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# PI3K regulates branch initiation and extension of cultured mammary epithelia via Akt and Rac1 respectively



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## ABSTRACT

The tree-like architecture of the mammary gland is generated by branching morphogenesis, which is regulated by many signals from the microenvironment. Here we examined how signaling downstream of phosphoinositide 3-kinase (PI3K) regulates different steps of mammary branching using three-dimensional culture models of the mammary epithelial duct. We found that PI3K was required for both branch initiation and elongation. Activated Akt was enhanced at branch initiation sites where its negative regulator, PTEN, was blocked by signaling via Sprouty2 (SPRY2); inhibiting Akt prevented branch initiation. The pattern of SPRY2 expression, and thus of Akt activation and branch initiation, was controlled by mechanical signaling from endogenous cytoskeletal contractility. In contrast, activated GTP-bound Rac1 localized to the leading edge of nascent branches and was required for branch elongation. These data suggest that the PI3K network integrates mechanical and biochemical signaling to control branching morphogenesis of mammary epithelial cells.

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## Introduction

Ramified epithelia such as the mammary gland develop via branching morphogenesis, which generates functionally efficient, complex, but well-ordered tissue architectures (Hogan, 1999). Epithelial branching morphogenesis is a reiterative process that can be divided into several unique steps, including primary bud formation, branch initiation, and branch elongation (Affolter et al., 2003; Zhu and Nelson, 2012). During branching of the murine mammary gland, terminal end buds (TEBs) form at the tips of the ducts and invade into the surrounding stroma in response to stimulation from ovarian hormones. New primary branches then form through bifurcation of the TEBs and secondary side branches sprout laterally from the primary ducts. This process is reiterated until the entire mammary fatpad is filled with the epithelial tree (Sternlicht, 2006).

Branching of an epithelium typically arises through changes in cell shape, size, division, motility, invasiveness, or combinations

thereof (Affolter et al., 2003; Zhang and Vande Woude, 2003; Zhu and Nelson, 2012). These cellular level processes are regulated by different signals including hormones, growth factors, receptor tyrosine kinases (RTKs), extracellular matrix (ECM) molecules, and endogenous mechanical stress (Gjorevski and Nelson, 2011; Metzger and Krasnow, 1999). The phosphoinositide 3-kinase (PI3K)/Akt signaling network can be activated by several RTKs to regulate a wide array of cellular functions (Martelli et al., 2006). Upon activation by growth factor stimulation, PI3K produces the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>), which in turn binds to the serine/threonine protein kinase Akt (Matsui et al., 2003), recruiting it to the membrane for subsequent activation by phosphorylation. Akt regulates a variety of transcription factors as well as several cytoplasmic proteins (Manning and Cantley, 2007). For example, Akt promotes cell invasion by increasing the production of matrix metalloproteinase-9 (MMP9) (Kim et al., 2001) and modulates cell migration by phosphorylating p21-activated kinase (PAK) (Zhou et al., 2003).

In normal tissue, PI3K signaling can be attenuated by PTEN, a ubiquitously expressed tumor suppressor protein that dephosphorylates PIP<sub>3</sub> into PIP<sub>2</sub> (Polak and Buitenhuis, 2012). PTEN is subject to regulation at the transcriptional level, as well as at the post-translational level by phosphorylation (Tamguney and Stokoe, 2007). The phosphorylated carboxy terminal tail of PTEN interacts with its catalytic domain, serving as a pseudosubstrate and therefore causing auto-inhibition (Odriozola et al., 2007; Tamguney and Stokoe, 2007). One of the proteins that regulates

**Abbreviations:** ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; GEF, guanine nucleotide exchange factor; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; PAK, p21-activated kinase; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; ROCK, Rho kinase; RTK, receptor tyrosine kinase; SPRY2, sprouty2; TEB, terminal end bud; 3D, three-dimensional.

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PTEN post-translationally is Sprouty2 (SPRY2), leads to dephosphorylation of PTEN at several sites, increasing its activity and thereby decreasing PI3K signaling (Edwin et al., 2006).

This regulation of the PI3K pathway is potentially relevant for branching morphogenesis. SPRY2 encodes an RTK inhibitor (Hacohen et al., 1998; Kramer et al., 1999), and was first identified as a novel antagonist of fibroblast growth factor (FGF) signaling during development of the *Drosophila* trachea. In SPRY mutants, overactivation of the FGF pathway induces ectopic branching from the stalks of the primary trachea (Hacohen et al., 1998). SPRY2 similarly acts as a negative regulator of branching morphogenesis in several other organs. Targeted overexpression of SPRY2 in mouse peripheral lung epithelium using the surfactant protein-C (SP-C) promoter reduces branching and inhibits epithelial proliferation (Mailleux et al., 2001). Conversely, transiently reducing the levels of SPRY2 in cultured mouse lung explants results in a dramatic increase in branching as well as in the expression of lung epithelial marker genes (Tefft et al., 1999). Consistently, tissue-specific overexpression of SPRY2 in the ureteric bud causes severe defects in kidney development including a reduction in organ size owing to inhibition of ureteric branching (Chi et al., 2004). SPRY2 is thus considered as a negative regulator of branching morphogenesis (Mailleux et al., 2001; Tefft et al., 1999) and is well poised to modulate PI3K signaling via PTEN.

Whereas the PI3K pathway has also been implicated in branching of the mammary gland (Li et al., 2002; Renner et al., 2008; Utermark et al., 2012), it remains unclear what downstream signals are involved in the regulation of the different steps of the branching process. Conditional deletion of PTEN in the mouse mammary gland via the use of MMTV-Cre leads to accelerated ductal extension and enhanced lateral branching during puberty (Li et al., 2002). Similarly, MMTV-mediated transgenic expression of an activated myristoylated p110 $\alpha$  protein, the catalytic subunit of PI3K, leads to increased ductal branching (Renner et al., 2008). In addition to Akt, PI3K activates Rac1 (Kolsch et al., 2008), which promotes the formation of lamellipodia (Ridley et al., 1992) and induces the expression of various MMPs (Mack et al., 2011), which are themselves required for mammary branching morphogenesis (Fata et al., 2007; Simian et al., 2001; Wiseman et al., 2003). PI3K is thus considered to be a positive regulator of branching by mammary epithelial cells (Zhu and Nelson, 2012).

Here we used organotypic culture models to characterize how signaling downstream of PI3K regulates the branch initiation and extension steps of mammary epithelial branching morphogenesis. We found that Akt is required for branch initiation downstream of growth factor signaling, and its activity is controlled by the levels of phosphorylated PTEN. In contrast, activated Rac1 found at the tips of the branches is required for branch elongation. Phosphorylated PTEN and Akt are regulated by endogenous mechanical stress, which signals through SPRY2. Therefore, the PI3K pathway couples mechanical signaling and biochemical signaling in its regulation of branching morphogenesis by mammary epithelial cells.

## Materials and methods

### Cell culture and reagents

Functionally normal EpH4 mouse mammary epithelial cells were cultured in DMEM/F12 growth medium (Hyclone) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals), 50  $\mu$ g/ml gentamicin (Sigma), and 5  $\mu$ g/ml insulin (Sigma). NMuMG mouse mammary epithelial cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 50  $\mu$ g/ml gentamicin, and 10  $\mu$ g/ml insulin. EpH4 and NMuMG cells have been widely used in studies of mammary epithelial branching morphogenesis. Cells were grown

in a 37 °C incubator with 5% CO<sub>2</sub> and treated with the following reagents diluted to the concentrations indicated: LY294002 (50  $\mu$ M; Cell Signaling); wortmannin (1  $\mu$ M; Cell Signaling); Akt inhibitor IV (5-(2-benzothiazolyl)-3-ethyl-2-[2-(methylphenylamino)ethenyl]-1-phenyl-1H-benzimidazolium iodide, 20  $\mu$ M; EMD Millipore); Y27632 (10  $\mu$ M; Tocris); blebbistatin (25  $\mu$ M; Sigma); calyculin A (0.5 nM; Calbiochem); NSC23766 (100  $\mu$ M; Tocris).

### shRNA and expression constructs

shRNAs targeting the *Mus musculus* sequence of SPRY2 (NM\_011897) were purchased from Open Biosystems (Table S1). Control scrambled shRNA (Sarbassov et al., 2005), constitutively active PI3K plasmid pCAG-Myr-p110-IH (Takahashi et al., 2003), and control plasmid pCAGEN (Matsuda and Cepko, 2004) were obtained from AddGene.

### Transfections

One day before transfection, cells were seeded in 10-cm dishes at 60–70% confluence. The following day, transfections were performed using 10  $\mu$ g of each relevant plasmid and SuperFect Transfection Reagent (Qiagen).

### Preparation and branching of cell clusters

Clusters of mouse mammary epithelial cells were prepared by overnight shaking (180 rpm at 37 °C for 15 h) in the presence of 0.083% (w/v) of pluronic F108 (BASF) as previously described (Lee et al., 2011). Cell clusters ~100  $\mu$ m in diameter were collected by centrifugation (200 rpm for 1 min) and embedded within 4 mg/ml of non-pepsinized native type I collagen (Koken), which was gelled as described previously (Pavlovich et al., 2011). A cell-free layer of collagen was placed underneath the layer containing clusters. Growth medium including no growth factor, EGF (4.2 nM, Invitrogen), or HGF (4.2 nM, Sigma) was added to the samples to induce branching.

### Engineered mammary epithelial tubules

Elastomeric stamps of poly(dimethylsiloxane) (PDMS; Sylgard 184, Ellsworth Adhesives) containing a relief of the desired tissue geometry were fabricated using a combination of photolithography and soft lithography (Gomez and Nelson, 2011; Nelson et al., 2008). Briefly, stamps were rendered non-adhesive by coating with 1% (w/v) bovine serum albumin in phosphate-buffered saline (PBS). Stamps were placed atop a drop of collagen, which was then gelled as described above. After removing the stamps, a suspension of mammary epithelial cells was allowed to settle within the molded collagen cavities. Any extra cells were washed away with growth medium and a gelled collagen lid was placed on top of the sample. The epithelial cells formed cylindrical tissues of the shape and size of the collagen cavities, and remained quiescent until induced to branch by addition of medium containing EGF or HGF.

### Imaging and immunofluorescence analysis

The branching pattern of microfabricated tubules was measured as described previously (Nelson et al., 2006). Briefly, samples were fixed in 4% paraformaldehyde for 15 min, stained for nuclei using Hoechst 33342 (Invitrogen), and visualized using a Hamamatsu Orca CCD camera attached to a Nikon Eclipse Ti microscope. The binarized images of 50 tubules were stacked with ImageJ software to obtain a pixel frequency map, which was subsequently color-coded in Adobe Photoshop.

For immunofluorescence analysis, samples were fixed, permeabilized (0.5% Igepal CA-630 for 10 min, then 0.1% Triton X-100 in PBS for 15 min), and blocked with 10% goat serum (Atlanta Biologicals) overnight. Samples were then incubated with primary antibodies against pAkt (Cell Signaling), pPTEN (Sigma), or cleaved caspase-3 (Cell Signaling), followed by incubation with Alexa Fluor-conjugated secondary antibodies (Invitrogen). Signal intensity as a function of spatial location was measured by taking a line scan through the midline of each tubule, and the end/middle ratio was calculated by dividing the signal intensity at the two ends by the signal intensity in the middle.

To visualize active GTP-bound Rac1, samples were fixed and permeabilized as described above. After blocking with 10% goat serum, the tissues were incubated with GST-PAK-PBD (Cytoskeleton) (final concentration 2.5  $\mu$ g/100  $\mu$ l of blocking solution) overnight, followed by rabbit anti-GST antibody (Sigma) and secondary goat anti-rabbit antibody.

### Immunoblotting

Whole cell lysates were prepared from mammary epithelial cells transfected with pCAG-Myr-p110-IH or pCAGEN control (AddGene) using RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, pH 8.0, and protease inhibitor cocktail). Immunoblot analysis was performed using antibodies against p110 $\alpha$  (Cell Signaling), pAkt (Cell Signaling), Akt (Cell Signaling), pPTEN (Sigma), PTEN (Sigma), SPRY2 (Abcam) or  $\beta$ -actin (Cell Signaling).

### Statistical analysis

All experiments were conducted independently at least three times. The Student's *t*-test was used to compare branch lengths and the percentage of branching clusters between conditions. *P* values < 0.05 were considered to be statistically significant.

## Results

### *The PI3K pathway is required for branching morphogenesis of mammary epithelial cells*

We used two complementary three-dimensional (3D) culture models to recapitulate the mammary epithelium and investigate the role of the PI3K pathway in the multi-step process of branching morphogenesis. In the first model, clusters of EpH4 mouse mammary epithelial cells were embedded within 3D ECM gels of type I collagen (Chen et al., 2009; Hirai et al., 1998; Lee et al., 2011; Pavlovich et al., 2011; Simian et al., 2001). Robust branching morphogenesis occurred after the addition of epidermal growth factor (EGF) or hepatocyte growth factor (HGF) (Fig. 1A and B). These branches were multicellular in morphology and extended up to 100  $\mu$ m into the surrounding collagen gel, as reported by several previous studies using this system (Chen et al., 2009; Hirai et al., 2007; Lee et al., 2011; Mori et al., 2013; Simian et al., 2001). Strikingly, we observed that branching was completely blocked in the presence of 50  $\mu$ M LY294002 or 1  $\mu$ M wortmannin (Fig. 1A and B), both of which are potent inhibitors of PI3K (Walker et al., 2000). We observed similar results in clusters of NMuMG mouse mammary epithelial cells (Fig. S1), a widely used model for the mammary epithelium (Owens et al., 1974).

In the second 3D culture system, mammary epithelial tubules of specific and reproducible pre-defined geometries were engineered within collagen gels using replica micromolding (Gjorevski and Nelson, 2010, 2012; Lee et al., 2011; Nelson et al., 2006, 2008; Pavlovich et al., 2011). When treated with HGF, branches formed

specifically at the ends of cylindrical tubules (Fig. 1C and D). Consistent with the results in 3D clusters, branching morphogenesis of the engineered tubules was blocked in the presence of LY294002 or wortmannin (Fig. 1C and D). Together, these results suggest that the PI3K pathway is necessary for mammary epithelial cells to branch in response to either EGF or HGF.

### *Inhibiting Akt blocks branching morphogenesis of mammary epithelial cells*

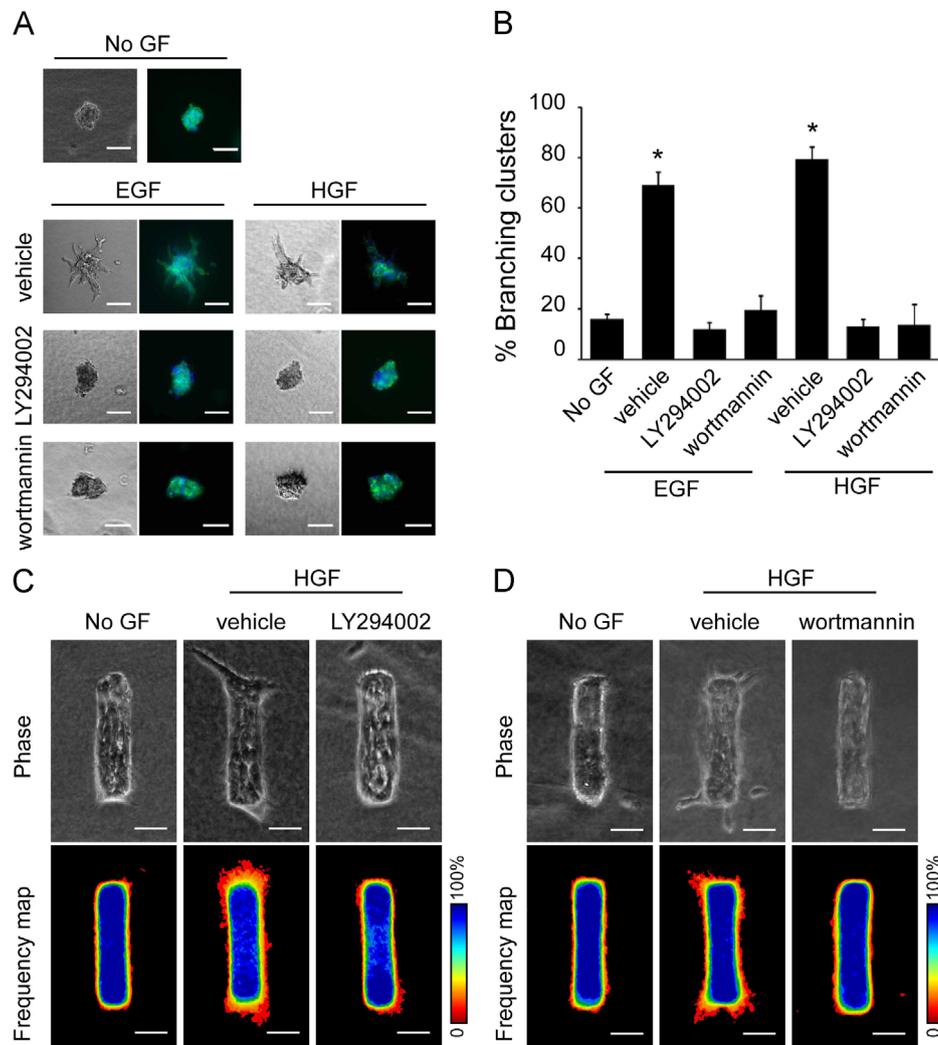
The PI3K pathway regulates divergent physiological processes that include cell proliferation, growth, differentiation, apoptosis, metabolism, and motility (Martelli et al., 2006). Many of these functions relate to the ability of PI3K to lead to activation of the serine/threonine kinase, Akt (Engelman, 2009). Akt thus acts as a critical signaling node downstream of PI3K.

To explore the role of downstream effectors of PI3K in the regulation of branching of mammary epithelial cells, we first disrupted the activity of Akt using a benzimidazole-derivative pharmacological inhibitor. Branching morphogenesis was evaluated using both the 3D cluster and engineered tubule assays, as described above. In both assays, branching was blocked in the presence of the Akt inhibitor (Fig. 2A and B; Fig. S1). To confirm that PI3K regulates branching of mammary epithelial cells via Akt, we expressed ectopically an activated form of the p110 $\alpha$  subunit of PI3K while simultaneously disrupting Akt activity with the pharmacological inhibitor. Expression of activated p110 $\alpha$  was confirmed by immunoblotting analysis (Fig. 2C). Surprisingly, expression of activated p110 $\alpha$  was not sufficient, by itself, to induce branching in the absence of treatment with growth factors (Fig. 2D). However, when induced to branch, tubules expressing activated p110 $\alpha$  extended branches that were significantly longer than those of controls (Fig. 2E). The initiation of these branches was completely blocked when tubules expressing activated p110 $\alpha$  were treated with the Akt inhibitor (Fig. 2D). These data suggest that activation of Akt is required for PI3K-mediated branching morphogenesis of mammary epithelial cells.

### *Akt is activated by HGF and phosphorylated Akt (pAkt) is sustained at future branch sites*

One advantage of the engineered tubule assay is that the initial geometry of the tubules is tightly controlled and the positions at which they branch are highly predictable and reproducible. This predictability permits examination of the signals that influence branching within the tissues at time points before branches have initiated. In the cylindrical tubules, branches initiate specifically at the two ends. That the Akt inhibitor completely blocked branching led us to examine the spatial distribution of activated Akt, which is characterized by phosphorylation at its carboxy terminal tail (Yang et al., 2002). Immunofluorescence analysis revealed that the phosphorylation of Akt increased rapidly and homogeneously throughout the mammary epithelial tubules within 30 min after treatment with HGF (Fig. 3A). At 2 h after induction with HGF, the pAkt signal in the body of the tubules had decreased, whereas the signal at the ends of the tubules was sustained (Fig. 3A and B). By contrast, total Akt was evenly distributed throughout the tubules (Fig. 3C). At this time point, we also observed that cells at the ends of the tubules had formed lamellipodia that projected out into the surrounding collagen (Fig. 3A, arrow). Thereafter, the pAkt signal gradually decreased and had reduced to background levels by 4 h after addition of growth factor (Fig. 3A).

PTEN is the primary inhibitory regulator of Akt phosphorylation and the PI3K pathway. Phosphorylation of PTEN decreases its membrane localization and activity (Tamguney and Stokoe, 2007). To investigate how the spatial distribution of pAkt is regulated,



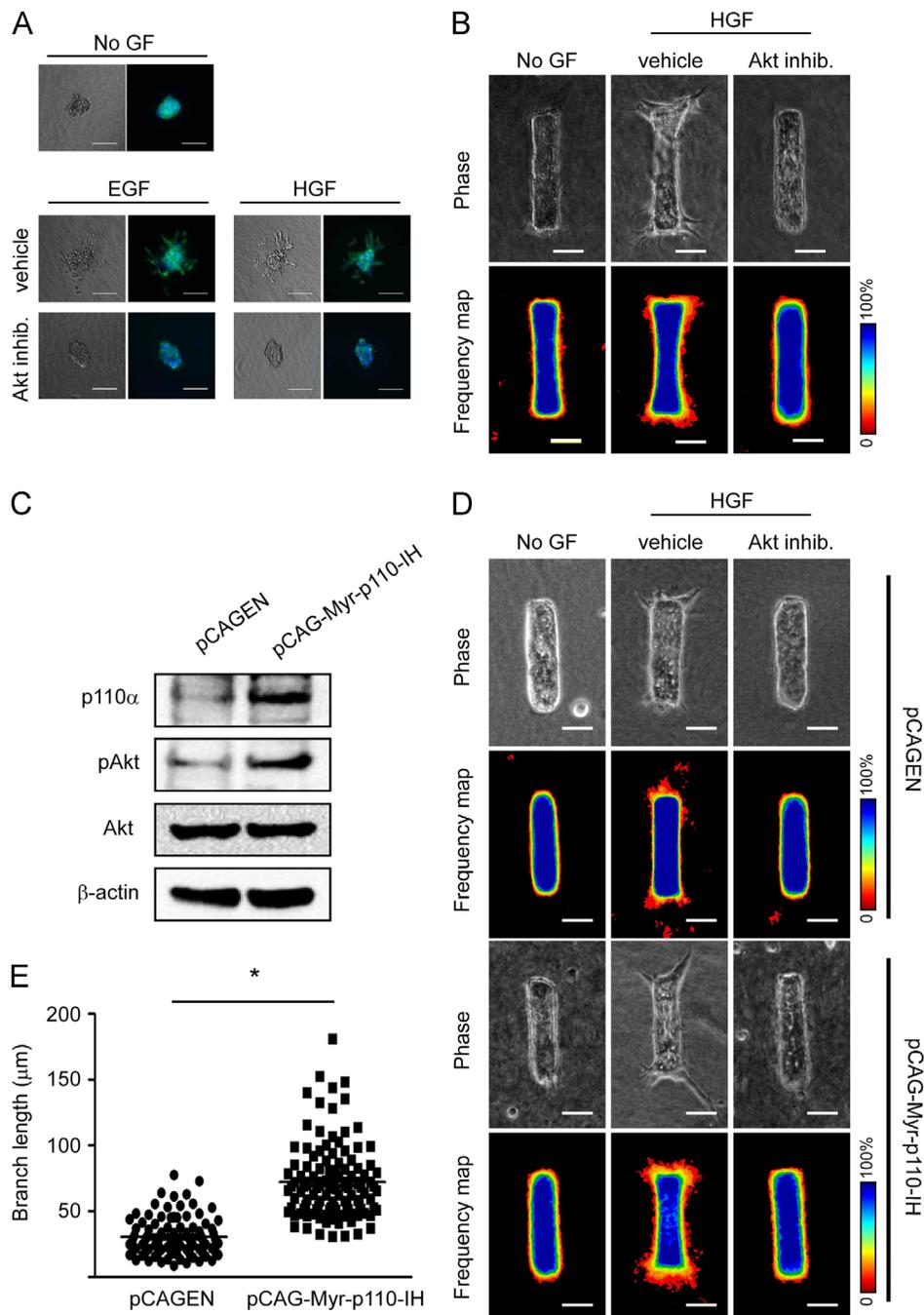
**Fig. 1.** Branching morphogenesis induced by EGF or HGF is blocked by PI3K inhibitors. (A) Clusters of mammary epithelial cells were embedded in collagen gel and treated with no growth factor (No GF), EGF or HGF with or without PI3K inhibitors and monitored for branching after 24 h. Shown are phase contrast and fluorescence images depicting nuclei (blue) and actin (green). Scale bars, 100  $\mu$ m. (B) Branching in the cluster assay was quantified by counting the percentage of clusters that branched in each well. \*Indicates  $p < 0.05$  compared with No GF control. (C, D) Mammary epithelial tubules were treated with No GF or HGF with or without LY294002 (C) or wortmannin (D) for 24 h. Shown are phase contrast images and frequency maps of 50 tubules stained for nuclei. Scale bars, 50  $\mu$ m. Color bars indicate frequency.

we examined the levels of phosphorylated PTEN (pPTEN) in the tubules. We found that pPTEN was also enhanced in the cells located at the ends of the tubules (Fig. 3D), which was similar to the spatial distribution of pAkt. In contrast, total PTEN showed an even distribution within the tubules (Fig. 3E). These data suggest that a local reduction in PTEN activity at the ends of the tubules leads to enhanced phosphorylation and activation of Akt, thus permitting branch initiation.

#### *High mechanical stress at the ends of the tubules sustains activation of the PI3K pathway*

The mechanical properties of the microenvironment have been shown to alter activation of the PI3K pathway (Hoshino et al., 2007; Rice et al., 2010). Shear stress activates Akt and Akt-related signaling proteins in vascular smooth muscle cells and this activation is closely correlated with shear-induced vascular smooth muscle cell reorientation (Rice et al., 2010). When bovine aortic endothelial cells are exposed to cyclic strain, the levels of pPTEN and pAkt increase in a time-dependent manner (Hoshino et al., 2007). We found previously that mechanical stress is distributed non-uniformly across engineered epithelial tissues, with higher stress concentrated at the ends of the tubules

(Gjorevski and Nelson, 2010, 2012). These observations led us to hypothesize that the high mechanical stress at the ends of the tubules was responsible for the locally sustained activation of the PI3K pathway. To explore this possibility, we modulated cytoskeletal contractility to perturb the endogenous mechanical stress profile within the tissue. Contractility of the actin cytoskeleton is enhanced by signaling through the Rho effector, Rho kinase (ROCK) and phosphorylation of myosin light chain. Treatment with either Y27632, which inhibits the activity of ROCK (Davies et al., 2000), or with blebbistatin, a small molecule inhibitor of non-muscle myosin II (Kovacs et al., 2004), reduce actomyosin-mediated contractility and thus mechanical stress at the ends of the engineered tubules (Gjorevski and Nelson, 2010). In vehicle-treated control samples, we observed elevated levels of pAkt and pPTEN at the ends of the tubules (Fig. 4A–C). However, treatment with either blebbistatin or Y27632 led to a homogenous distribution of both pAkt (Fig. 4A and B) and pPTEN (Fig. 4A and C) with the tubules. Conversely, increasing contractility with the myosin light chain phosphatase inhibitor calyculin A (Ishihara et al., 1989) led to an increase in the relative levels of pAkt at the ends of the tubules (Fig. S2). These data suggest that the spatial distributions of pAkt and pPTEN are regulated in part by endogenous mechanical stress within the epithelial tissues.

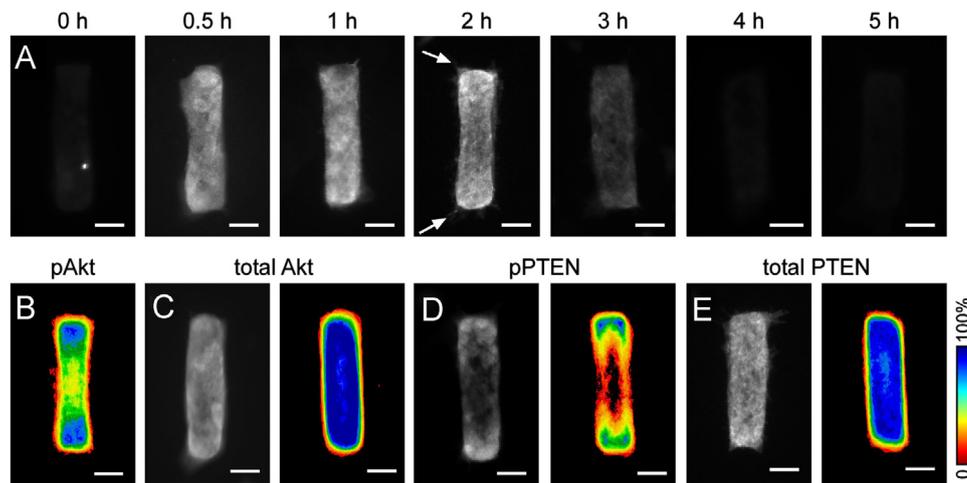


**Fig. 2.** Inhibiting Akt blocks branching morphogenesis of mammary epithelial cells. (A) Clusters of mammary epithelial cells were embedded in collagen gel and treated with no growth factor (No GF), EGF or HGF with or without Akt inhibitor (20  $\mu$ M) and monitored for branching after 24 h. Shown are phase contrast and fluorescence images depicting nuclei (blue) and actin (green). Scale bars, 100  $\mu$ m. (B) Mammary epithelial tubules were treated with No GF or HGF with or without Akt inhibitor for 24 h. Shown are phase contrast images and frequency maps of 50 tubules stained for nuclei. Scale bars, 50  $\mu$ m. Color bars indicate frequency. (C) Mammary epithelial cells were transfected with pCAGEN control vector or pCAG-Myr-p110-IH plasmid. Total protein was assessed by immunoblotting to determine the expression levels of p110 $\alpha$ , pAkt, total Akt, and  $\beta$ -actin. (D) Transfected mammary epithelial cells were used to form tubules, which were treated with No GF or HGF with or without Akt inhibitor for 24 h. Shown are phase contrast images and frequency maps of 50 tubules stained for nuclei. Scale bars, 50  $\mu$ m. Color bars indicate frequency. (E) Quantification of branch lengths from engineered tubules transfected with pCAGEN or pCAG-Myr-p110-IH plasmid. \* $p < 0.05$ .

*The PI3K pathway is negatively regulated by SPRY2 at branch sites during branching morphogenesis of mammary epithelial cells*

Previous studies have suggested that SPRY2 negatively regulates branching morphogenesis of the *Drosophila* trachea and several mammalian organs (Hacohen et al., 1998; Mailleux et al., 2001; Tefft et al., 1999). We were able to confirm a similar inhibitory role for SPRY2 in our 3D culture model. Small hairpin RNA (shRNA)-mediated knockdown of SPRY2 (shSPRY2) in mouse mammary epithelial cells led to ectopic and unpatterned

branching along the entire length of the tubules (Fig. S3). SPRY2 has been reported to lead to dephosphorylation of the inhibitory phosphates on the carboxy terminus of PTEN, thereby increasing PTEN levels and activity (Edwin et al., 2006). Indeed, we found that shRNA-mediated knockdown of SPRY2 increased the levels of pPTEN and pAkt (Fig. 5A). We therefore sought to determine whether SPRY2 affects the spatial distribution of pPTEN and pAkt within the 3D engineered tissues. Tubules comprised of mammary epithelial cells expressing shSPRY2, but not scrambled shRNA, showed higher relative levels of pAkt and pPTEN when treated



**Fig. 3.** pAkt and pPTEN are sustained at future branch sites. (A) Tubules were treated with HGF for different time periods and stained for pAkt. Scale bars, 50  $\mu$ m. (B) Frequency map of 50 tubules stained for pAkt after 2 h of treatment with HGF. (C–E) Immunofluorescence images and frequency maps of 50 tubules stained for total Akt (C), phosphorylated PTEN (D) or total PTEN (E) after 2 h of treatment with HGF. Scale bars, 50  $\mu$ m. Color bars indicate frequency.

with HGF (Fig. 5B and C). We obtained similar results using a second set of SPRY2-specific shRNAs (Fig. S4). These results suggest that SPRY2 blocks mammary epithelial branching morphogenesis in part by activating PTEN and thus inhibiting PI3K signaling along the trunk of the tissue.

#### *Inhibiting Rac1 blocks branch extension*

Our data revealed that Akt was activated soon after growth factor stimulation to promote branch initiation, and that the pAkt signal reduced to background levels after about 4 h, suggesting that signaling through effectors other than Akt were responsible for branch elongation. In addition to Akt, Rac1 has been implicated as a key effector downstream of PI3K (Kolsch et al., 2008). To investigate the role of Rac1 in the regulation of mammary branching by PI3K, we treated mammary epithelial tubules with the Rac inhibitor NSC23766, which prevents Rac1 activation by Rac-specific guanine nucleotide exchange factors (GEFs) without affecting the activation of Cdc42 or RhoA (Nassar et al., 2006). Treatment with NSC23766 did not completely block branching from the tubules, but instead significantly reduced the lengths of the branches that formed (Fig. 6A). We obtained similar results using the 3D cluster assay (Fig. 6B and C; Fig. S1). These data suggest that activation of Rac1 is required for growth factor-stimulated branch extension, but not branch initiation.

Our experiments showed that inhibiting Akt prevented branching completely whereas inhibiting Rac1 permitted branch initiation but prevented branch extension. These findings, along with the evidence that the activation of Akt reduced to background levels within 4 h of growth factor stimulation, led us to hypothesize that PI3K regulates branch extension through Rac1. To test this hypothesis directly, we transfected activated p110 $\alpha$  into mammary epithelial cells while simultaneously blocking Rac activity using NSC23766. In the presence of HGF, expression of activated p110 $\alpha$  resulted in longer branches than control plasmid-transfected cells (Fig. 6D and E). However, this effect was blocked by treatment with NSC23766 (Fig. 6D and E). These data suggest that activated p110 $\alpha$  induces branch extension through Rac1.

#### *Activated Rac1 is found at the leading edge of cells in the elongating branches*

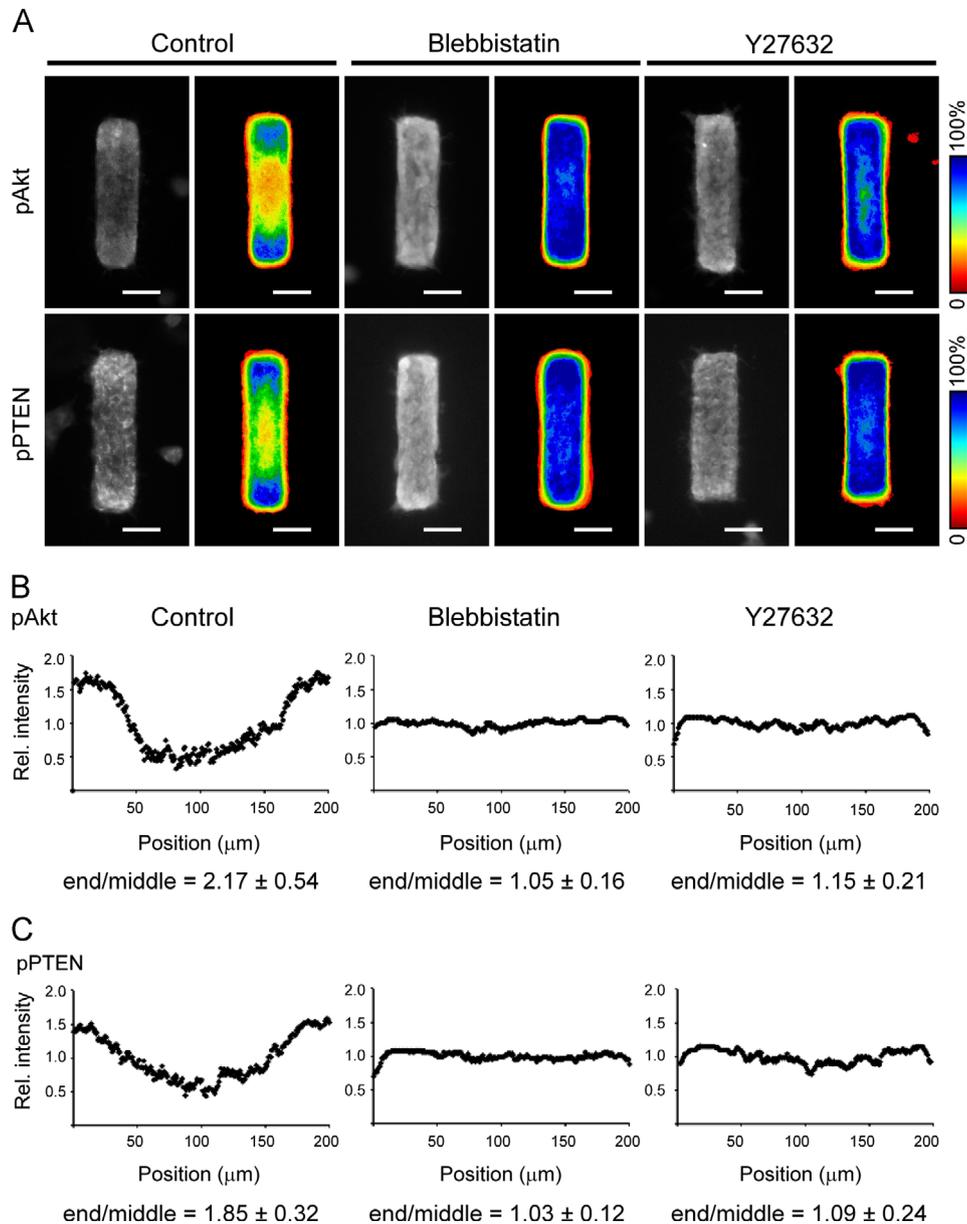
As shown by many studies, PI3K signaling through Rac1 is important for cell motility and invasion (Ridley et al., 1992). Rac1

has been implicated in establishing and maintaining the leading edge of motile cells, and Rac1 is dynamically activated at specific locations in the extending leading edge in different cell types including neutrophils and T cells (Cernuda-Morollon et al., 2010; Gardiner et al., 2002). However, no studies have reported the localization of activated Rac1 within morphogenetic tissues. Activated GTP-bound Rac1 can be detected in whole cell lysates by virtue of its ability to associate with the p21-binding domain (PBD) of the Rac1 effector protein PAK. To examine the spatial localization of activated Rac1 during branch extension, we took advantage of a GST-PAK-PBD fusion protein which only recognizes GTP-bound Rac1 (Cernuda-Morollon et al., 2010; Joffe et al., 2011). This in situ analysis revealed that Rac1 was activated at the tips of the cells leading the branches (Fig. 6F), consistent with a requirement for the activation of this GTPase during branch extension. These data thus suggest that activated Rac1 is spatially positioned to mediate PI3K-induced branch extension.

## **Discussion**

Here we investigated how the PI3K pathway regulates different steps of branching morphogenesis of mammary epithelial cells in culture. Our results suggest that PI3K regulates branch initiation and extension through distinct pathways (Fig. 7). High mechanical stress leads to sustained levels of phosphorylated activated Akt at future branch sites, and this activation is required for branch initiation. In contrast, Rac1 is activated at the leading edge of nascent branches and required for branch extension. The levels of pAkt are controlled by pPTEN, which in turn is regulated by mechanical signaling via SPRY2. Manipulating PI3K, Akt, Rac1, or SPRY2 thus alters branching morphogenesis of cultured mammary epithelial cells by tuning branch initiation or extension without inducing major alterations in cell death (Fig. S5).

The PI3K pathway has also been implicated in the regulation of branching morphogenesis of several other epithelial tissues. In a study which used the PTEN<sup>loxP/loxP</sup>; SP-C-rtTA<sup>+/-</sup>; TetO-Cre<sup>+/-</sup> mice, loss of PTEN resulted in impaired branching morphogenesis of the airways of the lung (Yanagi et al., 2007). Organ cultures of the ureteric bud taken from E10.5 mouse embryos revealed that inhibiting PI3K, but not MEK1 or p38 MAPK, completely blocks GDNF-dependent outgrowth, which suggests that induction of the ureteric bud requires PI3K (Tang et al., 2002). PI3K is also essential for branching after the ureteric bud has invaded the metanephric

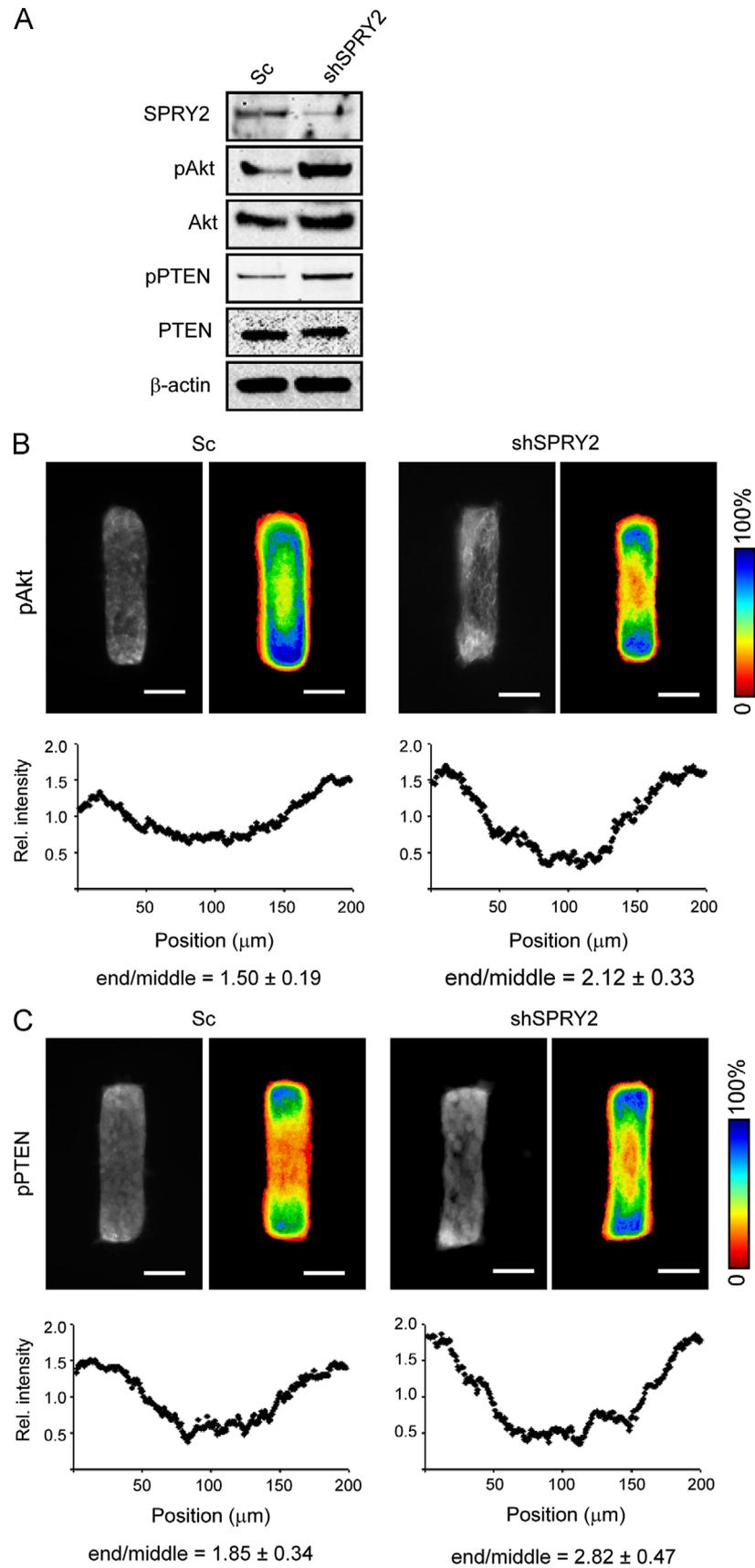


**Fig. 4.** Spatial distribution of pAkt and pPTEN is affected by altering cellular contractility. (A) Mammary epithelial tubules were treated with HGF with or without blebbistatin or Y27632 for 2 h. Shown are immunofluorescence images and frequency maps of 50 tubules stained for pAkt and pPTEN. Scale bars, 50  $\mu$ m. Color bars indicate frequency. (B, C) Average line scan of intensity of pAkt (B) or pPTEN (C) along the tubules.  $P < 0.0001$  for blebbistatin or Y27632 as compared to control.

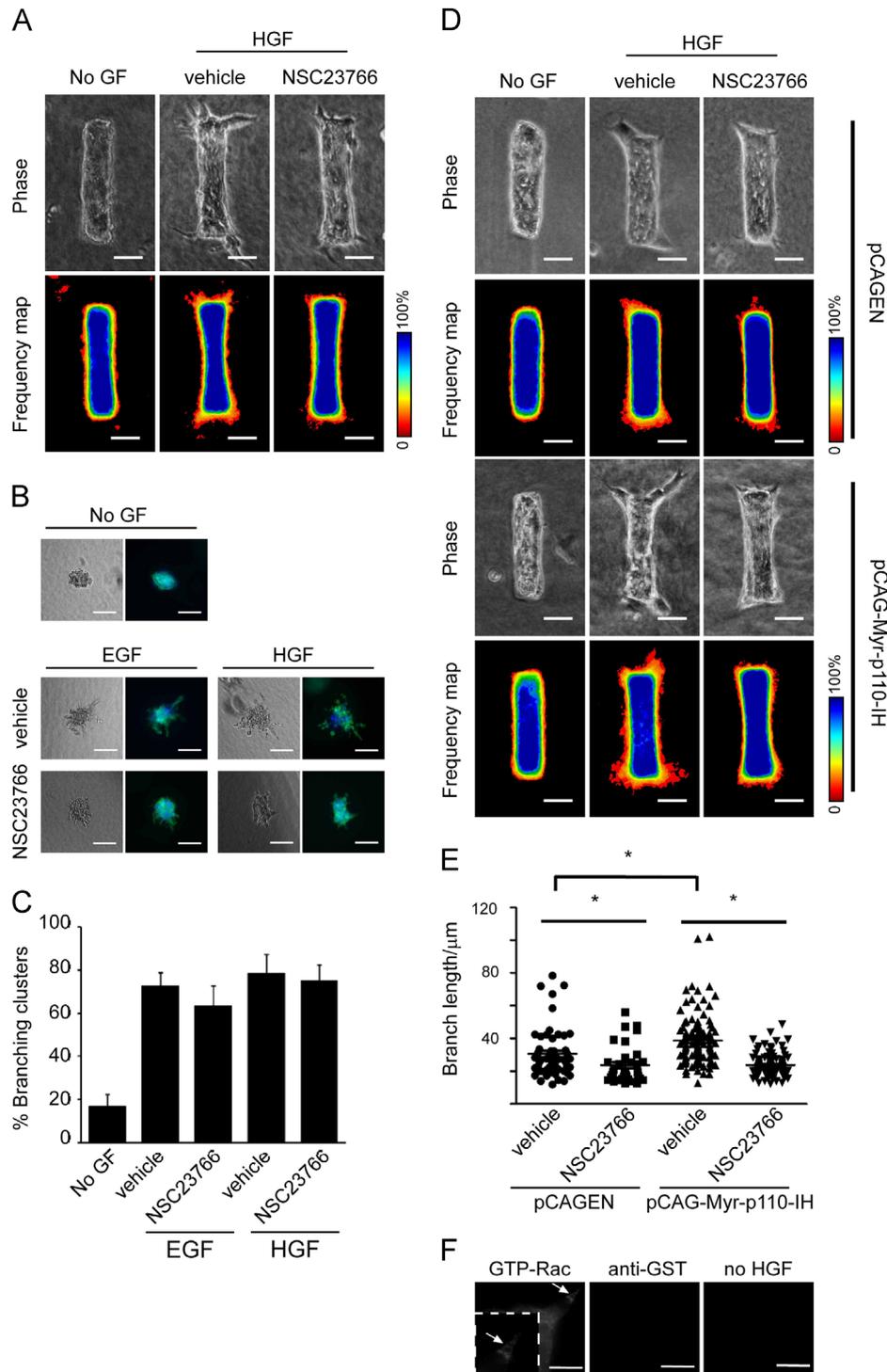
mesenchyme, since LY294002 inhibits branching in E11.5 metanephric kidneys (Tang et al., 2002). It is interesting to note that in our 3D culture models, the PI3K pathway is necessary but not sufficient to induce branching. In the absence of growth factors, we did not observe any branching in the tubules comprised of cells ectopically expressing activated p110 $\alpha$ . However, in the presence of growth factors, activated p110 $\alpha$  induced the extension of significantly longer branches. Transgenic mice overexpressing PI3K in the mammary gland also do not display excessive ductal branching until stimulated by hormones at puberty (Renner et al., 2008), even though complete knockout of p110 $\alpha$  significantly blocks mammary branching and ductal extension (Utermark et al., 2012). It is therefore likely that, in parallel to PI3K, other growth factor-induced signaling pathways are required to induce branching morphogenesis of mammary epithelial cells.

Our data suggest an important link between PI3K signaling and SPRY2 expression in the regulation of branching morphogenesis of

mammary epithelial cells. SPRY proteins are well appreciated as antagonists of FGF signaling during branching morphogenesis of the *Drosophila* tracheal system (Hacohen et al., 1998; Kramer et al., 1999). In that organ, SPRY is expressed in the tip cell (the actively branching cell) and antagonizes FGF-induced signaling in the adjacent stalk cells, thereby inhibiting ectopic secondary branching (Hacohen et al., 1998). Here, we found a similar pattern of expression in mammary epithelial tubules, with high levels of SPRY2 in cells located at future branch sites. We also found a similar inhibitory phenotype, although in this case, SPRY2 at branch sites activates PTEN in the trunk of the tubules (analogous to the stalk cells), thus leading to inhibition of Akt in those cells and preventing ectopic branching. How SPRY2 accomplishes this non-cell autonomous inhibition of branching in any organ remains a mystery, but our data suggest its reach is wider than the MAPK pathway previously defined. Indeed, SPRY2 was recently shown to signal through both MAPK and PI3K in the prostate epithelium (Schutzman and Martin, 2012).



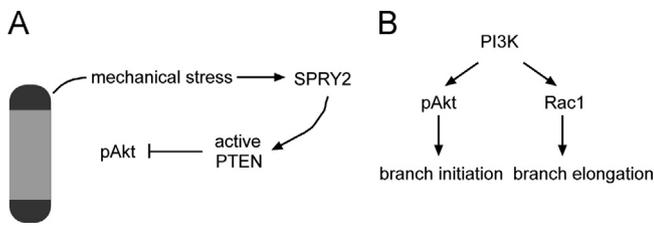
**Fig. 5.** Loss of SPRY2 leads to elevated levels of pPTEN and pAkt. (A) Mammary epithelial cells were transfected with scrambled shRNA (Sc) or shRNA targeting SPRY2 (shSPRY2). Levels of total and phosphorylated Akt and PTEN were analyzed by immunoblot. (B, C) Mammary epithelial cells were transfected with Sc or shSPRY2 and used to generate mammary epithelial tubules. Tubules were treated with HGF for 2 h and stained for pAkt (B) or pPTEN (C). Scale bars, 50  $\mu\text{m}$ . Color bars indicate frequency.  $P < 0.0001$  for shSPRY2 as compared to Sc control.



**Fig. 6.** Inhibiting Rac1 activity blocks branch extension. (A) Mammary epithelial tubules were treated with no growth factor (No GF) or HGF with or without the Rac1 inhibitor NSC23766 for 24 h. Shown are phase contrast images and frequency maps of 50 tubules stained for nuclei. Scale bars, 50  $\mu$ m. Color bars indicate frequency. (B) Clusters of mammary epithelial cells were embedded in collagen gel and treated with No GF, EGF or HGF with or without NSC23766 and monitored for branching after 24 h. Shown are phase contrast and fluorescence images depicting nuclei (blue) and actin (green). Scale bars, 100  $\mu$ m. (C) Branching in the cluster assay was quantified by counting the percentage of clusters that branched in each well. (D) Mammary epithelial cells were transfected with pCAGEN control vector or pCAG-Myr-p110-IH plasmid. Transfected cells were used to form tubules, which were treated with No GF or HGF with or without NSC23766 for 24 h. Shown are phase contrast images and frequency maps of 50 tubules stained for nuclei. Scale bars, 50  $\mu$ m. Color bars indicate frequency. (E) Quantification of branch length from engineered tubules transfected with pCAGEN or pCAG-Myr-p110-IH vector and treated with or without NSC23766. \* $P < 0.05$ . (F) Staining for activated Rac1. Shown is active GTP-bound Rac1 in HGF-treated, untreated, and GST-only control samples. Scale bars, 50  $\mu$ m.

Since the initial identification of SPRY in *Drosophila*, four mammalian SPRY proteins (SPRY1–4) have been discovered based on sequence similarity (Edwin et al., 2009; Guy et al., 2009). The expression of SPRY1, 2 and 4 is ubiquitous in both embryos and adults, whereas SPRY3 is restricted to the brain and testes

(Leeksa et al., 2002; Minowada et al., 1999; Panagiotaki et al., 2010). Though evidence suggests that RTK signaling regulates the expression levels of SPRY2 (Cabrita and Christofori, 2008), it is not clear whether other types of regulation are involved. Our data suggest a novel and prominent role for endogenous mechanical



**Fig. 7.** Roles of Akt, Rac1, PTEN, and SPRY2 in mammary epithelial branching morphogenesis. (A) pAkt at the ends of the tubules is required for branch initiation. In contrast, Rac1 is activated at the leading edge of nascent branches and required for branch extension. (B) High mechanical stress leads to high levels of SPRY2 at future branch sites, which leads to dephosphorylation and activation of PTEN along the trunk of the tissue. This in turn results in less pAkt in the body of the tubules, thus blocking ectopic branching from this location.

stress. Indeed, we found that SPRY2 expression increases in the presence of calyculin A, which enhances cytoskeletal contractility, and decreases in the presence of blebbistatin, which reduces contractility (Fig. S6). Although mechanical stress is well appreciated to regulate signal transduction and gene expression in several tissues, its effects on SPRY proteins are only beginning to be uncovered. SPRY1 is upregulated following mechanical loading in the heart (Huebert et al., 2004), and mechanical strain results in increased expression of Spred2, a SPRY-related protein, in osteoblasts (Ott et al., 2009). Our data suggest that mechanical stress regulates the expression of SPRY2 in mammary epithelial cells, thus controlling PTEN activation, Akt phosphorylation, and branch initiation. Global changes in tissue contractility lead to concentrated spatial patterns of mechanical stress (Gjorevski and Nelson, 2010), which explains why mechanical stress and the PI3K pathway are linked specifically and preferentially at branch sites. It will be interesting to determine precisely how mechanical stress alters the expression of SPRY2, both in the context of normal morphogenesis as well as in disease.

In summary, the work presented here demonstrates that the PI3K pathway integrates biochemical signaling from growth factors with mechanical signaling from endogenous cytoskeletal contractility to regulate the branching morphogenesis of mammary epithelial cells in culture. Given the subtle differences between conditions and the fact that pathways appear to intersect in a time-dependent manner during branching morphogenesis (Lee et al., 2011), we expect that the integration between these signaling pathways is complex. The ability to parse spatial and temporal changes in signaling is a key advantage of 3D epithelial culture models such as those used here; these simplified systems recapitulate many aspects of mammary epithelial morphogenesis (Lo et al., 2012; Nelson and Bissell, 2005), but lack the heterogeneous mesenchymal environment that also contributes to development of native tissues. Future studies exploring how PI3K and mechanical signaling are specifically regulated and how they work together to define the pattern of branching in vivo will provide insight into their respective roles during physiological and pathological development.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.04.029>.

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