Epithelial and Non-Epithelial *Patched-1 (Ptch1)* Play Opposing Roles to Regulate Proliferation and Morphogenesis of the Mouse Mammary Gland

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Keywords: Epithelial-stromal interactions, Patched-1, Smoothened, Hedgehog signaling

Summary Statement: Monkkonen et al define functions of *Patched-1* in the epithelium, stroma, and systemically (i.e. mammary gland extrinsic) in virgin mammary gland ductal patterning, proliferation, and histology.

List of Abbreviations: *Patched-1 (Ptch1)*, *Smoothened (Smo)*, Terminal End Bud (TEB), Adenovirus-*Cre* (Ad-*Cre*), Cleaved Caspase 3 (CC3), Estrogen Receptor (ER), Progesterone Receptor (PR)
Abstract

_Patched-1 (Ptc1)_ has epithelial, stromal, and systemic roles in murine mammary gland organogenesis, yet specific functions remain undefined. _Cre_-recombinase-mediated _Ptch1_ ablation in mammary epithelium increased proliferation and branching, but did not phenocopy transgenic expression of activated _Smoothened (SmoM2)._ Epithelium showed no evidence of canonical hedgehog signaling, and hyperproliferation was not blocked by SMO inhibition, suggesting a non-canonical function of PTCH1. Consistent with this possibility, nuclear localization of Cyclin B1 was increased. In non-epithelial cells, heterozygous _Fsp-Cre_-mediated _Ptch1_ ablation increased proliferation and branching, with dysplastic terminal end buds (TEB) and ducts. In contrast, homozygous _Ptch1_ ablation decreased proliferation and branching, producing stunted ducts filled with luminal cells showing altered ovarian hormone receptor expression. Whole gland transplantation into wild-type hosts, or estrogen/progesterone treatment rescued outgrowth and hormone receptor expression, but not histology. Bone marrow transplantation failed to rescue outgrowth. Ducts of _Fsp-Cre;Ptch1^{fl/fl}_ mice were similar to _Fsp-Cre;SmoM2_ ducts, but _Fsp-Cre;SmoM2_ outgrowths were not stunted, suggesting that the histology may be mediated by _Smo_ in the local stroma, with systemic _Ptc1_ required for ductal outgrowth and proper hormone receptor expression in mammary epithelium.
Introduction

Organogenesis is the developmental process by which organs are constructed from undifferentiated germ layers. This process requires coordinated interactions between cells and tissues, and for endocrine-targeted organs, cellular responses to extrinsic hormonal signals. These developmental processes are studied extensively, since they are often perturbed in cancer, and other diseases.

The hedgehog signaling network regulates cellular and tissue interactions essential for metazoan organogenesis (Briscoe and Thérond, 2013; Johnson et al., 2011; Robbins et al., 2012). In "canonical" mammalian hedgehog signaling, Patched-1 (PTCH1) and Patched-2 (PTCH2) inhibit downstream signaling by Smoothened (SMO), an effector protein, in the absence of ligands. When SMO is inhibited, the GLI3, and to a lesser extent GLI2, transcription factors are proteolytically cleaved into transcriptional repressors. With Hedgehog ligand (Sonic (SHH), Indian (IHH), or Desert (DHH) hedgehog) binding to PTCH1/2 on a responding cell, PTCH1/2 inhibition of SMO is released, and GLI transcription factors (GLI1/2/3) remain full-length transcriptional activators. GLI-mediated transcription regulates proliferation, survival, cell fate, and autoregulatory feedback.

Some hedgehog network members function "non-canonically", independent of the signaling cascade described above. For example, PTCH1 can sequester hedgehog ligand to restrict the range of signaling, sequester Cyclin B1 in the cytoplasm to inhibit cell cycle progression, or induce Caspase 9/3-mediated apoptosis in the absence of hedgehog ligands (Barnes et al., 2001; Chen and Struhl, 1996; Mille et al., 2009). In mammary epithelial cells, SHH-stimulated PTCH1 promotes ERK1/2 phosphorylation independent of SMO (Chang et al., 2010). In the mouse mammary epithelium, constitutively activated Smo (SmoM2) acts as a G-protein-coupled receptor (GPCR) via Gαi2 to induce proliferation independent of GLI activity, since hyperproliferation was not blocked by pharmacological inhibition of GLI1/2 (Villanueva et al., 2015), consistent with observations by Riobo et al (Riobo et al., 2006). TGFβ induces Gli2 to regulate osteolysis independent of Smo (Johnson et al., 2011), while K-Ras inhibits GLI2 function and GLI3 processing in the context of Smo activation (Lauth et al., 2010). A long non-coding RNA induced by the Twist transcription factor upregulates Gli1 and Gas1 (canonical hedgehog target genes) in vitro (Zhou et al., 2015). These non-canonical functions necessitate the evaluation of multiple network genes to fully understand hedgehog network function in a given organ.

The murine mammary gland is an excellent model for organogenesis (Daniel and Smith, 1999). In this system, organogenesis is initiated in the embryo, yielding a rudimentary ductal tree at birth, which remains relatively growth quiescent until puberty begins at 3-4 weeks.
of age. With puberty, systemic hormones (e.g. estrogen, progesterone, and other hormones) drive ductal outgrowth via terminal end buds (TEBs). TEBs are transient structures that migrate and proliferate to produce a branched ductal tree filling the mammary fat pad by 8-10 weeks of age. With conception, pregnancy hormones induce alveolar development to prepare for lactation. After lactation, the gland involutes and remodels to resemble the adult virgin (Hennighausen and Robinson, 2005; Macias and Hinck, 2012).

Previously, analysis of mammary glands from mice heterozygous for a germline knockout allele (Ptch1Δ/Δ), or homozygous for a hypomorphic Ptch1 allele (Ptch1mes), demonstrated distinct functions for Ptch1 in the mammary epithelium, local stroma, and systemically (mammary gland extrinsic) during postnatal virgin development (Lewis et al., 1999; Moraes et al., 2009). Neither the specific functions of Ptch1, nor the association of these phenotypes with canonical hedgehog signaling were investigated. Here, we employ tissue-compartment specific ablation of Ptch1, transplantation, and tissue-specific expression of an activated Smo allele, to specify epithelial, stromal, and systemic Ptch1 functions in virgin mammary gland development.

**Results**

Ptch1 *Inhibits Proliferation and Branching of Mammary Epithelium*

To determine the null phenotype of Ptch1 in mammary epithelium, mTmG-tagged primary mammary epithelial cells homo- or heterozygous for a Ptch1 conditional ablation allele (Ptch1fl) were treated with Adenovirus-Cre (Ad-Cre), and transplanted into the mammary fat pads of SCID/bg recipients (wild-type for Ptch1). Ad-Cre treated, Ptch1+/+ mTmG+ primary cells were transplanted to contralateral fat pads. This approach increased recombination compared to MMTV-Cre (Wagner et al., 2001).

Eight weeks post transplantation, we observed that while Ptch1+/+ glands had 75±11 branch points (Fig. 1A) and Ptch1+/+ glands had a comparable 65±5 branch points (Fig. 1B), Ptch1fl glands showed an increase in branch points with 131±14 (Fig. 1C) (p<0.011, paired t test; quantification Fig. 1D). Increased branching was present with increased proliferation by Ki67 expression. Eight weeks post transplantation, 1.4±0.2% of Ptch1+/+ cells were Ki67 positive (Fig. 1E). Ptch1fl ducts were 2.8±0.6% Ki67 positive (Fig. 1F) (n.s., paired t test), while proliferation in Ptch1fl cells increased significantly to 4.1±0.7% Ki67 positive (Fig. 1G) (p<0.01, paired t test; quantification, Fig. 1H). Apoptosis was comparable between Ptch1+/+ (Fig. 1I), Ptch1fl (Fig. 1J), or Ptch1fl ducts (Fig. 1K), by cleaved caspase 3 (CC3) staining, while CC3 positive cells were observed in positive control lymph nodes (Fig. 1K, inset).
Previously, the histological defects of Ptch1\(^{\Delta/+}\) or Ptch1\(^{mes}\) ducts (Lewis et al., 1999; Moraes et al., 2009) were resolved with epithelial fragment transplantation. Consistently, histology was normal in Ad-Cre;Ptch1\(^{fl/+}\) and Ad-Cre;Ptch1\(^{fl/fl}\) outgrowths (Fig. 1F-G), showing definitively that histological defects were not due to epithelial Ptch1 loss.

To ensure that the phenotypes were not due to differences in Cre-dependent recombination, we determined that GFP positive cells contributed similarly to ductal outgrowths by immunofluorescence. An average of 81±4% of Ptch1\(^{+/+}\), 69±6% of Ptch1\(^{fl/+}\), and 85±2% of Ptch1\(^{fl/fl}\) mammary epithelial cells were GFP positive (no difference, paired t test) (Fig. 1L).

To investigate whether Ptch1\(^{fl/fl}\) outgrowths displayed activated canonical hedgehog signaling due to reduced Smo inhibition, Ptch1\(^{+/+}\) and Ptch1\(^{fl/fl}\) epithelium was evaluated by qPCR for hedgehog network gene expression. Of the genes evaluated, only Ptch2 mRNA was slightly upregulated (Fig. 2F) (p<0.016), suggesting that canonical hedgehog signaling was not activated.

**Increased proliferation in Ad-Cre;Ptch1\(^{fl/fl}\) Ducts is Not Due to Activated Canonical Hedgehog Signaling**

Gene expression analysis indicated that phenotypes from Ptch1 loss may not be due to increased SMO activity (Fig. 1M), consistent with unique mammary gland phenotypes elicited by epithelium-limited ablation of Ptch1 and activation of Smo (Visbal et al., 2011). To test whether hyperproliferation requires Smo activity, we evaluated hyperproliferation due to Ptch1 loss in the context of pharmacological inhibition of SMO.

To confirm SMO inhibitor (IPI926) efficacy, we tested whether IPI926 would blunt uterine scratch-induced decidualization, since canonical hedgehog signaling is required for decidualization (Matsumoto et al., 2002; Villanueva et al., 2015). The unscratched, vehicle and IPI926-treated uteri displayed comparable histology (Fig. 2A, top panels). The scratched, vehicle treated tissue displayed histological changes consistent with decidualization (Fig. 2A, bottom left) that were absent with IPI926 treatment (Fig. 2A, bottom right). QPCR supported IPI926 efficacy: Ptch1, Ptch2, Gli1, and Hhip mRNA levels were significantly reduced in the IPI926-treated, scratched tissue relative to the vehicle treated, scratched tissue (Fig. 2B).

Given the efficacy of IPI926 in vivo at the chosen dose, we treated mice bearing Ad-Cre;Ptch1\(^{+/+}\) and Ptch1\(^{fl/fl}\) outgrowths with IPI926 three days before harvest at 8 weeks. We assayed for Ki67 to determine whether hyperproliferation was blocked by SMO inhibition.
Vehicle-treated $Ptch1^{+/+}$ ducts were 0.8±0.25% Ki67 positive, and $Ptch1^{fl/fl}$ ducts increased to 2.6±0.35% Ki67 positive (Fig. 2C, left panels (p<0.0019, paired t test). With IPI926 treatment, $Ptch1^{+/+}$ ducts were 1.2±0.6% Ki67 positive, while IPI926-treated $Ptch1^{fl/fl}$ ducts retained increased proliferation with 3.2±0.71% Ki67 positive cells (Fig. 2C, right panels) (p<0.02, paired t test). No significant differences were observed between vehicle and IPI926-treated $Ptch1^{+/+}$, or vehicle and IPI926-treated $Ptch1^{fl/fl}$ outgrowths (quantification, Fig. 2D). Since IPI926 did not block hyperproliferation, this phenotype is not likely due to SMO activation.

Since the hyperproliferation with Ad-Cre-mediated $Ptch1$ loss was not blocked by SMO inhibition, we assayed whether the non-canonical function of PTCH1 in cytoplasmic retention of Cyclin B1 could be involved (Barnes et al., 2001). Immunofluorescence showed that while $Ptch1^{+/+}$ ducts displayed 0.17±0.05% cells with nuclear Cyclin B1 (Fig. 2E), $Ptch1^{fl/fl}$ outgrowths showed a significant increase to 0.45±0.11% (Fig. 2 F) (p<0.0085, paired t test) (quantification, Fig. 2G).

Fsp-Cre-Mediated Disruption of $Ptch1$ in Non-epithelial Cells Alters Mammary Gland Histology, Proliferation and Morphology

To investigate non-epithelial functions of $Ptch1$, we crossed $mTmG$-tagged, $Ptch1^{fl/+}$ and $Ptch1^{fl/fl}$ with Fsp-Cre mice to ablate $Ptch1$ in fibroblasts and myeloid cells. At 6 weeks of age, control mice (Fsp-Cre;$Ptch1^{+/+}$, and $Ptch1^{fl/+}$ or $Ptch1^{fl/fl}$ mice lacking Fsp-Cre) displayed histologically normal TEBs (Fig. 3A), while many Fsp-Cre;$Ptch1^{fl/+}$ TEBs had irregular shape, microlumens, and an ill-defined cap cell layer (Fig. 3B). Histologically normal TEBs were also observed (Fig. 3B, inset). Additionally, Fsp-Cre;$Ptch1^{fl/fl}$ mice showed body cells detached from the cap cells (Fig. 3C), with few histologically normal TEBs (Fig. 3C, inset). Both mutants showed increased stromal condensation adjacent to TEBs relative to controls (Fig. 3B-C) (quantification, Fig. 3D).

To test whether the dysmorphic TEBs had altered proliferation, we assayed BrdU labeling. Control TEBs were 29±2% BrdU positive (Fig. 3E), Fsp-Cre;$Ptch1^{fl/+}$ TEBs were 18±2% positive (Fig. 3F), and Fsp-Cre;$Ptch1^{fl/fl}$ were 15±2% positive (Fig. 3G). Both mutants had less BrdU labeling than controls (controls versus Fsp-Cre;$Ptch1^{fl/+}$ p<0.01, controls versus Fsp-Cre;$Ptch1^{fl/fl}$ p<0.001; ANOVA/Tukey’s test, Fig. 3H).

With respect to apoptosis, control TEBs were 1.9±0.3% CC3 positive (Fig. 3I), while Fsp-Cre;$Ptch1^{fl/+}$ mice had reduced (0.79±0.14%) CC3 positivity (Fig. 3J) (p<0.001, ANOVA/
Tukey’s test). Fsp-Cre;Ptch1fl/+ TEBs had comparable apoptosis rates of 1.5±0.4% (Fig. 3K) (quantification, Fig. 3L).

In eight week old mice, control glands displayed normal branching (66±8 per 2x field) (Fig. 4A). Despite reduced TEB proliferation, Fsp-Cre;Ptch1fl/+ glands were hyperbranched (129±11) (Fig. 4B). In contrast, Fsp-Cre;Ptch1fl/fl mice had reduced branching (18±4) (Fig. 4C). (Fig. 4A-C show a portion of the fat pad; quantification in Fig. S1). Control and Fsp-Cre;Ptch1fl/+ fat pads were 95±3% and 95±4% filled with epithelium, respectively, while Fsp-Cre;Ptch1fl/fl ducts were dramatically stunted with 39±5% fat pad filled (p<0.0001, ANOVA/ Tukey’s test) (Fig. 4D). Time points after eight weeks were not evaluated due to skin phenotypes and low mutant survival.

Control ducts had normal histology at eight weeks (Fig. 4E). However, Fsp-Cre;Ptch1fl/+ ducts displayed microlumens and partially filled ducts (Fig. 4F). Histologically normal ducts were also observed (Fig. 4F, inset). Fsp-Cre;Ptch1fl/fl ducts were more frequently filled (Fig. 4G), with less frequent normal histology (Fig. 4G, inset). There was increased ductal filling in Fsp-Cre;Ptch1fl/+ (p<0.01) or Fsp-Cre;Ptch1fl/fl ducts (p<0.001, ANOVA/ Tukey’s test) (Fig. 4H).

Ductal filling was confirmed by confocal microscopy of control (Fig. S2A), Fsp-Cre;Ptch1fl/+ (Fig. S2B) and Fsp-Cre;Ptch1fl/fl glands (Fig. S2C) (Supplemental movies 1 and 2 for control and Fsp-Cre;Ptch1fl/fl ducts). To determine which cell type filled the ducts, we performed immunostaining for cytokeratin 8 (K8) (luminal cells), and cytokeratin 5 (K5) (basal cells). Control ducts had K8+ cells surrounded by K5+ cells as expected (Fig. S2D). Fsp-Cre;Ptch1fl/+ (Fig. S2E) and Fsp-Cre;Ptch1fl/fl ducts (Fig. S2F) displayed K8+ cells filling ducts. Insets show histologically normal ducts. ZO-1 (zona occludens 1) expression, which stains tight junctions and apical surfaces of luminal cells, was also assayed by immunofluorescence. While ZO-1 stained the control duct lumens as expected (Fig. S2G), stained Fsp-Cre;Ptch1fl/+ ducts confirmed the presence of microlumens (Fig. S2H), while Fsp-Cre;Ptch1fl/fl ducts displayed abnormal, concentric patterning (Fig. S2I).

We observed a significant reduction in mammary gland mass at 8 weeks of age in homozygous mutants (0.04±0.01g) versus controls (0.16±0.02g) or heterozygotes (0.13±0.03g) (Fig. S3A) (p<0.01, ANOVA/Tukey’s test). Mammary glands of homozygous mutants were also smaller than controls when normalized to body weight (Fig. S3B) (p<0.05). Fsp-Cre;Ptch1fl/fl body weights (14±0.5g) were also decreased versus controls (23±1.2g) and heterozygotes (22±0.7g) (p<0.0001, Fig. S3C). Heterozygotes displayed no significant changes.
With respect to proliferation at 8 weeks, control ducts were 2.0±0.8% BrdU positive (Fig. 4I), while Fsp-Cre;Ptch1fl/+ ducts were hyperproliferative (5.4±1.2%) (p<0.0334, ANOVA/Tukey’s test) (Fig. 4J). In contrast, ducts of Fsp-Cre;Ptch1fl/fl mice showed virtually no proliferation (0.04±0.03%) (p<0.0001, ANOVA/ Tukey’s test; quantification Fig. 4K). Thus, Fsp-Cre;Ptch1fl/+ mammary ducts had increased proliferation and branching, while the stunted ducts of Fsp-Cre;Ptch1fl/fl animals lacked proliferation.

Given that Fsp-Cre induces recombination in mammary gland extrinsic cells, and that the stunted duct and hypoproliferative phenotypes observed in Fsp-Cre;Ptch1fl/fl mice were similar to the stunted, hypoproliferative ducts of estrogen receptor alpha (ERα) knockout mice, and reduced side branching and proliferation similar to the progesterone receptor (PR) knockout mice, we hypothesized that hormone signaling in Fsp-Cre;Ptch1fl/fl mice was disrupted (Bocchinfuso and Korach, 1997; Lydon et al., 1995).

At eight weeks of age, ER and PR expression was perturbed in Fsp-Cre;Ptch1fl/fl mice. While controls had 36±6% ERα positive cells (Fig. 4M), and heterozygotes had comparable levels (38±6%) (Fig. 4N), ERα expression in Fsp-Cre;Ptch1fl/fl ducts increased to 62±2% (Fig. 4O) (p<0.05, ANOVA/ Tukey’s test; quantification, Fig. 4P). Control ducts were 18±3% PR positive (Fig. 4Q), and heterozygotes were comparable with 27±4% (Fig. 4R). However, PR expression was abolished in homozygotes (0.9±0.3%) (Fig. 4S) (p<0.01, ANOVA/ Tukey’s test; quantified in Fig. 4T).

Whole Gland Transplantation Rescues Ductal Growth and ER/PR Expression, but Not Histological Defects of Fsp-Cre;Ptch1fl/fl Mice

To determine whether phenotypes caused by Fsp-Cre-mediated disruption of Ptch1 were due to Ptch1 functions in mammary gland extrinsic cells, Cre- control and Fsp-Cre;Ptch1fl/fl donor glands were transplanted contralaterally into pre-pubertal recipient SCID/bg animals wildtype for Ptch1. Eight weeks post-transplantation, the stunted duct phenotype was rescued, with similar fat pad filling between Cre- (61±11%) (Fig. 5A) and Fsp-Cre;Ptch1fl/fl donor glands (64±7%) (Fig. 5B) (quantification, Fig. 5C; p<0.8285, paired t test). In contrast to eight week old homozygous mutants from genetic crosses (Fig. 4C), TEBs were observed in transplanted Fsp-Cre;Ptch1fl/fl glands (Fig. 5B). While Cre- donor ducts displayed normal histology (Fig. 5D), Fsp-Cre;Ptch1fl/fl glands were frequently filled-in (Fig. 5E), with some histologically normal ducts (Fig. 5E inset). Cre- donor ducts were 4±0.8% filled, while ducts of Fsp-Cre;Ptch1fl/fl donors were 26±5% filled (p<0.0252, paired t test) (quantification, Fig. 5F). Thus, the filled-in duct phenotype is due to loss of Ptch1 in the local mammary stroma, while
the stunted duct growth was due to Ptc1 disruption in mammary gland extrinsic, Fsp positive cells.

In whole gland transplants, ERα positivity was comparable between Cre- (29±2%) (Fig. 5G) and Fsp-Cre;Ptc1fl/fl donor ducts by immunostaining (32±1%) (Fig. 5H) (n.s., paired t test; quantification, Fig. 5I). Similarly, ducts of Cre- donors were 36±3% PR positive (Fig. 5J), while Fsp-Cre;Ptc1fl/fl donors were 46±3% (Fig. 5K) (quantification, Fig. 5L). This modest increase was significant (p<0.023, paired t test). The normalization of ER and PR levels by whole gland transplantation demonstrates that mammary gland extrinsic Ptc1 regulates ductal outgrowth and the characteristic ER/PR patterning of the mammary epithelium.

Stunted Ducts, but not Histological Defects of Fsp-Cre;Ptc1fl/fl Mutants are Rescued by E+P Treatment

Since whole gland transplantation showed that ‘systemic’ Ptc1 regulates mammary ductal elongation (Fig. 5), and Fsp-Cre;Ptc1fl/fl mutants had altered ER/PR patterning (Fig. 4), we tested whether estrogen and progesterone (E+P) treatment would rescue the stunted ducts. Relative to the control, vehicle-treated glands, the E+P-treated control glands had increased tertiary branching (Fig. 6A, left panels) as expected. Here, Fsp-Cre;Ptc1+/+, and Ptc1fl/+ or Ptc1fl/fl mice lacking Fsp-Cre were used as controls. While the vehicle-treated control fat pads were 86±4% filled, vehicle-treated mutants displayed reduced fat pad filling (57±4%) and side branching as previously (Fig. 6A, upper right) (p<0.01, ANOVA/Tukey’s vs. control). E+P-treated control fat pads were 90±3% filled, and E+P-treated Fsp-Cre;Ptc1fl/fl fat pads were 85±6% filled, consistent with rescue of the stunted ducts (quantification, Fig. 6B; p<0.01, ANOVA/Tukey’s test vs. vehicle-treated mutants; no difference, E+P-treated control versus E+P-treated mutants). E+P-treated Fsp-Cre;Ptc1fl/fl outgrowths still displayed reduced branching than the E+P-treated controls (Fig 6A, lower left). Thus, Ptc1 may regulate estrogen and/or progesterone production to drive pubertal ductal elongation.

We also evaluated proliferation in response to E+P. While vehicle-treated, control glands displayed 1.3±0.3% BrdU positivity, vehicle-treated Fsp-Cre;Ptc1fl/fl mutants displayed reduced positivity (0±0%) as previously (Fig. 6C). E+P-treated, control ducts were 14.5±1.8% BrdU positive (p<0.0001, ANOVA/Tukey’s test versus vehicle-treated controls), whereas E+P-treated Fsp-Cre;Ptc1fl/fl ducts were 2.5±0.5% BrdU positive (Fig. 6C) (significantly reduced vs. E+P-treated controls, p<0.0001 by ANOVA). Thus, while E+P induced proliferation and branching, the response in Fsp-Cre;Ptc1fl/fl mutants was attenuated compared to controls. The attenuated proliferation and tertiary branching displayed by the Fsp-Cre;Ptc1fl/fl mutants in response to E+P suggests that alveologenesis would be perturbed in these animals. Consistent with E+P rescue of ductal outgrowth suggesting
functional defects in the ovary, *Fsp-Cre;Ptch1<sup>fl/fl</sup>* animals displayed a disrupted estrous cycle (Fig. S4,A-C) and dramatically reduced fertility over 6.5 weeks (Fig. S4D).

**Bone Marrow Transplantation does not Rescue Outgrowth of Fsp-Cre;Ptch1<sup>fl/fl</sup> mutants**

Myeloid cells regulate pubertal ductal outgrowth of the mammary epithelium (Gouon-Evans et al., 2000), a subset of which are *Fsp-Cre* positive (Bhowmick et al., 2004). We therefore tested whether bone marrow transplantation could rescue ductal elongation in the *Fsp-Cre;Ptch1<sup>fl/fl</sup>* mutants. Six weeks after transplantation, control recipients of control bone marrow displayed 89±1.5% fat pad filled, and a normal ductal structure (Fig. 6D, upper left). Here, controls consisted of *Fsp-Cre;Ptch1<sup>+/+</sup>* and *Ptch1<sup>fl/+</sup>* or *Ptch1<sup>fl/fl</sup>* mice lacking *Fsp-Cre*. We observed engraftment of the transplanted cells, since GFP+ cells were present with transplantation of *Fsp-Cre;Ptch1<sup>+/+</sup>* cells to a Cre- recipient (Fig. 6D, inset). Control bone marrow transplanted to *Fsp-Cre;Ptch1<sup>fl/fl</sup>* recipients filled only 61±2% of the fat pad (Fig. 6D, upper right). Glands from control recipients of *Fsp-Cre;Ptch1<sup>fl/fl</sup>* bone marrow displayed 89±1.2% fat pad filled (Fig. 6D, bottom). Control bone marrow transplanted to *Fsp-Cre;Ptch1<sup>fl/fl</sup>* mutants displayed reduced fat pad filling relative to the other groups (quantification Fig. 6D, lower right; p<0.0001, ANOVA/Tukey's test). The inability of control bone marrow to rescue the mutant phenotype, or of mutant bone marrow to induce stunted ducts in controls indicates that *Ptch1* does not regulate ductal elongation in myeloid cells.

**Fsp-Cre-Mediated Expression of Activated Smoothened Phenocopies Histological Defects in Fsp-Cre;Ptch1<sup>fl/fl</sup> Mice**

To evaluate whether the non-epithelial effects of *Ptch1* loss were possibly mediated by *Smo*, we assessed whether *Fsp-Cre*-mediated expression of activated *Smo* could recapitulate phenotypes in *Fsp-Cre;Ptch1<sup>fl/fl</sup>* mice. At 6 weeks of age, Cre- control TEBs had normal histology (Fig. 7A), while *Fsp-Cre;SmoM2* TEBs displayed dysmorphia (Fig. 7B and inset). Dysmorphic TEBs had irregular shape, microlumens, and increased periductal stromal condensation (quantification, Fig. 7C). These TEBs were similar to *Fsp-Cre;Ptch1<sup>fl/+</sup>* (Fig.3B), *Fsp-Cre;Ptch1<sup>fl/fl</sup>* (Fig. 3C) and *Ptch1<sup>Δ/+</sup>* TEBs (Lewis et al., 1999).

At eight weeks of age, whole mounts of Cre- (Fig. 7D) and *Fsp-Cre;SmoM2*+ glands (Fig. 7E) were comparable. Cre- fat pads were 85±2% filled, and *Fsp-Cre;SmoM2*+ fat pads were slightly less filled(74±2%) (quantification, Fig. 7F) (p<0.0225, t test). This reduction was less than in *Fsp-Cre;Ptch1<sup>fl/fl</sup>* mutants, which displayed ~40% filled fat pads at 8 weeks (Fig.4D). While Cre- ducts at 8 weeks displayed normal histology (Fig. 7G), *Fsp-Cre;SmoM2*+ ducts often contained extra cells and microlumens (Fig. 7H), with some ducts appearing
normal (Fig. 7H inset). Neither mutant TEBs nor mature ducts displayed altered proliferation relative to Cre- ducts (Fig. 7I).

We tested whether the ERα and PR expression phenotypes of the Fsp-Cre;Ptch1fl/fl mutants are phenocopied by the Fsp-Cre;SmoM2 mutants. At 8 weeks of age, Cre- ducts were 33.2±2.5% ER positive, and Fsp-Cre;SmoM2 ducts were 35.1±2.3% positive (Fig. 7J), (not different by t test). While PR positivity was 35.9±2.7% in Cre- ducts, Fsp-Cre;SmoM2 ducts displayed slightly higher PR positivity (44.0±1.6%) (Fig. 7K, p<0.033, unpaired t test). Thus, Fsp-Cre;SmoM2 mutants do not display the increased ER or reduced PR expression present in the Fsp-Cre;Ptch1fl/fl mutants.

**Discussion**

Here, we elucidate tissue compartment-specific roles of Ptch1 in virgin mammary gland development using improved mouse models, and offer insight into signaling downstream of Ptch1. Ptch1 loss in the mammary epithelium elicits hyperproliferation and hyperbranching, likely independent of Smo. Data from Fsp-Cre;Ptch1fl/fl mutants indicate Ptch1 in Fsp+ fibroblasts regulates ductal histology, perhaps via Smo. We also show critical systemic roles of Ptch1 in ductal elongation and ER/PR expression in the mammary epithelium (see Fig. 8, model).

The Ad-Cre;Ptch1fl/fl model displayed hyperbranching and hyperproliferation in adult virgins. While mammary glands expressing SmoM2 also displayed hyperproliferation and hyperbranching (Moraes et al., 2009; Visbal et al., 2011), the Ptch1 loss and SmoM2 phenotypes diverge. SmoM2 expression yielded hyperproliferation and hyperbranching via a mixture of SmoM2+ and SmoM2- cells (Visbal et al., 2011), and elicited precocious alveolar budding—which are not the case with Ptch1 loss. Recently, we found that SmoM2-dependent hyperproliferation in the mammary gland requires Gαi2-dependent signaling (Villanueva et al., 2015). Hyperproliferation was blocked by inhibiting some Gαi subunits, but not inhibiting GLI1/2 (Villanueva et al., 2015). The differences between these models suggests that Ptch1 loss increases proliferation independent of Smo. However, we cannot exclude that divergent phenotypes could be due to different functions of SmoM2 (an allele identified in human basal cell carcinoma (Xie et al., 1998)) versus endogenous Smo. The phenotypic differences between SmoM2 conditional expression and Ptch1 loss in the mammary epithelium agree with the lack of canonical hedgehog target gene upregulation in Ad-Cre;Ptch1fl/fl ducts, and the inability of IPI926 to block hyperproliferation (Fig. 1-2), suggesting that hyperproliferation is SMO-independent. These data fit with reports that SMO (Moraes et al., 2009) and activated hedgehog signaling are absent from the normal mammary epithelium (Chang et al., 2010;
Hatsell and Cowin, 2006). From our data, it is possible that Ptch1 loss-induced hyperproliferation is due to reduced sequestration of Cyclin B1 outside the nucleus.

Data here confirm that non-epithelial Ptch1 regulates ductal histology. Analysis of Ptch1<sup>Δ/+</sup> (Lewis et al., 1999) and Ptch1<sup>mes/mes</sup> animals (Moraes et al., 2009) indicated that Ptch1 mediates ductal development; virgin Ptch1<sup>Δ/+</sup> mice had dysmorphic TEBs and filled-in ducts (Lewis et al., 1999). Whole Ptch1<sup>Δ/+</sup> glands transplanted to a wild-type host displayed filled-in ducts, while transplanted epithelial fragments did not, indicating that local stromal Ptch1 controls histology. From the Fsp-Cre model and transplantation experiments, we conclude that Ptch1 in the mammary fat pad fibroblasts- not myeloid cells- regulates histology. Based on the similar histology of Fsp-Cre;Ptch1<sup>fl/fl</sup> and Fsp-Cre;SmoM2 ducts, it seems that Ptch1 may regulate histology via Smo. Taken together, the Fsp-Cre and Ad-Cre studies indicate that most phenotypes of the Ptch1<sup>mes/mes</sup> mice, including altered TEB and ductal histology, and defective ductal elongation, were due to non-epithelial functions of Ptch1.

Aside from defining local stromal Ptch1 function, we have uncovered a role for mammary extrinsic, non-epithelial Ptch1 in pubertal mammary ductal outgrowth and ER/PR patterning in the mammary epithelium. The Fsp-Cre;Ptch1<sup>fl/fl</sup> mutant diverges from the Ptch1<sup>mes/mes</sup> mutant (Moraes et al., 2009), which displayed reduced ER and PR expression in stunted ducts. The differences between the Ptch1<sup>mes/mes</sup> and Fsp-Cre;Ptch1<sup>fl/fl</sup> models could be due to conditional ablation versus a hypomorphic allele, and/or global genetic manipulation versus loss of Ptch1 in Fsp positive cells. Altered ER/PR patterning may be due to abrogated hormone production by the ovary or pituitary, which may have been differentially effected in these models.

We have also further defined the ‘systemic’ function of Ptch1. Since E+P rescued the stunted ducts, Ptch1 may regulate E+P production and ovarian function to regulate pubertal outgrowth and proliferation. Indeed, the Fsp-Cre;Ptch1<sup>fl/fl</sup> mutants displayed functional defects, including abrogated cycling and fertility. Since the stunted duct phenotype was not rescued by bone marrow transplantation, Ptch1 does not function in myeloid cells to control ductal elongation.

Since Fsp-Cre-mediated Ptch1 loss reduced mammary gland mass, and the mammary fat pad consists primarily of adipocytes, it could be hypothesized that off-target Cre activity in adipocytes contributed to stunted ductal outgrowth. Mice with loss of adipocytes displayed stunted ducts (Landskroner-Eiger et al., 2010). While we cannot exclude that changes in the mutant adipocytes contributed to the stunted ducts, we did not observe Cre-dependent GFP expression in adipocytes, consistent with previous reports (Cheng et al., 2005); thus, such effects would likely be due to paracrine signaling.
Data here show stroma-to-epithelium and epithelium intrinsic \textit{Ptch1} functions in mammary gland development. It would be pertinent to determine whether bi-directional hedgehog mediated tissue interactions exist in other organs where only uni-directional signaling is reported, for example prostate and pancreas (Hebrok et al., 2000; Wang et al., 2003). Dissecting these tissue-tissue interactions is critical, since these developmental programs are inappropriately re-activated in cancer, and correlate with poor prognosis, for instance in prostate and pancreatic cancer (Bailey et al., 2009; Fan et al., 2004).

\textit{Implications for Ptch1 and Smo in Breast Cancer}

The hedgehog network is misregulated in many cancers including breast (Moraes et al., 2007; Rubin and de Sauvage, 2006). While hedgehog network activation induces basal cell carcinoma and medulloblastoma, data connecting hedgehog signaling and breast tumorigenesis are largely correlative, although \textit{Gli1} overexpression in mice induces tumorigenesis (Fiaschi et al., 2009).

\textit{PTCH1} protein levels are reduced in 50% of DCIS and invasive breast cancer (IBC), while 70% of DCIS and 30% of IBC display aberrant SMO, suggesting that hedgehog activation occurs frequently and early in human breast cancer (Moraes et al., 2007). Further, \textit{PTCH1} underexpression correlated with \textit{Ptch1} promoter methylation (Wolf et al., 2007). However, neither \textit{Ptch1}^{Δ/+} nor \textit{MMTV-SmoM2} mice show mammary tumors (Moraes et al., 2007; Moraes et al., 2009). Our data suggest that perhaps in the case of \textit{Ptch1}^{Δ/+}, the opposing functions of epithelial and systemic \textit{Ptch1} offset one another. These observations may explain why breast cancer incidence in Gorlin syndrome patients (Gorlin, 1987), who are heterozygous for germline \textit{Ptch1} loss-of-function and display higher risk for other cancers, is not higher than in the general population. Our Ad-\textit{Cre;Ptch1}^{fl/+} data suggest that \textit{Ptch1} heterozygosity would not alter mammary epithelial histology or proliferation.

Previous data suggest that high hedgehog ligand expression in tumor epithelium induces GLI1 (indicative of activated hedgehog signaling) in the adjacent stroma, which correlates with invasiveness and poor patient prognosis (O’Toole et al., 2011). Since local stromal loss of \textit{Ptch1} and non-epithelial activation of \textit{Smo} promote a DCIS-like phenotype in mammary epithelium, perhaps stromal \textit{Ptch1} loss promotes cancer-associated phenotypes. The data presented here suggest that loss of \textit{Ptch1} in fibroblasts may increase survival, reduce non-apoptotic cell death, or alter lumen formation. It would be interesting to determine whether \textit{Ptch1} heterozygosity correlates with DCIS in patients.
**Experimental Procedures**

**Animal models**

Mice carrying the *Ptch1<sup>c</sup>* allele, here termed *Ptch1<sup>f</sup>*, *Cre*-dependent conditional ablation allele were a gift from Dr. Brandon Wainwright (Ellis et al., 2003). Mice expressing *Cre* recombinase under the *Fsp1* (S100A4) promoter were a gift from Dr. Eric Neilson. These mice express *Cre* in fibroblasts and myeloid-derived leukocytes (Bhowmick et al., 2004). Mice carrying the *Gt(Rosa)26Sortm1(Smo/YFP)Amc/J SmoM2* conditional activation allele were obtained from Jackson Labs (Jeong et al., 2004). All animals were genetically tagged with the *mTmG Cre*-dependent reporter at the Rosa26 locus, *Gt(Rosa)26Sortm4(ACTB-tdTomato,−EGFP)Luo/J*. Cells lacking *Cre* recombinase express tdTomato Red, while cells expressing *Cre* recombinase display membrane-bound eGFP (Jackson Labs #007576)(Muzumdar et al., 2007).

For studies of *Ptch1<sup>f</sup>*, *Fsp-Cre;Ptch1<sup>f/+</sup>* males were crossed to *Ptch1<sup>f/+</sup>* or *Ptch1<sup>f/fl</sup>* females. *Fsp-Cre;SmoM2* mice were obtained by crossing *Fsp-Cre, mTmG* positive males to *SmoM2<sup>−/−</sup>* females (Xie et al., 1998). Genotyping for *Ptch1<sup>f</sup>*, *SmoM2*, and *Fsp-Cre* was performed as previously (Bhowmick et al., 2004; Ellis et al., 2003; Jeong et al., 2004). CB.17/1crHsd-Prkdc-scid-Lyst-bg (*SCID/beige*) mice (Harlan Laboratories) used for transplantation were from a breeding colony at Baylor College of Medicine. Animals were maintained according to the NIH Guide for the Care and Use of Experimental Animals with approval from Baylor College of Medicine Institutional Animal Care and Use Committee. For some analyses, 5-Bromo-2′-deoxyuridine (BrdU) (Sigma, B5002) in PBS was administered I.P. 2 hours prior to harvest at 0.01cc/ g body weight of 25 mg/mL solution.

**Adenoviral transduction and transplantation**

For epithelial ablation of *Ptch1*, mammary epithelial cells were harvested from #1, 3, 4, and 5 glands of 8 week old *Ptch1<sup>+/+</sup>* and *Ptch1<sup>f/fl</sup>* females with the lymph nodes removed. Glands were minced, digested with collagenase A (Roche Applied Science), 0.05% trypsin-EDTA, and strained into single cells (Visbal et al., 2011). Cells were infected at MOI 50 with Adenovirus-*Cre* (Ad-*Cre*) from the Vector Development Laboratory Core Facility at Baylor College of Medicine. Cells were recounted, resuspended in 50%PBS/50% Matrigel (BD Biosciences), and one hundred thousand *Ptch1<sup>+/+</sup>* and *Ptch1<sup>f/+</sup> or *Ptch1<sup>f/fl</sup>* cells were injected contralaterally into epithelium-free ‘cleared’ inguinal fat pads of 3 week old SCID/beige recipient mice (Deome et al., 1959) using a Hamilton syringe. Outgrowths were harvested 8 weeks later.
Whole mount Analysis

For fluorescent whole mount analysis, glands were agitated in 1 mL of 50% PBS/50% glycerol solution at 4°C overnight as described previously (Landua et al., 2009), and imaged using a Leica MZFL16 fluorescence stereomicroscope with a DFC300 FX camera. Branch points were counted manually using Metamorph software. Confocal microscopy was performed with a Leica TCS SP5 microscope. Non-fluorescent whole mounts were analyzed using neutral red (Sigma) staining, and imaged with a Leica MZ12.5 stereomicroscope with a Lumenera Infinity 1 camera, as described previously (Landua et al., 2009).

Immunofluorescence

Tissues were fixed in 4% paraformaldehyde in PBS for 3 hours at 4°C, paraffin-embedded, and sectioned at 3 μm thickness. Slides were rehydrated with a series of ethanols. Immunostaining was done using antigen retrieval in 0.1M Sodium Citrate Buffer, pH 6.0, and heating to 120°C in a decloaker (Biocare Medical). Primary antibodies were incubated overnight at 4°C with 8% M.O.M. protein reagent (Vector Labs BMK2202) and 1.5% goat serum. See Table S1 for antibody information. Micrographs were taken with a Zeiss Leica Axioskop 2 Plus with an AxioCam MRm FX camera. Cells from ten 40x fields, or ~1000 mammary epithelial cells were quantified per animal using Metamorph software. Each TEB was a data point, with ~300 cells/TEB.

Whole Gland Transplantation

Control (Ptch1fl/fl only, or Fsp-Cre only) and Fsp-Cre;Ptch1fl/fl donor glands at 3 weeks of age were transplanted contralaterally into 3 week old SCID/bg recipient mice as described previously (Lewis et al., 2001; Moraes et al., 2009). Glands were analyzed 8 weeks after transplantation.

Estrogen and Progesterone Treatment

Daily subcutaneous treatments of 1 μg β-estradiol (Sigma) and 1 mg (Sigma) progesterone in sesame oil, or sesame oil only, were administered for 14 days prior to animal harvest.

IPI926 Treatment (Inhibition of SMO)

Either IPI926 (Infinity) dissolved in 13% ethanol in Tween-20 (Sigma), or vehicle alone were administered by oral gavage. IPI926 doses were 40 mg/kg. For the mammary gland experiment, three daily treatments of vehicle or IPI926 were given prior to harvest.
Uterine Scratch

After ovarectomy post weaning and one week rest, a prescribed course of estrogen (0.1µg in 100 uL sesame oil for 3 days), 2 days rest, then estrogen + progesterone (1 mg progesterone + 6.7 ng estrogen daily until harvest) was administered prior to scratch of one uterine horn by blunted needle as described previously (Finn and Martin, 1972). Vehicle or IPI926 was administered for 7 days prior to, and the day of harvest 9 days after the first estrogen treatment. Hormone and IPI926 doses were timed as described previously (Villanueva et al., 2015).

QPCR

Tissues were collected into RNA Later (Qiagen) and frozen at -80 degrees Celsius. RNA was extracted with the Qiagen RNeasy Kit, and cDNA was synthesized with the Superscript III kit (Thermo Fischer) using random hexamers. The cDNA was analyzed using an Applied Biosystems 7500-Fast thermocycler for TaqMan quantitative PCR with standard conditions. Product accumulation was represented as $2^{\Delta Ct}$, with ANOVA of ΔCt values used for statistical comparison. 18S rRNA was used for normalization. Primers: see Table S2.

Bone Marrow Transplantation

Recipient animals 4-5 weeks of age received Baytril water 24 hours prior to irradiation and up to six days post-irradiation. Recipients received a dose of 400 centigray, and 24 hours later, bone marrow cells were harvested and isolated from 4-5 week old donor mice. Irradiated recipients received two million donor cells injected retro-orbitally. Recipients were harvested 6 weeks post transplantation.
**Author Contributions**

JDL participated in imaging, animal work, and bone marrow transplantation. APV assisted with experimental design, animal acquisition, and breeding. TM performed all other experiments, statistics, interpretation, and manuscript preparation. MTL participated in project conception, experimental design, data interpretation, and manuscript preparation, and was principle investigator.

**Acknowledgements**

We thank Andrew Ta, Rupali Sood, and Sydnee Spruiell for technical assistance. Thanks to Dr. Brandon Wainwright (University of Queensland) and Dr. Eric Neilson (Vanderbilt University) for mouse lines. We thank Drs. Tao Wang and Susan Hilsenbeck for advice regarding statistical analyses. We acknowledge Dr. Chad Shaw, Lukas Simon, and David Henke for bioinformatics assistance. We thank Yi Athena Ren and JoAnne Richards for critical advice. Thanks to Dr. Hugo Villanueva for experimental assistance. This work was supported by the National Institutes of Health and National Cancer Institute (RO1 CA127857 to M.T.L. and P30 CA125123 grant for the Dan L. Duncan Cancer Center to C. Kent Osborne). This project was supported by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the National Institutes of Health/ National Institutes of Allergy and Infectious disease, National Cancer Institute, and National Center for Research Resources (P30AI036211, P30CA125123, and S10RR024574 to Joel M. Sederstrom, Director).

Michael T. Lewis is a founder of, and Limited Partner in StemMed Ltd., and is a Manager of StemMed LLP, its General Partner.
References


Figure 1. Loss of \textit{Ptch1} in mammary epithelium increases branching and proliferation in adult virgin glands.

A-D: Fluorescent whole mounted A) Ad-Cre;\textit{Ptch1}\textsuperscript{+/+}, B) Ad-Cre;\textit{Ptch1}\textsuperscript{fl/+}, and C) Ad-Cre;\textit{Ptch1}\textsuperscript{fl/fl} outgrowths. GFP identifies Cre+ cells. The insets show tdTomato Red+, Cre-cells. Scale bar is 1 mm. D) Quantification showing increased branching in \textit{Ptch1}\textsuperscript{fl/fl} epithelium.

E-H: GFP and Ki67 stained E) Ad-Cre;\textit{Ptch1}\textsuperscript{+/+}, F) Ad-Cre;\textit{Ptch1}\textsuperscript{fl/+}, and G) Ad-Cre;\textit{Ptch1}\textsuperscript{fl/fl} ducts. H) Quantification showing increased proliferation in \textit{Ptch1}\textsuperscript{fl/fl} epithelium.

I-K: CC3 stained I) Ad-Cre; \textit{Ptch1}\textsuperscript{+/+}, J) Ad-Cre;\textit{Ptch1}\textsuperscript{fl/+}, K) Ad-Cre;\textit{Ptch1}\textsuperscript{fl/fl} ducts—all negative for CC3. The inset shows a CC3-stained lymph node used as a positive control.

L: Quantification showing similar percent GFP positivity in outgrowths of different genotypes.
M: Relative expression of hedgehog target genes in Ad-Cre;Ptch1+/+ and Ptch10/0 tissues. Data displayed as 2^-dCt with min and max values. Ptch2 expression is significantly higher in Ptch10/0 tissues by unpaired t test.

Graphs show data as mean ± SEM. Paired t tests were used to compare Ptch10/0 glands with contralateral Ptch1+/+ controls. * indicates p<0.05, and ** indicates p<0.01. Scale bar is 50 µm except A-C.
Figure 2. Hyperproliferation due to Ad-Cre-mediated Ptch1 loss is not due to SMO activation.

A: H and E stained vehicle-treated (left panels) and IPI926-treated (right panels) of unscratched (upper panels) and scratched (lower panels) uterine tissue, showing decidualization in the vehicle treated, scratched uterus only.

B: qPCR of Ptch1+/+ and Ptch1fl/fl outgrowths showing no significant changes in hedgehog activation, aside from upregulation of Ptch2. Unpaired t test used for statistics. Data represented as 2^dCt.
C-D: Ki67 and GFP costained vehicle treated \( \text{Ptch1}^{+/+} \) (upper left), vehicle treated \( \text{Ptch1}^{fl/fl} \) (lower left), IPI926 treated \( \text{Ptch1}^{+/+} \) (upper right), and IPI926 treated \( \text{Ptch1}^{fl/fl} \) ducts (lower right). D) Quantification of %Ki67 positive cells by genotype, showing increased proliferation in vehicle and IPI926- treated mutant ducts relative to controls by paired t test.

E-G: Confocal images of Cyclin B1 stained \( \text{Ptch1}^{+/+} \) and \( \text{Ptch1}^{fl/fl} \) ducts showing increased nuclear localization (G) in \( \text{Ptch1}^{fl/fl} \) ducts compared to controls by paired t test. Scale bar indicates 10 \( \mu \text{m} \).

The scatterplot shows data as mean ± SEM. Boxplots show data as mean ± SEM, with min and max values. * indicates \( p<0.05 \), ** indicates \( p<0.01 \), *** indicates \( p<0.001 \), and **** indicates \( p<0.0001 \). Scale bars are 50 \( \mu \text{m} \), except E-F.
Figure 3. Pubertal animals (6 weeks) with *Fsp-Cre* mediated loss of *Ptch1* display dysmorphic, hyperproliferative TEBs.

A-D: Hematoxylin and eosin (H and E) stained A) control, B) *Fsp-Cre;Ptch1*\(^{fl/+}\), and C) *Fsp-Cre;Ptch1*\(^{fl/fl}\) TEBs. D) Percent perturbed TEBs by genotype.

E-H: BrdU stained E) control, F) *Fsp-Cre;Ptch1*\(^{fl/+}\), and G) *Fsp-Cre;Ptch1*\(^{fl/fl}\) TEBs. (H) Quantification of BrdU in TEBs showing decreased proliferation in both mutants. One data point represents one TEB.

I-L: CC3 stained I) control, J) *Fsp-Cre;Ptch1*\(^{fl/+}\), and K) *Fsp-Cre;Ptch1*\(^{fl/fl}\) TEBs. L) Quantification of CC3 by genotype. Only *Fsp-Cre;Ptch1*\(^{fl/+}\) has reduced apoptosis.

Graphs show data as mean ± SEM. ** indicates p<0.01, and *** indicates p<0.001 by ANOVA/Tukey’s test. Scale bars indicate 50 µm. Insets show histologically normal TEBs.
Figure 4. Eight week old control, *Fsp-Cre;Ptch1^+/−*, and *Fsp-Cre;Ptch1^fl/fl* animals display altered branching, histology, and epithelial proliferation.

A-D: Whole mounted A) control, B) *Fsp-Cre;Ptch1^fl/+*, and C) *Fsp-Cre;Ptch1^fl/fl* glands showing branching. Heterozygotes are hyperbranched, while homozygotes display reduced branching. Scale bar is 1 mm. D) Quantification of fat pad filling, showing that *Fsp-Cre;Ptch1^fl/fl* outgrowths are severely stunted.

E-H: H and E stained E) control, F) *Fsp-Cre;Ptch1^fl/+*, and G) *Fsp-Cre;Ptch1^fl/fl* ducts showing frequent partial filling in heterozygotes, and complete filling in homozygotes. H) Quantification of ductal filling frequency by genotype.

I-L: BrdU stained I) control, J) *Fsp-Cre;Ptch1^fl/+*, and K) *Fsp-Cre;Ptch1^fl/fl* ducts. Scale bar is 50 µm. L) Quantification of BrdU showing hyperproliferation in heterozygotes, and hypoproliferation in homozygotes.
M-P: ERα stained M) control N) Fsp-Cre;Ptch1\textsuperscript{+/+}, and O) Fsp-Cre;Ptch1\textsuperscript{fl/+} ducts showing upregulated ERα expression in Fsp-Cre;Ptch1\textsuperscript{fl/+} ducts. P) Quantification of ERα in the mammary epithelium.

Q-T: PR stained Q) control R) Fsp-Cre;Ptch1\textsuperscript{+/+}, and S) Fsp-Cre;Ptch1\textsuperscript{fl/fl} ducts showing ablation of PR in Fsp-Cre;Ptch1\textsuperscript{fl/fl} ducts. T) Quantification of PR in the mammary epithelium.

Graphs show data as mean ± SEM. Insets display histologically normal ducts. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, and **** indicates p<0.0001 by ANOVA/Tukey’s test. The scale bars are 50 µm except for A-D.
Figure 5. Whole gland transplantation rescues stunted ducts and ER/PR levels, but not histology of Fsp-Cre;Ptch1\textsuperscript{fl/fl} animals. Genotype indicates donor glands transplanted to SCID/bg recipients wild-type for Ptch1.

A-C: Fluorescent whole mounted A) control and B) Fsp-Cre;Ptch1\textsuperscript{fl/fl} donor glands, 8 weeks post-transplantation. Scale bar is 0.5 mm. C) Quantification of fat pad filling, indicating no difference between groups.

D-F: H and E stained D) control and E) Fsp-Cre;Ptch1\textsuperscript{fl/fl} donor ducts. Scale bar is 50 µm. F) Quantification of ductal filling, showing increased ductal filling in mutant donors.

G-I: ERα stained G) control and H) Fsp-Cre;Ptch1\textsuperscript{fl/fl} donor ducts. Scale bar is 50 µm. I) ERα quantification showing similar expression between groups.
J-L: PR stained G) control and H) Fsp-Cre;Ptch1^{fl/fl} donor ducts. Scale bar is 50 µm. I) PR quantification showing a small increase in Fsp-Cre;Ptch1^{fl/fl} donor ducts.

Graphs show data as mean ± SEM. Insets display histologically normal ducts. * indicates p<0.05 by paired t test. Insets display histologically normal ducts (E,G,H).
Figure 6. Ptch1 may regulate estrogen/progesterone production, but not myeloid cell function to promote mammary ductal elongation.

A: Whole mounted vehicle or E+P treated control, or Fsp-Cre;Ptch1\textsuperscript{fl/fl} glands. Top panels: vehicle treatment. The bottom panels show E+P increases branching.

B: Quantification showing E+P rescue of stunted ducts of Fsp-Cre;Ptch1\textsuperscript{fl/fl} mutants (here, “mutant”).

C: BrdU labeling quantification in vehicle or E+P-treated control and Fsp-Cre;Ptch1\textsuperscript{fl/fl} ducts. E+P induced proliferation, albeit attenuated, in Fsp-Cre;Ptch1\textsuperscript{fl/fl} mutants.

D: Whole mounted glands of control to control (upper left), control to Fsp-Cre;Ptch1\textsuperscript{fl/fl} (upper right), or Fsp-Cre;Ptch1\textsuperscript{fl/fl} to control bone marrow (lower left) transplanted animals. Inset: Inguinal mammary lymph node of Cre- recipient showing colonization by Cre+, mTmG+ cells.
Scale bar, 50 µm. Lower right: Quantification showing that donor bone marrow does not change mammary ductal outgrowth.

Graphs show data as mean ± SEM. Scale bar shows 0.5 mm. ** indicates p<0.01; **** indicates p<0.0001 by ANOVA/Tukey’s test.
Figure 7. Aberrant *Fsp-Cre;Ptch1*fl/fl histology may be due to activated canonical hedgehog signaling.

A-C: H and E-stained A) Cre- and B) *Fsp-Cre; SmoM2* TEBs from 6 week old mice, showing perturbed histology and increased stromal condensation. C) Quantification of perturbed TEBs.

D-F: Fluorescent mount of D) Cre- and E) *Fsp-Cre; SmoM2* glands at 8 weeks. Scale bar is 0.5 mm. F) Quantification showing a slight reduction of fat pad filling in mutants.

I: BrdU quantification showing no difference in TEBs at 6 weeks, and ducts at 8 weeks.

J: ERα quantification showing no difference at 8 weeks.

K: PR quantification showing a small increase in mutants at 8 weeks.

Data displayed as mean ± SEM. Unpaired t test used for analysis. * signifies p<0.05. Scale bar is 50 µm except E-G. Insets (B,H) show histologically normal structures.
Figure 8. *Ptch1* Functions in Mammary Gland Morphogenesis and Histogenesis.

*Ptch1* in the mammary epithelium inhibits proliferation and branching, independent of Smo. *Ptch1* is essential in a mammary gland extrinsic *Fsp* positive cell (fibroblasts) for mammary ductal ER/PR patterning and pubertal outgrowth. *Ptch1* acts locally in an *Fsp* positive stromal cell (likely fibroblast) to inhibit Smo and elicit normal TEB and ductal histology.
**Fig. S1.** Quantification demonstrating increased branching in *Fsp-Cre;Ptch1fl/+* mutants, but reduced branching in *Fsp-Cre;Ptch1fl/fl* mutants.

Graph shows mean ± SEM. * indicates p<0.05, and *** indicates p<0.001 by ANOVA/Tukey’s test. Branch points were quantified with a representative 2x field for each gland. Cont
Figure S2. *Fsp-Cre*-mediated ablation of *Ptch1* produces ducts filled with K8+ luminal cells, and aberrant microlumens.

A-C: 3D confocal reconstruction of A) *Fsp-Cre, mTmG B) Fsp-Cre;Ptch1fl/+ and C) Fsp-Cre;Ptch1fl/fl ducts. *Cre* negative cells express TdTomato Red, and *Cre* positive cells express GFP. There are increased luminal cells inside the ducts of heterozygotes, while the homozygote displays completely filled-in ducts. Scale bar is 100 µm.

D-E: K5 (basal), and K8 (luminal) coimmunofluorescence of D) control E) *Fsp-Cre;Ptch1fl/+ and F) Fsp-Cre;Ptch1fl/fl ducts, indicating that the cells filling the ducts of mutants are K8 positive. Insets display ducts with normal histology. Scale bar is 50 µm.

G-I: ZO-1 (apical and tight junction marker) and K5 costained G) control H) *Fsp-Cre;Ptch1fl/+ and I) Fsp-Cre;Ptch1fl/fl ducts. The heterozygote displays extra staining in microlumens, while the homozygous mutant displays aberrant concentric staining.
Figure S3. *Fsp-Cre* mediated loss of *Ptch1* reduces mammary gland and body weight.

A) Mass of inguinal mammary gland of control and mutant animals at 8 weeks, showing a decrease in *Fsp-Cre;Ptch1 fl/fl* mutants.

B) Mass of control and mutant animals at 8 weeks as percent body weight, showing a decrease in *Fsp-Cre;Ptch1 fl/fl* mutants.

C) Body weight of control and mutant animals at 8 weeks, showing a decrease in *Fsp-Cre;Ptch1 fl/fl* mutants.

Graphs show data as mean ± SEM, and weights are given in grams. * indicates p<0.05, ** indicates p<0.01, and **** indicates p<0.0001 by ANOVA/Tukey’s test.
Figure S4. *Fsp-Cre;Ptch1*fl/fl animals do not proceed through the estrous cycle normally, and have severely compromised fertility.

A-C: representative estrous cycle for A) control B) *Fsp-Cre;Ptch1*fl/+ and C) *Fsp-Cre;Ptch1*fl/fl animals. Homozygous mutants do not cycle normally as assayed by vaginal smear in ≥4 animals per group. D indicates diestrous, M metestrous, E estrous, and P proestrous.

D: Number of pups produced by females of different genotypes over 6.5 weeks showing severe loss of fertility in *Fsp-Cre;Ptch1*fl/fl animals. Control animals produced 14.8±1.5, heterozygotes produced 14.5±1.7, and homozygotes produced no pups. **** denotes p <0.0001 by ANOVA/Tukey’s test.
Table S1. Antibodies used for immunostaining.

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<th>Antibody</th>
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<td>Ki67 (Vector Labs, Rabbit Polyclonal VP K451)</td>
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<td>Cyclin B1 (Cell Signaling 4138)</td>
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<td>TE pH 9.0</td>
</tr>
<tr>
<td>Anti-Rabbit 594 (Secondary) (AlexaFluor)</td>
<td>1:500</td>
<td>either</td>
</tr>
<tr>
<td>Anti-Mouse 488 (Secondary) (AlexaFluor)</td>
<td>1:500</td>
<td>either</td>
</tr>
</tbody>
</table>
### Table S2: Primers used for QPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Ref No. (Taqman)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Mm03928990_g1</td>
<td></td>
</tr>
<tr>
<td>Ptch1</td>
<td>Mm00436026_m1</td>
<td>Exon 17-18 (start base 2846)</td>
</tr>
<tr>
<td>Ptch2</td>
<td>Mm00436047_m1</td>
<td>Exon 19-20 (start 3115)</td>
</tr>
<tr>
<td>Smo</td>
<td>Mm01162704_m1</td>
<td>Exon 1-2 (start 856)</td>
</tr>
<tr>
<td>Gli1</td>
<td>Mm00494654_m1</td>
<td>Exon 11-12 (start 1476)</td>
</tr>
<tr>
<td>Gli2</td>
<td>Mm01293111_m1</td>
<td>Exon 13-14 (start 2598)</td>
</tr>
<tr>
<td>Gli3</td>
<td>Mm00492345_m1</td>
<td>Exon 14-15 (start 2867)</td>
</tr>
<tr>
<td>Hhip</td>
<td>Mm00469580_m1</td>
<td>Exon 12-13 (start 2406)</td>
</tr>
</tbody>
</table>
Supplemental Movie 1:

This movie is a confocal Z-stack from an eight-week-old $Fsp-Cre;Ptch1^{+/+};mTmG^+$ mammary duct showing a clear ductal lumen. GFP and RFP are endogenous fluorescence from the $mTmG$ reporter; GFP indicates $Fsp-Cre^+$ stromal cells, while RFP is expressed by $Fsp^-$ cells.
Supplemental Movie 2:

This movie is a confocal Z-stack of eight-week-old Fsp-Cre; Ptch1^{fl/fl}; mTmG+ mammary ducts showing some ducts with clear lumens, and a duct filled with RFP+ cells (upper right). GFP and RFP are endogenous fluorescence from the mTmG reporter; GFP indicates Fsp-Cre+ cells, while RFP is expressed by non-Fsp+ cells.