Defects in mouse mammary gland development caused by conditional haploinsufficiency of *Patched-1*

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**SUMMARY**

In vertebrates, the hedgehog family of cell signaling proteins and associated downstream network components play an essential role in mediating tissue interactions during development and organogenesis. Loss-of-function or misexpression mutation of hedgehog network components can cause birth defects, skin cancer and other tumors. The mammary gland is a specialized skin derivative requiring epithelial-epithelial and epithelial-stromal tissue interactions similar to those required for development of other organs, where these interactions are often controlled by hedgehog signaling. We have investigated the role of the *Patched-1* (*Ptc1*) hedgehog receptor gene in mammary development and neoplasia. Haploinsufficiency at the *Ptc1* locus results in severe histological defects in ductal structure, and minor morphological changes in terminal end buds in heterozygous postpubescent virgin animals. Defects are mainly ductal hyperplasias and dysplasias characterized by multilayered ductal walls and dissociated cells impacting ductal lumens. This phenotype is 100% penetrant. Remarkably, defects are reverted during late pregnancy and lactation but return upon involution and gland remodeling. Whole mammary gland transplants into athymic mice demonstrates that the observed dysplasias reflect an intrinsic developmental defect within the gland. However, *Ptc1*-induced epithelial dysplasias are not stable upon transplantation into a wild-type epithelium-free fat pad, suggesting stromal (or epithelial and stromal) function of *Ptc1*. Mammary expression of *Ptc1* mRNA is both epithelial and stromal and is developmentally regulated. Phenotypic reversion correlates with developmentally regulated and enhanced expression of *Indian hedgehog* (*Ihh*) during pregnancy and lactation. Data demonstrate a critical mammary role for at least one component of the hedgehog signaling network and suggest that *Ihh* is the primary hedgehog gene active in the gland.

**Key words:** Hedgehog signal transduction, Organogenesis, Breast cancer, Mammary gland, Mouse

**INTRODUCTION**

Mammary gland development (Fig. 1), like that of many organs, requires interactions between an epithelium and a surrounding mesenchyme (embryonic) or stroma (postnatal) (Cunha, 1994; Daniel and Silberstein, 1987; Howlett and Bissell, 1993; Imagawa et al., 1994; Russo and Russo, 1987; Sakakura, 1987; Schmeichel et al., 1998) and between epithelial cells themselves (Brisken et al., 1998). Such interactions control growth, govern overall patterning of the ductal tree, and influence the function of the gland. Most mammary development occurs in the subadult animal, where its embryonic-like growth characteristics can be readily examined and manipulated. This fact coupled with the similarities between tissue interactions critical to mammary gland development and those in other organs make the mammary gland an attractive model for the study of basic questions in developmental biology.

Mouse mammary development begins at approximately embryonic day 10 (E10) (Fig. 1), with the definition of the nipple region and subsequent invasion of the underlying mammary mesenchyme by the presumptive mammary epithelium to establish a bulb of epithelial cells. After approximately E16, the bulb elongates and invades a second type of mesenchyme, the mammary fat pad precursor mesenchyme. The gland then initiates a small amount of ductal growth and branching morphogenesis, after which it becomes growth quiescent until puberty.

Stimulated by ovarian hormones at puberty, the gland begins a proliferative phase of development, growing rapidly via the terminal end bud (TEB). The TEB is a bulb-like structure consisting of relatively undifferentiated epithelial cells at the tip.
Upon HH binding, PTC is inactivated allowing SMO downstream signaling mediated by the smoothened (SMO) protein, located in the membrane of nearby cells. In the absence of HH binding, PTC acts as a molecular brake to inhibit (PTC), located in the membrane of nearby cells. In the absence of HH binding, PTC acts as a molecular brake to inhibit

In flies, the signaling network consists of a single secreted hedgehog (HH) protein which binds to a receptor, patched (PTC), located in the membrane of nearby cells. In the absence of HH binding, PTC acts as a molecular brake to inhibit downstream signaling mediated by the smoothened (SMO) protein. Upon HH binding, PTC is inactivated allowing SMO to function. These events ultimately favor the conversion of a transcription factor, cubitus interruptus (CI) to a full-length activator form CI(act) over an alternative repressor form CI(rep). CI, in turn, controls expression of target genes that contribute to establishment of cell identity and to patterning of the fly body.

In mammals the signaling network is more complex, with many of the fruitfly genes being duplicated to form multigene families (Ingham, 1998b). For example, instead of one hedgehog gene, there are three related genes Shh, Ihh and Dhh (Kumar et al., 1996) among which Shh and Ihh mediate most known signaling functions (Bitgood et al., 1996; Hammerschmidt et al., 1997). Similarly, instead of one ptc receptor gene there are at least two Ptc1 and Ptc2 (Carpenter et al., 1998; Goodrich et al., 1996; Motoyama et al., 1998); and instead of a single ci transcription factor gene, there are at least three (designated Gli1, Gli2 and Gli3) (Hughes et al., 1997; Ruppert et al., 1990; Walterhouse et al., 1993). Despite this increase in complexity, the mammalian network appears to act in similar fashion to the system in flies.

To exercise its control during vertebrate development, the hedgehog network regulates, or interacts with, a battery of gene families. Depending on the organ, these gene families include those encoding Fibroblast Growth Factors (FGFs), Wnt proteins (wingless homologs), transforming growth factor-β (TGF-β) family members (including TGF-β, Bone Morphogenic Proteins (BMPs), activins and inhibins), homeodomain transcription factors (including Hox and Pax), and parathyroid hormone-related protein (PthRP) and its receptor (Hammerschmidt et al., 1997). Importantly, members of each of these gene families have known or suspected roles in mammary development or neoplastic progression (Daniel et al., 1996; Edwards, 1998; Robinson and Hennighausen, 1997; Wysolmerski et al., 1998). This association provides a compelling reason to investigate hedgehog family signal transduction in the mammary gland.

A promising candidate regulatory system for mediating the tissue interactions during mammary development is hedgehog signal transduction. In mammals, the genes encoding the hedgehog family of secreted signaling proteins (Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh)) and associated signaling network components are important regulators of cellular identity, patterning and tissue interactions during embryogenesis and organogenesis. These molecules are typically expressed in regions of inductive tissue interactions and are involved in diverse processes such as the development of skin, limbs, lung, eye, nervous system and tooth, the differentiation of cartilage and sperm, and the establishment of left-right asymmetry (Hammerschmidt et al., 1997; Ingham, 1998b; Levin, 1997).

Whereas the range of vertebrate developmental processes dependent on hedgehog signaling testifies to its critical importance, the mechanics of hedgehog signaling are best understood from genetic studies in the fruitfly Drosophila melanogaster (Hammerschmidt et al., 1997; Ingham, 1998b). In flies, the signaling network consists of a single secreted hedgehog (HH) protein which binds to a receptor, patched (PTC), located in the membrane of nearby cells. In the absence of HH binding, PTC acts as a molecular brake to inhibit downstream signaling mediated by the smoothened (SMO) protein. Upon HH binding, PTC is inactivated allowing SMO to function. These events ultimately favor the conversion of a transcription factor, cubitus interruptus (CI) to a full-length activator form CI(act) over an alternative repressor form CI(rep). CI, in turn, controls expression of target genes that contribute to establishment of cell identity and to patterning of the fly body.

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Another compelling reason to study the hedgehog signaling network in the mammary gland is the issue of breast cancer. Several of the genes in the mammalian hedgehog signaling network have been identified as either protooncogenes or tumor suppressor genes. A number of these genes, including Ptc1, Smo, Shh and Gli1, contribute to the development of skin cancers, most notably basal cell carcinomas (Dahmane et al., 1997; Fan et al., 1997; Ingham, 1998a; Johnson et al., 1996; Oro et al., 1997; Reifenberger et al., 1998; Xie et al., 1998). Ptc1 has also been causally implicated in the development of medulloblastomas (brain tumors) and other soft tissue tumors (Goodrich et al., 1997; Hahn et al., 1998). Gli1 was originally identified as an amplified gene in human glioblastomas (brain tumors) and amplification has since been observed in other tumor types (Dahmane et al., 1997; Kinzler et al., 1988; Rao et al., 1998). While Ptc1 mutations have been identified in a small fraction of human breast cancers (Xie et al., 1997), no general role for the hedgehog network has been established in the mammary gland, nor has the tumorigenic potential for altered network function in the mammary gland been explored.

Of the two known hedgehog receptors, Ptc1 is most fully characterized. Animals homozygous for targeted disruption of Ptc1 show early embryonic lethality (around embryonic day 9.5) with, among other alterations, severe defects in nervous system development accompanied by changes in neural cell...
fates. Heterozygous animals can also show defects including skeletal abnormalities, failure of neural tube closure, medulloblastomas (brain tumors), rhabdomyosarcomas, and strain-dependent embryonic lethality (Goodrich et al., 1996; Hahn et al., 1998).

If the hedgehog network plays a role in mammary development, components of the network should be expressed in developmentally regulated patterns and disruption of their function should have developmental consequences. In this paper we demonstrate cell-type specific and developmentally regulated mammary expression of two hedgehog network genes, \textit{Ptc1} and \textit{Ihh}. Further, we show that wild-type levels of \textit{Ptc1} function are essential for proper mammary histogenesis, with heterozygous virgin animals developing ductal dysplasias that are reversible during pregnancy and lactation, allowing normal secretory function. Phenotypic reversion correlates with enhanced expression of \textit{Ihh} during these stages of development. Coupled with expression and functional analysis of other hedgehog network genes (M. T. L. and C. W. D., unpublished) our data provide the first model for hedgehog signaling function in the mammary gland.

**MATERIALS AND METHODS**

**Animals**

The inbred mouse strains Balb/C and C57/B16 are maintained in our laboratory. C57/B16 × DBA2 F1 (B6D2F1) female mice were obtained from Taconic. Athymic Balb/C nude (nude) female mice were obtained from Simonsen.

Two breeding pairs of mice heterozygous for a disrupted \textit{Ptc1} gene were used to initiate a breeding colony and have been previously described (Goodrich et al., 1997). The original \textit{Ptc1} mutation was maintained in a 129Sv:C57/B16 background with subsequent backcross to B6D2F1. In our laboratory, the mutation was likewise maintained in a B6D2F1 background by serial backcross but this background is still mixed (as evidenced by segregation of coat color markers) which precluded epithelial or whole mammary gland transplants between animals (see below). Genotyping was performed by PCR as per Goodrich (1997).

For expression studies (northern hybridization, and in situ hybridization) Balb/C animals were used to correlate results with expression of other genes in the hedgehog signaling network currently under study. In situ hybridizations for \textit{Ptc-1} were replicated using C57/B16 mice to demonstrate consistency between strains (data not shown).

**Developmental stages**

Except for the northern hybridizations, the developmental stages examined were: 3 weeks, 5 weeks, 7 weeks, 10 weeks, early pregnant (5.5-9.5 d.p.c.), late pregnant (15.5-19.5 d.p.c.), lactating (days 6-7), involuting (days 2, 10 and 14). For 5-7- and 10-week timepoints, animals were taken from different litters on different dates to minimize possible complications due to the estrus cycle. For pregnancy, lactation and involution studies, mice were matured to 10 weeks of age prior to mating. For involution stages in expression studies, mice were allowed to lactate 10 days prior to pup removal to ensure that the dams were still actively feeding pups. Not all stages were examined with all techniques, as noted.

**mRNA isolation**

No. 4 mammary glands of female Balb/C mice were used for RNA extractions. Lymph nodes were removed using forceps and the gland flash-frozen in liquid nitrogen immediately upon removal. Glands were stored at –80°C prior to use. Total RNA was isolated by column chromatography (Qiagen). Each sample represents pooled RNA from at least 6 animals taken from different cages to minimize the possibility of estrus cycle synchronization. Embryonic (14 day) RNA was isolated in a similar fashion.

**Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

Reverse transcription reactions to produce first strand cDNA used total RNA (10 µg) from either mouse mammary gland or 14-day embryo essentially as described by Silberstein et al. (1997). Amplification was performed on a Perkin-Elmer 9600 as follows: 94°C for 1 minute followed by 30 cycles of 94 for 1 minute, 65°C for 2 minutes, and 72°C for 3 minutes and thereafter maintained at 4°C. Amplifications for \textit{Dhh} and \textit{Ihh} were optimized by adding DMSO to 5%.

Gene specific primers for \textit{Dhh} (accession no. X76292), \textit{Ihh} (accession no. U85610), \textit{Shh} (accession no. X76290), \textit{Ptc1} (accession no. AA080038) and \textit{Ptc2} (accession no. AB000847) were designed to avoid highly conserved regions in either gene family. With the exception of those for \textit{Ptc2}, primers were designed over introns to control for DNA contamination. The primer pairs used for this study are as follows: (\textit{Dhh}) (sense) mDhhF1 5'GACCTCTCGATCCCAACTACAACCCCG-3', (antisense) mDhhR1 5’-ACGGTCGTGAGCAGCTTCTGCTACAAG-3'. (antisense) mIhhR3 5’-GCACATCAGTAGGGTGGGTTCC-3' (\textit{Ihh}) (sense) mIhhF4 5’-CAAGCTCTGGCTCTTGCTCAGTAAAG-3', (antisense) mIhhR3 5’-GCACATCAGTAGGGTGGGTTCC-3', (\textit{Shh}) (sense) mShhF1 5’-TCAGGAAGATTAAAGGAACACCC-3', (antisense) mShhR1 5’-GGCTCCAGCCTCTGCAGTACGTA-3', (\textit{Ptc1}) (sense) mPtc/R2 5’-GGCCGCTGGAAGGTAAAG-3', (antisense) mPtc/R2 5’-CGCTCCACGCAGTACGTA-3', (\textit{Ptc2}) (sense) mPtcR2 5’-GTGAGGTAGCTCTGACCCCGTGACTGG-3', (antisense) mPtcR2 5’-GTGAGGTAGCTCTGACCCCGTGACTGG-3', (antisense) mPtcR2 5’-TCGCTCCACGCGATGTCAGTTC-3' Specificity of the hedgehog family RT-PCR was confirmed by Southern hybridization of the reaction products according to standard techniques (Sambrook et al., 1989) using digoxigenin-11-dUTP-labeled plasmid-derived probes (Boehringer Mannheim, Genius System) for each of the cloned genes.

**Northern hybridizations**

Probe preparation and northern hybridization was performed as described by Friedmann and Daniel (1996). The probe used for \textit{Ptc1} was a 350 bp fragment derived from of the \textit{Ptc1} cDNA (nt 3740-4099) which does not have a counterpart in the \textit{Ptc2} cDNA and does not cross-hybridize with \textit{Ptc2} mRNA.

**In situ hybridization**

The no. 2 and no. 3 mammary glands of Balb/C mice were used. Experiments using the \textit{Ptc1} probes were also repeated using glands of C57/B16 mice to ensure consistency between strains. Glands were fixed in ice-cold 4% paraformaldehyde:PBS for 3 hours and processed for in situ hybridization (Friedmann and Daniel, 1996). Digoxigenin-labeled riboprobes for \textit{Ptc1}, \textit{Shh} and \textit{Ihh} corresponded to the same cDNA fragments used in the Southern and northern hybridizations and were prepared using T7 and SP6 RNA polymerases and hybridized essentially as described (Friedmann and Daniel, 1996). The following stages were not examined: 3 week, 7 week.

In situ hybridization in the mammary gland is not an efficient semi-quantitative method; the qualitative statements made regarding relative staining intensity (expression) are based on exhaustive replication over a one year period using multiple serial sections of tissue samples taken from different animals at each developmental stage.

**Whole gland morphological analysis**

Backcross-derived \textit{Ptc1} heterozygotes and wild-type littermate or age matched females were used. B6D2F1 animals were also examined as controls. Mammary glands 1-5 were harvested at various developmental stages (at least 5 mice each stage), fixed in ice-cold 4% paraformaldehyde:PBS, and hematoxylin stained as described by
Daniel et al. (1989). Each gland was examined for developmental abnormalities under a dissecting scope.

**Histological analysis**

The no. 2 or no. 3 mammary glands were used. At least 3 representative animals were examined for each developmental stage. Gland fragments were embedded in paraffin wax, sectioned at 7 μm and hematoxylin/eosin stained. Propidium iodide (nuclear DNA) and phallloidin (actin) staining was performed using frozen sections as described by Aumuller et al. (1991).

**Hormone injection studies**

4-week old virgin female heterozygotes (n=4) and wild-type littermates (n=4) were injected subcutaneously with 1 mg progesterone and 1 μg estradiol (in cottonseed oil) daily for 9 consecutive days (Tonelli and Sorof, 1980). Mammary glands were removed immediately thereafter and processed for whole gland and histological analysis.

**Whole mammary gland transplantation studies**

Whole mammary gland transplantation experiments were performed in similar fashion to those described previously (Brisken et al., 1998) to determine if the defects observed in Ptc1 heterozygotes were intrinsic to the gland. Entire no. 4 mammary glands containing both epithelium and stroma were removed from 3-week old wild-type and heterozygous animals and contraterally transplanted between the skin and abdominal wall (their normal position) of 3-week old Balb/C nu/nu mice and allowed to revascularize and grow for 4 weeks. Glands were removed and processed for whole gland and histological analysis.

**Epithelial transplantation studies**

Transplantation experiments were performed to determine whether dysplastic and hyperplastic epithelium from Ptc1 heterozygotes maintained an altered phenotype upon transplant into wild-type stroma of virgin female Balb/C nu/nu mice whose endogenous epithelium had been surgically removed (cleared), as previously described (DeOme et al., 1958). Small fragments epithelium from virgin or early pregnant wild-type and heterozygous animals were contraterally transplanted and allowed to regenerate a ductal tree for 6 weeks to 8 months. Glands were removed and processed for whole gland and histological analysis.

Transplant outgrowths are easily discriminated from ingrowths resulting from incomplete removal of endogenous epithelium by identification of a growth center at the site of transplantation in the middle of the fat pad (outgrowth); ingrowths are characterized by invasion of the fat pad from the cut end. Nevertheless, cleared gland fragments were routinely fixed and stained to ensure complete removal of endogenous epithelium.

Behavior of transplanted epithelium during pregnancy and lactation was not investigated in this study since Balb/C nu/nu females are not efficiently impregnated.

Since the genetic background of this strain is still mixed, reciprocal epithelial transplantation between Ptc1 heterozygotes and wild-type littermates could not be performed due to histoincompatibility.

**RESULTS**

**Components of the hedgehog signal transduction network are expressed in the mouse mammary gland**

RT-PCR experiments demonstrated that several components of the hedgehog signal transduction network were expressed in the mammary gland throughout postnatal development, including all three hedgehog genes (Fig. 2A), Ptc1 and Ptc2 (Fig. 2B), as well as the Gli1, Gli2 and Gli3 genes (data not shown). Given the profound effect of targeted disruption of Ptc1 in embryos (Goodrich et al., 1996; Hahn et al., 1998) and the pivotal position of the gene in the signal transduction network, we chose to investigate the expression and function of the Ptc1 gene in mammary gland development.

To confirm Ptc1 expression in the mammary gland and to examine whether or not Ptc1 expression is regulated through

![Figure 2](image-url)

**Fig. 2.** Hedgehog network component expression – non-quantitative RT-PCR and northern blot hybridization. (A) Hedgehog gene expression detected by Southern blot hybridization of products from RT-PCR at different stages of mammary development. Each hybridizing band was of the expected size for each of the gene-specific primer pairs, as shown. (B) Ptc1 and Ptc2 gene expression detected by RT-PCR at selected stages of mammary development. Panels depict ethidium bromide-stained agarose gel separations of RT-PCR products. Ptc1 products using RNA derived from mature animals were generally not observed but were detected in replicate experiments. Variable detection of an amplified band in 12-week samples is consistent with the reduction in Ptc1 mRNA observed by northern hybridization relative to 5-week samples (below) but suggests that further optimization of the reaction conditions is required for consistent visible detection of this amplicon. (C) Northern blot hybridization for Ptc1 expression during proliferative development. Transcript sizes are noted at the right side for Ptc1. Even loading of RNA for each sample was confirmed by hybridization with a probe for the L7 ribosomal protein mRNA.
mammary proliferative development, we performed developmental Northern hybridization (Fig. 2C). At least two transcripts of the expected sizes (9.5 kb and 10.0 kb) were readily detected in RNA from glands of 5-week old animals, a proliferative stage characterized by both rapid ductal growth and differentiation of epithelial and stromal elements. By contrast, expression was reduced in prepubescent glands of 3-week animals and mature glands of 12-week virgin animals. Data suggested that Ptc1 expression is developmentally regulated.

**Ptc1 is differentially expressed in mammary epithelial cell types**

To further investigate developmental regulation suggested by Northern analysis and to determine which cell types express Ptc1, in situ hybridization was performed at variousdevelopmental stages. In all tissues examined to date, Ptc1 transcriptionally autoregulates, repressing its own transcription to low levels in the absence of Hedgehog signal. Therefore higher level Ptc1 transcription (often the only detectable expression) is an indication that cells have received Hedgehog signal. Ptc1 function, however, may be active in cells where little transcription can be detected (Goodrich et al., 1997).

During embryonic development, Ptc1 is expressed at least as early as E14 in the epithelial bulb (Fig. 3A). Expression in the bulb is reduced relative to that in the overlying epidermis and approximately equal to that detected in the surrounding mammary mesenchyme.

During puberty, the pattern of expression in terminal end buds at 5 weeks of age is of particular interest in that these rapidly growing structures are largely responsible for growth and patterning of the mammary ductal tree (Fig. 3B). Body cells (relatively undifferentiated luminal epithelial cells) of the terminal end bud express a comparatively high level of Ptc1 relative to cap cells (myoepithelial stem cells) and subtending ducts. This cell-type specific expression is retained as these two cell populations differentiate into luminal epithelium and myoepithelium, respectively, along the subtending duct formed by the advancing end buds (Fig. 3B) and in mature ducts of 10-week animals (Fig. 3C).

At 5 weeks, 10 weeks and in early pregnancy (Fig. 3D), low levels of Ptc1 expression can also be detected in periductal stroma, but not in the fat pad immediately in front of growing end buds or distant from epithelial structures. These data suggest Ptc-1 may function in both epithelium and stroma to mediate epithelial-stromal or epithelial-epithelial interactions, or both. Presumptive periductal fibroblasts are pre-existent in the mammary fat pad ahead of growing terminal end buds and are induced to divide, differentiate, and condense around the subtending duct behind the endbud (Williams and Daniel, 1983). Since no stromal cells distant from epithelium detectably express Ptc1, these data also indicate that Ptc1 expression in the stroma is induced by the presence of mammary epithelium.

Throughout pregnancy Ptc1 expression becomes progressively elevated in developing lobule-alveolar structures relative to associated ducts (Fig. 3D, E). Highest levels of Ptc1 expression are found during lactation (Fig. 3F) as evidenced by significantly more rapid and heavy accumulation of the blue-black precipitate relative to all other tissue samples examined. Ptc1 expression becomes undetectable as early as 2 days of involution (Fig. 3G) but returns to the near mature virgin pattern in both epithelium and periductal stroma by 10 days of involution (Fig. 3H). Sense strand control hybridizations showed no staining (Fig. 3I).

**Ihh expression is enhanced during pregnancy and lactation**

Ptc1 appears to be a universal target for transcriptional up-regulation in response to hedgehog signaling (Hammerschmidt et al., 1997). Enhanced expression of Ptc1 during pregnancy and lactation coupled with the timing of phenotypic reversion during these developmental stages (see below) suggested that there may be fundamental differences in hedgehog signaling status between virgin, pregnant and lactating states. To address this possibility, we performed in situ hybridization with probes for Shh and Ihh through mammary gland development.

Shh was not detectable by in situ hybridization at any stage of development nor was it detected by subsequent northern hybridization (data not shown). By contrast, Ihh expression was detectable in situ hybridization and its expression was shown to be both epithelium-limited and developmentally regulated.

During virgin stages, Ihh expression was relatively low showing epithelium-limited expression in body cells of the TEB and low-to-undetectable expression in cap cells and differentiating myoepithelial cells at 5-weeks postpartum (Fig. 4A). Weak epithelial expression was maintained in ducts of mature animals at 12-weeks postpartum (Fig. 4B).

By contrast during both early (Fig. 4C) and late pregnancy (Fig. 4D), expression of Ihh appeared enhanced in both ducts and developing alveoli. As with Ptc-1, Ihh expression appeared to be highest during lactation (Fig. 4E).

Expression of Ihh during involution paralleled that of Ptc1, being undetectable by 2 days of involution (Fig. 4F) and becoming detectable in remodeling epithelium at least as early as 14 days of involution (Fig. 4G). Sense strand hybridization showed no staining (Fig. 4H).

Coordinated and enhanced transcription of Ihh and Ptc1 during pregnancy and lactation suggest Ihh functions to inactivate the PTC1 protein and thereby induce Ptc1 transcription. Results are consistent with both an autocrine or paracrine Ihh signal in the epithelium of developing and lactating alveoli and signaling to the surrounding stroma, particularly in early pregnancy. These observations coupled with the lack of an overt phenotype of any kind in Dhh homozygous null females (Bitgood et al., 1996) suggest that Ihh may be the primary hedgehog family member mediating hedgehog signaling in the mammary gland.

**Targeted disruption of the Ptc1 gene results in defective tissue organization during development in virgins**

In situ hybridization demonstrated that Ptc1 expression was both spatially and temporally regulated during mammary development, suggesting a functional role. To determine whether or not disruption of the Ptc1 gene resulted in developmental defects in the mammary gland, glands were examined at several stages of development. No alterations were observed in overall patterning of the mammary tree at 3 weeks
of age (data not shown). At 5 weeks of age, terminal end buds in wild-type animals appeared normal in whole-mount preparations of glands (Fig. 5A), whereas up to approximately 30% of terminal end buds in heterozygous animals appeared misshapen or disrupted (Fig. 5B). These morphological changes do not lead to overt patterning defects, in that disruption of TEB at 5 weeks did not lead to alterations in ductal patterning in adult animals at 10 weeks of age. No morphological distinctions could be made between wild-type (Fig. 5C) and heterozygous (Fig. 5D) glands.

The small morphological changes belie dramatic changes in the properties of the tissues. Histological analysis revealed ductal dysplasias and hyperplasias in 100% of heterozygous animals by 5 weeks of age. While not apparent in glands taken from 3-week old wild-type and heterozygous animals (Fig. 6A and 6B, respectively), severe histological abnormalities were observed at 5-weeks of age when compared with wild-type controls (Fig. 6C versus 6D). In some ducts, the multilayered lumenal epithelial cells (body cells) of the TEB failed to thin to a monolayer as the subtending duct was established and, in
many cases, the lumenal space was completely occluded by epithelial cells (Fig. 6D). Condensation of the periductal stroma around the neck of the TEB appeared altered in some cases such that adipocytes were included within the condensate and condensation appeared to occur at an unusual distance away from the duct (Fig. 6D). At higher magnification, body cells of wild-type end buds appear well ordered and cap cells form a distinct, organized layer as they differentiate into myoepithelial cells (Fig. 6E). By contrast in some end buds of heterozygous animals, body cells were disordered (Fig. 6F) and the cap cell layer was visibly altered (Fig. 6F).

**Fig. 5.** Whole gland morphological analysis during proliferative development in virgin animals. (A) Wild-type, 5-weeks old. Terminal end buds (black arrows) and subtending ducts appear well formed. (B) Ptc1 heterozygote, 5-weeks old. TEB are generally normal (black arrows) but a subset of TEBs are clearly disrupted (asterisks) (up to approximately 30% in individual glands of some animals). (C) Wild type, 10-weeks old. Ducts and terminal structures. (D) Ptc1 heterozygote, 10-weeks old. Ducts and terminal structures.

**Fig. 6.** Histological comparison of glands during development in virgin animals. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column. A-H are stained with hematoxylin and eosin; I-J are stained with phalloidin (yellow-green, actin) and propidium iodide (red, nuclei). Red asterisks denote ductal lumens; a red letter ‘s’, adipose stroma. (A) Longitudinal section through a mammary duct. Lumenal epithelium is generally a monolayer of darkly staining cells surrounding the ductal lumen. Eosinophilic (pink) periductal stroma adjoins the duct and consists mainly of fibroblasts. Bar, 80 μm. (B) Mammary duct which is indistinguishable from its normal counterpart. Bar, 80 μm. (C) Terminal end bud with characteristic body cell layer composed of 3-6 layers of epithelial cells thinning to a monolayer surrounding a well-defined lumen in the subtending duct (red arrowhead). A thin, uniform layer of condensing periductal stroma is shown at the neck of the TEB and along the duct. Bar, 200 μm. (D) Terminal end bud. Body cell layer fails to thin to a monolayer in the subtending duct (red arrowhead) resulting in ductal occlusion. Stromal condensation may occur at unusual distances from the TEB and can also appear disrupted with the inclusion of adipocytes within the condensate (black asterisks). Bar, 200 μm. (E) Terminal end bud at increased magnification. Body cell layer appears well ordered, surrounded by a well-defined monolayer of cap cells (black arrow). Bar, 80 μm. (F) Terminal end bud at increased magnification. Body cell layer appears less well organized with a clearly disrupted cap cell layer (black arrows). Note the unusual inclusion of adipocytes (black asterisks) within the condensed stroma at the tip of this end bud. Bar, 80 μm. (G) Normal mammary duct. Bar, 80 μm. (H) Severely affected mammary duct showing complete occlusion by epithelial cells. Bar, 80 μm. (I) Normal mammary duct. Lumen is denoted by a white asterisk. A uniform layer of myoepithelial cells is identifiable (white arrows) as a line of yellow cells lining the outer surface of the duct. Bar, 80 μm. (J) Severely affected mammary duct showing complete occlusion by epithelial cells. The myoepithelial cell layer (white arrows) appears unaffected. Clusters of epithelial cells which form microlumens within the ducts can be identified (circled by white dots) with inappropriate actin localization at the microluminal surface. Bar, 80 μm.
The ductal defects observed at 5 weeks of age become more pronounced when animals reach 10 weeks. Whereas wild-type ducts have a clear lumen within a monolayer of luminal epithelial cells (Fig. 6G), a majority of ducts in glands from heterozygous animals are partially or completely filled with loosely associated epithelial cells, presumably arising by an alteration in cell-cell adhesion within the ductal wall (Fig. 6H). Examination of serial sections through entire ducts showed some areas appearing relatively unaffected (data not shown). Cells in occluded ducts are not uniform with respect to nuclear morphology and can include large cells with round nuclei and clear cytoplasm suggesting that multiple epithelial subtypes contribute to the dysplasias (Chepko and Smith, 1997; Smith, 1996).

To further characterize cells within the dysplasias, propidium iodide (nuclear stain) and phalloidin (actin stain) were used to examine actin localization in the myoepithelial and epithelial cell layers. In wild-type ducts (Fig. 6I), actin staining clearly identified the myoepithelial cell layer as well as the terminal web and microvilli at the apical (luminal) surface of luminal epithelial cells. Faint actin staining was also observed on the lateral surfaces of luminal cells. In affected ducts of heterozygous animals (Fig. 6J), myoepithelial cells did not appear to contribute to the cell population of the dysplasias but remained associated with the basal lamina surrounding the impacted ducts. By contrast, actin staining within the dysplasia was generally disorganized but could be observed at the apical cell surface around microlumens formed by circular clusters of epithelial cells (Fig. 6J). Data suggest that only luminal epithelial cells contribute to the dysplasias and that cells can become polarized, albeit inappropriately, around microluminal spaces within the dysplasias.

Ptc1-induced dysplasias are reversible during pregnancy and lactation

Given the severity of the mammary phenotype in virgin Ptc1 heterozygotes, the question arises: why does cellular occlusion of ducts in mature animals not impair their ability to lactate? To investigate this, we examined glands at various stages of pregnancy, lactation, and involution.

Morphological alterations were not detected in whole-mount preparations at any stage of cyclical development. By histological analysis it is apparent that many ducts in early pregnancy remain filled, or nearly filled, with cells and are qualitatively similar to those of mature individuals (data not shown). However, by comparison with wild-type ducts in late pregnancy (Fig. 7A), most ducts of heterozygotes show phenotypic reversion toward a wild-type histarchoarchitecture, becoming cleared of epithelial blockages with duct walls thinned to form a single layer of luminal epithelial cells (Fig. 7B). Only sporadic cellular impaction of ducts remained evident (Fig. 7C). Late pregnancy alveolar development appears normal in both wild-type (Fig. 7D) and heterozygous animals (Fig. 7E). By 6 days of lactation, ducts and alveoli are phenotypically normal in both wild-type and heterozygous animals (Fig. 7F and 7G, respectively) with little to no evidence of ductal hyperplasia. Ducts in heterozygous animals remain open in early stages of involution (data not shown) and in late involution as do wild-type ducts (Fig. 7H) but elements of the impacted phenotype are re-established in some ducts by late involution (14 days) (Fig. 7I and 7J). Severe stromal overgrowth (Fig. 7J) was also observed occasionally.

The onset of the mutant phenotype at about 5 weeks of age, its progression during the virgin stages, and its reversion during pregnancy and lactation suggests that ovarian hormones (estrogen, progesterone, or both) may contribute to the phenotype after the onset of puberty. To begin to address this question, we injected Ptc1 heterozygotes (and wild-type littermates) with estradiol and progesterone for 9 consecutive days and examined the mammary glands immediately thereafter. In animals of both genotypes, hormone treatment stimulated growth and side branching similarly indicating no overt differences in hormone responses. However, treatment with both hormones enhanced the mutant histological phenotype with three of four heterozygotes showing characteristic disruption of a majority of terminal end buds and ducts examined (data not shown). Wild-type control animals showed no defects.

Ptc1-induced dysplasias reflect intrinsic defects in mammary gland development

Since heterozygous disruption of Ptc1 could affect expression of systemic mammotrophic factors, the next question was whether the mammary defects observed in Ptc1 heterozygotes were due to developmental alterations within the gland itself or due to extrinsic influences acting on the gland. To answer this question we used whole mammary gland transplantation in which entire mammary glands (containing both fat pad stroma and ductal epithelium) from wild-type and heterozygous donors were contralaterally transplanted into athymic mice and allowed to revascularize and grow for 4 weeks.

As expected, mammary glands from wild-type donors showed normal terminal end bud structure (Fig. 8A) with highly ordered cap cell and body cell layers. As was observed with intact heterozygotes, in transplanted mammary glands derived from heterozygous donors approximately 20% of terminal end buds demonstrated characteristic histological defects in cap cell and body cell layer organization and periductal stromal condensation (Fig. 8B). Mature ducts of wild-type mammary glands had normal structure with clear lumens (Fig. 8C). Mature ducts of heterozygous mammary glands had predominantly normal histoarchitecture but had multiple focal regions of cellular impaction within ductal lumens (Fig. 8D). These defects are consistent with, but less severe than, defects observed in intact heterozygotes at the identical developmental stage (7 weeks postpartum) (data not shown). The characteristic dysplasias in terminal end buds and mature ducts of the transplanted heterozygous mammary gland demonstrate intrinsic defects in mammary gland development in Ptc1 heterozygotes. These data also demonstrate that, at least under present conditions, the heterozygous fat pad is capable of sustaining generally normal ductal growth. The less severe phenotype in transplanted glands in Balb/C nu/nu hosts suggests influences either by local or systemic factors that may be expressed differently in these animals or by the transplantation and revascularization process.

Ptc1-induced defects are not stable upon epithelial transplantation into cleared fat pads of wild-type recipients

We next wished to determine whether the Ptc1-induced dysplasias reflect an intrinsic defect in the epithelium or whether there may be a stromal function as well. In addition, with respect
to a possible role in breast cancer, an important question is whether or not the dysplasias represent a preneoplastic or neoplastic state. Most mouse mammary tumors and preneoplastic lesions characterized to date are immortalized and capable of being serially transplanted with relative phenotypic stability and varying tumorigenic potentials (Said et al., 1995). To address these questions, wild-type and heterozygous mammary epithelium were transplanted contralaterally into both number 4 epithelium-free (cleared) fat pads of athymic mice and allowed to regenerate a ductal tree for 6 weeks to 8 months, permitting comparison of both epithelial genotypes under identical physiological and environmental conditions.

Heterozygous donor epithelium from the region surrounding the transplanted area showed mild-to-severe histological defects (Fig. 9B), while donor epithelium from wild-type animals was normal (Fig. 9A). Upon transplantation, wild-type epithelium produced normal ductal outgrowths, as expected (Fig. 9C). Epithelium transplanted from affected heterozygous animals were also histologically normal even after 8 months post-transplantation (Fig. 9D). Qualitatively similar results were obtained in the 6 week transplants though very limited evidence of terminal end bud disruption and focal ductal dysplasia was observed (data not shown). Consistent with the whole mammary gland transplants, the results suggest that \textit{Ptc1} function may be required in both epithelium and stroma (or stroma only) for transplanted heterozygous epithelium to recapitulate the mutant phenotype observed in virgin animals. Further, these data indicate that \textit{Ptc1}-induced dysplasias are not stable upon transplantation, in contrast to most characterized hyperplasias and neoplasias.

**DISCUSSION**

We have demonstrated that several components of the hedgehog signaling network are expressed in the mouse mammary gland. By expression and functional analysis, we have shown that one of these components, the \textit{Ptc1} hedgehog receptor, is developmentally regulated at the mRNA level, and is conditionally required for proper histogenesis during virgin stages of development and late-stage involution. In \textit{Ptc1} heterozygotes, body cells of the terminal end bud appear to fail to thin to a single cell layer in the subtending duct. This failure is compounded by progressive duct wall thickening resulting in obstruction of the lumen in a majority of mammary ducts by 10 weeks of age. Lumenal obstruction is reversible during late pregnancy and lactation allowing successful milk secretion to occur, an event that correlates with enhanced epithelial expression of \textit{Ihh} mRNA. Whole mammary gland transplantation demonstrates that mammary defects are intrinsic to the gland but dysplasias are not stable on epithelial transplantation, suggesting both epithelial and stromal function of \textit{Ptc1}.

**Pattern formation is genetically separable from ductal morphogenesis**

An unusual aspect of the \textit{Ptc1} phenotype is that it illuminates a distinction between the genetic regulation of two fundamental aspects of mammary ductal development, namely pattern formation and ductal morphogenesis. The patterning of the branched, mammary ductal system and the development of its component ducts have tacitly been considered interdependent; without proper ductal morphogenesis, it is assumed that overall gland architecture would be altered. The \textit{Ptc1} phenotype demonstrates genetic separation of these two developmental processes. Ductal patterning is a highly regulative process that results from end bud bifurcations and turning maneuvers in response to local environmental signals from the stroma and from nearby mammary epithelium. In the \textit{Ptc1} animals, a normal branching pattern is established even though the internal structure of individual ducts is severely disrupted indicating that reception and interpretation of these environmental signals is not impaired.

**Novel aspects of the \textit{Ptc1} phenotype**

There are at least two additional novel features of the \textit{Ptc1} phenotype herein described. First, with the exception of a small size difference between wild-type and heterozygous animals, ductal dysplasia is the only 100% penetrant heterozygous phenotype described to date. Each of the other phenotypes reported previously, including medulloblastomas and other soft tissue tumors, appear in a significantly lower percentage of mutant animals and may take several months to develop (Goodrich et al., 1997; Hahn et al., 1998).

The second novel feature is that phenotypic reversion during a specific developmental phase of the mammary gland has been described for only one other targeted disruption. This similar reversion occurs in mice heterozygous for a disrupted prolactin receptor gene in which the first lactation cycle in young mice was affected but the second lactation (or first lactation in older mice) was successful (Ormandy et al., 1997). These results indicate that certain phenotypes are strongly influenced by physiological changes during reproduction, and suggests that the hedgehog network is regulated by, or interacts with, hormone- or growth factor-mediated signal transduction pathways. Since levels of several mammotropic hormones and growth factors (e.g. estrogen, progesterone, prolactin, TGF-\beta family members etc) are dramatically altered during these stages, and disruption of each of these signaling networks independently disrupts gland development and function, identification of the interactions involved in phenotypic reversion is likely to be complex.

Our finding that haploinsufficiency during virgin proliferative development results in severe histological dysplasias suggests that complete loss-of-function at the \textit{Ptc1} locus might have more severe consequences for the mammary gland. Unfortunately, \textit{Ptc1} disruption is an early embryonic (~E9.5) homozygous lethal mutation which precludes analysis since overt mammary gland development does not begin until E10. In this light, it will be of interest to perform tissue-specific disruption of \textit{Ptc1} (Wagner et al., 1997) or to rescue the proximal causes of the embryonic lethal phenotype thereby allowing the homozygotes to progress to a later stage of development (Wyssolmerski et al., 1998) at which time whole-mount analysis and transplant rescue experiments can be performed.

**Possible mechanisms underlying the \textit{Ptc}-1 phenotype**

Given that many genes under hedgehog network control in other organs are known to function in the mammary gland, it is possible that no single downstream alteration is solely responsible for the phenotypes observed and that they may,
instead, be the cumulative result of relatively minor alterations in multiple cellular functions.

At least one mechanism underlying the *Ptc1* phenotype is suggested by the disorganization of the ductal cells. Proper ductal morphogenesis requires that cell-cell and cell-substrate adhesion systems be coordinated spatially and temporally with epithelial differentiation and apoptosis. Among many other candidates including integrins and laminins, P-cadherin emerges as a strong candidate for a cell adhesion molecule that may be influenced by *Ptc1* disruption. P-cadherin is primarily localized in the cap cell layer of the end bud and in differentiated myoepithelial cells in mature ducts. Disruption of P-cadherin function by antibodies delivered to the gland via slow-release plastic implants, caused disorganization of the end bud and impaction of the duct with dissociated cells, an effect reversible upon depletion of the antibody (Daniel et al., 1995). Further, a phenotype similar to *Ptc1* has been reported for animals homozygous for a targeted disruption of P-cadherin. In this mutant, loosely associated epithelial cells aggregating or floating within the lumen and excessive alveolar development have been observed (Radice et al., 1997). Preliminary immunohistochemical analysis of both E- and P-cadherin using wild-type and *Ptc-1* heterozygous animals at 10 weeks postpartum do not show consistent alterations in labeling between the two genotypes.

**Support for a role of hedgehog signaling in tissue interactions in the mammary gland**

In our experiments, severely affected epithelium from donor...

**Fig. 7.** Histological analysis of glands during the mammary cycle. Developmental stages are shown on the left. Genotype of the animal from which the glands are derived is shown at the top of each column of panels. Red asterisks denote ductal lumens; a red letter ‘s’, adipose stroma. (A) Mammary duct. Bar, 80 μm. (B) Mammary duct. Bar, 80 μm. (C) Mammary duct impacted with cells. Cells within the lumen are not attached to the duct wall proper which suggests alterations in cell adhesions play a role in duct clearing. Bar, 80 μm. (D) Alveoli. Bar, 80 μm. (E) Alveoli. Bar, 80 μm. (F) Duct wall (red arrow) and alveoli. Bar, 80 μm. (G) Duct wall (red arrow) and alveoli. Duct walls thin to a monolayer and lumens appear free of epithelial cells. Bar, 80 μm. (H) Mammary duct. Bar, 80 μm. (I) Mammary duct impacted with epithelial cells Bar, 80 μm. (J) Mammary duct and sidebranch surrounded by unusually dense layer of peri ductal fibroblastic stroma (red arrow). Bar, 200 μm.

**Fig. 8.** Whole mammary gland transplant into nude mice. Genotype of the transplanted mammary gland is noted above the panel columns to which they refer. Red asterisks denote lumenal spaces; a red letter ‘s’, adipose stroma. (A) Terminal end bud showing well-ordered cap and body cell layers. (B) Terminal end bud showing disrupted body cell layer with a prominent microlumen (black arrowhead). Cap cell layer disruption is also apparent. (C) Mammary duct showing a monolayer of luminal epithelial cells along the duct wall and a clear lumen. (D) Mammary duct showing localized cellular impaction characteristic of intact *Ptc1* heterozygotes at comparable age. Bars, 80 μm.
animals underwent overly normal differentiation and morphogenesis when it repopulated the recipient cleared fat pad. These results, coupled with the whole mammary gland transplant data showing partial recapitulation of the mutant phenotype, suggest that wild-type *Ptc1* function is required either in both the stroma and epithelium or in the stroma only during virgin stages of development. The possibility of both epithelial and stromal functions for *Ptc1* is consistent with its own expression pattern and with the expression patterns of both *Gli2* and *Ihh* which have developmentally regulated and cell type-specific mRNA expression in the mammary gland. *Gli2* is expressed exclusively in the periductal stroma through virgin stages of development but becomes both epithelial and stromal during pregnancy and lactation (M. T. L. and C. W. D., unpublished). In contrast, *Ihh* is expressed exclusively in the epithelium throughout development and is low during virgin stages but appears elevated during pregnancy and lactation (Fig. 4). Further, phenotypic and transplantation analysis of a targeted disruption strain of *Gli2* confirms that its function is also required for proper virgin mammary gland development (M. T. L. and C. W. D., unpublished). Together, these data support a general role for hedgehog signaling, and for *Ptc1*, *Ihh* and *Gli2* specifically, in mediating tissue interactions during mammary gland development.

A working model for hedgehog signaling in the mammary gland

Interpretations regarding the conditional haploinsufficiency in *Ptc1* heterozygotes during virgin stages of development are complicated by the unusual characteristics of *Ptc1* gene expression and function demonstrated primarily in the developing nervous system (Goodrich et al., 1997). Under the current general model, the function of PTC1 protein is to inhibit signaling by SMO and the function of the hedgehog proteins is to relieve this inhibition (Fig. 10) permitting downstream GLI-mediated gene activation. Mice homozygous for a disrupted *Ptc1* gene (loss-of-function) showed derepression of *Gli1* mRNA (a hedgehog signaling target) and *Ptc1* itself, as evidenced by increased and ectopic expression of β-gal derived from the *Ptc1* knockout allele. These observations lead to the following paradox: increased *Ptc1* mRNA and protein levels are inversely correlated with PTC1 activity (inhibition of SMO).

One interpretation of our results is that wild-type levels of *Ptc1* function are required during proliferative development and gland remodeling but are not required during pregnancy and lactation. If the general model holds true in the mammary gland, the overall increase in *Ptc1* expression observed during pregnancy and lactation is the result of *Ihh*-mediated PTC1 inactivation and subsequent increased SMO-mediated downstream signaling.

It is possible that *Ptc1* function in the gland is not regulated at the level of *Ptc1* mRNA or protein expression but rather at the level of hedgehog family ligand availability. Thus in the virgin stages PTC1 protein might be active as the result of low hedgehog family expression, whereas during cyclical development (pregnancy and lactation) PTC1 may be normally inactivated by the presence of high levels of hedgehog family ligands. This hypothesis is supported by the observed expression pattern of *Ihh* (Fig. 4) in which *Ihh* expression is low in the virgin and increased during pregnancy and lactation. Thus, reduced PTC1 protein levels in ducts in virgins might be predicted to have functional consequences, whereas reduction in PTC1 levels during cyclical stages would be predicted to have no effect, leaving the gland free to function normally.

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**Fig. 9.** Epithelial transplantation into cleared fat pads. Tissue source is noted on the left; genotype of the epithelium is noted above the panel columns to which they refer. Red asterisks denote luminal spaces; a red letter ‘s’, adipose stroma. (A) Mammary duct showing an open lumen and monolayer of epithelial cells along the duct wall. (B) Mammary duct from the region of transplant source showing characteristic cellular impaction. (C) Mammary duct of an 8-month old transplant of wild-type epithelium showing expected monolayer of epithelial cells along the duct wall. (D) Mammary duct of an 8-month old transplant of *Ptc1* heterozygous epithelium also showing a monolayer of epithelial cells along the duct wall. Transplants harvested at 6 weeks were qualitatively similar though limited evidence of terminal end bud disruption and ductal impaction was observed and duct walls were not thinned as observed in glands harvested at 8 month posttransplantation. Bars, 80 μm.

**Fig. 10.** Summary of a portion of the model for hedgehog signal transduction in mammals. (A) Schematic of functional interactions among the hedgehog proteins, PTC1, PTC2 and SMO leading to GLI-mediated control of target gene expression. (B) A generalized summary of *Ptc1* gene expression relative to protein activity and downstream hedgehog signal transduction highlighting the reciprocal nature of *Ptc1* expression and PTC1 protein activity (Goodrich et al., 1997; Hammerschmidt et al., 1997; Ingham, 1998b).
Is \textit{Ptc1} a mammary tumor suppressor gene?

The presence of dissociated cell masses in mammary ductal lumen is reminiscent of the histology of human ductal carcinoma in situ (DCIS) and functionally suggests a lost of contact inhibition commonly associated with uncontrolled, neoplastic cell division. In fact, cellular impactions strikingly similar to the \textit{Ptc1} phenotype have been observed in ductal outgrowths from transplants of hormone-dependent tumors which arise during pregnancy and regress during involution (Aidells and Daniel, 1974). The cause of these tumors is unknown but given the remarkable similarity in the ductal phenotype, a possible contributory role for \textit{Ptc1} should be investigated. However, it is also important to note that the behavior of hormone-dependent tumors is reciprocal to that of \textit{Ptc1}-induced dysplasias during pregnancy and lactation. That is, during pregnancy, hormone-dependent tumors worsen in severity while \textit{Ptc1}-induced dysplasias revert to wild-type histoarchitecture. Thus, while the ductal phenotypes are similar, they may have arisen by unrelated mechanisms.

The behavior of \textit{Ptc1}-induced mammary dysplasias in these initial epithelial transplant experiments is remarkably similar to the behavior of basal cell carcinomas, a skin cancer that can be \textit{Ptc1}-induced (Cooper and Pinkus, 1977; Grimwood et al., 1985; Stamp et al., 1988). In most cases when human basal cell carcinomas are transplanted into athymic mice, the cells within the tumor fail to form a new tumor in the recipient. Instead, they appear to differentiate into normal skin cells. Successful transplant of basal cell carcinoma into athymic mice required further immunosuppression by splenectomy and injection of anti-lymphocyte serum suggesting that the physiological state of the animal profoundly influences the phenotype of the affected epithelium on transplantation. These transplant experiments also demonstrated that, despite the difficulty in achieving transplantable basal cell carcinomas, the defect is intrinsic to the epithelium. We anticipate that the heterogeneous mammary epithelium will ultimately show similar transplant behavior.

Still another possibility is that transplant timing and stromal environment both contribute to the stability of the mutant phenotype suggesting a form of ‘stromal permissiveness’ must be present for the mutant phenotype to be recapitulated on transplantation. Further transplantation studies including tissue recombination and immunosuppression are necessary to determine the epithelial vs. stromal contribution to the phenotype and to determine what effect transplant timing and physiological state have on recapitulation of the mutant phenotype.

These epithelial transplantation data could also serve as an in vivo correlate to in vitro observations in which tumor cells could be phenotypically reverted by altering their interaction with extracellular matrix components (Schmeichel et al., 1998; Sun et al., 1998; Weaver et al., 1997). In one set of experiments (Weaver et al., 1997) tumor cells were maintained in three-dimensional cultures to closely mimic the in vivo state and treated with function-blocking antibodies to β1 integrin. Cells treated in such a way showed phenotypic reversion toward that of normal cells. These experiments further demonstrate the plasticity of mammary epithelium and that the microenvironment strongly influences the phenotypic behavior and is capable of overriding the genotype of the cell.

Given the \textit{Ptc1} phenotype, its expression pattern, the expression patterns of \textit{Ihh} and \textit{Gli2}, and our understanding of how the pathway functions in other organ systems, we can predict that disruption or overexpression of other network components should have significant consequences to mammary gland development. For example, overexpression of hedgehog genes, \textit{Smo} or one of the \textit{Gli} genes (possibly in the stroma) may mimic the \textit{Ptc1} heterozygous phenotype due to inappropriate activation of the signaling network. Similarly, overexpression of \textit{Ptc1} may inhibit gland development or epithelial proliferation. In addition, we should be able to ask important questions concerning the role of the \textit{Gli} genes and known hedgehog signaling targets in gland development and function. Further genetic analyses coupled with the exceptional repertoire of techniques to experimentally manipulate the gland both in vivo and in vitro should allow us to dissect hedgehog network function in the mammary gland and to determine how this network interacts with other signal transduction pathways, particularly those of the TGF-β and \textit{Wnt} families.

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