Epithelial and non-epithelial *Ptch1* play opposing roles to regulate proliferation and morphogenesis of the mouse mammary gland

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**ABSTRACT**

**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, as they are often perturbed in cancer and other diseases.

The hedgehog signaling network regulates cellular and tissue interactions that are 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, 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* and/or *Ptch2* (*Ptch1/2*) on a responding cell, GLI1/2/3 are released, and GLI transcription factors (GLI1, GLI2 and GLI3) 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- or caspase 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 and ERK2 phosphorylation independently of SMO (Chang et al., 2010). In the mouse mammary epithelium, constitutively activated *Smo* (*SmoM2*) acts as a G-protein-coupled receptor (GPCR) via G_{i2q} to induce proliferation independently of GLI activity, as hyperproliferation was not blocked by pharmacological inhibition of GLI1 or GLI2 (Villanueva et al., 2015), consistent with observations by Riobo et al. (Riobo et al., 2006). TGFβ induces *Gli2* to regulate osteolysis independently of Smo (Johnson et al., 2011), whereas 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 that fills 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 (\(Ptch1^{\Delta/}\)), or homozygous for a hypomorphic \(Ptch1\) allele (\(Ptch1^{mes}\)), 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 was 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 (\(Ptch1^{fl}\)) 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 with \(MMTV-Cre\) (Wagner et al., 2001).

Eight weeks post-transplantation, we observed that whereas \(Ptch1^{+/}\) glands had 75±11 branch points (Fig. 1A) and \(Ptch1^{fl/+}\) glands had a comparable 65±5 branch points (Fig. 1B), \(Ptch1^{fl/fl}\) glands showed an increase in branch points with 131±14 (Fig. 1C) \((P<0.011, \text{paired } t\text{-test; quantification Fig. 1D})\). 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). \(Ptch1^{fl/+}\) ducts were 2.8±0.6% Ki67 positive (Fig. 1F) (n.s., paired \(t\)-test), whereas proliferation in \(Ptch1^{fl/fl}\) cells increased significantly to 4.1±0.7% Ki67 positive (Fig. 1G) \((P<0.01, \text{paired } t\text{-test; quantification, Fig. 1H})\). Apoptosis was comparable between \(Ptch1^{+/}\) (Fig. 1I), \(Ptch1^{fl/+}\) (Fig. 1J) or \(Ptch1^{fl/fl}\) ducts (Fig. 1K) using cleaved caspase 3 (CC3) staining, whereas 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.

**Fig. 1.** Loss of \(Ptch1\) in mammary epithelium increases branching and proliferation in adult virgin glands. (A-C) Fluorescent whole-mount (A) Ad-\(Cre;Ptch1^{+/}\), (B) Ad-\(Cre;Ptch1^{fl/+}\) and (C) Ad-\(Cre;Ptch1^{fl/fl}\) outgrowths. GFP identifies \(Cre^{+}\) cells. The insets show tdTomato Red+ \(Cre^{-}\) cells. (D) Quantification showing increased branching in \(Ptch1^{fl/fl}\) epithelium. (E-G) GFP- and Ki67-stained (E) Ad-\(Cre;Ptch1^{+/}\), (F) Ad-\(Cre;Ptch1^{fl/+}\) and (G) Ad-\(Cre;Ptch1^{fl/fl}\) ducts. (H) Quantification showing increased proliferation in \(Ptch1^{fl/fl}\) epithelium. (I-K) CC3-stained (I) Ad-\(Cre;Ptch1^{+/}\), (J) Ad-\(Cre;Ptch1^{fl/+}\) and (K) Ad-\(Cre;Ptch1^{fl/fl}\) ducts—all negative for CC3. The inset shows a CC3-stained lymph node used as a positive control. (L) Quantification showing similar percentage GFP positivity in outgrowths of different genotypes. (M) Relative expression of hedgehog target genes in Ad-\(Cre;Ptch1^{+/}\) and \(Ptch1^{fl/fl}\) tissues. Data displayed as 2\(^{-\Delta\text{Ct}}\) with minimum and maximum values. \(Ptch2\) expression is significantly higher in \(Ptch1^{fl/fl}\) tissues (unpaired \(t\)-test). Graphs show data as mean±s.e.m. Paired \(t\)-tests were used to compare \(Ptch1^{fl/fl}\) glands with contralateral \(Ptch1^{+/}\) controls. \(*P<0.05\) and \(**P<0.01\). Scale bars: 1 mm in A-C; 50 µm in E-G,I-K.
An average of \(81\pm4\%\) of \(Ptch1^{+/+}\), \(69\pm6\%\) of \(Ptch1^{fl/+}\) and \(85\pm2\%\) 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 demonstrate SMO inhibitor (IPI926) efficacy, we tested whether IPI926 would blunt uterine scratch-induced decidualization, as 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). The scratched vehicle-treated tissue displayed histological changes consistent with decidualization (Fig. 2A) that were absent with IPI926 treatment (Fig. 2A). 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 3 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; \(P<0.0019\), paired t-test). With IPI926 treatment, \(Ptch1^{+/+}\) ducts were 1.2±0.6% Ki67 positive, whereas IPI926-treated \(Ptch1^{fl/fl}\) ducts retained increased proliferation with 3.2±0.71% Ki67-positive cells (Fig. 2C) \((P<0.01,\) 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). As IPI926 did not block hyperproliferation, this phenotype is not likely due to SMO activation.

As 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 whereas *Ptc1*+/− ducts displayed 0.17±0.05% cells with nuclear cyclin B1 (Fig. 2E), *Ptc1*+/⊕ outgrowths showed a significant increase to 0.45±0.11% (Fig. 2F) (P<0.0085, paired t-test) (Fig. 2G).

**Fsp-cre-mediated disruption of Ptch1 in non-epithelial cells alters mammmary gland histology, proliferation and morphology**

To investigate non-epithelial functions of *Ptch1*, we crossed *mTmG-*tagged, *Ptc1*+/− and *Ptc1*+/⊕ with Fsp-Cre mice to ablate *Ptc1* in fibroblasts and myeloid cells. At 6 weeks of age, control mice (*Fsp-Cre;Ptch1fl/+) displayed histologically normal TEBs. At 6 weeks of age, control mice (Fig. 3B, inset). Additionally, ill-defined cap cell layer (Fig. 3B). Histologically normal TEBs. Histologically normal TEBs.

To test whether the dysmorphic TEBs had altered proliferation, we assayed BrdU labeling. Control TEBs were 29±2% BrdU positive (Fig. 3E), *Fsp-Cre;Ptch1fl/+* TEBs were 18±2% positive (Fig. 3F) and *Fsp-Cre;Ptch1fl/fl* TEBs were 15±2% positive (Fig. 3G). Both mutants had less BrdU labeling than controls (controls versus *Fsp-Cre;Ptch1fl/+* P=0.01, controls versus *Fsp-Cre;Ptch1fl/fl* P<0.001; ANOVA/Tukey’s test). With respect to apoptosis, control TEBs were 1.9±0.3% CC3 positive (Fig. 3I), whereas *Fsp-Cre;Ptch1fl/+* mice had reduced (0.79±0.14%) CC3 positivity (Fig. 3J) (P<0.001, ANOVA/Tukey’s test). *Fsp-Cre;Ptch1fl/fl* TEBs had comparable apoptosis rates of 1.5 ±0.4% (Fig. 3K,L).

In 8-week-old mice, control glands displayed normal branching (66±8 per 2× field) (Fig. 4A). Despite reduced TEB proliferation, *Fsp-Cre;Ptch1fl/+* glands were hyperbranched (129±11) (Fig. 4B). By contrast, *Fsp-Cre;Ptch1fl/fl* mice had reduced branching (18±4) (Fig. 4C). (Fig. 4A-C show part 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, whereas *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 8 weeks were not evaluated owing to skin phenotypes and low mutant survival.

Control ducts had normal histology at 8 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) (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

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**Fig. 3. Pubertal animals (6 weeks) with Fsp-Cre-mediated loss of Ptch1 display dysmorphic hyperproliferative TEBs.** (A-C) Hematoxylin and Eosin-stained (A) control, (B) *Fsp-Cre;Ptch1fl/+* and (C) *Fsp-Cre;Ptch1fl/fl* TEBs. (D) Percentage perturbed TEBs by genotype. (E-G) BrdU-stained (E) control, (F) *Fsp-Cre;Ptch1fl/+* and (G) *Fsp-Cre;Ptch1fl/fl* TEBs. (H) Quantification of BrdU in TEBs showing decreased proliferation in both mutants. One data point represents one TEB. (I-K) CC3-stained (I) control, (J) *Fsp-Cre;Ptch1fl/+* and (K) *Fsp-Cre;Ptch1fl/fl* TEBs. (L) Quantification of CC3 by genotype. Only *Fsp-Cre;Ptch1fl/+* has reduced apoptosis. Graphs show data as mean±s.e.m. **P<0.01 and ***P<0.001 by ANOVA/Tukey’s test. n.s., not significant. Scale bars: 50 μm. Insets show histologically normal TEBs.
immunofluorescence. Although ZO-1 stained the control duct lumens as expected (Fig. S2G), stained Fsp-Cre;Ptch1fl/+ ducts confirmed the presence of microlumens (Fig. S2H), whereas 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±0.01 g) versus controls (0.16±0.02 g) or heterozygotes (0.13±0.03 g) (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 ±0.5 g) were also decreased versus controls (23±1.2 g) and heterozygotes (22±0.7 g) (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), whereas Fsp-Cre;Ptch1fl/+ ducts were hyperproliferative (5.4±1.2%) (P<0.0334, ANOVA/Tukey’s test) (Fig. 4J). By 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. 4L). Thus, Fsp-Cre;Ptch1fl/+ mammary ducts had increased proliferation and branching, whereas 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 hypoproliferation phenotypes observed in Fsp-Cre;Ptch1fl/fl mice were similar to the stunted hypoproliferative ducts of estrogen receptor α (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;Ptch1<sup>B/G</sup> mice was disrupted (Bocchinfuso and Korach, 1997; Lydon et al., 1995).

At 8 weeks of age, ER and PR expression was perturbed in Fsp-Cre;Ptch1<sup>B/G</sup> mice. Although controls had 36±6% ERα-positive cells (Fig. 4M) and heterozygotes had comparable levels (38±6%) (Fig. 4N), ERα expression in Fsp-Cre;Ptch1<sup>B/G</sup> 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 (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;Ptch1<sup>B/G</sup> 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;Ptch1<sup>B/G</sup> donor glands were transplanted contralaterally into pre-pubertal recipient SCID/bg animals that were wild type 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;Ptch1<sup>B/G</sup> donor glands (64±7%) (Fig. 5B) (quantification, Fig. 5C; P<0.8285, paired t-test). In contrast to 8-week-old homozygous mutants from genetic crosses (Fig. 4C), TEBs were observed in transplanted Fsp-Cre;Ptch1<sup>B/G</sup> glands (Fig. 5B). Although Cre- donor ducts displayed normal histology (Fig. 5D), Fsp-Cre;Ptch1<sup>B/G</sup> glands were frequently filled-in (Fig. 5E), with some histologically normal ducts (Fig. 5E inset). Cre- donor ducts were 4±0.8% filled, whereas ducts of Fsp-Cre;Ptch1<sup>B/G</sup> 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, whereas the stunted duct growth was due to Ptch1 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;Ptch1<sup>B/G</sup> 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), whereas Fsp-Cre;Ptch1<sup>B/G</sup> 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 Ptch1 regulates ductal outgrowth and the characteristic ER/PR patterning of the mammary epithelium.

Stunted ducts, but not histological defects, of Fsp-Cre;Ptch1<sup>B/G</sup> mutants are rescued by E+P treatment

As whole-gland transplantation showed that ‘systemic’ Ptch1 regulates mammary ductal elongation (Fig. 5) and Fsp-Cre;Ptch1<sup>B/G</sup> 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), as expected. Here, Fsp-Cre;Ptch1<sup>+/+</sup> and Ptch1<sup>+/+</sup> or Ptch1<sup>B/G</sup> mice lacking Fsp-Cre were used as controls. Although 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 described (Fig. 6A) (P<0.01, ANOVA/Tukey’s test; quantification showing a small increase in Fsp-Cre;Ptch1<sup>B/G</sup> donor ducts. Graphs show data as means±s.e.m. Insets display histologically normal ducts. *P<0.05 by paired t-test. Insets display histologically normal ducts (E,H,K). n.s., not significant. Scale bars: 0.5 mm in A,B; 50 µm in D, E,G,H,J,K.

Fig. 5. Whole-gland transplantation rescues stunted ducts and ER/PR levels, but not histology, of Fsp-Cre;Ptch1<sup>B/G</sup> animals. Genotype indicates donor glands transplanted to SCID/bg recipients that are wild type for Ptch1. (A,B) Fluorescent whole-mount (A) control and (B) Fsp-Cre;Ptch1<sup>B/G</sup> donor ducts, 8 weeks post-transplantation. (C) Quantification of fat pad filling, indicating no difference between groups. (D,E) Hematoxylin and Eosin-stained (D) control and (E) Fsp-Cre;Ptch1<sup>B/G</sup> donor ducts. (F) Quantification of ductal ERα expression between groups. (G) 26±5% filled (P<0.0252, paired t-test). E+P-treated control glands had increased tertiary branching (Fig. 5A), as expected. Here, Fsp-Cre;Ptch1<sup>+/+</sup> and Ptch1<sup>+/+</sup> or Ptch1<sup>B/G</sup> mice lacking Fsp-Cre were used as controls. Although 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 described (Fig. 6A) (P<0.01, ANOVA/Tukey’s test; quantification showing a small increase in Fsp-Cre;Ptch1<sup>B/G</sup> donor ducts. Graphs show data as means±s.e.m. Insets display histologically normal ducts. *P<0.05 by paired t-test. Insets display histologically normal ducts (E,H,K). n.s., not significant. Scale bars: 0.5 mm in A,B; 50 µm in D, E,G,H,J,K.

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Myeloid cells regulate pubertal ductal outgrowth of the mammary glands. E+P increases branching (compare top and bottom panels). (B) Quantification showing E+P-mediated rescue of stunted ducts of Fsp-Cre;Ptch1<sup>fl/fl</sup> mutants (Mutant). (C) BrdU labeling quantification in vehicle- or E+P-treated control and Fsp-Cre;Ptch1<sup>fl/fl</sup> ducts. E+P induced proliferation, albeit attenuated, in Fsp-Cre;Ptch1<sup>fl/fl</sup> mutants. (D) Whole-mound glands of control (upper left), control to Fsp-Cre;Ptch1<sup>fl/fl</sup> (upper right) or Fsp-Cre;Ptch1<sup>fl/fl</sup> to control (lower left) bone marrow-transplanted animals. Inset: inguinal mammary lymph node of Cre- recipient showing colonization by Cre+; mTmG+ cells. Lower right: quantification showing that donor bone marrow does not change mammary ductal outgrowth. Graphs show data as means±s.e.m. Scale bars: 0.5 mm in A, D; 50 μm in D, inset. **P<0.01, ****P<0.0001 by ANOVA/Tukey’s test.

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;Ptch1<sup>fl/fl</sup> 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;Ptch1<sup>fl/fl</sup> ducts were 2.5±0.5% BrdU positive (Fig. 6C) (significantly reduced versus E+P-treated controls, P<0.0001 by ANOVA). Thus, although E+P induced proliferation and branching, the response in Fsp-Cre;Ptch1<sup>fl/fl</sup> mutants was attenuated compared with controls. The attenuated proliferation and tertiary branching displayed by the Fsp-Cre;Ptch1<sup>fl/fl</sup> 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. S4A-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). Here, controls consisted of Fsp-Cre;Ptch1<sup>+/+</sup> and Ptch1<sup>+/+</sup> or Ptch1<sup>+/+</sup> mice lacking Fsp-Cre. We observed engraftment of the transplanted cells, as GFP+ cells were present with transplantation of Fsp-Cre;Ptch1<sup>+/+</sup> cells to a Cre- recipient (Fig. 6D). Control bone marrow transplanted to Fsp-Cre;Ptch1<sup>fl/fl</sup> recipients filled only 61±2% of the fat pad (Fig. 6D). Glands from control recipients of Fsp-Cre;Ptch1<sup>fl/fl</sup> bone marrow displayed 89±1.2% of the fat pad filled (Fig. 6D). Control bone marrow transplanted to Fsp-Cre;Ptch1<sup>fl/fl</sup> mutants displayed reduced fat pad filling relative to the other groups (Fig. 6D; 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 SMO 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), whereas Fsp-Cre;Smo<sup>M2</sup> TEBs displayed dysmorphia (Fig. 7B). Dysmorphic TEBs had irregular shape, microlumens and increased periductal stromal condensations (Fig. 7C). These TEBs were similar to Fsp-Cre;Ptch1<sup>+/+</sup> (Fig. 3B), Fsp-Cre;Ptch1<sup>fl/fl</sup> (Fig. 3C) and Ptch1<sup>+/+</sup> TEBs (Lewis et al., 1999).
At 8 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%) (Fig. 7F) (P=0.0225, t-test). This reduction was less than in Fsp-Cre;Ptch1β/β 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). Neither mutant TEBs nor mature ducts displayed altered proliferation relative to Cre- ducts (Fig. 7J).

We tested whether the ERα and PR expression phenotypes of the Fsp-Cre;Ptch1β/β 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. 7K) (not different by t-test). PR positivity was 35.9±2.7% in Cre- ducts, whereas 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;Ptch1β/β 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; Ptch1β/β mutants indicate Ptch1 in Fsp+ fibroblasts regulates ductal histology, perhaps via Smo. We also show the crucial systemic roles of Ptch1 in ductal elongation and ER/PR expression in the mammary epithelium (see Fig. 8, model).

The Ad-Cre;Ptch1β/β model displayed hyperbranching and hyperproliferation in adult virgins. Whereas 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 G1α2-dependent signaling (Villanueva et al., 2015). Hyperproliferation was blocked by inhibiting some G1α subunits, but not by inhibiting GLI1 and GLI2 (Villanueva et al., 2015). The differences between these models suggests that Ptch1 loss increases proliferation independently of Smo. However, we cannot exclude the possibility 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;Ptch1β/β ducts, and the inability of IPI926 to block hyperproliferation (Figs 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*^A+/c^ (Lewis et al., 1999) and *Ptch1*^mes/mes^ animals (Moraes et al., 2009) indicated that *Ptch1* mediates ductal development; virgin *Ptch1*^A+/c^ mice had dysmorphic TEBs and filled-in ducts (Lewis et al., 1999). Whole *Ptch1*^A+/c^ glands transplanted to a wild-type host displayed filled-in ducts, whereas 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*^fl/fl^ 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*^mes/mes^ 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*^fl/fl^ mutant diverges from the *Ptch1*^mes/mes^ mutant (Moraes et al., 2009), which displayed reduced ER and PR expression in stunted ducts. The differences between the *Ptch1*^mes/mes^ and *Fsp-Cre;Ptch1*^fl/fl^ 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 affected in these models.

We have also further defined the ‘systemic’ function of *Ptch1*. As 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*^fl/fl^ mutants displayed functional defects, including abrogated cycling and fertility. As the stunted duct phenotype was not rescued by bone marrow transplantation, *Ptch1* does not function in myeloid cells to control ductal elongation.

As *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). Although we cannot exclude the possibility 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 *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 unidirectional signaling is reported, e.g. prostate and pancreas (Hebrok et al., 2000; Wang et al., 2003). Dissecting these tissue-tissue interactions is crucial, as these developmental programs are inappropriately re-activated in cancer, and correlate with poor prognosis, e.g. in prostate and pancreatic cancer (Bailey et al., 2009; Fan et al., 2004).

**Implications for Ptch1 and Smo in breast cancer**

The hedgehog network is misregulated in many cancers, including breast (Moraes et al., 2007, 2009). Although hedgehog network activation induces basal cell carcinoma and medulloblastoma, data connecting hedgehog signaling and breast tumorigenesis are largely correlative, although Gli1 overexpression in mice induces tumorigenesis (Fiaschi et al., 2009).

*Ptch1* protein levels are reduced in 50% of DCIS and invasive breast cancer (IBC), whereas 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). Furthermore, *Ptch1* underexpression correlated with *Ptch1* promoter methylation (Wolf et al., 2007). However, neither *Ptch1*^A+/c^ nor *MMTV-SmoM2* mice show mammary tumors (Moraes et al., 2007, 2009). Our data suggest that perhaps, in the case of *Ptch1*^A+/c^, the opposing functions of epithelial and systemic *Ptch1* offset one another. These observations may explain why breast cancer incidence in individuals with Gorlin syndrome (Gorlin, 1987), who are heterozygous for germline *Ptch1* loss-of-function and display higher risk for other cancers, is not higher than in the general population. Our *Ad-Cre;Ptch1*^fl/fl^ data suggest that *Ptch1* heterozygosity would not alter mammary epithelial histology or proliferation.

Previous data suggest that high hedgehog ligand expression in tumor epithelium induces Gli1 (which is indicative of activated hedgehog signaling) in the adjacent stroma, which correlates with invasiveness and poor patient prognosis (O’Toole et al., 2011). As local stromal loss of *Ptch1* and non-epithelial activation of Smo promote a DCIS-like phenotype in mammary epithelium, perhaps
stromal Ptch1 loss promotes cancer-associated phenotypes. The data presented here suggest that loss of Ptch1 in fibroblasts may increase survival, reduce non-apoptotic cell death or alter lumen formation. It would be interesting to determine whether Ptch1 heterozygosity correlates with DCIS in patients.

MATERIALS AND METHODS

Animal models

Mice carrying Ptch1<sup>Cre</sup>, here termed Ptch1<sup>Cre<ref>/sup> dependent conditional ablation allele were a gift from Dr Brandon Wainwright (University of Queensland, Australia) (Ellis et al., 2003). Mice expressing Cre-recombinase under the Esp1 (S100A4) promoter were a gift from Dr Eric Neilson (Vanderbilt University, Nashville, TN, USA). These mice express Cre in fibroblasts and myeloid-derived leukocytes (Bhowmick et al., 2004). Mice carrying the Gt(Rosa)26SORT1(mSmoYFP)Amc/J SmoM2 conditional activation allele were obtained from Jackson Labs (#005130) (Jeong et al., 2004). All animals were genetically tagged with the mTmG Cre-dependent reporter at the Rosa26 locus, Gt(Rosa)26SORT4 (ACTBtdTomato−EGFP)Luo/J. Cells lacking Cre-recombinase express tdTomato Red, whereas cells expressing Cre-recombinase display membrane-bound eGFP (Jackson Labs, #007576) (Muzumdar et al., 2007).

For studies of Ptch1<sup>Cre</sup>, Fsp-Cre;Ptch1<sup>Cre<ref>/sup> males were crossed to Ptch1<sup>fl/fl</sup> or Ptch1<sup>fl/fl<ref>/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>Cre</sup>, SmoM2 and Fsp-Cre was performed as previously described (Bhowmick et al., 2004; Ellis et al., 2003; Jeong et al., 2004). CB.17/ICRsd-Pkd2-scid-Lyst-bg (SCID/beige) mice (Harlan Laboratories) were used for transplantation in a National Disease Research Interchange Core Facility 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 intraperitoneally 2 h prior to harvest at 250 mg/kg.

Adenoviral transduction and transplantation

For epithelial ablation of Ptch1, mammary epithelial cells were harvested from glands 1, 3, 4 and 5 of 8-week-old Ptch1<sup>fl/fl<ref>/sup> and Ptch1<sup>fl/fl<ref>/sup> females with the lymph nodes removed. Glands were minced, digested with collagenase A (Roche Applied Science) and 0.05% trypsin-EDTA, and strained into single cells (Vishal 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. 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 intraperitoneally 2 h prior to harvest at 250 mg/kg.

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 h at 4°C, embedded in paraffin wax and sectioned at 3 μm. Slides were rehydrated using decreasing concentrations of ethanol. Immunostaining was carried out using antigen retrieval in 0.1 M 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% MOM protein reagent (Vector Labs, BPK2202) 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 40× 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 (Ptch1<sup>fl/fl</sup> only or Fsp-Cre only) and Fsp-Cre;Ptch1<sup>fl/fl</sup> 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; Morales 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.

IP1926 treatment (inhibition of SMO)

Either IP1926 (Infinity) dissolved in 13% ethanol in Tween-20 (Sigma) or vehicle alone were administered by oral gavage. IP1926 doses were 40 mg/kg. For the mammary gland experiment, three daily treatments of vehicle or IP1926 were given prior to harvest.

Uterine scratch

After ovariectomy post-weaning and a 1-week rest, a prescribed course of estrogen (0.1 μg in 100 μl sesame oil for 3 days), 2 days rest, then estrogen +progesterone (1 mg progesterone +6.7 mg 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 IP1926 was administered for 7 days prior to, and the day of harvest 9 days after the first estrogen treatment. Hormone and IP1926 doses were timed as described previously (Villanueva et al., 2015).

QPCR

Tissues were collected into RNA Later (Qiagen) and frozen at −80°C. RNA was extracted with the Qiagen RNeasy Kit, and cDNA was synthesized with the Superscript III kit (Thermo Fisher) using random hexamers. The cDNA was analyzed using an Applied Biosystems 7500-Fast thermocycler for TaqMan quantitative PCR under standard conditions. Product accumulation was represented as 2<sup>−ΔΔCt</sup>, with ANOVA of Δct values used for statistical comparison. 18S RNA was used for normalization. See Table S2 for primers.

Bone marrow transplantation

Recipient animals 4–5 weeks of age received Baytril water 24 h prior to irradiation and up to 6 days post-irradiation. Recipients received a dose of 400 centigray, and 24 h later, bone marrow cells were harvested and isolated from 4–5-week-old donor mice. Irradiated recipients received 2 million donor cells injected retro-orbitally. Recipients were harvested 6 weeks post-transplantation.

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Competing interests

M.T.L. is a founder of, and Limited Partner in, StemMed Ltd, and is a Manager of StemMed Holdings LLC, its General Partner.

Author contributions

J.D.L. participated in imaging, animal work and bone marrow transplantation. A.P.V. assisted with experimental design, animal acquisition and breeding. T.M. performed all other experiments, statistics, interpretation and manuscript preparation. M.T.L. participated in project conception, experimental design, data interpretation and manuscript preparation, and was principle investigator.
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