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Research Article

Mammary branch initiation and extension are inhibited by separate pathways downstream of TGFβ in culture

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ABSTRACT

During the branching morphogenesis process that builds epithelial trees, signaling from stimulatory and inhibitory growth factors is integrated to control branch initiation and extension into the surrounding stroma. Here, we examined the relative roles played by these stimulatory and inhibitory signals in the patterning of branch initiation and extension of model mammary epithelial tubules in culture. We found that although several growth factors could stimulate branching, they did not determine the sites at which new branches formed or the lengths to which branches extended. Instead, branch initiation and extension were defined by two separate signals downstream of the inhibitory morphogen, transforming growth factor (TGF)-β. Branch initiation was controlled by signaling through p38 mitogen-activated protein kinase, whereas branch extension was controlled by Smad-mediated induction of a second diffusible inhibitor, Wnt5a. These data suggest that mammary epithelial branching is patterned predominately by repulsive signaling, and that TGFβ activates multiple inhibitory pathways to refine the architecture of the tree.

Introduction

Several epithelial organs, including the lung, kidney, salivary gland, and mammary gland, develop into tree-like structures through branching morphogenesis. During branching, a subpopulation of epithelial cells is instructed to form a nascent branch and extend into the surrounding stroma, while neighboring cells are instructed to remain within the subtending duct or trunk. In general, this patterning process requires stimulatory and inhibitory cues that are interpreted by the epithelium to initiate and refine these branching and non-branching regions, but precisely how these cues determine patterning during mammary epithelial branching is still unclear. Systemic endocrine hormones stimulate pubertal mammary branching by inducing local expression of peptide growth factors. Disrupting signaling through the epidermal growth factor (EGF) receptor blocks mammary gland development [1–3], and disrupting fibroblast growth factor (FGF) receptor-2 prevents formation of embryonic mammary placodes [4] and delays

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Abbreviations: BMP, bone morphogenetic protein; DDR, discoidin domain receptor; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; FEM, finite element method; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; JNK, Jun N-terminal kinase; KGF, keratinocyte growth factor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; SBE, Smad-binding element; TβRI, TGFβ receptor type I; TβRII, TGFβ receptor type II; TGF, transforming growth factor; 3D, three-dimensional.

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pubertal branching [5,6]. These stimulatory growth factors thus appear to be required for mammary branching morphogenesis.

In addition to stimulatory growth factors, branching morphogenesis is regulated by inhibitory signaling from members of the transforming growth factor (TGF)-β family. During mammary gland development, TGFβ1 is highly enriched in periductal extracellular matrix (ECM) and focally depleted in regions surrounding incipient lateral branches and advancing end buds [7–9]. Exogenous addition of TGFβ via slow-release implants dramatically inhibits formation and extension of branches *in vivo* [10–13]. Mice that overexpress TGFβ1 in the mammary epithelium have a hypoplastic mammary tree [14], whereas those that express a dominant negative receptor [15,16] or that are heterozygous for a null allele of TGFβ (TGFβ+/−) show increased branching morphogenesis [7]. The effects of TGFβ are likely mediated by both autocrine and paracrine interactions, as disrupting TGFβ signaling in stromal fibroblasts leads to increased production of multiple stimulatory growth factors, including hepatocyte growth factor (HGF), resulting in hyperplastic development of the epithelial tree and eventual formation of tumors [16–18]. TGFβ1 has therefore long been postulated to aid in the maintenance of proper ductal spacing by enabling ducts to avoid one another [11]. Nonetheless, despite decades of study, the mechanisms underlying the effects of TGFβ on branch initiation and extension remain enigmatic.

Although several stimulatory growth factors can induce branching of mammary epithelium, their influence on the pattern of branch initiation is also unclear. Whereas concentration gradients of FGFs specify branch sites in the *Drosophila* trachea and vertebrate lung, there is no evidence for chemoattraction in patterning branching morphogenesis of the mammary gland [19]. Also unlike other branched organs, the mammary gland does not have a stereotyped pattern of branching; this lack of a predictable pattern represents one of the major challenges of examining branch site initiation in the intact gland. Pubertal mammary branching can be recapitulated by culturing mammary epithelial cells in three-dimensional (3D) gels of ECM and providing purified growth factors such as EGF or HGF [20–25]. These simple models mimic the invasive behavior of mammary branching morphogenesis, and are also regulated by both stimulatory and inhibitory growth factors. Antibodies against TGFβ augment the branching of mammary epithelial cells in culture, whereas exogenous addition of recombinant TGFβ inhibits branching [24,26]. Here, we describe experiments aimed at uncovering the relative influences of stimulatory growth factors and inhibitory TGFβ on branch initiation and extension of mammary epithelial cells. We took advantage of a culture model in which we could vary the predicted concentration of TGFβ by altering the initial geometry of mammary epithelial tubules. We found that although stimulatory growth factors could induce the branching process, they had no discernible effect on the branching pattern. The major patterning signal came instead from autocrine-secreted TGFβ, which inhibited branch initiation and extension through two separate pathways.

Materials and methods

Cell culture and reagents

Functionally normal EpH4 mouse mammary epithelial cells [27] were cultured in 1:1 DMEM:F12 supplemented with 2% fetal bovine serum, 5 μg/ml insulin, and 50 μg/ml gentamycin (Sigma). Microfabricated tubules were treated with the following reagents diluted to the concentrations indicated in the text: p38 MAPK inhibitors SB202190 [28,29], SB203580, PD169316, JNK inhibitor SP600125, PI3K inhibitor LY294002 (all from Sigma), Alk5 inhibitor [3-(pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole (Calbiochem), control chicken IgG, chicken anti-TGFβ1, and recombinant Wnt5a (all from R&D Systems).

Constructs and transfections

For knockdown studies, predesigned small interfering RNA (siRNA) sequences (Ambion) were verified for specific knockdown by quantitative RT/PCR and transfected into mammary epithelial cells using Lipofectamine 2000 (Invitrogen) 1 day prior to microfabricating tissues. For overexpression studies, constructs were transfected into mammary epithelial cells using Lipofectamine 2000 as above. Constructs used included activated porcine TGFβ1 [30], dominant negative TGFβ receptor type II (TβRII) [30], Smad6 and Smad7 (AddGene), and their respective vector controls.

Branching morphogenesis assays

Branching from cell clusters was performed as previously described [23,31], with minor modifications. Briefly, mammary epithelial cells suspended in 500 μl of growth medium containing 0.1% (w/v) of Pluronics F108 (BASF) were incubated in 24-well plates at 37 °C with gentle rotation for 16 h, which yielded rounded and well-packed clusters of cells. Clusters were embedded in liquid neutralized type I collagen (BD Biosciences or Koken), the collagen was allowed to gel, and incubated in growth medium overnight. Samples were treated with 4.2 nM EGF, TGFα, amphiregulin, IGF1, HGF, FGF2, FGF7, FGF10 (all from Sigma), or 0.08 pM (20 pg/ml) TGFβ1 (R&D Systems) as indicated in the text.

Microfabricated tubules of epithelial cells were formed by replica micromolding of collagen as previously described [30,32]. Briefly, elastomeric stamps of poly(dimethylsiloxane) (PDMS; Sylgard 184, Ellsworth Adhesives, Germantown, WI) containing features in bas relief were rendered non-adhesive by coating with a 1% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Coated stamps were placed upon a drop of liquid neutralized collagen (BD Biosciences or Koken) at 37 °C until gelation. After removing stamps, a concentrated suspension of cells was allowed to settle within the micromolded cavities. Excess cells were removed, and a second layer of collagen gel placed on top of the sample. Cells formed tubules overnight, at which time the samples were treated with 4.2 nM EGF, TGFα, amphiregulin, IGF1, HGF, FGF2, FGF7, FGF10, or 0.08 pM (20 pg/ml) TGFβ1.

Quantitative real-time RT/PCR analysis

Total RNA was extracted from cells using either Trizol reagent (Invitrogen) or an RNeasy kit (Qiagen). cDNA was synthesized using Superscript II first strand synthesis kit (Invitrogen) from equal amounts of RNA. Quantitative real-time PCR analysis was performed with the MJ MiniOpticon Real-Time PCR system (BioRad) using the iQ SYBR Green Supermix (BioRad). The

following primers were used to amplify Smad6, Smad7, Wnt5a, and GAPDH cDNA sequences: Smad6 forward primer 5′-CCC CAA GCC AGA CAG TCC CCG-3′ and reverse primer 5′-GAG TTG GTG GCC TCG GTT TCA GTG-3′; Smad7 forward primer 5′-GAG CTC GAG TTC AGG ACC AAA CGA TCT GCG CTC-3′ and reverse primer 5′-GAG GAA TTC CTA CCG GCT GTT GAA GAT GAC CTC-3′; Wnt5a forward primer 5′-GCC TGC TTC ATT GTT GTG TAA G-3′ and reverse primer 5′-CGC TTC GCC AAG GAG TTC-3′; GAPDH forward primer 5′-GCC TTC CGT GTT CCT ACC-3′ and reverse primer 5′-GCC TGC TTC ACC ACC TTC-3′. Amplification was followed by melting curve analysis to verify the presence of a single PCR product.

Imaging and statistical analysis

Samples were fixed, stained for nuclei with Hoechst 33258 (Invitrogen), and visualized using a Hamamatsu Orca CCD camera attached to a Ti-U Nikon microscope. Total cumulative data were represented by stacking aligned binarized images of total nuclei from 50 tubules, obtaining relative pixel frequency with Scion image software, and color-coding images in Adobe Photoshop. All experiments were conducted at least three times.

For immunofluorescence analysis of cell surface receptors and intracellular signaling molecules, the following primary antibodies were used: pEGFR (Y^{1173}) (clone 53A5, Cell Signaling); Met (clone H-190, Santa Cruz Biotechnology); pMet (Y^{1230}/Y^{1234}/Y^{1235}) (Millipore); TβRI (Santa Cruz); TβRII (Santa Cruz); pSmad1/5/8 (Cell Signaling); pSmad2/3 (Santa Cruz); cleaved caspase-3 (Cell Signaling). For analysis of cell proliferation, EdU incorporation was performed using the Click-iT EdU Alexa Fluor 594 imaging kit (Invitrogen) according to the manufacturer's protocol.

For analysis of branch lengths, samples fixed and stained with Hoechst 33258 were imaged and processed using ImageJ software. Branch lengths were defined as the length from the tubule to the most distal nucleus in the branch. Lengths were measured from at least 50 tubules.

Computational modeling

We used the finite element method (FEM) to solve a 3D model of TGFβ diffusion from different tubule geometries using COMSOL Multiphysics 3.4 software. The culture dish was represented using a cylinder with diameter of 40 mm and height of 6 mm. The walls and the top of the cylinder were set as diffusion sinks with a concentration equal to 0. The bottom of the cylinder could be set to be inert with a flux equal to 0 or as a diffusion sink with a concentration equal to 0 without affecting the results. Tubules of varying geometries with a height of 50 μm were drawn; these tubules were centered in the culture dish, representing immersion in collagen. Initially the normal pre-defined mesh conditions were used. However, in order to increase the accuracy around the tubule, the mesh on the tubule boundary was defined to have a maximum element size of 0.005 mm with a growth rate of 1.5. This mesh was refined once. The diffusion equation was then solved using the UMFPACK linear system solver. The concentration and concentration gradient profile figures were obtained by examining a slice through the midplane of each tubule. Mesh independence was verified by determining that additional refinement of the mesh did not change the concentration appreciably ($\leq$0.1%).

Results

Stimulatory growth factors induce branching but do not determine sites of branch initiation

HGF and members of the EGF and FGF families are all expressed in the mouse mammary gland during puberty [6,25,33–36], and exogenous administration of EGF, TGFα, and insulin-like growth factor (IGF)-1 induce mammary branching *in vivo* [33,37–39]. Although tissue recombination experiments have suggested that specific mitogens act on stromal receptors and are required in the stroma, mammary epithelium remains competent to respond to these growth factors [40]. Indeed, branching of mammary epithelial cells can be replicated in culture in the absence of mesenchyme and steroid hormones by the addition of growth factors. Here, we found that clusters of EpH4 mouse mammary epithelial cells were induced to branch into gels of type I collagen upon treatment with EGF, TGFα, amphiregulin, IGF1, HGF, FGF2, FGF7, or FGF10 (Fig. 1A–K), consistent with previous studies using a variety of mammary epithelial cell lines and primary cells [23,24,39–44]. There thus appear to exist "multiple pathways to ductal growth" in culture, as there do *in vivo* [45].

We recently developed a 3D engineered culture model that recapitulates mammary tubules and permits examination of the signals that influence branch site selection during morphogenesis [30,32]. Multicellular branches emerge from specific locations depending on the initial geometry of the tubule (Supplementary Fig. 1). We previously found that cells branched from the ends of cylindrical tubules when treated with either EGF or HGF (Fig. 2A–D, H); this pattern of branching required both the exogenous stimulatory growth factor and TGFβ, an endogenous antagonist that acts as an autocrine inhibitor of branching [30]. Here, we examined the growth factors identified in Fig. 1 as capable of inducing branching of mammary epithelial cells. We found that cylindrical tubules always branched from the ends, regardless of the stimulatory growth factor used to induce the process (Fig. 2D–K). These data suggest that growth factors may stimulate the tissue to branch but do not play a major role in defining the locations at which branches form.

These results also suggest that stimulatory and inhibitory cues are integrated at a level downstream of the growth factor receptors. Consistent with this interpretation, we found even distribution of the EGF and HGF receptors, EGFR and cMet, along the length of the tubules (Fig. 3A–B). Growth factor addition also led to homogeneous activation of these receptors in all cells of the tubules, as determined by immunofluorescence analysis of the phosphorylated forms of the receptors (Fig. 3C–D). Activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade has been previously shown to be required for branching morphogenesis of mammary epithelial cells [41]. Activation of ERK was found to be homogeneous across the cylindrical tubules, and inhibiting signaling through EGFR or ERK by treatment with pharmacological inhibitors totally abrogates branching [30]. These results suggest that patterned branching is not a function of patterned localization or activation of stimulatory growth factor receptors.

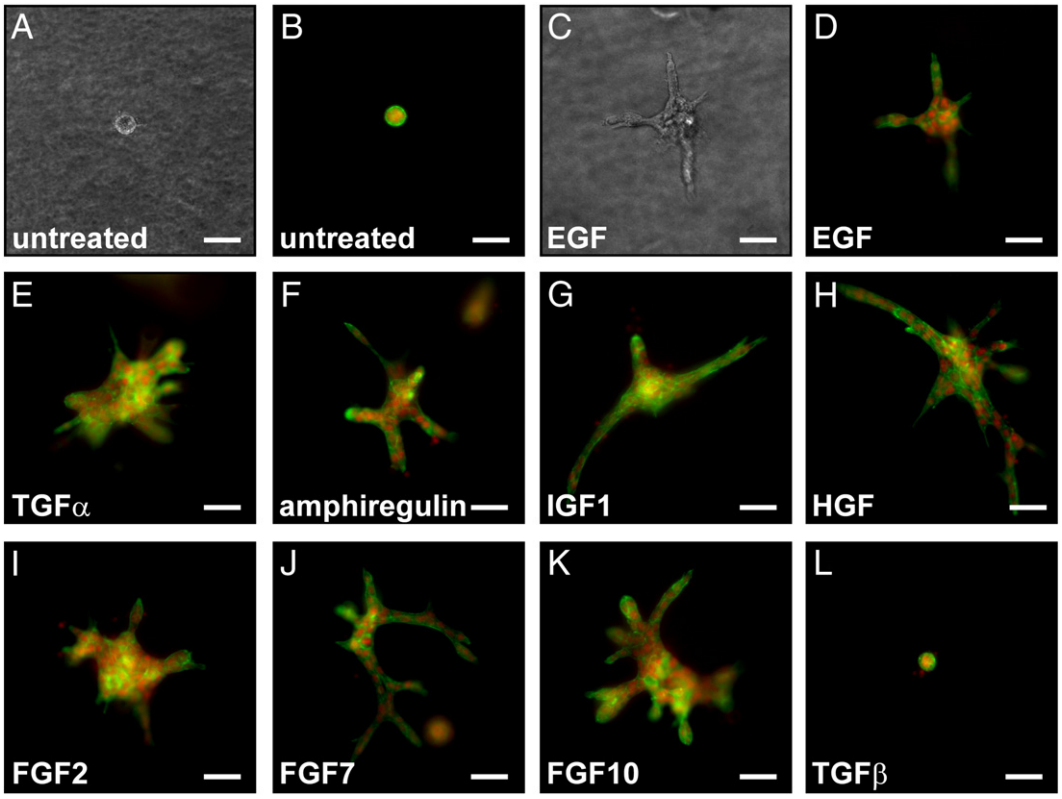

Fig. 1 – Mammary epithelial cells branch in response to several different growth factors. (A) Phase contrast image of mammary epithelial cluster before induction of branching. (B) Fluorescence image of F-actin (green) and nuclei (red) in mammary epithelial cluster before induction of branching. (C) Phase contrast image of mammary epithelial cluster after induction of branching with EGF. (D-L) Fluorescence images of F-actin (green) and nuclei (red) in mammary epithelial clusters after treatment with (D) EGF, (E) TGFα, (F) amphiregulin, (G) IGF1, (H) HGF, (I) FGF2, (J) FGF7, (K) FGF10, or (L) TGFβ. Scale bars, 100 μm.

TGFβ affects both branching pattern and length

Branching pattern requires both stimulatory growth factors to induce subpopulations of cells to form a branch, and inhibitors to prevent spurious branching from neighboring cells within the trunk. In addition to having no effect on branch site selection, the different stimulatory growth factors also appeared to have minimal differential effect on cell proliferation or death within the tubules (Supplementary Fig. 2), or on the lengths of the branches that formed (Fig. 4A). In the engineered tissues and in the mammary gland *in vivo*, TGFβ acts as an autocrine-secreted antagonist to inhibit branching [7,13,30]. Exogenous addition of TGFβ1 completely inhibited branching of mammary epithelial clusters and engineered tubules (Figs. 1L and 2L); the few branches that emerged in TGFβ-treated samples were short and not significantly different in length or number from those in untreated samples (Fig. 4A). Similarly, ectopic expression of activated TGFβ1 inhibited the initiation and extension of branches from EGF-treated engineered tubules (Fig. 4B) [30]. Blocking TGFβ signaling at the ligand/receptor level by treating with a function-blocking antibody against TGFβ1, a pharmacological inhibitor of Alk5 receptor kinase activity, or ectopic expression of a dominant negative type II receptor all resulted in unpatterned branching from the engineered tubules [30], and also led to a marked and significant increase in branch length (Fig. 4C). TGFβ therefore appears to control both the sites at which branches form, as well as the lengths of branch extensions.

TGFβ controls branch site selection through p38 MAPK

TGFβ inhibits branching morphogenesis of many organs, but the molecular mechanism by which TGFβ exerts its inhibitory effects on branching is unclear. Active TGFβ signals through a multimeric complex of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. Ligand-bound TβRII recruits, phosphorylates, and activates TβRI. In the canonical TGFβ signaling pathway, activated TβRI phosphorylates receptor-regulated R-Smads and thereby directs their translocation to the nucleus. Immunofluorescence analysis demonstrated homogeneous distribution of both TβRI and TβRII along the tubules (Fig. 5A–B). Staining for pSmad1/5/8 or pSmad2/3 also revealed nuclear localization in all cells along the length of the tubules (Fig. 5C–D), suggesting that Smad-mediated signaling was homogeneous in these tissues, at least at the resolution afforded by immunofluorescence analysis. Signaling through the canonical pathway is antagonized by the inhibitory I-Smads, Smad6 and Smad7, which can inhibit the phosphorylation of R-Smads through a plethora of mechanisms including competitive binding, ubiquitination, and dephosphorylation of the type I receptors [46,47]. Consistent with homogeneous nuclear R-Smads, preventing signaling through Smads by

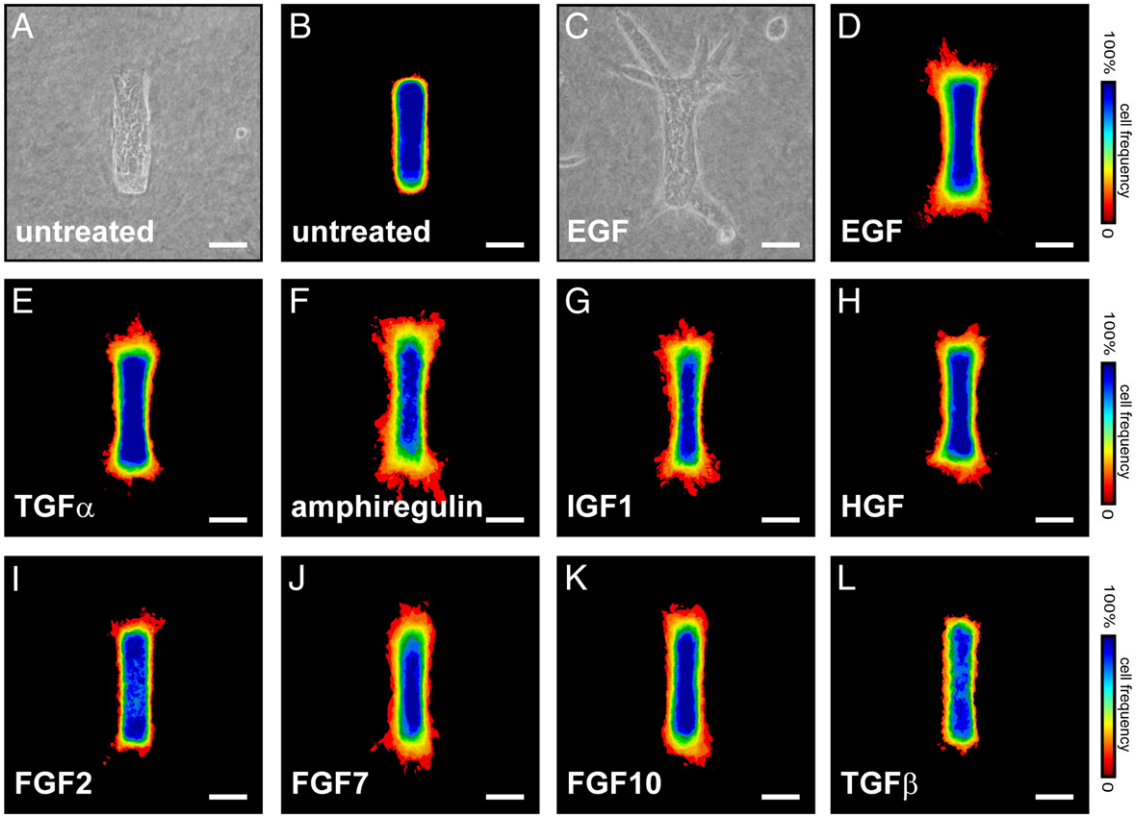

Fig. 2 – Pattern of branching is independent of growth factor used to induce morphogenesis. (A) Phase contrast image of mammary epithelial tubule before induction of branching. (B) Frequency map depicting average location of cells within tubule before induction of branching. (C) Phase contrast image of mammary epithelial tubule 24 h after treatment with EGF. (D–L) Frequency map of tubules after treatment with (D) EGF, (E) TGFα, (F) amphiregulin, (G) IGF1, (H) HGF, (I) FGF2, (J) FGF7, (K) FGF10, or (L) TGFβ. Frequency represents percent of tubules that have cells at that location. Scale bars, 50 μm.

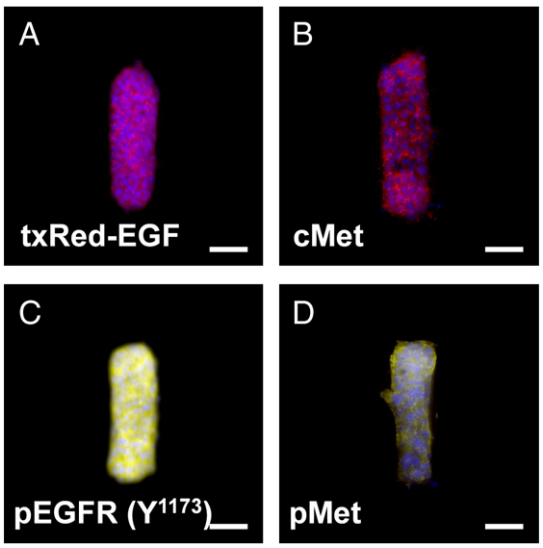

Fig. 3 – Treatment with growth factors leads to unpatterned activation of receptors. (A) Fluorescence image of uptake of TexasRed-conjugated EGF by tubule. (B–D) Fluorescence images of (B) cMet, (C) pEGFR (Y^{1173}) and (D) pMet ($Y^{1230}/Y^{1234}/Y^{1235}$). Tubules were treated with EGF or HGF for 15 min prior to fixation. Scale bars, 50 μm.

overexpressing Smad6 or Smad7 had no effect on pattern of branching from the tubules (Fig. 5E–J). Altogether, these data suggest that TGFβ does not pattern the initiation of branches through induction of Smad signaling.

How then does TGFβ inhibit spurious branching and specify pattern? In addition to the canonical Smad-mediated signaling pathway, TGFβ can activate downstream signaling through several kinase cascades, including Jun N-terminal kinase (JNK), phosphoinositide-3 kinase (PI3K), and p38 MAPK. Treating tubules with the JNK inhibitor SP600125 (Fig. 6A–C) or the PI3K inhibitor LY294002 (Fig. 6D–F) had no effect on the pattern of branching, suggesting that neither signaling pathway is involved in branch site selection. However, treatment with the p38 MAPK inhibitor SB202190 led to unpatterned branching along the entire length of the tubule (Fig. 6G–I). Treatment with other p38 MAPK inhibitors, SB203580 and PD169316, also led to unpatterned branching (Supplementary Fig. 3). These data suggest that TGFβ inhibits branch initiation by signaling through p38 MAPK.

TGFβ affects branch length through Smads and Wnt5a

Although p38 MAPK blocked spurious branching, pharmacological inhibition of p38 MAPK had no effect on branch length (Fig. 6J). Furthermore, Smad-mediated signaling did not affect branch site selection, although ectopic expression of Smad7, but not Smad6,

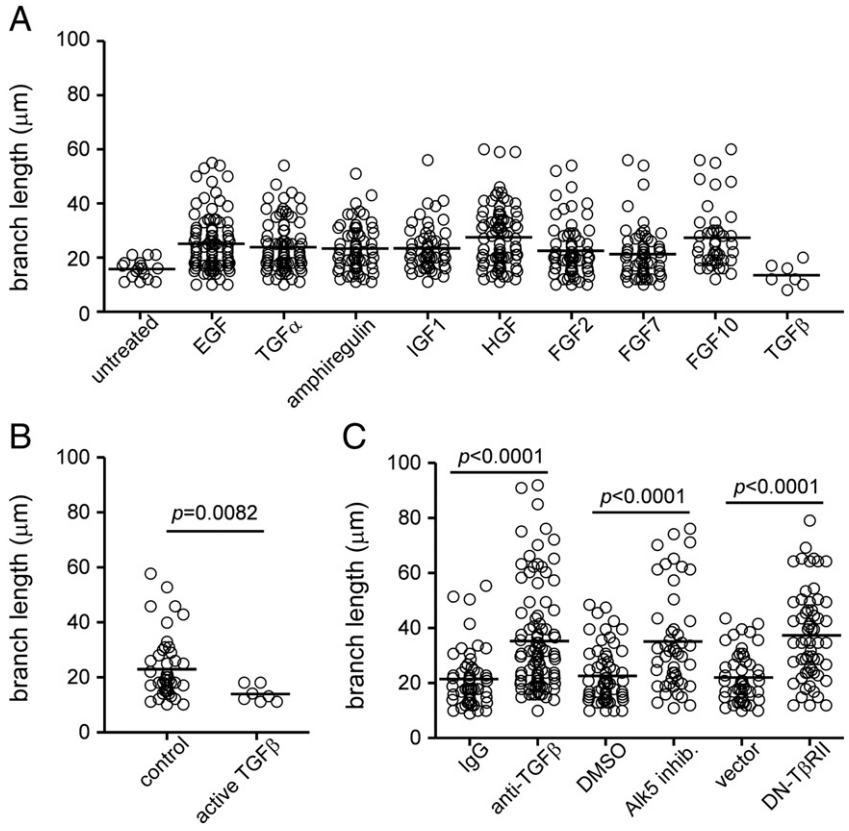

Fig. 4 – Branch length is affected by the endogenous inhibitor, TGFβ, but not by stimulatory growth factors. (A) Plot of branch length from engineered tubules under various conditions. (B) Plot of branch length from engineered tubules ectopically expressing activated TGFβ. (C) Plot of branch length from engineered tubules treated with function blocking antibody against TGFβ1, TβRI (Alk5) inhibitor, or ectopically expressing dominant negative TβRII, and the associated control tubules. *p* values calculated using Mann–Whitney test.

caused a statistically significant increase in the lengths of the branches (Fig. 5K). These data suggest that TGFβ affects branch initiation and branch extension through separate pathways. Once in the nucleus, activated Smad complexes associate with other transcription factors, bind to Smad-binding elements (SBEs) on chromatin, and thereby regulate transcription [48]. Several genes are regulated by Smad signaling, including Wnt5a, which contains a total of eight predicted SBEs within its promoter and first intron [49]. Wnt5a is a non-canonical member of the Wnt family that has recently been shown to act as an inhibitor of branching morphogenesis in several organs, including the lung and prostate gland [50–53]. Wnt5a is downregulated during HGF-induced branching of mammary epithelial cells [54], can block mammary branching in culture [55], and was recently shown to be involved in TGFβ-mediated inhibition of branching morphogenesis *in vivo* [56]. Here, we found that TGFβ1 induced the expression of Wnt5a in mammary epithelial tubules (Fig. 7A). This induction was apparently independent of p38 MAPK activation, as treatment with the p38 inhibitor had no effect on Wnt5a expression at the mRNA level (Fig. 7A). Conversely, ectopic expression of Smad7 led to a decrease in Wnt5a expression (Fig. 7B). Mammary epithelial clusters treated simultaneously with EGF and Wnt5a grew into irregular masses that failed to branch (Fig. 7C, D); Wnt5a similarly inhibited branching from engineered mammary epithelial tubules (Fig. 7E, F). Conversely, siRNA-mediated depletion of Wnt5a increased branch lengths (Fig. 7I), but did not alter sites at which new branches form (Fig. 7G, H). Altogether, these data suggest that TGFβ signals through Smads to upregulate the expression of a second diffusible signal, Wnt5a, in order to control branch extension.

Branch site selection is controlled by concentration of TGFβ

Although TGFβ has long been recognized as an inhibitor of branching morphogenesis, it is unclear from experiments *in vivo* and cell clusters in culture whether the cells are sensing the absolute concentration of TGFβ or its local gradient. We took advantage of the engineered culture model to create tubules in which we could vary TGFβ concentration independently of TGFβ gradient. Using a computational model of TGFβ secretion and diffusion, bent tubules were predicted to have local minima in the concentration of TGFβ at the ends and "elbow" (Fig. 8A–C), and local maxima in the concentration gradient of TGFβ at these same regions (Fig. 8D–F). Increasing the angle of the bend increased the concentration and decreased the gradient of TGFβ at the elbow (Fig. 8G). Conversely, increasing the angle of the bend decreased the concentration of TGFβ at the ends, without affecting the gradient (Fig. 8G). From these shapes, we make two predictions. If cells sense the absolute concentration of TGFβ, then branching should decrease at the elbow and increase at the ends as the angle

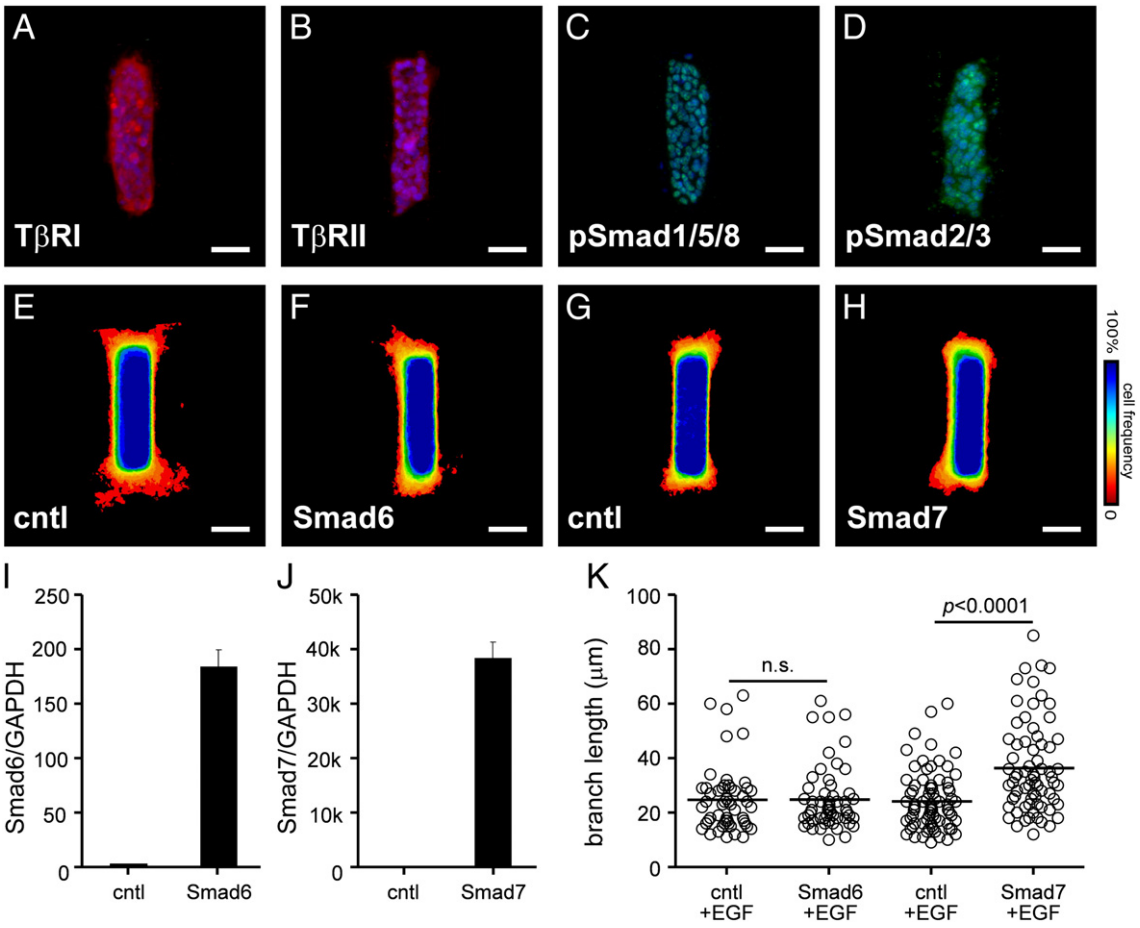

Fig. 5 – Inhibiting Smad-mediated signaling has no effect on pattern of branching. (A, B) Fluorescence images of receptors (red) and nuclei (blue) in tubules stained for (A) TβRI or (B) TβRII. (C, D) Fluorescence images of phosphorylated Smads (green) and nuclei (blue) in tubules stained for (C) pSmad1/5/8 or (D) pSmad2/3. (E–H) Frequency maps of (E, G) control tubules, (F) tubules ectopically expressing Smad6, and (H) tubules ectopically expressing Smad7. (I, J) Quantitative RT/PCR analysis of mammary epithelial cells transfected for (I) Smad6 or (J) Smad7, and analyzed for respective expression. (K) Plot of branch length from engineered tubules ectopically expressing Smad6, Smad7, and the associated control tubules. *p* values calculated using Mann–Whitney test. Scale bars, 50 μm.

of the bend is increased. However, if cells sense the high local gradient in TGFβ concentration, then branching should increase at the elbow and remain the same at the ends as the angle of the bend is increased. We engineered bent mammary epithelial tubules in culture, induced branching by treating with EGF, and found that as the angle of the bend increased, branching decreased at the elbow and increased at the ends (Fig. 8H–J). These data suggest that mammary epithelial cells sense the local concentration of TGFβ, and as the concentration increases, their branching is inhibited.

Discussion

Here we investigated how stimulatory and inhibitory growth factors determine the sites of incipient branch formation and branch extension during morphogenesis of mammary epithelial cells in culture. We found that although eight different growth factors representing four different families can induce the branching process, none was individually responsible for specifying pattern or length of branches. Instead, the sites of branch initiation and the lengths of the branches that formed were regulated by TGFβ, an endogenous inhibitor in this system. Our data are consistent with *in vivo* studies showing that stimulatory growth factors do not act as chemoattractants or induce ectopic branches in the mammary gland [39,57]. Our data are also consistent with the longstanding hypothesis first proposed by Daniel and colleagues that the pattern of branching is sculpted not by stimulatory cues but instead by the inhibitory activity of TGFβ [13], which at high concentrations blocks new branch formation and at low concentrations permits branch extension. This mechanism of pattern formation contrasts with that of other branched organs. In the mammalian lung, epithelial buds are induced by and extend toward FGF secreted by the surrounding mesenchyme, and are inhibited by the TGFβ superfamily member, bone morphogenetic protein (BMP)-4 [58]. In the *Drosophila* trachea, new branches initiate and extend toward Branchless, an FGF-family ligand [59]. Likewise, in the mammalian kidney, branching of the ureteric bud is guided by glial cell-derived

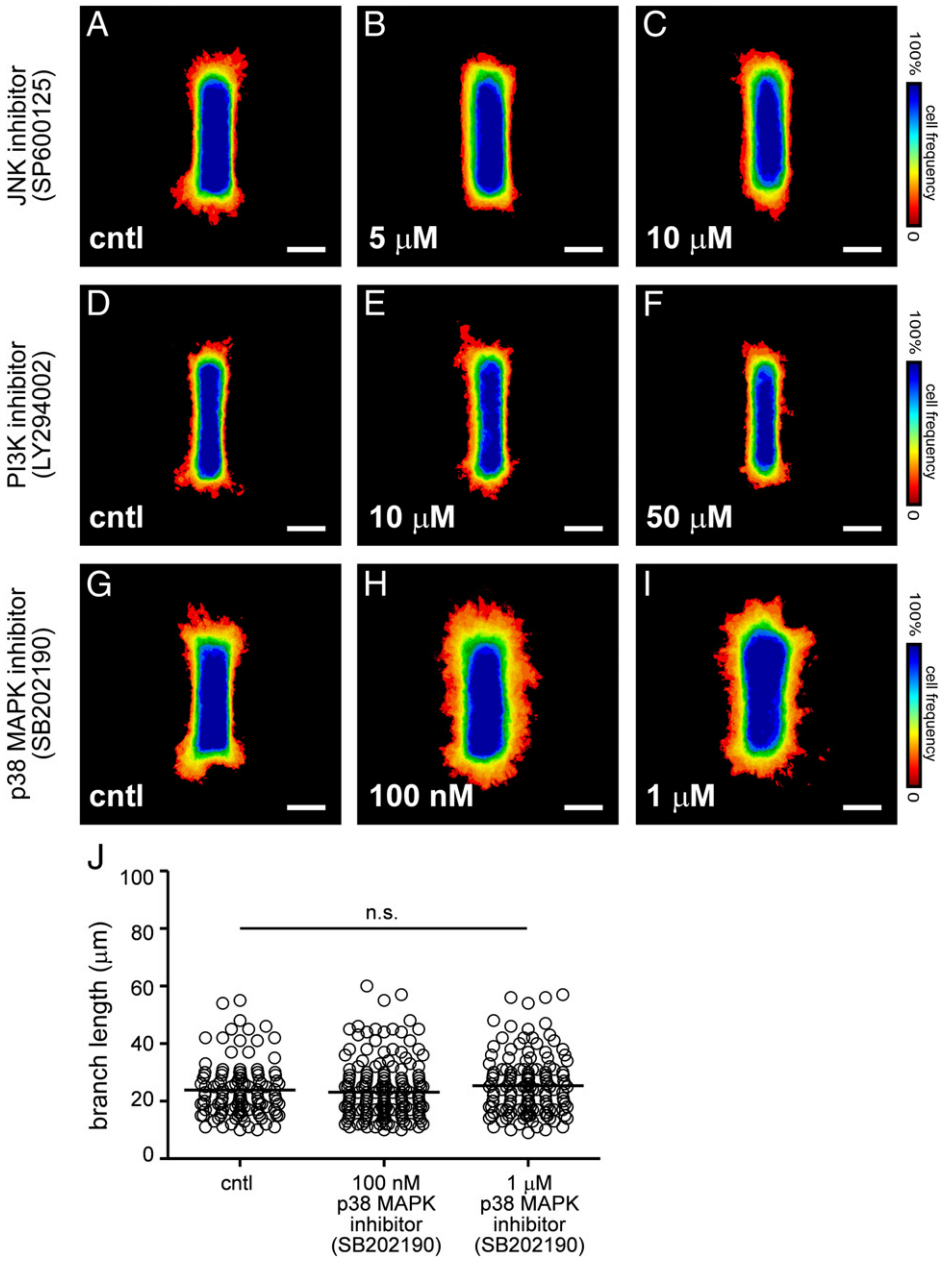

Fig. 6 – Inhibiting p38 MAPK, but not JNK or PI3K, causes unpatterned branching. (A–C) Frequency maps of tubules treated with the JNK inhibitor, SP600125. (D–F) Frequency maps of tubules treated with the PI3K inhibitor, LY294002. (G–I) Frequency maps of tubules treated with the p38 MAPK inhibitor, SB202190. (J) Plot of branch length from engineered tubules treated with the p38 MAPK inhibitor, SB202190, at the given concentrations. *p* values calculated using Mann–Whitney test. Scale bars, 50 μm.

neurotrophic factor (GDNF) [60]. Thus, although many of the signals and biochemical pathways are evolutionarily conserved and expressed throughout development of branching organs [61], patterning of mammary epithelial branching appears to be unique in its reliance on repulsion rather than attraction.

What, then, is the role of the stimulatory growth factors in pubertal mammary branching? Unlike branching of other organs such as the *Drosophila* trachea and salivary gland, branching of mammary epithelium requires cell proliferation [30]. Rather than induce new branches to form in a subpopulation of cells within the duct, stimulatory growth factors may induce the proliferation and survival of the entire population, thereby increasing the number of epithelial cells and allowing for both morphogenesis and growth of the tree. Analysis of mammary branching in transgenic mice supports this hypothesis. Signaling through the FGF receptor, FGFR2, promotes mammary epithelial cell proliferation during puberty [5]. Even though several different growth factors are capable of inducing branching in culture and *in vivo*, there is some evidence to suggest that they play non-redundant roles during mammary branching. Bissell and colleagues found that, in primary mammary organoids, TGFα and FGF7 differentially activate the ERK pathway and thereby have different effects on branching [41], and furthermore that these effects changed as the mice aged [40,41]. The mammary gland is one organ that continues to

undergo branching throughout adulthood, namely during the hormone and pregnancy cycles, so it will be interesting to determine whether aging has an effect on how the mammary gland is patterned.

We found that TGFβ inhibits branch initiation and branch extension through two separate pathways, p38 MAPK and Smad/Wnt5a respectively. There are four isoforms of p38 MAPK in mammals: p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12), and p38δ (MAPK13). The pharmacological inhibitors of p38 MAPK used here (the pyridinyl imidazoles SB202190, SB203580, and PD169316) inhibit both p38α and p38β with similar specificities [62,63]. Knockout of p38α is embryonic lethal [64–67], whereas p38β knockout mice are viable with no apparent defects [68]. This suggests that if p38 MAPK is downstream of TGFβ in the inhibition of mammary branching *in vivo*, then the p38α isoform is a promising candidate. A role for p38 MAPK has previously been implicated in the branching of other organs, although in these cases p38 MAPK appears to signal downstream of stimulatory growth factors such as BMP7 to induce rather than inhibit morphogenesis. Knockdown of p38α disrupts branching of cultured embryonic lungs [69] and treatment with the pharmacological inhibitor SB203580 blocks growth of cultured embryonic kidneys [70] and tubulogenesis of collecting duct epithelial cells [71,72]. One group has also reported that TGFβ induces branching morphogenesis of mammary epithelial cells in culture through p38 MAPK [73]; it is important to note that the concentrations of TGFβ used in that study (100–500 pg/ml) are 5- to 25-fold higher than those used here, and in our hands lead to massive apoptosis of mammary epithelial cells under all culture conditions tested (data not shown). Whether p38 MAPK plays a role in the inhibition of mammary branching by TGFβ *in vivo* will require further testing and the generation of targeted deletions or conditional mutants.

Although blocking p38 MAPK signaling led to branch initiation in an unpatterned fashion along the entire surface of the ducts, it had no effect on the final lengths of the branches that formed. Instead, we found that TGFβ induced the expression of Wnt5a in a Smad-dependent manner, and that overexpressing the inhibitory Smad7 or downregulating expression of Wnt5a led to an increase in branch length. Smads1–5 are all expressed in the mouse mammary gland during pubertal branching [74]. That we found Smad7, but not Smad6, affected branch length suggests a role for Smad2 or Smad3 in the induction of Wnt5a and inhibition of branch extension. Smad2 is the likely candidate for regulating Wnt5a during pubertal branching, as analysis of Smad3-null animals treated with exogenous hormones revealed normal mammary branching morphogenesis [74]. Knockouts of Smad2 are embryonic lethal [75–78], necessitating in the future the use of targeted and conditional approaches to determine its role in the regulation of Wnt5a expression in the pubertal mammary gland.

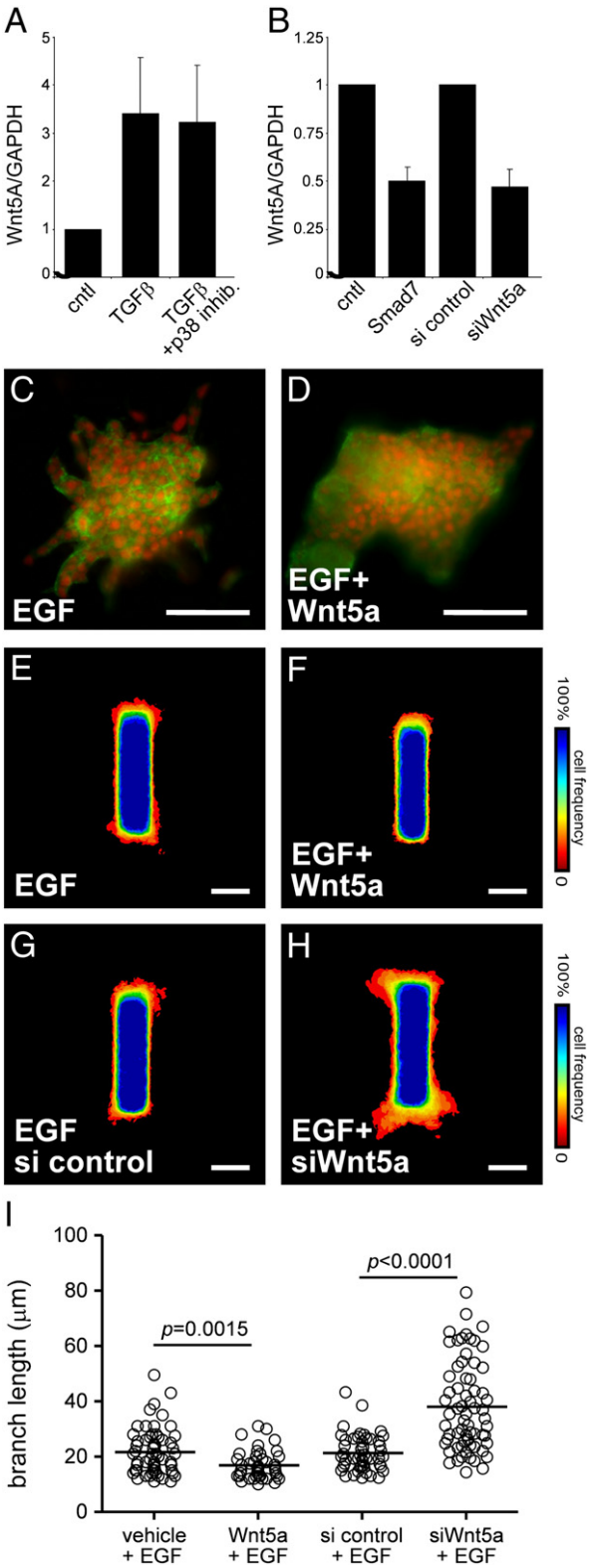

Fig. 7 – Inhibiting Wnt5a increases branch length without affecting branch initiation. (A) Quantitative RT/PCR analysis for Wnt5a in mammary epithelial tubules treated with TGFβ. (B) Quantitative RT/PCR analysis for Wnt5a in mammary epithelial tubules transfected with siRNA against Wnt5a (siWnt5a). (C, D) Fluorescence images of F-actin (green) and nuclei (red) in mammary epithelial clusters treated with (C) EGF or (D) EGF and Wnt5a. (E–H) Frequency maps of engineered tubules treated with (E) EGF, (F) EGF and Wnt5a, (G) EGF and transfected with control siRNA, or (H) EGF and transfected with siWnt5a. (I) Plot of branch length from engineered tubules treated with Wnt5a or transfected with siWnt5a and the associated controls. *p* Values calculated using Mann–Whitney test. Scale bars, 50 μm.

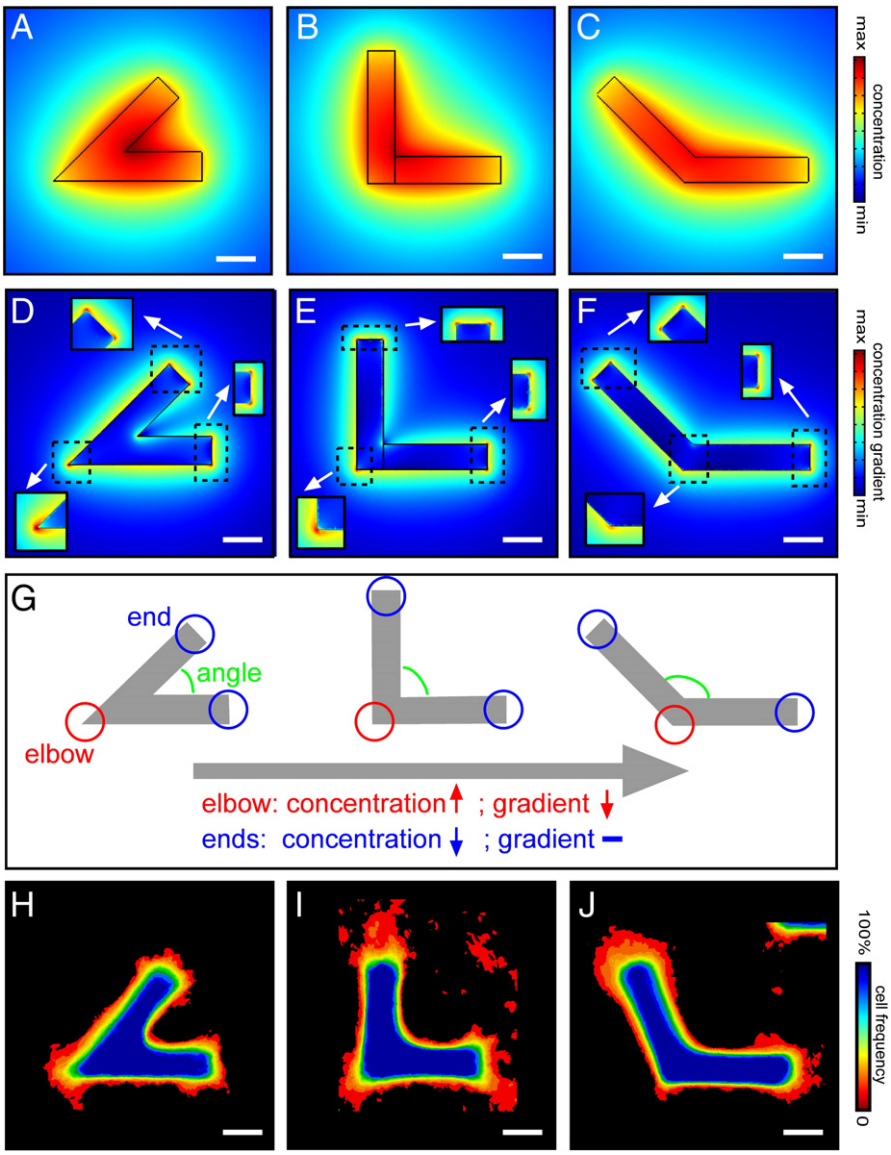

Fig. 8 – Cells sense and respond to changes in TGFβ concentration but not concentration gradient. (A–C) FEM predictions of TGFβ concentration in bent tubules. (D–F) FEM predictions of TGFβ concentration gradient in bent tubules. (G) Schematic of changes in concentration and gradient as angle of bend increases. (H–J) Frequency maps of engineered tubules treated with EGF. Scale bars, 50 μm.

Nonetheless, Serra and colleagues recently reported that Wnt5a mediates a subset of the inhibitory actions of TGFβ in the mammary gland, and in particular that Wnt5a−/− mammary glands show enhanced branch extension [56], consistent with our findings. These authors also showed that Wnt5a activated a collagen receptor, discoidin domain receptor (DDR)-1, and surmised that activation of DDR1 inhibited mammary branching. Consistent with this hypothesis, DDR1 has been implicated in control of collective cell migration during branching morphogenesis in culture [79] and DDR1-null animals show increased branching in the mammary gland at 3 months of age [80]. It remains to be determined whether the Smad-mediated inhibitory effects of TGFβ in the mammary gland occur through DDR1.

The signaling pathways activated by TGFβ *in vivo* to inhibit mammary branching morphogenesis may, of course, be different than those we have found to be activated in culture. Nonetheless, our results suggest that this endogenous morphogen may accomplish its inhibitory effects through more than one pathway. Our results also suggest that branch initiation may use a different set of signaling pathways from branch extension, just as limb bud initiation and extension are controlled by separate pathways (reviewed by [19]). We were only able to distinguish between these fundamentally different aspects of morphogenesis (initiation and extension) by using culture models in which we could build hundreds of identical tissues with predictable and reproducible branching patterns. Using this system, we were able to test for the first time whether mammary epithelial cells are inhibited from branching in response to the local concentration or gradient of TGFβ, and found that TGFβ concentration is responsible for the inhibitory effect. This adds to the evidence that epithelial cells

modify their microenvironment and condition it with multi-functional factors such as TGFβ that allow branches to sense and repel each other during the sculpting of the epithelial tree.

Supplementary materials related to this article can be found online at doi: 10.1016/j.yexcr.2011.03.017.

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