Supporting Online Material for

Tissue Geometry Determines Sites of Mammary Branching Morphogenesis in Organotypic Cultures

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Materials and Methods

Cell culture and reagents. Functionally normal EpH4 mouse mammary epithelial cells (*S1*) were cultured in 1:1 Dulbecco's Modified Eagle's Medium:Ham's F12 Nutrient Mixture (DMEM/F12), 2% fetal bovine serum, 5 μg/mL insulin, 50 μg/mL gentamycin (Sigma). EpH4 cells stably expressing GFP under control of the human vimentin gene promoter (*S2*)(a gift from C. Gilles, University of Liege) were maintained in growth medium supplemented with geneticin (G418)(400 μg/mL). Primary epithelial organoids consisting primarily of luminal epithelial and myoepithelial cells were prepared from 8-week-old virgin CD-1 mice as previously described (*S3*). Inguinal glands were removed aseptically, minced with scalpel blades and incubated with agitation (100 rpm) for 30 min at 37ºC in growth medium supplemented with trypsin and collagenase A. The digested cells were centrifuged at 1500 rpm for 10 min, supernatant containing fat tissue discarded, and cell pellets resuspended in growth medium supplemented with 1000 U DNase I (Sigma). Single cells (mainly fibroblasts) were removed from the organoids by differential centrifugation in DMEM/F12. Organoids were plated on tissue culture polystyrene in DMEM/F12 supplemented with 20% fetal bovine serum, insulin/transferrin/sodium selenite (ITS; Sigma), and penicillin/streptomycin (UCSF Cell Culture Facility). The following day, the monolayer of primary epithelial cells was treated with trypsin for 3 min at 37°C. The reaction was stopped with soybean trypsin inhibitor, cells were spun down at 800 rpm, resuspended in DMEM/F12, and plated in micropatterns. Patterned cells were grown in DMEM/F12 supplemented with ITS and penicillin/streptomycin.

Tubules were treated at the time of induction of branching with the following reagents diluted to the concentrations indicated in the text: GM6001 (Calbiochem); SS7 peptide (*S4*) which inhibits EpH4 cell binding to epimorphin (a gift from Y. Hirai, Kyoto University); the EGFR inhibitor AG1478 (Calbiochem); control chicken immunoglobulin-G (IgG)(R&D Systems); chicken anti-TGFβ1 (R&D Systems); and TGFβ type I receptor (Alk-5) kinase inhibitor [3-(pyridine-2-yl)-4-(4-quinonyl)]-1H-pyrazole (Calbiochem). To inhibit cell proliferation, tubules were exposed to mitomycin C (50 μg/mL; Sigma) for 4 hours prior to induction with EGF. For cell proliferation assays, tubules were exposed to 5-bromo-2-deoxyuridine (BrdU) at the time of induction with EGF and processed using a commercially available kit (Amersham). For TGFβ overexpression studies, EpH4 cells were transiently co-transfected with wild type or activated porcine TGFβ1 (a gift from A. Roberts, National Cancer Institute) and yellow fluorescent protein (YFP) or YFP alone using Lipofectamine 2000 (Invitrogen) one day prior to micropatterning. For dominant negative receptor studies, EpH4 cells stably expressing dominant negative TGFβ type II receptor (a gift from K. Luo, UC Berkeley) and YFP or vector and YFP were maintained in growth medium supplemented with G418 (400 μg/mL).

Micropatterned tubules. Micropatterned cultures of epithelial cells embedded within collagen gels were formed by replica micromolding of collagen (*S5*). Patterned

elastomeric stamps of poly(dimethylsiloxane) (PDMS; Sylgard 184, Ellsworth Adhesives, Germantown, WI) were rendered nonadhesive by coating with a 1% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Modified stamps were placed upon a drop of liquid neutralized collagen (ICN Biomedicals, Costa Mesa, CA) at 37ºC until gelation. After removing stamps, a concentrated suspension of EpH4 cells, primary cells, or primary organoids was allowed to settle within the micromolded collagen cavities. Excess cells were rinsed away with culture medium, and a second layer of collagen gel was gently placed on top of the pattern. Within 24 hours, cells either formed a hollow lumen or completely filled the cavities. Thus, the culture system was comprised of tubules (50-μm in diameter) embedded in a 3D volume of collagen gel (~2 mm thick by ~4-mm in length and width) bathed in growth medium. Branching was induced by the addition of recombinant EGF (20 ng/mL; Sigma) or HGF (1 ng/mL; Sigma). Branches were apparent within 24 hours after induction.

Calculations of concentration gradients. A 3D model of the different tubule geometries was defined using Comsol Multiphysics 3.2a software (Comsol Inc, Palo Alto, CA). The geometries were confined by a cylindrical bounding box that was 40-mm in diameter and 6-mm in height (to represent the culture dish). All outer boundaries except the bottom were set up as diffusion sinks with a concentration of 0, while the bottom surface was inert with a flux condition of 0. Constant uniform secretion of the inhibitor from the tubule was assumed by defining a flux condition of 1 at the tubule surface. The partial differential equations were solved using the finite element method, yielding steady state concentrations in 3D. These concentrations were visualized as a section through the midplane of the tubules. The concentration profiles from the numerical models presented in the figures are relative, and should not be compared directly with each other.

Reverse transcription followed by polymerase chain reaction (RT/PCR) analysis. Total RNA was extracted from cells using an RNeasy kit (Qiagen). cDNA was synthesized using Superscript III first strand synthesis kit (Invitrogen) from equal amounts of RNA, and then used as a template for amplification of TGFβ1, TGFβ2, TGFβ3, TGFβRI, TGFβRII, TGFβRIII, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an equal loading control, using established primer sequences (*S6-9*). Annealing temperatures of 56 ºC (TGFβ1) and 55 ºC (TGFβ2, TGFβ3, TGFβRI, TGFβRII, TGFβRIII, and GAPDH) were used for 38 cycles.

Imaging and statistical analysis. Samples were fixed, stained for nuclei with Hoechst 33258 (Molecular Probes, Eugene, OR), and visualized using a Spot CCD camera attached to a Nikon Diaphot microscope. Total cumulative data were represented by stacking in registration binarized images from 50 samples, obtaining relative pixel frequency with Scion Image software, and color-coding images in Adobe Photoshop. All experiments were conducted at least three times, generating >1000 tubules for each condition. To calculate certainty of data, we determined the approximate 95% confidence intervals (~95% CI) for the proportion of branches that initiated from specific locations within each tubule geometry using: 95% CI ~ $p \pm 1.96 \sqrt{p(1-p)/N}$, where p

is the fraction of branches initiating at a specific location, and N is the total number of branches (*S10*).

For immunofluorescence analysis of the α1 chain of laminin-111, samples were washed in PBS and fixed in 50:50 methanol:acetone for 20 min at –20°C. Samples were blocked in PBS + 5% goat serum (PBS-S), then incubated in primary antibody (#198, which strongly recognizes the polymerized form of the laminin-111 α1 chain; gift from L. Sorokin, Lund University) diluted in PBS-S overnight at 4°C. Samples were washed extensively, and then incubated in secondary antibody diluted in PBS-S overnight at 4°C. Washed samples were stained for nuclei with Hoechst 33258 and imaged using a confocal microscope.

For immunofluorescence analysis of TGFβ1, samples were washed in PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Samples were permeabilized 2 x 10 min in 0.5% NP40 in PBS followed by a 15 min incubation in 0.1% Triton X-100 in PBS. Samples were blocked in PBS-S + 0.1% Triton-X-100 (PBS-ST), then incubated in primary antibody diluted in PBS-ST overnight at 4°C. Samples were washed extensively, and then incubated in secondary antibody diluted in PBS-ST overnight at 4°C. Washed samples were stained for nuclei with Hoechst 33258 and imaged using a confocal microscope. Relative pixel intensities as a function of distance along and away from the tubules were obtained using Scion Image software. Tubules comprised of primary mammary epithelial cells and those comprised of EpH4 cells produced similar results. As a control, we analyzed relative pixel intensities of samples stained for keratin-8 and Hoechst 33258 (Fig. S6). These experiments indicated that gradient formation was not an artifact of immunostaining, confocal analysis, or local cell density.

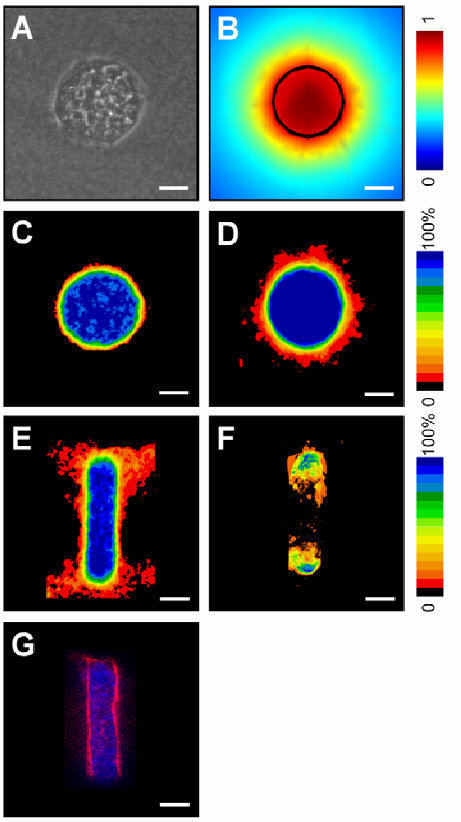

Fig. S1. Engineered tubules comprised of mouse mammary epithelial cells assume the shape of the collagen cavities in which they are embedded. **(A)** Phase contrast image, **(B)** calculation of diffusible inhibitory gradient, and frequency maps **(C)** before and **(D)** after stimulation of branching of spheroid tubules. These structures branched randomly, with ~4-16% of branching from any point around the structure. Similar effects are observed in **(E)** branching and **(F)** vimentin gene promoter activation when cells are stimulated with HGF. Mammary epithelial cells within collagen gels synthesize, secrete, and assemble a basement membrane (*S11*) that surrounds the tubule, as demonstrated by **(G)** confocal analysis of polymerized laminin-111 α1 chain (red) and nuclei (blue) in patterned tubule of primary mammary epithelial cells. Scale bar, 50 μm.

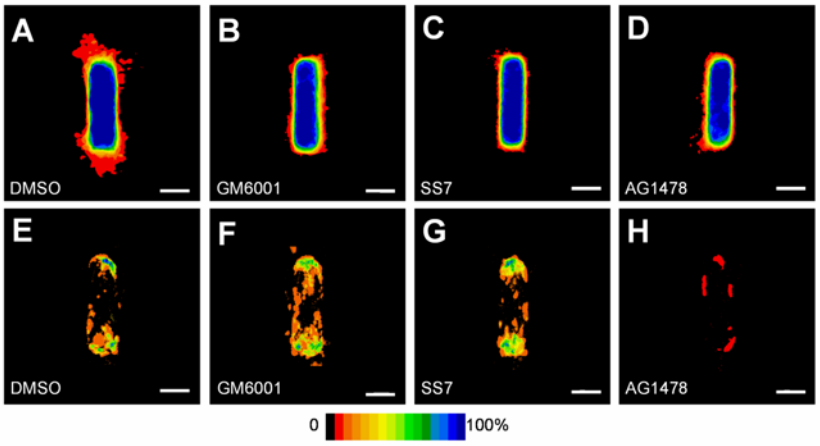

Fig. S2. Frequency maps 24 hours after induction of branching with EGF and treatment with **(A)** dimethylsulfoxide (DMSO) vehicle control, **(B)** broad spectrum MMP inhibitor GM6001 (40 µM), **(C)** epimorphin antagonist SS7 (4 µg/mL), **(D)** EGFR kinase inhibitor AG1478 (80 µM). Frequency maps of vimentin gene promoter-GFP expression 8 hours after stimulation with EGF and treatment with **(E)** DMSO control, **(F)** MMP inhibitor GM6001 (40 µM), **(G)** epimorphin antagonist SS7 (4 µg/mL), **(H)** EGFR kinase inhibitor AG1478 (80 µM). Scale bar, 50 µm.

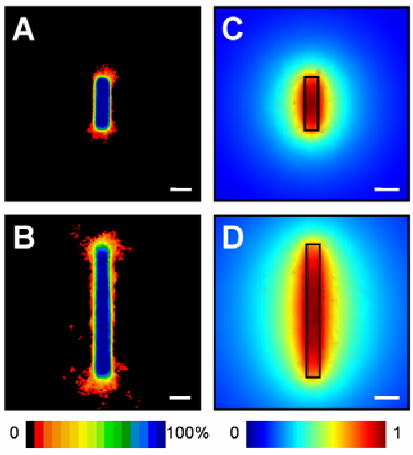

Fig. S3. Frequency maps 24 hours after induction of branching from **(A)** short tubules and **(B)** long tubules. Calculated concentration gradients of secreted diffusible inhibitors from **(C)** short tubules and **(D)** long tubules. Scale bars, 50 μm.

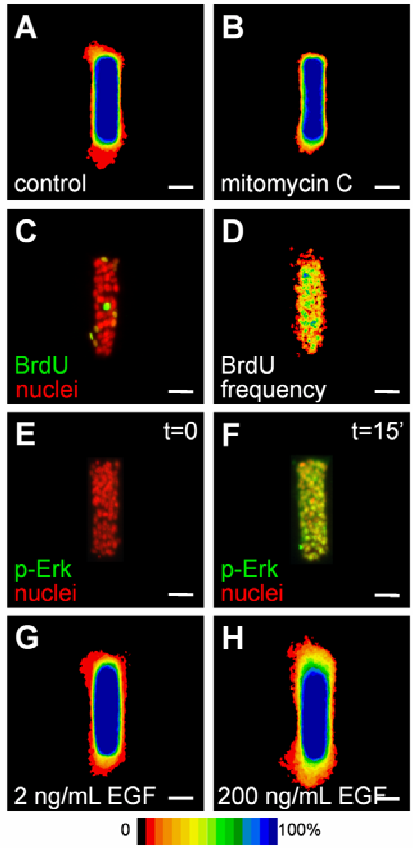

Fig. S4. Frequency maps 24 hours after induction of branching **(A)** without or **(B)** with mitomycin C treatment to block cell proliferation. Cell proliferation is uniformly distributed across the tubule, as demonstrated by **(C)** immunofluorescence analysis and **(D)** frequency map of BrdU incorporation at 12 hours after addition of EGF. Similar results were observed using 2 hour pulses of BrdU along a time course up to 24 hours after addition of EGF. EGF receptor signaling is also even across the tubule, as demonstrated by staining for phosphorylated Erk (p-Erk) at **(E)** time 0 and **(F)** 15 min after addition of EGF. Similar results were observed at later time points. **(G, H)** Frequency maps 24 hours after induction of branching show that altering the concentration of EGF affects the magnitude but not the position of branching. Scale bar, 50 µm.

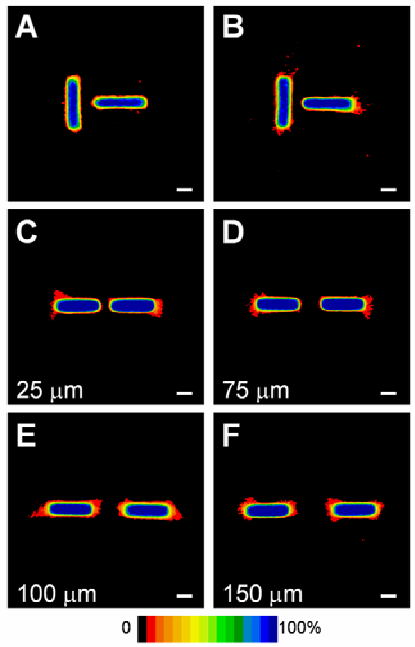

Fig. S5. Frequency maps of perpendicular pairs of tubules of primary mammary epithelial cells that were **(A)** untreated or **(B)** treated with EGF for 24 hours. Frequency maps 24 hours after induction of branching in pairs of tubules of EpH4 cells separated by distances of **(C)** 25 µm, **(D)** 75 µm, **(E)** 100 µm, and **(F)** 150 µm. Scale bar, 50 µm.

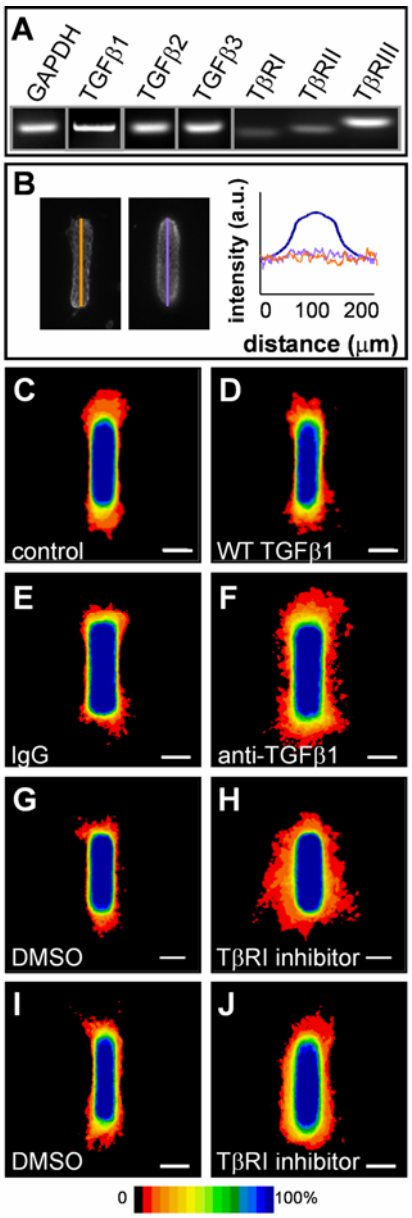

Fig. S6. (A) RT/PCR analysis shows that TGFβ family ligands and receptors are expressed in EpH4 tubules. **(B)** Confocal sections of primary mammary epithelial tubules stained for keratin-8 (left) and nuclei using Hoechst 33258 (right), with graphs representing relative pixel intensity (arbitrary units, a.u.) along tubules (orange: keratin-8; violet: nuclei). Graphs are scaled the same as that in Fig. 4A. Numerical prediction of secreted gradient is shown as blue curve. Frequency maps 24 hours after induction of branching in tubules of **(C)** control cells and **(D)** cells over-expressing wild type (latent) TGFβ1. Frequency maps 24 hours after induction of branching in EpH4 tubules treated with **(E)** control chicken IgG (10 ng/mL), **(F)** chicken anti-TGFβ1 (10 ng/mL), **(G)** DMSO, or **(H)** TGFβ type I receptor kinase inhibitor (TβRI inhibitor; 1.8 μM). Frequency maps 24 hours after induction of branching in primary mammary epithelial cell tubules treated with **(I)** DMSO, or **(J)** TGFβ type I receptor kinase inhibitor (TβRI inhibitor; 1.8 μM). Scale bar, 50 μm.

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