**Ptch1** is required locally for mammary gland morphogenesis and systemically for ductal elongation

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Systemic hormones and local growth factor-mediated tissue interactions are essential for mammary gland development. Using phenotypic and transplantation analyses of mice carrying the mesenchymal dysplasia (mes) allele of patched 1 (**Ptch1**<sub>mes</sub>), we found that **Ptch1**<sub>mes</sub> homozygosity led to either complete failure of gland development, failure of post-pubertal ductal elongation, or delayed growth with ductal dysplasia. All ductal phenotypes could be present in the same animal. Whole gland and epithelial fragment transplantation each yielded unique morphological defects indicating both epithelial and stromal functions for **Ptch1**. However, ductal elongation was rescued in all cases, suggesting an additional systemic function. Epithelial function was confirmed using a conditional null **Ptch1** allele via MMTV-Cre-mediated disruption. In **Ptch1**<sub>mes</sub> homozygotes, failure of ductal elongation correlated with diminished estrogen and progesterone receptor expression, but could not be rescued by exogenous ovarian hormone treatment. By contrast, pituitary isografts were able to rescue the ductal elongation phenotype. Thus, **Ptch1** functions in the mammary epithelium and stroma to regulate ductal morphogenesis, and in the pituitary to regulate ductal elongation and ovarian hormone responsiveness.

**KEY WORDS:** Hedgehog, SMO, Ductal morphogenesis, Hyperplasia, Pituitary isograft, Tissue interaction, Mouse

**INTRODUCTION**

Mammary gland development requires both systemic hormones and local growth factor-mediated tissue interactions. Classical hormone ablation/replacement experiments, and more-recent genetic analyses in mice, have shown that post-pubertal gland development requires systemic hormones from ovary [estrogen (E) and progesterone (P)], pituitary [growth hormone (GH) and prolactin (PRL)] and adrenal gland (glucocorticoids) (Topper and Freeman, 1980). Loss of ovarian or pituitary function leads to failure of hormone-dependent ductal elongation after puberty, with E and GH participating primarily in ductal elongation and P and PRL participating primarily in alveolar development. Glucocorticoids enhance (but are not essential for) ductal elongation and are required for alveolar function in lactation.

In addition to systemic hormones, local growth factor signaling, both within and among tissue compartments, is essential for many aspects of normal embryonic and postnatal mammary gland development, as well as for organ maintenance and function in the adult (Sternlicht, 2006; Wiseman and Werb, 2002). For example, insulin-like growth factor 1 (IGF1) functions downstream of GH and PRL to promote ductal elongation. Amphiregulin, a member of the epidermal growth factor (EGF) family, is a major mediator of estrogen-stimulated growth. Other local growth factors contributing to ductal elongation or branching morphogenesis include members of the EGF, fibroblast growth factor (FGF), IGF, Wnt, Notch and TGFβ families, as well as factors such as colony stimulating factor 1 (CSF1), hepatocyte growth factor (HGF), and parathyroid hormone-related protein (PTHrP; PTHLH – Mouse Genome Informatics). Altered tissue interactions mediated by these local growth factors and their receptors contribute significantly to breast pathologies including mastitis and cancer.

In addition to these growth factor signaling systems, one of the primary signaling networks mediating cell-cell interactions during embryonic and postnatal mammary gland development is the hedgehog signaling network (reviewed by Hatsell and Frost, 2007; Lewis and Visbal, 2006). However, it has been unclear in which tissue compartments hedgehog network genes function to regulate gland development, and whether there might be a systemic requirement for hedgehog network gene activity.

In the mouse, mammary gland development begins at about embryonic day 10 (E10) with the induction of the milk line, along which the five pairs of mammary glands will be placed. At E11, mammary placodes (the presumptive nipple) are induced in characteristic positions along the milk line. Induction of the milk line and mammary placodes requires inductive tissue interactions from somites, as well as from a mesodermally derived mammary mesenchyme, to an overlying ectodermally derived epithelium (Chu et al., 2004; Sakakura, 1987; Veltmaat et al., 2003; Veltmaat et al., 2006). Between ~E12 and ~E16, the mammary gland consists of a small bulb of epithelium that begins to invade the fat pad precursor mesenchyme (which gives rise to the mammary fat pad stroma in the postnatal animal) (Hens et al., 2007; Sternlicht, 2006). This early gland development is hormone-independent. At birth, the ductal tree is a rudimentary branched structure, and remains relatively growth quiescent until puberty.

Most mammary gland development occurs after puberty and is under both systemic and local control (Daniel and Silberstein, 1987; Topper and Freeman, 1980). At puberty, systemic reproductive hormones (mainly E and P produced in the ovaries, along with PRL and GH produced in the pituitary) stimulate rapid and invasive ductal elongation driven by growth of the terminal end bud (TEB). Ductal elongation also requires paracrine or juxtacrine cell-cell interactions...
between the epithelial cells themselves (Brisken et al., 1999; Brisen et al., 1998; Mallepell et al., 2006), as well as interactions between the epithelium and surrounding mammary fat pad stroma proper (e.g. fibroblasts, adipocytes) (Sternlicht, 2006; Wiseman and Werb, 2002). Finally, epithelial cells must interact with ‘non-mammary’ cell types that are produced elsewhere and are recruited into the mammary fat pad. These cell types include vascular cells, macrophages, eosinophils and neuronal cells (Gouon-Evans et al., 2002; Parnelly and Manning, 1983; Roubian and Blair, 1977).

Canonical hedgehog signaling (Cohen, 2003; Evangelista et al., 2006; Hooper and Scott, 2005; Nusse, 2003) typically involves two types of cells, a signaling cell expressing a member of the hedgehog family of secreted ligands [sonic hedgehog (SHH), Indian hedgehog (IHH) or desert hedgehog (DHH)], and a responding cell expressing one or more patched family hedgehog receptors [patched 1 (PTCH1) and patched 2 (PTCH2)]. In the absence of ligand, PTCH1 and PTCH2 can function to inhibit downstream signaling by antagonizing the function of the smoothened (SMO) transmembrane effector protein. Under these conditions, expression of hedgehog target genes is inhibited by repressor forms of one or more members of the Gli family of transcription factors (Gli2 or Gli3). In the presence of ligand, PTCH1 releases inhibition of SMO, which leads to the induction of target genes by transcriptional activator forms of Gli transcription factors (Gli1, Gli2 or Gli3) (Ellis et al., 2003). Gli activity is not functionally equivalent to SMO activation. Epithelial fragment transplantation experiments suggested that Gli1 functions primarily in the stroma to influence epithelial cell behavior, but epithelial function was not ruled out. However, the mutant phenotype was only partially recapitulated in whole mammary gland transplantation, suggesting that Gli1 might have a systemic function as well (Lewis et al., 1999).

To address the tissue compartment-specific requirement for Gli1 more fully, we exploited an allelic series of mutations consisting of a targeted disruption allele (ΔGli1) (Goodrich et al., 1997), a conditional disruption allele (Gli1f) (Ellis et al., 2003), as well as the mesenchymal dysplasia (mes) allele (Makino et al., 2001) (herein designated Ptch1mes), for phenotypic and transplantation analysis of the mouse mammary gland during virgin development. The Ptch1mes allele arose as a spontaneous deletion mutation and is haploinsufficient over the null allele, suggesting that it is hypomorphic. However, unlike homozygous ΔPtch1 mice, which die at E9.5, homozygous Ptch1mes mice are viable and show dysplastic growth of mesenchymal tissues, polydactyly, white belly spot, and prior to this study have shown sterility in both sexes. Homozygous Ptch1mes pups are initially smaller than their wild-type littermates, but become significantly larger than the wild type by 8-10 weeks of age, consistent with the role of Ptch1 in body size regulation (Milenkovic et al., 1999).

Using phenotypic and transplantation analyses in conjunction with endocrine manipulation, we find that Ptch1 is required in both the epithelium and mammary stroma to regulate multiple aspects of gland development. In addition, Ptch1 functions systemically in the pituitary to promote ductal elongation.

MATERIALS AND METHODS

Animals

Mice were maintained as breeding colonies in our laboratory, and were maintained and used in accordance with the NIH Guide for the Care and Use of Experimental Animals with approval from our Institutional Animal Care and Use Committee.

The mes allele of Ptch1 (Ptch1mes) arose spontaneously on the CBA/J background and is the result of a 32 bp deletion in the Ptch1 locus. This deletion causes a frameshift, such that the resulting PTCH1 protein lacks 271 C-terminal amino acids of the normal protein, which are replaced by 68 unrelated residues. The derivative strain B6C3Fe-a/a-Ptch1mes (stock number 001430) was obtained for this study. Homozygous Ptch1mes mice of both sexes are sterile in this genetic background (Makino et al., 2001).

Two breeding pairs of heterozygous B6C3Fe-a/a-Ptch1+/- mice were used to initiate a breeding colony by serially backcrossing heterozygous males to C57BL/6J females for at least eight generations. Backcross-derived heterozygous mice were then crossed with transgenic mice expressing enhanced cyan fluorescent protein (ECFP) under the chicken β-actin/CMV immediate early enhancer promoter in an inbred C57BL/6J background [strain B6.129(ICR)-Tgt(ACCT-ECFP)Ck6Nagy/J; stock number 004218] (herein designated ACTB-ECFP) (Hadjantonakis et al., 2002) to tag the lineage genetically to facilitate transplantation experiments and gland imaging. Homozygous Ptch1mes females and wild-type controls used experimentally were generated by intercrossing backcross-derived heterozygous mice. Homozygous Ptch1mes mice in an inbred C57BL/6J background are poorly fertile, with females frequently failing to deliver viable pups (data not shown).

Ptch1mes mice were genotyped by PCR analysis of tail DNA (DNeasy, Qiagen). Primers used were: forward, 5'-TCCAAATGTGCTCCTGGTGTG-3'; reverse, 5'-GTGGCTTCCACAATCAGTTT-3'. Step-down cycling conditions were: 94°C for 60 seconds, 64°C for 30 seconds, and 72°C for 90 seconds (5 cycles), followed by 94°C for 60 seconds, 62°C for 30 seconds, and 72°C for 90 seconds (5 cycles), followed by 94°C for 60 seconds, 60°C for 30 seconds, and 72°C for 90 seconds (25 cycles). A 142 bp product indicated the presence of the wild-type allele, and a 172 bp product indicated the presence of the mutant allele.

Mice carrying a Ptch1 targeted disruption allele (Ptch1Δ/Δ), here designated ΔPtch1 were described previously (Goodrich et al., 1997) and were a generous gift from Dr Matthew Scott. The ΔPtch1 allele was maintained in a C57BL/6J × DBA2 hybrid background by periodic intercrossing with B6D2F1 mice. Genotyping for the disruption allele was modified from that published previously (Goodrich et al., 1997) because of conflicts resulting from the presence of other Ptch1 alleles. Primers used were: forward, 5'-CAGAGCGGGTAAACTGGCTCGGATTAG-3'; reverse, 5'-CACCGGTTGGATGTGGAATGTGTGCG-3'. Conditions were: 94°C for 60 seconds, 57°C for 60 seconds, and 72°C for 90 seconds (35 cycles). A 1100 bp product indicated the presence of the ΔPtch1 allele.
Mice carrying a Ptch1 Cre-recombinase-dependent conditional disruption allele (Ptch1<sup>Cre</sup>) were described previously (Ellis et al., 2003) and were maintained by serial backcrossing to C57BL/6J. Genotyping for the conditional allele and the recombinated allele was performed as described (Ellis et al., 2003).

Mice carrying a Cre-recombinase-dependent β-galactosidase (lacZ) reporter allele targeted to the Rosa locus were described previously (Soriano, 1999) and were obtained from the Jackson Laboratories [strain B6.129S4-Gt(Rosa)26Sor<sup>tm1Sor</sup>]; stock number 003474] (herein designated R26R) and were maintained by serial backcrossing to C57BL/6J mice. Genotyping for the presence of the R26R allele was performed as described (Soriano, 1999).

A transgenic mouse line expressing Cre recombinase under the control of the mouse mammary tumor virus (MMTV) promoter (MMTV-Cre<sup>+</sup>) was generated previously (Li et al., 2002). Genotyping for the presence of the MMTV-Cre transgene was performed as described previously (Li et al., 2002). MMTV-Cre mice were intercrossed with R26R, and backcrossed to C57BL/6J at least once prior to use in this study to allow analysis of mutant phenotypes in a predominately C57BL/6J genetic background.

Immunocompromised B6.129S7-Rag2<sup>tm1Mom</sup>/J (stock number 00216) (herein designated ΔRag1) homozygous female mice used as transplantation hosts were not genetically tagged, so as to allow transplanted ECFP-expressing epithelium to be easily distinguished from endogenous epithelium.

**Whole gland morphological analysis**

For analysis of the Ptch1<sup>mes</sup> allele, homozygous, heterozygous and wild-type littermate or age-matched females were used. Mammary glands #1-5 were harvested from the right side of at least ten female mice at 5 and 10 weeks of age. Additional mice were examined at 20 weeks and at older than 52 weeks of age. Glands were fixed in ice-cold 4% paraformaldehyde in PBS, and examined as whole-mount preparations using Neutral Red staining as described previously (Moraes et al., 2007). Some glands were examined by whole-mount preparations using the ECFP tag for imaging using a Leica MZFLE16 fluorescence stereomicroscope equipped with an Optronics Maginfire camera. For display, fluorescence images were exposure-reversed so as to appear similar to Neutral Red-stained preparations. The percentage of fat pad filled was estimated from the whole-mount preparations.

For analysis of the Ptch1<sup>+</sup> allele, we examined glands derived from mice in which Ptch1 function was conditionally disrupted in the mammary epithelium via MMTV-Cre-mediated deletion (Li et al., 2002). To accomplish these analyses, Ptch1<sup>+/−</sup>; R26R<sup>+/−</sup> mice were crossed with ΔPtch1<sup>+/−</sup>; MMTV-Cre<sup>+/−</sup>; R26R mice to yield the required Ptch1 genotypes for analysis, either with, or without, MMTV-Cre mediated deletion.

For β-galactosidase staining to detect the recombined R26R reporter gene, mammary glands were removed and fixed in cold paraformaldehyde for 2 hours, and then stained as described (Ismael et al., 2002). Glands not staining for lacZ expression were stained subsequently with Neutral Red (Moraes et al., 2007).

**Histology and immunohistochemistry**

For histological analysis, the #2 and #3 mammary glands from the left side of the animal were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned at 3 μm, and either Hematoxylin/Eosin-stained or used for immunolocalization studies. Antibodies against estrogen receptor (ER), progesterone receptor (PR) and BrdU used for immunolocalization studies were as described (Moraes et al., 2007). All immunostaining was performed with antigen retrieval in 0.1 M Tris–HCl (pH 9.0) containing 10% Tween 20, by heating to 120°C for 10 minutes in a pressure cooker. For immunohistochemistry, detection was by standard peroxidase staining using the ABC System (Vector Laboratories).

For transplantation into immunocompromised hosts, we introgressed the Ptch<sup>mes</sup> allele into a C57BL/6J inbred background by serial backcross of heterozygous Ptch<sup>mes</sup>; ACTB-ECFP males to C57BL/6J females for at least eight generations. Selected progeny were then tested for histocompatibility by transplantation of epithelial fragments into C57BL/6J hosts. Thereafter, experimental animals were generated by a backcross-intercross strategy, in which backcross-derived heterozygotes were intercrossed to generate homozygous and wild-type mice used for transplantation donors and hosts. For whole gland transplantation, mammary glands from ACTB-ECFP-tagged 3-week-old female homozygous Ptch<sup>mes</sup> or wild-type donors were transplanted between the skin and body wall of 3-week-old female ΔRag1 host mice between the endogeneous #3 and #4 mammary glands (Brisken et al., 1998; Lewis et al., 2001). Transplanted mammary glands were allowed to regenerate ductal trees for 8 weeks. Glands were removed and processed for whole gland and histological analysis.

**Ovarian hormone treatments**

Virgin mice, at least six per genotype, were treated at 9 weeks of age for a period of 9 days to assay the ability of ovarian hormones to rescue the Ptch<sup>mes</sup> phenotype and to assay for differential hormone responses. Slow-release implants were constructed of silastic tubing (Cohen and Milligan, 1993) containing either estradiol (E2) alone (20 μg) (Sigma, E-2758), P alone (20 mg) (Sigma, P-130), or E2 and P in combination. Implants were placed under the skin in the suprascapular region. At the end of the treatment period, glands were removed and processed for whole gland and histological analysis.

**Pituitary isografts**

Virgin mice, at least four per genotype, were transplanted with a single pituitary isograft from a wild-type male donor, or sham-operated as control. The pituitary was placed into the kidney capsule of 4- to 5-week-old host mice (wild-type and Ptch<sup>mes</sup> homozygous females) as described previously (Said et al., 2001). Three weeks after transplantation, mammary glands were harvested and processed for whole gland and histological analysis.

**RESULTS**

**Ptch<sup>mes</sup> is a pleiotropic mutation that affects multiple aspects of mammary ductal development**

We showed previously that ΔPtch1 heterozygosity and MMTV-SmoM2 transgene overexpression led to two unique mammary hyperplasia phenotypes. Given that the primary function of PTCH1 in the absence of ligand is in downregulation of SMO function (Makino et al., 2001), and that the Ptch<sup>mes</sup> allele is hypomorphic, we predicted that Ptch<sup>mes</sup> homozygosity would affect mammary gland ductal development in a manner similar to, but more extensive than, ΔPtch1 heterozygosity. Alternatively, glands might appear similar to those observed in MMTV-SmoM2 transgenic mice (which show ductal hyperplasia and increased branching).

To test these predictions, we examined mammary glands of ACTB-ECFP-tagged wild-type and homozygous Ptch<sup>mes</sup> virgin mice at 5, 10 and 20 weeks of age as whole-mount and histological preparations. In whole-mount analysis, at 5 weeks of age glands from wild-type mice showed normal growth, with ~50% of the fat pad filled and multiple TEBs (Fig. 1A). By contrast, 32.4% of glands in Ptch<sup>mes</sup> homozygotes lacked ductal epithelium entirely (primarily glands #2 and #3) and 59.5% showed severely stunted ductal trees with no TEB present (Fig. 1B). In 8.1% of glands examined, ‘escape’ ducts formed (Fig. 1C), but in no case did the percentage of fat pad filled approach that of wild-type glands.

At 10 weeks of age, a similar distribution of mammary phenotypes was observed. Whereas glands of wild-type mice were completely filled with a ductal tree and TEBs were completely regressed (Fig. 1D), Ptch<sup>mes</sup> homozygotes showed 13.3% of glands that lacked mammary epithelium entirely (quantified in Fig. 1J), with 50.5% of glands stunted (Fig. 1E). The escape ducts observed...
in 5-week-old mice were also observed in 10-week-old mice (36.2%) (Fig. 1F,J), typically with multiple bifurcating or trifurcating TEBs (Fig. 1F). In no case did we observe a completely filled fat pad. Thus, at the level of whole-mount analysis, glands from Ptch1mes homozygotes did not resemble those from ΔPtch1/+ heterozygotes (Lewis et al., 1999) or MMTV-SmoM2 transgenic mice (Moraes et al., 2007).

At 20 weeks of age, glands of wild-type mice were 100% filled, with increased side-branching and alveolar budding relative to 10-week-old mice, and, as at other time points, several glands showed a complete lack of epithelium or remained stunted. However, for some glands, 100% of the fat pad was filled (Fig. 1I).

To characterize the observed gland-to-gland variation in the mammary phenotype, we compared the incidence rates for a given phenotype for gland pairs 1-5 from 16 mice at 10 weeks of age (eight Ptch1mes and eight wild type). Fig. 1K summarizes the frequency of mammary gland ductal phenotypes as a function of mammary gland position along the anterior-posterior axis, showing that all glands could be affected, and that ductal elongation varied from gland-to-gland within a given animal.

Abnormalities observed in ΔPtch1 heterozygotes are partially recapitulated in Ptch1mes homozygotes

The most prominent defect observed previously in ΔPtch1 heterozygotes was partial or complete occlusion of the ducts with multiple layers of luminal epithelial cells. Disorganization of the cap and body cell layers of the TEB was also observed. By histological analysis of Ptch1mes homozygous, as compared with wild-type, TEB at 5 weeks of age, stunted ducts showed no histological evidence of a TEB, and no obvious disruption of the epithelium (Fig. 2B versus 2A). By contrast, escape ducts showed some evidence of disruption, particularly in the cap cell layer (Fig. 2C). At 5 weeks of age, escape ducts appeared relatively normal histologically (Fig. 2D versus 2E). At 10 weeks of age, stunted ducts were frequently 3-4 cell layers thick (Fig. 2F), rather than the normal 1-2 cell layers observed in wild-type ducts (Fig. 2E). These ductal dysplasias resembled those seen in Ptch1/+ mice (see Fig. 5E,F), but were not as extensive.

Ptch1 functions in both mammary epithelium and stroma

Previous phenotypic and transplantation analyses of ΔPtch1 heterozygotes suggested that Ptch1 functions in the stroma to influence epithelial cell function. To determine whether the Ptch1mes phenotype observed in intact animals was due to an intrinsic defect in the gland proper, we performed whole gland transplants into immunocompromised ΔRag1 mice. As expected, glands derived from wild-type mice grew normally (Fig. 3A,C). At 5 weeks of age, escape ducts appeared relatively normal histologically (Fig. 2D versus 2E). At 10 weeks of age, stunted ducts were frequently 3-4 cell layers thick (Fig. 2F), rather than the normal 1-2 cell layers observed in wild-type ducts (Fig. 2E). These ductal dysplasias resembled those seen in Ptch1/+ mice (see Fig. 5E,F), but were not as extensive.
immunocompromised ΔRag1 mice. Epithelial fragments from wild-type mice grew as expected, nearly filled the fat pad (Fig. 3E), and showed normal ductal patterning and termini (Fig. 3G). Similarly, and consistent with epithelial growth in whole gland transplantation, epithelial fragments derived from Ptch1<sup>mes</sup> homozygotes grown in the contralateral fat pad grew to a similar extent as those derived from the wild type (Fig. 3F). However, in contrast to glands derived from wild-type mice (Fig. 3G), duct termini from outgrowths derived from Ptch1<sup>mes</sup> homozygotes frequently showed aberrant termini with splayed ends (Fig. 3H). Epithelial fragment transplantation into immunocompetent hosts showed similar results (data not shown). These results demonstrate that Ptch1 has a function in mammary epithelium to regulate ductal morphogenesis, and indicate that the rounded duct termini observed in whole gland transplants derived from Ptch1 homozygotes were due to an additional defect in the mammary stroma.

### Conditional disruption of Ptch1 by MMTV-Cre confirms an epithelial role for Ptch1 in mammary ductal morphogenesis

To confirm the epithelial role in mammary gland growth, we examined glands derived from mice in which Ptch1 function was conditionally disrupted in the mammary epithelium via MMTV-Cre-mediated deletion (Li et al., 2002). To accomplish these analyses, Ptch1<sup>Δ</sup>/Δ; R26R/+ mice were crossed with ΔPtch1/+; MMTV-Cre/+; R26R mice to yield the required Ptch1 genotypes for analysis, either with, or without, MMTV-Cre. R26R-tagged glands that were wild-type for Ptch1 showed no morphological or histological defects (data not shown). Similarly, mammary glands heterozygous for either ΔPtch1 (Fig. 4A versus 4B) or Ptch1<sup>Δ</sup> (Fig. 4C versus 4D) showed no morphological alterations in the presence of MMTV-Cre. By contrast, mice carrying both the Ptch1<sup>Δ</sup> allele and the ΔPtch1 null allele showed dramatic morphological changes, but only in the presence of MMTV-Cre (Fig. 4E versus 4F), including hyperplasia, increased branching and alveolar budding.

In histological analysis, mammary glands heterozygous for either ΔPtch1 (Fig. 4G versus 4H) or MMTV-Cre; Ptch1<sup>Δ</sup> (Fig. 4I versus 4J) showed no obvious phenotype; this was unexpected based on our
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Morphological changes caused by ΔPtch1 heterozygosity are background dependent

During the course of the analysis of the Ptch1 allele and the ΔPtch1 allele showed histological changes, with some ducts showing more than two layers of luminal epithelial cells, but only in the presence of MMTV-Cre (Fig. 4K versus 4L). Thus, the phenotype of Ptch1+/ΔPtch1 was similar to, but less extensive than, that of ΔPtch1 heterozygosity in a B6D2F1 background.

Duct growth is not rescued by exogenous estrogen or progesterone

Gland-to-gland variability in an individual animal was not entirely consistent with a systemic hormonal defect, yet stunted duct growth in Ptch1mice homozygotes was reminiscent of mice carrying loss-of-function mutations in estrogen receptor α (ERα) (Korach, 1994; Mallepell et al., 2006). To test the ability of ovarian hormones to rescue the stunted growth phenotype, we treated wild-type and homozygous mutant mice with E2 alone, P alone, or both (E2+P), and compared them with untreated animals. Untreated wild-type animals showed normal gland morphology, with little alveolar development (Fig. 7A); there was increased alveolar budding with E2 alone (Fig. 7B), P alone (Fig. 7C), and E2+P treatments (Fig. 7D). By contrast, the stunted glands of Ptch1mice homozygotes showed little or no evidence of response to any treatment (Fig. 7H, inset). However, escape ducts (Fig. 7F versus Fig. 6H) did show evidence of hormone responsiveness, with E2 alone (Fig. 7F), P alone (Fig. 7G), and E2+P treatments (Fig. 7H) causing increased sidebranching and alveolar budding, such that they approached levels seen in wild-type mice.

To investigate further the reason for the differential hormone responses of stunted versus escape ducts in Ptch1mice homozygotes, we conducted an immunohistochemical analysis of ER and progesterone receptor (PR) expression, as well as of BrdU incorporation as an index of proliferation. Untreated wild-type ducts did not show expression of detectable receptor. Escape ducts also showed reduced ER expression (Fig. 7K) and an intermediate percentage of cells expressing detectable ER. PR showed a similar pattern of expression to ER (Fig. 7L,M). This reduced expression of both receptors was consistent with the reduced hormone responsiveness observed.

With respect to proliferation, untreated wild-type glands showed 0.5% BrdU labeling (Fig. 7O). Similarly, untreated stunted ducts of Ptch1mice homozygotes showed only 0.5% BrdU labeling (Fig. 7P), and untreated escape ducts of Ptch1mice homozygotes showed 7.2% BrdU labeling (Fig. 7Q). We detected a significant increase in BrdU incorporation caused by Ptch1mice only in glands expressing the near-normal range of estrogen receptors.
Ductal elongation defects caused by \textit{Ptch1\textsuperscript{mes}} homozygosity are rescued by pituitary isograft

To determine whether the ductal elongation failure in \textit{Ptch1\textsuperscript{mes}} homozygotes could be rescued by pituitary hormones, we transplanted a single pituitary into \textit{Ptch1\textsuperscript{mes}} and wild-type host animals at 4-5 weeks of age. Three weeks after transplantation, sham-operated wild-type animals showed ductal elongation characteristic of 7- to 8-week-old virgin mice, with extensive ductal elongation and visible TEBs (Fig. 8A). Wild-type animals hormonally stimulated by pituitary isografts exhibited a significant increase in ductal elongation, branching and alveolar development (Fig. 8B), with no TEBs remaining, and ducts reaching the periphery of the fat pad. Sham-operated \textit{Ptch1\textsuperscript{mes}} homozygotes showed the expected stunted or modest escape duct growth (Fig. 8C). However, \textit{Ptch1\textsuperscript{mes}} homozygotes receiving a pituitary transplant showed significantly enhanced ductal elongation (Fig. 8D), with prominent TEBs and an average of $\sim$55% of the fat pad filled (in contrast to an average of $\sim$26% in sham-operated homozygotes) (Fig. 8). These results indicate that \textit{Ptch1} function is required in the pituitary to drive ductal elongation and control hormone responsiveness.

DISCUSSION

It is not currently known in which tissue compartment(s) and cell type(s) \textit{Ptch1} function is required for normal mammary gland development, nor is it known which of the five or more functions of \textit{Ptch1} may be acting in a given location.

Our previous analysis of a \textit{Ptch1} loss-of-function allele (\textit{\textDelta}Ptch1) in a mixed genetic background (C57BL/6J × DBA2) demonstrated that heterozygous loss of \textit{Ptch1} in mouse led to increased proliferation and ductal dysplasias similar to ductal hyperplasias of the human breast. Transplantation of epithelial fragments into cleared fat pads of immunocompromised (\textit{Rag1}\textsuperscript{−/−}) or B6D2F1 host mice did not recapitulate the phenotype observed in intact animals. By contrast, whole mammary gland transplantation into immunocompromised or B6D2F1 host mice achieved a partial recapitulation of the mutant phenotype. These data led to the interpretation that \textit{Ptch1} functions primarily in the stroma to direct epithelial cell behavior. However, epithelial and systemic functions of \textit{Ptch1} were not ruled out.

**Fig. 5. Effect of \textit{\textDelta}Ptch1 heterozygosity in different genetic backgrounds.** The mouse background is shown above each column, the genotype to the left.

- (I-P) BrdU staining for evaluation of proliferation.
- (Q) BrdU labeling index for wild type and \textit{\textDelta}Ptch1/+ in different backgrounds. *$P=0.017$ and *$P=0.024$ for DBA2 and B6D2F1, respectively (Wilcoxon rank sum test).

Scale bar: 50 \textmu m.
In this study, we exploited two additional novel Ptch1 alleles to elucidate the roles of Ptch1 in virgin mammary gland development more fully. Using the hypomorphic Ptch1<sup>mes</sup> allele, we demonstrate that homozygosity leads to three main phenotypes in intact animals: (1) the complete absence of mammary epithelium; (2) a failure of ductal elongation leading to persistence of stunted, rudimentary glands similar to those observed in wild-type mice prior to puberty; or (3) dysplastic growth of escape ducts, which fail to fill the available fat pad by 10 weeks of age despite increased proliferation. All three phenotypes may be present in the same animal. Whole gland and epithelial transplantation showed that Ptch1 has functions in both the mammary epithelium and mammary stroma. However, the stunted growth phenotype was fully rescued by transplantation in all cases, indicating a systemic function.

Using the Ptch1<sup>c</sup> allele in combination with the ΔPtch1 allele analyzed previously, we now confirm a key role for epithelial Ptch1 in regulating ductal morphogenesis. However, the dysplastic ductal histology observed previously was not fully recapitulated when Ptch1 was disrupted solely in the epithelium in Ptch1<sup>c</sup>/ΔPtch1 mice, nor was it observed in a mixed genetic background. Although loss of Ptch1 function solely in mammary epithelium led to ductal dysplasia, this phenotype was distinctly different from the alveolar hyperplasias induced by ectopic expression of activated SMO in MMTV-SmoM2 transgenic mice. Thus, Ptch1 loss-of-function is not functionally equivalent to SMO activation in mammary epithelium, suggesting that Ptch1 might have SMO-independent functions in the gland.

In virgin female Ptch1<sup>mes</sup> homozygous mice, we show that ~30% of animals have one or more mammary glands that entirely lack mammary epithelium. However, the stunted growth phenotype was fully rescued by transplantation in all cases, indicating a systemic function.

Using the Ptch1<sup>c</sup> allele in combination with the ΔPtch1 allele analyzed previously, we now confirm a key role for epithelial Ptch1 in regulating ductal morphogenesis. However, the dysplastic ductal histology observed previously was not fully recapitulated when Ptch1 was disrupted solely in the epithelium in Ptch1<sup>c</sup>/ΔPtch1 mice, nor was it observed in a mixed genetic background. Although loss of Ptch1 function solely in mammary epithelium led to ductal dysplasia, this phenotype was distinctly different from the alveolar hyperplasias induced by ectopic expression of activated SMO in MMTV-SmoM2 transgenic mice. Thus, Ptch1 loss-of-function is not functionally equivalent to SMO activation in mammary epithelium, suggesting that Ptch1 might have SMO-independent functions in the gland.

In virgin female Ptch1<sup>mes</sup> homozygous mice, we show that ~30% of animals have one or more mammary glands that entirely lack mammary epithelium. However, we have not yet investigated whether this phenotype is due to a failure of development in the embryo, lack of mammary structures is similar to the embryonic phenotype of ΔGli3 homozygotes, in which mammary glands #3...
and #5 fail to develop (Hatsell and Cowin, 2006; Veltmaat et al., 2006). GLI3 repressor function is required in the somites underlying the #3 mammary glands to allow FGFl0 signaling to the outlying ectoderm to induce a mammary placode. It will be of considerable interest to determine whether the Ptch1<sup>mes</sup> phenotype is indeed due to a similar failure to induce FGFl0 signaling, with a broader position specificity than GlI3 loss-of-function.

The ability of transplantation to rescue the stunted duct growth phenotype suggested a systemic role for Ptch1 in regulating gland development. If a defect is intrinsic to the epithelial compartment [e.g. disruption of the estrogen receptor α gene (Erec, Esr1 – Mouse Genome Informatics) (Mallepall et al., 2006)], one expects the mutant phenotype to be recapitulated in epithelial fragment transplantation. If a defect is intrinsic to the mammary gland stromal compartment proper [e.g. disruption of the parathyroid hormone-related peptide receptor (PTHrP1; PTH1R – Mouse Genome Informatics) (Dunbar et al., 1998)], one would expect the phenotype to be recapitulated in whole mammary gland transplants. Although we observed minor morphological alterations under these two conditions, mammary duct outgrowth was rescued in all cases, thereby demonstrating a systemic role for PTCH1. Using classical hormone treatment, we now show that Ptch1 is required in the pituitary for ductal elongation and hormone responsiveness.

The pituitary gland produces two hormones required for gland development, GH and PRL. GH acts on mammary stroma to produce IGF1, which in turn acts on mammary epithelium to promote ductal outgrowth. PRL also facilitates ductal morphogenesis via IGF1, and regulates ER and PR expression (Frasor and Gibori, 2003; Muldoon, 1987), but is primarily responsible for driving alveolar development and function during pregnancy and lactation (Ormandy et al., 1997). Preliminary experiments indicate that neither GH nor PRL treatment alone is sufficient to rescue the growth of mammary glands in Ptch1<sup>mes</sup> homozygotes, suggesting that a combinatorial function of pituitary-derived factors is required to promote ductal elongation and hormone responsiveness. We are currently exploring whether treatment with IGF1, amphiregulin and/or PTHrP is capable of altering the Ptch1<sup>mes</sup> homozygous phenotype, either alone or in combination. We are also testing whether forced expression of Erα in MMTV-Erα of IGF1 in MMTV-Igf1 transgenic mice will rescue the stunted growth phenotype.

Finally, we demonstrate that the ΔPtch1/+ hyperplastic phenotype described previously (Lewis et al., 1999; Moraes et al., 2007) is dependent on genetic background, with B6D2F1 and DBA2 mice expressing the mutant phenotype, whereas C57BL/6J and FVB mice do not show hyperplasia. The hyperplastic phenotype was also observed recently in a C57BL/6J/129 mixed genetic background (Li et al., 2007) is dependent on genetic background, with B6D2F1 and DBA2 mice expressing the mutant phenotype, whereas C57BL/6J and FVB mice do not show hyperplasia. The hyperplastic phenotype was also observed recently in a C57BL/6J/129 mixed genetic background (Li et al., 2007). These results provide an explanation for why two other groups did not observe a dysplastic phenotype in their alternative genetic backgrounds (Fiaschi et al., 2007; Hatsell and Frost, 2007).

Recently, a polymorphic variant in the Ptch1 gene has been identified in C57BL/6J versus FVB mice, the presence of which correlates with either resistance (C57BL/6J) or susceptibility (FVB) of mice to Hras-induced squamous cell carcinoma of the skin (Wakabayashi et al., 2007). This polymorphism cannot account fully for our observations, given that the ductal dysplasia phenotype was not observed in the FVB background, but was observed in DBA2 despite the presence of the same allelic variant. However, the disparity observed in the backgrounds might be explained by the presence of a genetic modifier in DBA and B6D2F1, which may be acting synergistically with the deletion of the Ptch1 gene to induce higher levels of cellular proliferation.

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