with PrS^{GFP}, stained with phalloidin and DAPI; showing some solid chords (zoom, z-60 yellow arrow) and branches that contain lumens (zoom, z-120 white arrow).

Supplemental Figure 3. Organoids exhibit inter-patient heterogeneity, related to Figure 2 and Table 1

Brightfield images of organoids from all 12 tissues (7 patients) used and RWPE-1 cells grown in mono- and co-culture 3D conditions. Mono and co-culture images from the same patient were captured on the same day. All images in the left panel were taken between days 7 and 10, co-culture images were grown with PrS mix 1. The right panel were taken on day 13 of culture, co-culture images for PrE-5/PrECa-5 was grown with PrS mix 5, and PrE-6/PrECa-6 and PrE-7/PrECa-7 were grown with PrS mix 5. Scale bars 500μm. See Table 1 for patient characteristics.

Supplemental Figure 4. Formation efficiency and area of organoids in co-culture with NIH-3T3 fibroblasts, related to Figure 4

(A) Formation efficiency of organoids in mono-culture and in co-culture with NIH-3T3 fibroblasts assessed on day 7. Triplicate wells per condition, error bars represent standard error of the mean.

(B) Area quantification of organoids grown in mono-culture and co-culture with NIH-3T3 fibroblasts after 7 and 14 days. Triplicate wells analyzed per condition, error bars show mean with 95% confidence.

Organoids were grown from PrE-1 cells. A p-value <0.1 was considered statistically significant where * = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$ (ns, not signficant).

Supplemental Figure 5. Images and AMACR staining in co-cultured organoids derived from cancer regions, related to Figure 5.

(A) Matched benign (PrE-3) and cancer (PrECa-3) tissue with hematoxylin and eosin staining (left, top panel) and presence of AMACR by immunohistochemistry (left, bottom panel). 10X image shown with 40X inset for AMACR. Organoids with hematoxylin and eosin staining (right, top panel) and AMACR by immunohistochemistry (right, bottom panel).

(B) Matched benign (PrE-7) and cancer (PrECa-7) tissue with hematoxylin and eosin staining (left, top panel) and presence of AMACR by immunohistochemistry (left, bottom panel). 10X image shown with 40X inset for AMACR. Organoids with hematoxylin and eosin staining (right, top panel) and AMACR by immunohistochemistry (right, bottom panel), organoids were not viable for RNA or embedding.

Supplemental Figure 6. Images and *AMACR* expression of co-cultured organoids derived from cancer regions at passage 0 and passage 2, related to Figure 5

(A) Whole-well images of organoids derived from cancer regions were obtained at day 7 on passage 0 (left) and passage 2 (right).

(B) Gene expression of AMACR in passage 0 and passage 2 organoids derived from cancer regions by RT-qPCR. A p-value <0.1 was considered statistically significant where * = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$ (ns, not signficant).

Transparent Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Larisa Nonn (lnonn@uic.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Primary cell lines
Primary prostate cells established from fresh male radical prostatectomy tissues were isolated as previously described (Giangreco et al., 2013; Nonn et al., 2006). Briefly, radical prostatectomy tissue from benign and cancer regions of the peripheral zone was collected according to UIC Internal Review Board-approved protocol and patients consented prior to surgery. See **Table 1** for primary cell patient characteristics. Tissue histology was confirmed by a board-certified pathologist. Tissue was digested in collagenase/trypsin to single cells and cultured in either Prostate Cell Growth Media (Lonza) or MCDB-105 media (Sigma-Aldrich) to select for epithelial (PrE and PrECa) or stromal cell (PrS) populations, respectively. When ~70% confluent, cells are cryopreserved singly and thawed prior to experimentation. Epithelial population purity was authenticated by expression of the epithelial specific markers *CK5*, *CK8*, *CK18*, and *p63* by RT-qPCR. Stromal cell purity was authenticated by lack of epithelial marker expression and expression of the stroma specific marker *TIMP3*. See Table 1 for patient characteristics.

Cell Lines
NIH-3T3 cells were generously provided by Dr. Alan Diamond, and originally purchased from ATCC. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). RWPE-1 cells were purchased from ATCC (2014), frozen in multiple low-passaged aliquots, re-authenticated in 2016, and used only at passages <20. RWPE-1 cells were cultured in Keratinocyte serum free medium (KSFM). All cells cultured at standard 37°C with 5% CO_2.

METHOD DETAILS

3D Organoid Mono- and Co-culture
Epithelial cells were grown at passage 2 from thaw in 100 mm collagen-coated dishes to ~70% confluent. To seed cells singly in 3D mono-cultures, the specified number of epithelial cells were mixed with media containing 33% growth factor reduced phenol red-free Matrigel (Corning). Cultures were grown using a flat-bottom 96-well microplate containing a 50 µL base layer of 50% Matrigel in media. Once the base layer solidified, 100 µL of the cell and Matrigel mixture was plated directly on top of the base layer and allowed to solidify for at least 1 hour at 37ºC. Once solid, 100 µL of keratinocyte serum-free media supplemented with 5% charcoal-stripped fetal bovine serum and 10 nM dihydrotestosterone were added to each well. Cultures were grown for up to 28 days and media replaced every 3 days.

For 3D co-cultures, stromal cells were grown from thaw up to 90% confluent. Epithelial and stromal cells were mixed at specified ratios in keratinocyte serum-free media supplemented with 5% charcoal-stripped fetal bovine serum and 10nM dihydrotestosterone containing 33%

Matrigel. Co-cultures were plated identically to mono-culture described above, with cells seeded singly and free of clumps.

Transwell Insert Co-culture

Primary stromal cells were grown from thaw up to 90% confluent then seeded at 5,000 cells/insert onto 0.4µm Transwell inserts (Corning) one day prior to co-culture. 3D cultures were prepared in 12-well microplates with a 125uL base layer of 50% Matrigel followed by addition of 250 epithelial cells in 250µL of 33% Matrigel per well. The microplate was tilted to promote Matrigel solidification in a crescent shape in the corner of each well for at least one hour at 37ºC. Once solid, Transwell inserts containing the stromal cells were transferred to epithelial containing wells. Epithelial cells were grown from thaw in 100mm collagen-coated dishes up to 70% confluent prior to 3D culture. For direct co-culture controls, 250 PrE cells were mixed with 5,000 PrS cells in 250uL of 33% Matrigel. All Transwell experiments completed in keratinocyte serum-free media supplemented with 5% charcoal-stripped fetal bovine serum and 10nM dihydrotestosterone. Cultures were imaged for analysis after 14 days of co-culture.

Brightfield Image Acquisition, Processing, and Analysis

Images of each culture were acquired at 4x magnification using the Evos FL Auto 2 Imaging System (Thermo Fisher Scientific). A whole-well enhanced depth-of-field image was generated for each well by acquiring up to 25 images in the z-plane in covering four quadrants of each well. Images from each quadrant at every z-position were stitched together and the z-stack compressed to a single enhanced depth-of-field image using Celleste™ Image Analysis Software (Thermo Fisher Scientific).

Organoid count, area, circularity, and maximum/minimum radius ratio metrics were generated by manual and automated assisted identification of each organoid. Image analysis and measurements were performed using Celleste™ Image Analysis Software (Thermo Fisher Scientific).

Immunofluorescence and Immunohistochemistry

PrS mix 3 cells were grown on a glass chamber slide, fixed in 4% paraformaldehyde for 1 hour, and incubated with rabbit polyclonal anti-vimentin (Proteintech Group) and monoclonal anti-smooth muscle actin (M0851)(Dako) antibodies, counterstained with DAPI and imaged on the Vectra3 confocal microscope (PerkinElmer).

Organoids were dissociated in Dispase (1 U/mL, STEMCELL Technologies), resuspended in HistoGel™ (Thermo Fisher Scientific), solidified at 4ºC, fixed in 4% paraformaldehyde for 1 hour, transferred to 70% ethanol, and paraffin-embedded. 5µm sections were incubated with polyclonal guinea pig anti-Cytokeratin 8/18 (03-GP11)(American Research Products, Inc), rabbit monoclonal anti-p63a antibody (D2K8X)(Cell Signaling Technology), counterstained with DAPI and imaged on the Vectra3 confocal microscope.

Formalin-fixed and paraffin embedded organoid sections of 5µm were also incubated with monoclonal rabbit anti-androgen receptor antibody (D6F11)(Cell Signaling Technology), 3,3'-Diaminobenzidine for visualization, and counterstained with hematoxylin immunohistochemical staining.

For whole mount immunofluorescence, organoids were transferred by pipette to a chamber slide (Nunc Lab-Tek II Chamber Slide System) and fixed in 4% paraformalydehyde. GFP fluorescence from GFP-labeled PrS cells was quenched with 50mM NH_4Cl (Fig 2C), quenching step was skipped for PrS-GFP whole mount observation (Fig2D, Fig S3). Fixed organoids were

permeabilized and incubated in polyclonal guinea pig anti-Cytokeratin 8/18 (03-GP11)(American Research Products, Inc), polyclonal rabbit anti-Keratin 5 (905501)(BioLegend), counterstained with Alexa Fluor™ 647 Phalloidin (Thermo Fisher Scientific) and DAPI, and imaged on the Zeiss LSM 710 confocal microscope.

Radical prostatectomy tissue sections of 5 µm and formalin-fixed and paraffin embedded organoid sections of 5µm were incubated with mouse monoclonal anti-AMACR antibody (13H4)(Genemed) and counterstained with hematoxylin for immunohistochemical staining.

Lentivirus Transduction

NIH-3T3 and PrS cells were transduced with pGreenPuro Scramble Hairpin lentivirus (System Biosciences) and selected by passaging at least once into media containing 1-3 µg/mL puromycin until all cells were GFP+. Cells at lowest passage were cryofrozen and thawed before use.

For determination of single cell origin of organoids, PrE-2 cells were transduced with lentiviral vectors for GFP (MZIP000-VA-1)(System Biosciences) at an MOI of 20 or RFP (LVP309)(GenTarget Inc.) at an MOI of 6.25 with puromycin resistance. Cells were selected with puromycin treatment for at least 1 passage, mixed, and seeded into 3D culture as described. Organoids were imaged after 18 days of culture using the Evos FL Auto 2 Imaging System.

Tissue Histology

Tissue from each patient isolated for primary cell culture was also used for histopathology. Briefly, a small piece was collected, formalin-fixed, and paraffin-embedded. Sections of 5 µm were obtained and stained with hematoxylin and eosin for pathological assessment.

Proliferation assay

Stromal cells grown in 3D mono-culture for 4 days were incubated with Click-iT™ EdU (Thermo Fisher Scientific) for 48 hours. Matrigel was dissociated with Dispase (1 U/mL) and cells visualized for EdU fluorescence and DAPI counterstain.

Organoids co-cultured with GFP-labeled PrS cells were incubated with Click-iT™ EdU (Thermo Fisher Scientific) for 24 hours. Whole organoids and associated stromal cells were collected by pipette and transferred to a chamber slide (Nunc Lab-Tek II Chamber Slide System). Cells were fixed in 3.7% formaldehyde and visualized for EdU fluorescence and Hoechst counterstain using the Zeiss LSM 710 confocal microscope.

The number of PrS cells after 7 of 3D culture was quantified by dissociating the Matrigel with Dispase (1 U/mL) and counted using a hemocytometer.

PCR Profiling Arrays and RT-qPCR gene expression

Stromal cells were grown to 90% confluent in 2D prior to seeding in 3D. A total 10,000 cells/well were plated in mono-culture conditions as described above. Matrigel in the 3D cultures was dissociated with Dispase (1 U/mL), washed with HBS, and collected in Trizol (Thermo Fisher Scientific). RNA was isolated using the Trizol extraction method, and RNA quantity and quality determined by NanoDrop Spectrophotometer (Thermo Fisher Scientific). cDNA was generated with Qiagen RT^2 First Strand Kit (Qiagen) and gene expression assessed using the Human Growth Factors and Human TGFß/BMP Signaling Pathway Plus RT^2 Profiler PCR Arrays (Qiagen). Arrays were run on a QuantStudio6 (Thermo Fisher Scientific) and normalized

independently using 5 reference genes according to the manufacture's protocol. The $\Delta\Delta C_T$ method was used for comparative analysis (Livak and Schmittgen, 2001).

Matched benign-cancer cells grown in 2D, 3D and 3D co-culture were harvested for RNA using the Trizol method described above. 500 ng of RNA was reverse transcribed using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific) and qPCR performed on the QuantStudio6 machine. Reference genes *GAPDH* with forward primer 5'-AAGGTCGGAGTCAACGGATTTGGT-3' and reverse 5'-TGATGACAAGCTTCCCGTTCTCAG-3', *KRT5* with forward primer 5'- ATCGCCACTTACCGCAAGC-3' and reverse 5'-CCATATCCAGAGGAAACACTGC-3', *KRT8* with forward primer 5'-GCTGGTGGAGGACTTCAAGA-3' and reverse 5'- TCGTTCTCCATCTCTGTACGC-3', and *CDH1* forward primer 5'-ATGAGTGTCCCCCGGTATCT-3' and reverse 5'-GGTCAGTATCAGCCGCTTTC-3' were used for normalization. Expression of *AMACR* was quantified using forward primer 5'-GTGCTGCTGGAGCCCTTC-3' and reverse primer 5'-CAGCTGGAGTTTCTCCATGA-3' and normalized to the reference gene *GAPDH* (Fig S5A PrE3-PrECa-3, PrE4-PrECa-4) or mean *KRT5, KRT8, CDH1* (Fig 5 D, Fig S5A PrE6-PrECa-6, PrE7PrECa-7). Presence of a TMPRSS2 fusion gene was tested using *TMPRSS2* forward primer 5'- TAGGCGCGAGCTAAGCAGGAG-3' in combination with reverse primers 5'-CCGCACATGGTCTGTACTCCA TA-3' or 5'- CCGTGGAGAGTTTTGTAAGGC T-3' for *ERG*, or reverse primer 5'- TCCCGATACATTCCTGGCTC-3' for *ETV1*.

Organoid Passaging

Organoids were harvested every 5-7 days in Dispase (1U/mL) to dissociate Matrigel and incubated in TrypLE™ Express Enzyme (Gibco) for single cell suspension. Single cells were re-plated in 33% matrigel over a 50% baselayer. Co-cultures were spiked with 1k additional stromal cells per well to account for cell loss during passaging. Media was supplemented overnight with 25 µ Rho-kinase inhibitor (Y-27632, STEMCELL) and replaced with fresh media the next day.

3D Co-culture Branching Inhibitor Assay

3D mono- and co-cultures were setup as described above and grown for 7 days prior to treatment with 2µg/mL of recombinant human Noggin (R&D Systems), 250nM FGFR inhibitor NVP-BGJ398 (Selleckchem), or monoclonal TGF beta-1,2,3 neutralizing antibody (1D11)(Thermo Fisher Scientific). Media was changed every 2-3 days with freshly prepared treatments and cultures imaged as previously described on day 14 after 7 days of treatment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software). Parameters and experimental details can be found in the figure legends. A two-sided unpaired t-test was used for mono- and co-culture comparisons of organoid formation efficiency, area, circularity, and maximum/minimum radius ratio, and RT-qPCR gene expression. One-way analysis of variance (ANOVA) across mono-culture and co-culture conditions was used for formation efficiency and area. The post-test for linear trend between means is reported when significance threshold was surpassed. Microarray results, heatmaps, and Wikipathways analyses were generated and analyzed using Transcriptome Analysis Console (TAC) Software (Thermo Fisher Scientific). Filtering criteria for differentially expressed genes were > 2 or < -2

fold-change and $p < 0.05$. For Wikipathways analysis the p-value was established using a Fisher's Exact Test from a contingency table of the number of overlapping genes, number of non-overlapping genes, number of genes filtered, and number of genes outside of filter criteria for each pathway. A p-value <0.1 was considered statistically significant where * = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$ (ns, not signficant).

DATA AND SOFTWARE AVAILABILITY

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE115052.