Materials and methods

Alginate hydrogel preparation.

Sodium alginate with an average molecular weight of 138 kDa (high molecular weight) was purchased from FMC Biopolymer (Protanal LF10/60) and used to prepare more elastic and viscoelastic gels as described previously[1, 2]. Briefly, alginate was irradiated with a 5mRad cobalt source to obtain a low molecular weight alginate (38 kDa). The adhesion peptide GGGGRGDSP (RGD – Peptide 2.0) was covalently coupled to alginate (RGD concentration 2.7mM) utilizing carbodiimide chemistry (Sulfo-NHS, Pierce Chemical; EDC, Sigma-Aldrich). Lissamine Rhodamine B Ethylenediamine (ThermoFisher) was also coupled to alginate using carbodiimide chemistry for experiments involving fluorescent alginate as described previously[3]. Next, modified alginate was dialyzed against deionized water for 3-4 days (molecular weight cutoff of 3.5kDa), treated with activated charcoal (Sigma-Aldrich), filter sterilized (0.22µm) and lyophilized for one week. The day before the experiment, alginate was reconstituted in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, Gibco). For the MCF10A spheroids experiments, two syringes per gel were prepared to get a 2% alginate gel. One containing 2.5% alginate. The second syringe contained normal medium and different amounts of calcium sulfate depending on the material mechanical properties. Calcium sulfate was previously diluted in media without supplements. Then, spheroids were gently added to the syringe with media and the syringe was turned up and down to mix well the calcium sulfate. Next, both syringes were connected together with a female-female Luer-lock coupler, taking care not to introduce bubbles or air into the mixture. After, the two solutions were mixed rapidly and immediately deposited the alginate gel on top of a plate. The recipes for all alginate hydrogels were the same except the calcium sulphate concentration that increased to increase the stiffness: 16.8, 28.8, 57.6, 82.4 mM and 33.6, 52.8, 96, 163.6 mM for elastic and viscoelastic hydrogels respectively. For intestinal organoids experiments, gels were prepared differently. First, the alginate and Matrigel solution was prepared. Alginate and Matrigel were left on ice for over an hour. Next, Matrigel was added to a 2.5% alginate

solution. As Matrigel concentration varies from batch to batch, the appropriate amount of media (with no supplements) was added to a final concentration of 1.25% alginate and 5mg/ml Matrigel. This solution was thoroughly mixed for 40-50 times with a pipette, being careful not to generate bubbles and maintained in ice. First, a syringe with alginate + Matrigel solution was prepared and left on top of the ice. A second syringe was prepared with medium and the appropriate concentration of calcium sulphate. In parallel, Matrigel with organoids was dissolved with cell recovery solution. The recipes for all alginate-matrigel hydrogels were the same except the calcium sulphate concentration that increased to increase the stiffness: 26.4, 48, 72.6 mM and 48, 96, 163.6 mM for elastic and viscoelastic hydrogels respectively.

Mechanical characterization of hydrogels.

The storage moduli of hydrogels were determined with an AR-G2 stress-controlled rheometer (TA instruments) as utilized previously[2, 4]. Briefly, a 20 mm parallel plate was used with a gap of 1mm. The circular plate was immediately placed on the polymer solution before the hydrogel started to gel, forming a 20 mm disk hydrogel. Oscillatory rheology (1Hz, 1% strain) was used to measure the storage modulus. Gels were maintained at 37°C until equilibrium was reached.

To measure the stress relaxation half time a compression test with an Instrom 3342 mechanical apparatus (Norwick, MA) was performed as described previously[2, 5]. Briefly, hydrogels were fabricated with a 2mm height, and allowed to equilibrate for 24h[4, 6, 7]. Then, gels were strained at a 1mm/min rate until a 15% strain was reached; the strain was then held constant. The stress relaxation half time was measured as the time at which the initial stress decreased by a factor of 2.

MCF10A cell culture

MCF10A breast cell line (ATCC, CRL-10317) were cultured following the protocols developed by Debnath and Brugge[8]. Briefly, cells were cultured in DMEM/F12 media (Gicbo) supplemented with 5% Horse Serum (Invitrogen), 1% Pen/Strep (Invitrogen), 20ng/ml EGF (Peprotech), 0.5 mg/ml

Hydrocortisone (Sigma-Aldrich), 100 ng/ml Cholera toxin (Sigma-Aldrich) and 10µg/ml Insulin (Sigma-Aldrich).

MCF10A spheroids experiments

To prepare MCF10A spheroids, cells were trypsinized from tissue culture flasks and resuspended in pretreated Aggrewell multi-well plates (Aggrewell 400) to generate spheroids of ~2000 cells. Plates were left overnight in the incubator to allow spheroids to form. The spheroids were then carefully removed from the Aggrewell plates and added to the polymer solution before gelation (see hydrogel preparation above). A plate was deposited on top of each gel to provide a final controlled height of 1mm, and gels were left in the incubator for 45min. Individual gel samples were then obtained with an 8mm puncher, and each gel was introduced into a separate well of a 24-well plate. The media was changed after 2 hr, and during experiments the media was changed every 2 days, except where indicated. For experiments with inhibitors, once spheroids were encapsulated in gels and gels equilibrated, media with the defined inhibitor concentration was added. The media with inhibitors was also changed every 2 days. The inhibitors used were: 10µM Y27632 (SIGMA-ALDRICH) to inhibit ROCK, 50µM NSC23766 (TOCRIS) to inhibit Rac1, 100µM CK666 to inhibit ARP 2/3, 10µM Gadolinium to block ion channels, 5µM PF 573228 (TOCRIS) to inhibit FAK, 2mM Thymidine (SIGMA-ALDRICH) to block cell cycle progression and 20µM SMIFH2 (SIGMA-ALDRICH) to inhibit formin.

Intestinal organoids culture.

Intestinal organoids were cultured from isolated jejunal crypts of $Lgr5^{CreERGFP}$ adult mice (Jackson Laboratory) in which the $Lgr5^{+}$ stem cells are labeled with GFP expression. Intestinal organoids were cultured in DMEM/F12 media (Invitrogen) supplemented with 10% RS2 condition medium (RS2 producer line is a gift from Dr. Xi He, Boston Children's Hospital), 10mM HEPES (ThermoFisher), 1X GlutaMAX™ supplement (ThermoFisher), 1X N2 supplement (ThermoFisher), 1X B27 supplement (ThermoFisher), 10 µM DMH1 (Cayman), 20 µM CHIR99021 (LC Laboratories), 50 ng/ml EGF (R&D), 10 µM Y27632 (LC Laboratories) and 0.1 mg/ml Primocin (invivoGen). For routine culture, medium

was changed every 2-3 days and organoids were passaged after 5 days at the latest. To passage the organoids, cell recovery solution (CORNING) was added to the wells containing intestinal organoids in Matrigel (CORNING) to disrupt matrigel. After adding the Cell Recovery Solution, the plate was left on ice until Matrigel was degraded. Then, organoids were gently disrupted using mechanical agitation. Disrupted organoids were added to a Matrigel containing solution and 30 µl droplets of Matrigel with organoids were deposited in pre-heated wells. These wells were left in an incubator for 30 min to allow Matrigel to solidify and before adding medium.

Intestinal organoids experiments.

Intestinal organoid encapsulation was similar to the procedure utilized for MCF10A spheroids, although in this case an IPN of alginate and Matrigel was used for encapsulation. Intestinal organoids were first cultured in Matrigel (BD Biosciences) for 1-2 weeks. Then, the Matrigel was dissolved with cell recovery solution (Corning) and organoids were dissociated with TrypLE (Gibco). After dissociation, cells were encapsulated in Matrigel for 24h. This process allows the size of organoids to be more homogeneous at the start of the experiment. After 24h, organoids were added to the syringe with Matrigel + alginate prior to gel formation. To control the thickness of the gels, a plate was deposited on top of each gel at a controlled height of 1mm. Gels were allowed to form inside the incubators for 45min, and individual gel samples were then punched with an 8mm puncher. Each gel was introduced into a separate well of a 24-well plate. Medium was changed after two hours, and subsequently every 2 days, except where indicated. For single-organoid experiments, organoids were dissociated and directly encapsulated in the Matrigel + alginate. For experiments with addition of 100 uM Ouabain (Sigma-Aldrich), media with ouabain was added after equilibration and was changed every day.

Bulk hydrogel immunostaining

Hydrogels were fixed with 4% paraformaldehyde for 30 min. After fixation, hydrogels were washed with PBS with 10mM EDTA to facilitate staining. Then, cells within hydrogels were permeabilized and blocked with 0.5% triton, 3% Goat serum in PBS with calcium (blocking buffer) for 24h. Once hydrogels were permeabilized and blocked, primary antibodies were added in blocking buffer for 24h. Primary antibodies used were YAP (Santa Cruz, 1:200), Cytokeratin 14 (Covance, 1:100), Vimentin (abcam, 1:200). After incubation with primary antibodies, Hydrogels were washed for 24h in blocking buffer. Next, secondary antibodies were added in blocking buffer. Then, hydrogels were washed for 3h and blocking buffer with phalloidin (ThermoFisher, 1:200) was then added for 24h to label F-actin. Hydrogels were then washed for 8 hours with blocking buffer with Hoechst (ThermoFisher, 1:2000) to label cell nuclei and, afterwards, washed with PBS overnight. Finally, Prolong (ThermoFisher) antifade reagent was added to the hydrogels.

Immunostaining of hydrogel sections

Hydrogels were fixed with 4% paraformaldehyde for 30 min. After fixation, hydrogels were washed 3 times with PBS containing calcium (cPBS), and then incubated overnight in cPBS containing 30% Sucrose. Hydrogels were then incubated in a solution consisting of equal volumes of a 30% Sucrose in cPBS containing solution, and OCT (Tissue-Tek) solution for 24h. Next, the solution was removed and hydrogels were embedded in OCT for several hours, and then frozen. The frozen hydrogels were sectioned with a cryostat (Leica CM1950) to a thickness of $15\ \mu m$. Sections were permeabilized with a PBS solution containing 0.2% triton and 3% Goat Serum. Next, pFAK (abcam,1:100), emerin (Santa Cruz, 1:100) or nesprin (ThermoFisher, 1:100) antibody was added for 3h. Then, after 6 washes, a secondary antibody with phalloidin was added for an hour. Last, ProLong (ThermoFisher) antifade reagent was added. After mounting, sections were imaged with 20x (NA=0.8), 40x (NA=1.0) or 63x (NA=1.4) water immersion objectives in an Upright laser-scanning confocal Zeiss LSM 710.

Bulk Organoid staining:

To follow the 3D structure and evolution of organoids, the F-actin and nuclei were stained with Phalloidin and Hoechst respectively. Hydrogels were fixed with 4% paraformaldehyde for 30 min. After fixation, hydrogels were washed with PBS containing 10mM EDTA to facilitate staining. Then, hydrogels were permeabilized and blocked with 0.5% triton, 3% Goat serum in PBS with calcium (blocking buffer) for 48h. Once hydrogels were permeabilized and blocked, phalloidin (ThermoFisher, 1:200) was added to blocking buffer to label F-actin and incubated with gels for 24h. Hydrogels were then washed for 8 hours with blocking buffer with Hoechst (ThermoFisher, 1:2000) to label the nuclei, and then washed with PBS overnight. Finally, Prolong (ThermoFisher) antifade reagent was added to the hydrogels. After mounting, organoids were imaged with a 40x (NA=1.0) water immersion objective in an Upright laser-scanning confocal Zeiss LSM 710.

Organoid immunostaining:

Hydrogels were incubated in cell recovery solution (CORNING) for 45 min on ice. The alginate in the gels was then degraded with 34 U/ml alginate lyase (Sigma-Aldrich), while maintaining gels on ice. Hydrogels were subsequently fixed with 4% paraformaldehyde for 30 min. After fixation, organoids were permeabilized for 30 min with 0.5% Triton. Once organoids were permeabilized, they were blocked with 3% Goat serum, 0.1% Triton in PBS for 3h. Then, primary antibodies: Lysozyme (Dako, 1:200), L-FABP (Santa Cruz, 1:50), Chromogramin A (Santa Cruz, 1:50), Sox9 (Abcam, 1:50), Mucin 2 (ThermoFisher, 1:50) was added in 3% Goat Serum, 0,1% Triton in PBS and left overnight at 4 degrees. Once the primary antibody was washed the next day, secondary antibodies (ThermoFisher, 1:200) and phalloidin (ThermoFisher, 1:200) were added to gels in a solution containing 3% Goat Serum, 0,1% Triton in PBS for 4h. Secondary antibodies were then washed, organoids incubated with Hoechst (ThermoFisher, 1:2000) for 4h, washed 6 times and, last, ProLong (ThermoFisher) was added. After mounting, organoids were imaged with a 40x (NA=1.0) water immersion objective in a laser-scanning confocal Upright Zeiss LSM 710.

Analysis of cell proliferation in tissues

In experiments with MCF10A spheroids, EdU (Click-iT™ EdU Cell Proliferation Kit, Invitrogen) was added for 4 hr to spheroids containing bulk hydrogels at day 5. For intestinal organoids experiments, EdU was added for 2h at day 7. After following the staining protocol provided by Invitrogen, ProLong mounting media was added. After mounting, spheroids or organoids were imaged with a 20x (NA=0.8) or 40x (NA=1.0) water immersion objectives in an Upright laser-scanning confocal Zeiss LSM 710. The percentage of EdU positive cells was quantified by determining the total number of cells from the Hoechst channel, and then the number of EdU positive nuclei. Custom MATLAB software was used to quantify the spatial distribution of EdU positive cells and cell density across the spheroids. In brief, the perimeter of a 2D slice of a spheroid was first defined. Then, the tissue area was divided into squares of defined area. To measure the local density and the percentage of EdU positive cells, the software measures the number of nuclei from the Hoechst staining and the number of EdU positive nuclei per square. With these measurements, the local density of cells and the percentage of EdU positive cells are calculated. The radial distribution of cell density and percentage of EdU positive cells was also quantified. To accomplish, the distance from the center to the edge of the tissue was normalized in order to compare all spheroids and conditions.

Spheroid area and circularity quantification

To measure spheroids or organoids circularity and area during experiments, phase contrast images were taken with a 4x and 10x objective with a Microscope (EVOS) every day or the last day of experiments. These images were quantified with Image J. Briefly, the perimeter of each individual spheroid/organoid was drawn manually, and the enclosed area and circularity was measured.

Cytokeratin 14 quantification

To measure cytokeratin 14 staining intensity, images were obtained after immunostaining with a 20x (NA=0.8) or 40x (NA=1.0) water immersion objective in an Upright laser-scanning confocal Zeiss LSM

710. Then, custom MATLAB software was used to quantify the average intensity of the cytokeratin 14 staining per spheroid. First, the perimeter of each spheroid was defined. Then, the perimeter ring width was widened inwards and outwards to include all pixels positive for cytokeratin 14 staining. The average cytokeratin 14 intensity was then determined, and all values were normalized to the average value of cytokeratin 14 staining in elastic hydrogels.

YAP quantification

To quantify YAP staining, images of immunostained spheroids were taken with a 100X (NA=1.40) oil immersion objective using a laser-scanning confocal Upright Zeiss LSM 710. The percentage of cells with nuclear YAP was quantified by counting the number of cells with nuclear YAP with respect to the total number of cells. These measurements were performed in the core of spheroids, the edges, and cells present at the initiation of fingers (in viscoelastic gels).

Mice experiments with MDA-MB-231 cells

Female, 3-week-old NOD SCID mice (NOD.Cg-Prkdc^{scid}/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). MDA-MB-231 cells (1 x 10^7 cells/mL) were added to alginate solutions (hydrogel preparation as noted above to yield the stiff viscoelastic and elastic gels), mixed and immediately injected subcutaneously at the left flank to allow gelation in situ. The dimensions of the growing tumors were measured externally using calipers, and the volume of an ellipsoid was calculated. All animal studies were performed in accordance with guidelines set by the National Institutes of Health and Harvard University Faculty of Arts and Sciences' Institutional Animal Care and Use Committee (IACUC).

Microfluidic device development and cell flux driven experiments

To explore the impact of pressure on tissue growth, gels containing cell spheroids were confined by placing a polydimethylsiloxane (PDMS) cover over gels contained within a petri dish. The PDMS cover was fabricated to allow continuous injection of a cell suspension into the center of a spheroid to model

pressure-drive tissue growth. The cover was fabricated by mixing PDMS (Sylgard 184, Down Corning, Midland, MI) base and cross-linker in a 5:1 weight ratio using a Thinky mixer (AR-100, Thinky Corp., Tokyo, Japan). The PDMS was degassed for 20 minutes and the mold was cured in the oven at 65C overnight. The device was then cut out of the mold and a hole through the device was created with a 1.2mm biopsy punch (Uni-Core, GE Healthcare Life Sciences, Pittsburgh, PA). The alginate solution was kept at room temperature. The PDMS cover was then surfaced treated with Aquapel (PPG Industries, Pittsburgh, PA) to make the gel-contacting surface hydrophobic. Once ready, hydrogel solution prepared as described above is poured onto a petri dish of 100mm diameter to allow gelation. Eight circular pillars were used to surround the forming hydrogel to control its thickness. Then, remaining air bubbles were carefully removed with a pipette. The PDMS cover was then placed on top of the forming gel, supported by the pillars, to create gels ~170μm thick. Hydrogels were allowed to cure at room temperature for 30 minutes. During this time, cells are stained (Hoechst nucleus stain -Thermo-Fisher-), suspended in cell medium at a density of $1x10^7$ cells/ml, and are loaded into a syringe. Once the hydrogel has formed, the syringe pump was used to inject the cell suspension into the center of the gel using a 200mm length (L) tubing of radius (R) 0.2mm inserted through the hole created in the PDMS cover. The tubing was cut parallel to the PDMS to favor even cell injection in all directions at a constant rate. Cells were injected for 9 minutes, at a flow rate of (Q) 1μl/min, to provide a constant pressure of 19 kPa . We calculated the pressure with the Hagen-Poiseuille Law. The dynamic viscosity (μ_t) of MCF10A cells is ~0.01Pa*s[9].

$$\Delta P = \frac{8\mu_t L Q}{\pi R^4}$$

Mice experiments with MCF10A cells

2mm-high alginate gels (LMW or HMW, stiff) containing MCF10A spheroids were prepared as previously described and 8mm-diameter gels were obtained using a biopsy punch. Gels were surgically implanted in the subcutaneous space on the flanks of NU/J athymic nude mice. For histological analysis, gels were excised seven days after implantation, fixed in 1% paraformaldehyde (Electron

Microscopy Sciences) and transferred to a 30% sucrose solution overnight. Gels were then embedded in Tissue-Tek OCT compound (VWR) and sectioned to 20um on a Leica CM1950 cryostat. Sections were stained for vimentin (abcam, 1:200), phalloidin (ThermoFisher,1:200) mithocondria (abcam, 1:200) and imaged using a Zeiss LSM 710 confocal microscope.

Flow cytometry of MCF10A cells from gels

Gels containing MCF10A spheroids were prepared as previously described. For flow cytometric analysis, gels from in vitro or in vivo experiments were collected and digested in MCF10A medium containing 1mg/mL alginate lyase (Sigma), 25mM EDTA (Sigma), and 100U/mL collagenase IV (STEMCELL Technologies). Retrieved spheroids were incubated for 60 seconds in 0.05% Trypsin-EDTA (Gibco), passed through a 70um filter, and counted using a Countess II FL (ThermoFisher). Single-cell suspensions were permeabilized and fixed using the eBioscience Foxp3/ Transcription Factor Staining Buffer Set (Invitrogen) and stained with antibodies against human ZEB1, Snail, Slug, and ZEB2 (R&D Systems), and TWIST1 (ThermoFisher). Cells were run on a BD LSRII flow cytometer and analyzed using FlowJo v10 software.

Characterization of interpenetration of alginate-matrigel networks

To characterize the interpenetration, homogeneity and the lack of phase separation we followed the same procedure as described previously[3]. We performed fluorescence microscopy of the IPNs to quantify the intensity distribution of alginate and Matrigel in the matrix. To study the intensity distribution of alginate, the intensity of fluorescently coupled rhodamine-alginate was analyzed. To study the intensity distribution of Matrigel, immunostaining of laminin (abcam, 1:200) was performed. Images were acquired with a Zeiss LSM 710 confocal microscope. The distribution observed for all conditions in Extended Data Fig. 19 show a single peak. If there would be phase separation, there would be two or more peaks that would indicate different concentrations of alginate or laminin.

Scanning Electron Microscopy Experiments

To prepare samples for scanning electron microscopy (SEM), the gels were first fixed in paraformaldehyde for 15 minutes and then rinsed three times with PBS with 10 mM CaCl2. Next, the samples were dehydrated in increasing ethanol baths as follows: 50% ethanol, 50% deionized water x1 change, 70% ethanol, 30% deionized water x1 change, 80% ethanol, 20% deionized water x2 changes, 90% ethanol, 10% deionized water x2 changes, 100% ethanol x2 changes. After the gels were left in the 100% ethanol bath, they were added to a 1:2 solution of HMDS:ethanol for 20 minutes, then transferred to a 1:1 HMDS:ethanol solution for 20 minutes, then transferred to a 2:1 HMDS:ethanol solution for 20 minutes, and finally left in a purely HMDS solution overnight. The lid of the container was left slightly ajar to allow the HMDS to evaporate. To prepare the samples for imaging, each sample was mounted to a SEM stub (Ted Pella) using conductive carbon tape. The samples were coated with 10 nm of Pt/Pd 80/20 using an EMS 150 T S Metal Sputter Coater (Quorum), and finally placed in a field emission SEM, FESEM Ultra55 (Zeiss). The samples were imaged at 2-3 keV using the Inlens SE detector.

Nanostring analysis

Three independent experiments were performed (n=3). For each experiment, 3 gels/condition (technical replicates) were included and samples from each (taken with a biopsy punch) were pooled together to obtain RNA. Cells were retrieved from the gels by alginate lyase digestion and then lysed with RLT buffer (Qiagen) with 1% β-mercaptoethanol. RNA was isolated with the RNAeasy Mini Kit and on-column DNA digestion (Qiagen) following the manufacturer's instructions. RNA quantity and quality was initially determined with a spectrophotometer (Nanodrop) and confirmed by electrophoresis (Agilent Tapestation 4200). All samples included had excellent RNA integrity (RIN≥9.6). 75 ng RNA per sample were used for Nanostring analysis (PanCancer Progression panel consisting of human 770 genes) following the manufacturer's recommendations. Raw data was analyzed with Nsolver 4.0 software.

Data availability

The data has been deposited in a public repository.

Code Availability

The computational model code is available at
https://github.com/anupamdata/ABM_VE_Matrix_Viscous_Tissue.git

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