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Differential Requirements for Shh in Mammary Tissue and Hair Follicle Morphogenesis

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Sonic Hedgehog (Shh) is a secreted morphogen that directs patterning and cellular differentiation through binding to its receptor Patched (Ptc). It is required for the development of skin-derived organs, such as hair, whiskers, and teeth. The mammary gland is a skin-derived organ that develops mainly during adult life in which Shh is expressed from puberty to lactation. We have investigated the role of Shh in mammary gland morphogenesis and differentiation by two transplantation approaches. Since Shh-null fetuses die at late embryogenesis, we transplanted Shh-null mammary anlagen into cleared fat pads and under the renal capsule of wild type host mice. Pregnancy-mediated functional differentiation of Shh-null mammary epithelium was indistinguishable from wild type transplants, while hair follicles derived from cotransplanted skin only developed in wild type transplants. Transplants of Ihh-null anlagen also developed normally. To assess the molecular consequences of Shh deletion in mammary tissue, we compared mRNA levels of *patched 1*, a target gene of Hedgehog signaling, in Shh-null and wild type mammary epithelial transplants. No reduction of Ptc1 transcripts was observed in Shh-null mammary tissues. Our results demonstrate that neither Shh nor Ihh is required for mammary gland morphogenesis and functional differentiation, suggesting that the two members of the Hedgehog family may have redundant function in activating the Ptc1 signaling pathway during mammary gland development.

Key Words: **hedgehog; mammary gland; tissue interaction.**

INTRODUCTION

Hedgehog proteins constitute a family of secreted signaling molecules that regulate patterning and cellular differentiation during embryogenesis (reviewed in Hammerschmidt *et al.*, 1997; Ingham, 1998). The *hedgehog* (*hh*) gene was first identified in *Drosophila*, where it directs patterning of larval segments and adult appendages (Nusslein-Volhard and Wieschaus, 1980). Two multipass membrane proteins, the hedgehog (Hh) receptor Patched (Ptc) and Smoothened (Smo), transduce Hh signals upon ligand binding. In the absence of ligand, Ptc inhibits the activity of Smo, which mediates all Hh signaling. Thus, Hh binding to Ptc represses Ptc activity, leading to the derepression of Smo and the constitutive activation of Hh target genes (reviewed in McMahon, 2000). In addition, Ptc itself is a target of Hh signaling, and in the absence of Hh signaling, Ptc mRNA levels decrease dramatically (Goodrich *et al.*, 1996; Marigo *et al.*, 1996). In mammals, three Hh homologues, Sonic (Shh), Indian (Ihh), and Desert (Dhh), and two Ptc homologues, Ptc1 and Ptc2, have been identified, and deletion mutants have been generated (Bitgood *et al.*, 1996; Chiang *et al.*, 1996; Goodrich *et al.*, 1997; Motoyama *et al.*, 1998; St-Jacques *et al.*, 1999).

Mammary gland morphogenesis and differentiation can be divided into three distinct stages (for reviews see Hennighausen and Robinson, 1998, 2001). First, at day E10–E11 in the mouse, five pairs of ectodermal placodes are developed ventrally at precise locations. From the ectodermal placodes, epithelial buds are formed through inductive epithelial–mesenchymal interactions. The buds proliferate becoming primary sprouts that penetrate the adjacent fat pad. The second stage occurs during puberty when mam-

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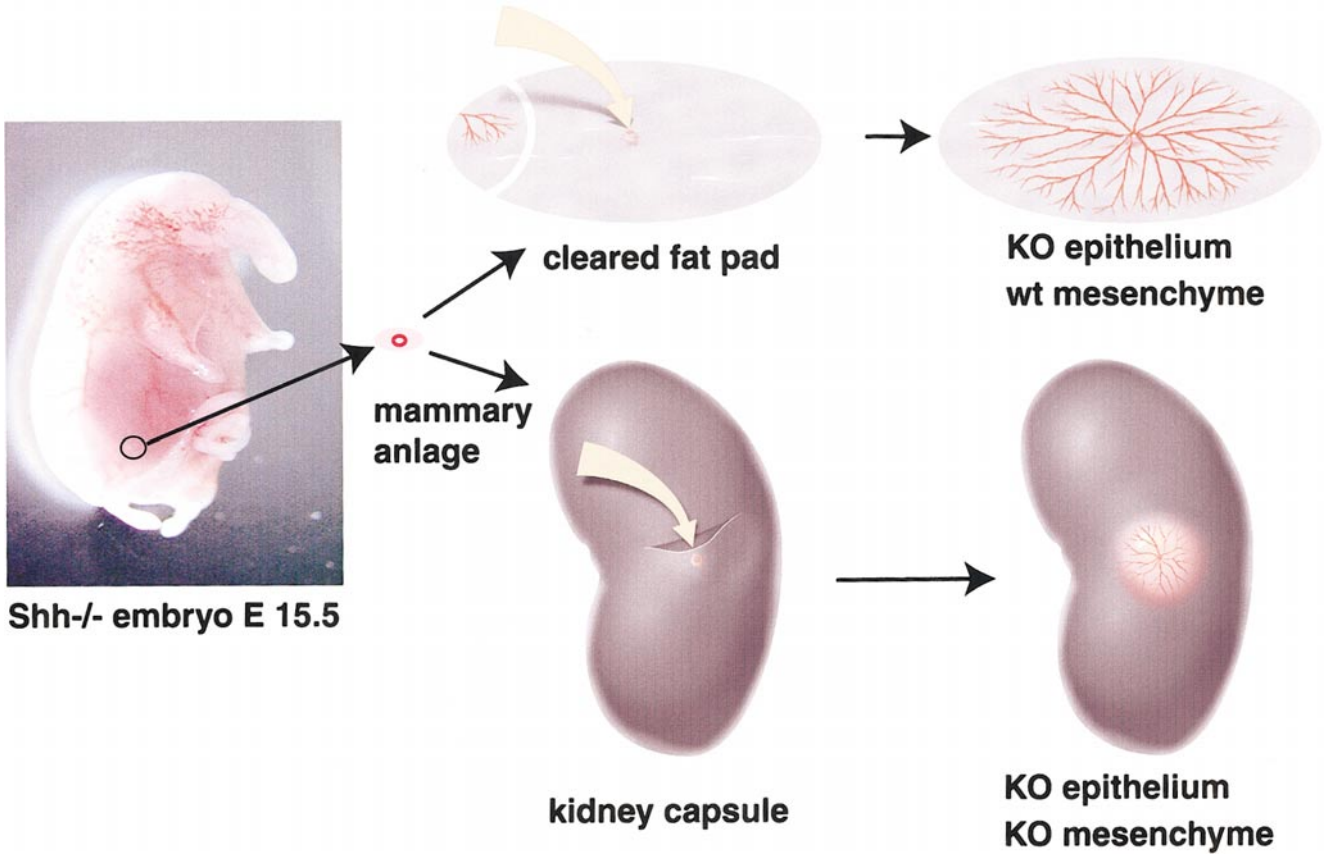

FIG. 1. Schematic presentation of the two types of transplantations of mammary anlagen. In the first type (top), the anlage from an E15.5 embryo is placed in a previously cleared fat pad of a host female. Circulating ovarian hormones stimulate the growth of the transplanted mammary epithelium, and the ducts penetrate the host fat pad. The result of this transplant is a mammary gland in which the transplanted epithelium abuts the fat pat of the host mouse. In the second type of transplant (bottom), the anlage from an E15.5 embryo is placed under the renal capsule of a wild type host female. Circulating ovarian hormones stimulate growth of the epithelial tree, but the growth is limited to the size of its own fat pad. In this case, both epithelium and fat pad are derived from the donor.

mary ducts elongate and ramify driven by the increase in circulating ovarian hormones. The third stage encompasses alveolar proliferation and functional differentiation during pregnancy and at the onset of lactation, respectively. These programs are controlled by progesterone, placental lactogens, and prolactin. After weaning, the mammary gland experiences a phase of extensive apoptosis and involutes. All aspects of mammopoiesis require epithelial–stromal interactions and paracrine interactions between epithelial cells (reviewed in Robinson *et al.*, 1999, 2000b).

Shh is a secreted morphogen that is required for patterning of the neural tube, development of the limbs, and morphogenesis of eye, lung, and skin-derived organs, such as hair, whiskers, and teeth (Chiang *et al.*, 1996; reviewed in Chuong *et al.*, 2000). The mammary gland is also a skin-derived organ. It shares with other skin appendages some of the signals required for epithelial–mesenchymal interactions (Robinson *et al.*, 1999). Shh is expressed in mammary tissue from puberty to lactation (Lewis *et al.*, 1999), but its role in mammary gland biology has not been explored. Another member of the family, Ihh is strongly expressed in alveolar cells during pregnancy (Lewis *et al.*, 1999 and 2001). Furthermore, haploinsufficiency in mammary gland development of Ptc1 and Gli2 mutants suggests a role for HH signaling (reviewed in Lewis, 2001). Shh- and Ihh-null fetuses die before birth, thus prohibiting the direct study of mammary gland development. We have therefore used Shh- and Ihh-null mammary tissue and hair follicles transplantation experiments to compare the requirements for Shh and Ihh in these two skin-derived organs.

MATERIALS AND METHODS

Animals

Shh- and Ihh-null embryos were produced by breeding Shh- and Ihh-hemizygous mice. Fifteen-day-old fetuses were identified by their altered morphology and confirmed by PCR (Chiang *et al.*,

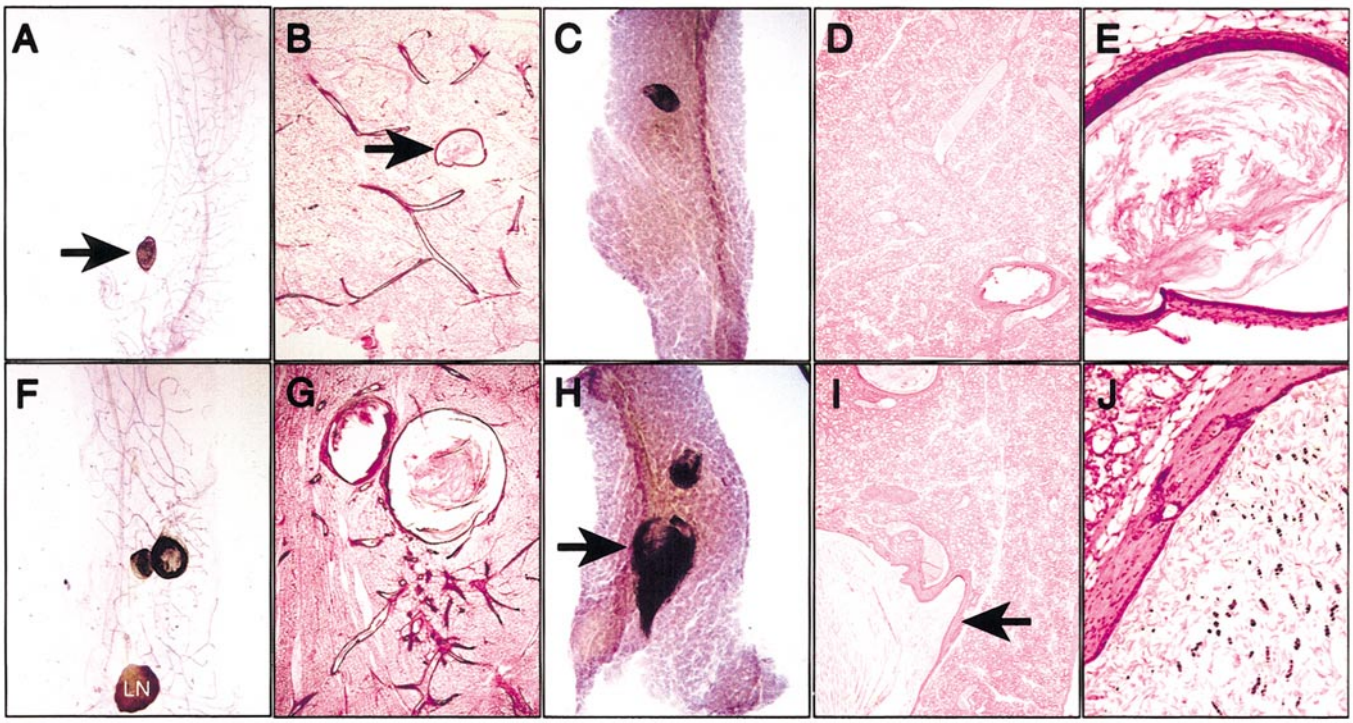

FIG. 2. Mammary gland development in Shh-null and control epithelial transplants. Virgin samples were harvested 8 weeks after transplantation and analyzed by whole mount (A, F) and H&E staining of histological sections (B, G). No difference was found between Shh-null (A, B) and wild type (F, G) epithelial transplants. Lactating samples were harvested within 12 h of parturition. Whole-mount (C, H) and histological analyses (D, I) of the transplants revealed no difference between Shh-null (C, D) and wild type (H, I) epithelial transplants. The whole mounts were stained with carmine alum and photographed at 10×. The histological sections in (B), (D), (G), and (I) were H&E-stained and photographed at 25×. Arrows in (A) and (H) point to skin patches included in the transplant. Arrows in (B) and (I) indicate the areas shown at higher magnification in (E) and (J), respectively (100×). Hair development occurs in the wild type (J) but not in Shh-null skin (E). Note hair follicles and dark cross-sections of hair in the lumen in (J). LN, lymph node.

1996; St-Jacques *et al.*, 1999). N/Cr athymic nu/nu mice were used as recipients in all transplants. Mice were housed in a 12-h day/night cycle at 22°C and 80% relative humidity with food and water *ad libitum*. For all pregnancy experiments, the day on which the vaginal plug was found was considered day 0 of pregnancy

Transplantations

Fetal mammary glands from 15-day-old Shh- and Ihh-null and control fetuses were transplanted into cleared fat pads as described (Robinson, 2000a) or under contralateral renal capsules of the same animal as described (Cunha and Hom, 1996). To induce lobuloalveolar development, females were mated 4 (kidney transplants) or 8 weeks (fat pad transplants) after transplantation and sacrificed at late pregnancy or within 12 h after parturition.

Histology and Immunohistochemistry

Mammary tissue was harvested from cervically dislocated mice. For whole-mount examination, the tissue was fixed in Carnoy's solution for 4 h and stained with carmine alum overnight. Whole mounts were embedded and sectioned by using standard methods. For histological analysis, 5-μm paraffin sections were stained with H&E. For immunostaining, tissues were fixed for 4 h with paraformaldehyde and processed by standard protocols. Paraffin sections (5 μm) were deparaffinized and boiled in antigen retrieval solution (Vector) following the manufacturer's conditions, incubated overnight with either anti-N-Shh rabbit polyclonal antibody (Chang *et al.*, 1994) or anti-C-Shh goat polyclonal antibody (R&D), 1 h with the appropriate biotinylated secondary antibody, and 30 min with Vetacastain ABC reagent (as per kit instructions). The slides were incubated for 2 min with the DAB developing solution and counterstained with methyl green.

Northern Blots and RT-PCR Assays

Total RNA was isolated and Northern blots were performed as described previously (Robinson *et al.*, 1995). Total cellular RNA was purified from Shh-null and wild type epithelial transplants harvested postpartum. Total RNA (4 μg) was reverse-transcribed by using Thermoscript (GIBCO/BRL, Life Technologies Inc.) with oligo(dT) primers at 55°C for 45 min. Complementary DNA was diluted to 20 μl, and 2 μl were used for the PCR with *Ptc1*-specific primers (Lewis *et al.*, 1999), which spanned one intron and ampli-

fied a fragment of 590 bp. The PCR conditions were performed as described (Lewis *et al.*, 1999). One μl of each cDNA reaction was used for amplification with keratin 18-specific primers: K18F, 5′-GCT GAG ACC AGT ACT TGT CCA G-3′; K18R, 5′-CGC ATC GTC TTG CAG ATC GAC A-3′. The keratin PCR fragment spanned one intron and amplified a fragment of 400 bp. Amplification conditions were: denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min.

RESULTS

Although Shh-null fetuses died prior to birth, it was possible to transplant mutant mammary anlagen into wild type mice. This permitted the analysis of Shh-null mammary epithelium and stroma in the context of wild type hosts. Two different types of transplants were performed (Fig. 1). First, embryonic anlagen were grafted into cleared mammary fat pads of host mice (Fig. 1, top). Second, the anlagen were grafted under the renal capsules (Cunha and Hom, 1996) of wild type hosts (Fig. 1, bottom). In the first strategy, embryonic mammary anlagen from the donor (mutant or control) were placed into epithelium divested mammary fat pads of wild type hosts. During puberty and pregnancy, the transplanted mammary epithelial tissue grew and filled the host's mammary fat pad. This permitted the reconstitution of a mammary gland in which the epithelium was derived from the donor and the mesenchyme from the host mouse. In the second type of transplant, the entire gland was derived from the embryonic anlagen. The epithelial component of the anlagen grew during puberty and pregnancy, but its growth was limited by the size of its own stroma. Although these transplants never reached the size of an endogenous mammary gland, they were instrumental in evaluating epithelial–mesenchymal interactions during puberty and pregnancy in the absence of Shh.

Embryonic Anlagen Transplants: Epithelial Shh Is Not Necessary for Mammary Gland Development and Differentiation

Despite their profound patterning abnormalities (Fig. 1), Shh-null fetuses presented five pairs of mammary buds at the correct location, along two symmetrical lines, ventral of the limbs. Shh-null and wild type anlagen from E15 fetuses were transplanted into 30 prepubescent nude female mice. We transplanted tissue from Shh-null and wild type littermates into contralateral, cleared fat pads to expose both transplants to the same physiological conditions. One-half of these mice were sacrificed after 8 weeks, and mammary transplants were harvested and analyzed by whole mount and H&E staining of histological sections. Based on whole-mount analyses, no differences were observed between Shh-null (Fig. 2A) and wild type (Fig. 2F) epithelial transplants. In nearly all wild type and Shh-null transplants, the ductal tree had reached the end of the fat pad and presented abundant branching. Ductal morphology was also normal on a histological level, and no differences were observed between Shh-null (Fig. 2B) and wild type transplants (Fig. 2G). The ductal lumina were open and lined by a single layer of epithelial cells.

The other half of the recipients were mated 8 weeks after transplantation, and mammary tissue was harvested after parturition. Whole-mount analyses revealed no difference between Shh-null (Fig. 2C) and wild type (Fig. 2H) mammary epithelial transplants. Both types of transplanted tissues were fully developed. Histological analyses showed expanded alveoli with milk secretion and fat droplets in both cases (Figs. 2D and 2I). Two independent lines of evidence proved that the epithelium in the transplanted fat pad was derived from the embryonic donor. First, complete clearing of the endogenous epithelium in the host was confirmed by an examination of the excised tissue. Second, since the embryonic anlage cannot be completely separated from the epidermis, many of the transplants included embryonic hair follicles from the skin surrounding the mammary gland. In the wild type transplants, these hair follicles gave rise to hair (arrows in Figs. 2H and 2I). In contrast, we never observed hair growth in the Shh-null transplants, even though hair follicles were deliberately included in Shh-null mammary gland embryonic transplants (arrows in Figs. 2A, 2B, and 2E).

To evaluate the differentiation status of transplanted wild type and Shh-null mammary epithelium after pregnancy, we analyzed β-casein mRNA levels by Northern blots (Fig. 3A). Similar levels of β-casein (Fig. 3A) and whey acidic protein (WAP) (data not shown) mRNA were observed in wild type and Shh-null tissue. Keratin 18 mRNA was used as an indicator of the epithelial content of the transplant (Fig. 3A).

Patched1 Expression Is Not Suppressed in Shh-Null Epithelial Transplants

Ptc1 is a receptor for the three hedgehog family members, and the gene is a target of hedgehog signaling. Ptc1 mRNA levels dramatically decrease in the absence of hedgehog signaling. We compared Ptc1 mRNA levels in Shh-null and wild type mammary epithelial transplants harvested after parturition, through the amplification of single-stranded cDNA with Ptc1-specific primers (Fig. 3B, top). The intensity of the bands from wild type (Shh +) and Shh-null (Shh −) epithelial transplant was compared after 32 (segment 1), 34 (segment 2), and 36 cycles (segment 3). We did not find diminished Ptc1 levels in Shh-null epithelial transplants compared with wild type epithelial transplants. As control for the efficiency of the reverse transcriptase reaction, the templates were also amplified with keratin 18-specific primers (Fig. 3B, bottom) and the products were compared after 22 (segment 1), 24 (segment 2), and 26 (segment 3) cycles. No significant differences were found in the intensity of the bands from the Shh-null (Shh −) and wild type (Shh +) epithelial transplant. No products were

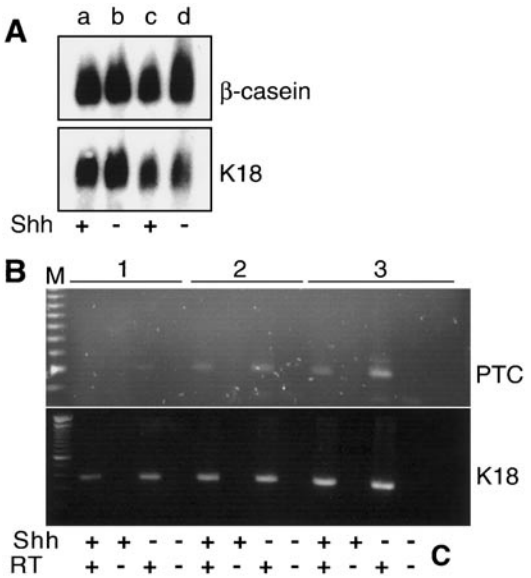

FIG. 3. (A). Functional differentiation of Shh-null epithelial transplants. Northern blot analysis of β-casein mRNA in two wild type lactating epithelial transplants (lanes a and c) and two Shh-null lactating epithelial transplants (lanes b and d) revealed no differences in β-casein expression. The membrane was probed with keratin 18 (K 18, bottom) to determine mammary epithelial content in the samples. (B). Ptc expression in lactating Shh-null and control mammary epithelial transplants. Ptc expression was compared by semiquantitative RT-PCR in Shh-null (Shh −) and wild type (Shh +) epithelial transplants post partum. The cDNA was prepared from 2 μg of total RNA and was used as template for amplification with Ptc-specific primers (top). A negative control reaction, in which the reverse transcriptase was omitted during the synthesis of the first strand of the Shh-null and wild type cDNAs, was included (RT −). The products were analyzed by agarose gel electrophoresis stained with ethidium bromide. The intensity of the bands from wild type and Shh-null epithelial transplant was compared at 30 (segment 1), 32 (segment 2), and 34 cycles (segment 3). A sample without DNA was also amplified with Ptc primers for 34 cycles (C) as negative control. Same amounts of these cDNAs were used as templates for amplification with keratin 18-specific primers (bottom), and the products were compared at 24 (segment 1), 26 (segment 2), and 28 (segment 3) cycles.

observed by using Ptc1 or keratin 18 primers when the reverse transcriptase was omitted from the RT reaction (RT −), indicating no amplification from contaminating genomic DNA. This demonstrates that Ptc1 mRNA levels are not reduced in Shh-null epithelial transplants.

Epithelial and Stromal Transplants: Shh Is Not Required for Mammary Gland Morphogenesis

Shh is only expressed in the epithelial compartment of skin-derived organs (Bitgood and McMahon, 1995) and it is not expected to be expressed in mammary stroma. Therefore, Shh-null epithelial mammary transplants should be adequate and sufficient to explore the requirements of Shh in mammary gland morphogenesis. Nevertheless, we could not rule out the possibility that Shh was supplied by the wild type stroma. To clarify this question, we also performed total mammary transplants under the renal capsules of host mice. Thirty recipients were transplanted with wild type and Shh-null fetal mammary tissue under the left and right kidney capsules, respectively. One-half of the host mice were sacrificed 4 weeks after the transplants were performed. Shh-null and wild type transplanted tissues were harvested, and histological analyses were performed. The H&E staining of histological sections revealed no differences between Shh-null (Fig. 4A) and control transplants (Fig. 4B). The only difference was, once more, the hair growths in many of the skin patches included in wild type transplants and the absence of hair growth in any of the Shh-null transplants (data not shown). This served as an internal control for the absence of Hh diffusion from the wild type kidney to the transplants, although permissivity for other Hh proteins may be different in hair follicles and mammary gland. Fifteen of the transplanted hosts were mated 4 weeks after the transplants were performed and were sacrificed within 12 h of parturition. Transplants were analyzed by H&E staining of histological sections. No differences were observed between Shh-null (Fig. 4C) and wild type (Fig. 4D) transplants. In both cases, we observed fully expanded alveoli lined by epithelial cells that displayed signs of secretory activity.

Ihh in the Epithelium Is Dispensable for Mammary Development

High levels of Ihh expression are observed in mammary epithelium during pregnancy and lactation (Lewis *et al.*, 2001), suggesting that Ihh might be required for alveolar development. We therefore performed transplantations of anlagen from Ihh-null embryos into the cleared fat pad. To assess ductal development, three animals were euthanized after 8 weeks. No differences in ductal outgrowth were found in fat pads carrying a transplant of Ihh-null anlagen compared with the contralateral wild type transplant. Embryonic hair follicles included in all Ihh-null and wild type transplants gave rise to well-differentiated hair (data not shown), indicating that Ihh, contrary to Shh, is not necessary for hair follicle development. In histological sections, the ductal morphology of transplants was indistinguishable from the endogenous gland of the host (data not shown). Similarly, no differences between Ihh-null and wt transplants and the endogenous gland of the host were detected on days 6 and 7 of pregnancy (one animal each). Transplants harvested after the host delivered pups displayed fully developed alveoli in whole-mount (Figs. 5A and 5B) and histological analyses (Figs. 5C–5F) independent of the genotype of the transplanted epithelium. These results demonstrate that absence of Ihh does not affect mammary epithelial development.

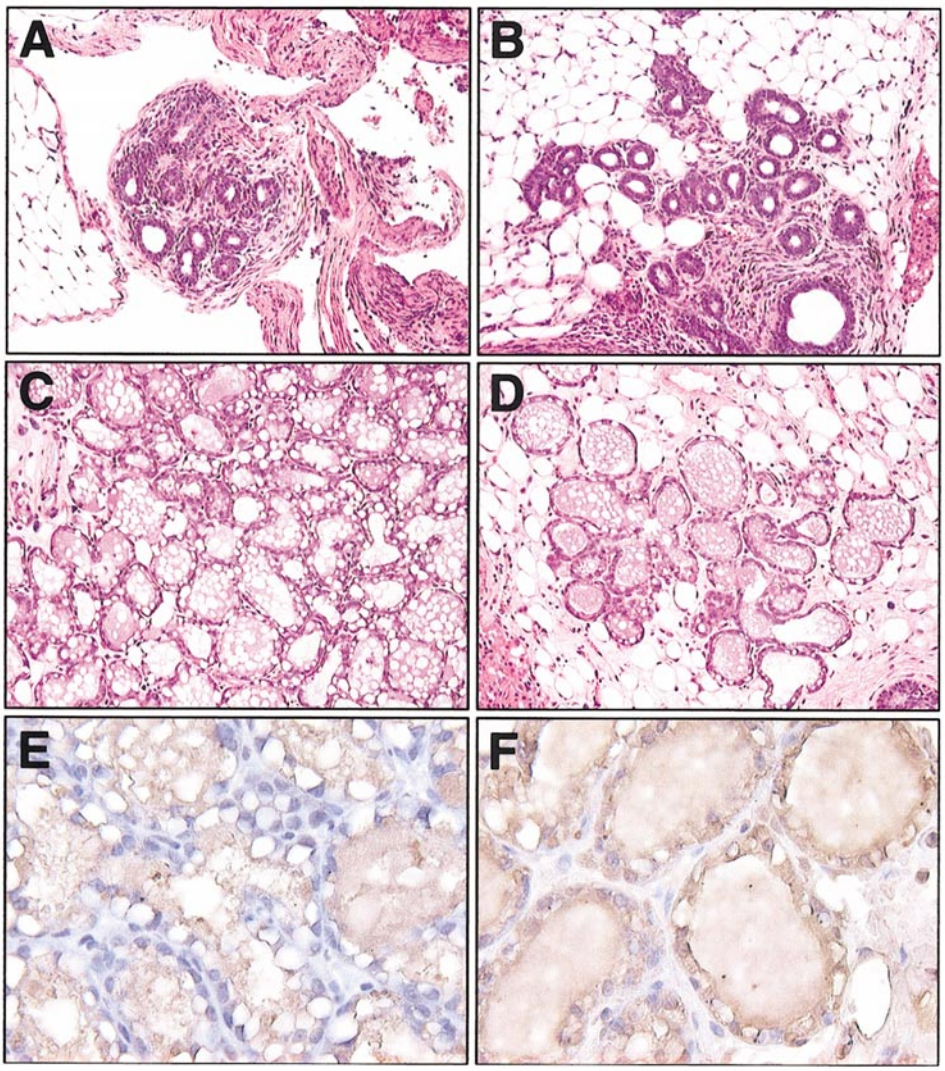

FIG. 4. Mammary development in Shh-null and control transplants (A–D) and immunostaining of tissues after parturition (E, F). Wild type and Shh-null anlagen were transplanted under the left and right renal capsules of wild type nude mice, respectively. For virgin analyses, the hosts were sacrificed and samples were harvested 4 weeks after the transplantation. Histological analysis did not reveal any difference between Shh-null (A) and wild type (B). For lactational analyses, host mice were mated 4 weeks after transplantation and sacrificed after parturition. Samples were harvested and histological analysis was performed. H&E staining of the transplants revealed no differences between Shh-null mammary tissues (C) and wild type mammary tissues (D). Histological paraffin sections, adjacent to the ones shown (C, D) were stained with an anti-C-Shh antibody and photographed at 650×. (E) Shh-null mammary tissues. (F) Wild type mammary tissues. Samples were processed in parallel under the same conditions. Nonspecific staining is seen in the lumina of Shh-null and wild type alveoli. Specific cytoplasmic staining is observed in alveolar cells from wild type mice (F).

Shh Expression in Wild Type but Not in Shh-Null Transplants

We performed immunohistochemistry and immunofluorescence with anti-N-Shh rabbit polyclonal antibodies (Chang *et al.*, 1994) (data not shown) and immunohistochemistry with anti-C-Shh goat polyclonal antibodies (R&D) on sections of wild type embryonic gland and sections of Shh-null and wild type virgin and postpartum mammary transplants (Figs. 4E and 4F). Both antibodies gave comparable results. No staining was observed in the wild type E14 embryonic anlagen; however, specific staining in the cytoplasm of alveolar cells from wild type virgin (not shown) and lactating mammary tissue (Fig. 4F) contrasted with the lack of staining in the alveolar cells from Shh-null lactating mammary tissue (Fig. 4E). Both Shh-null and wild type tissues presented nonspecific staining of milk within the lobuloalveolar structures.

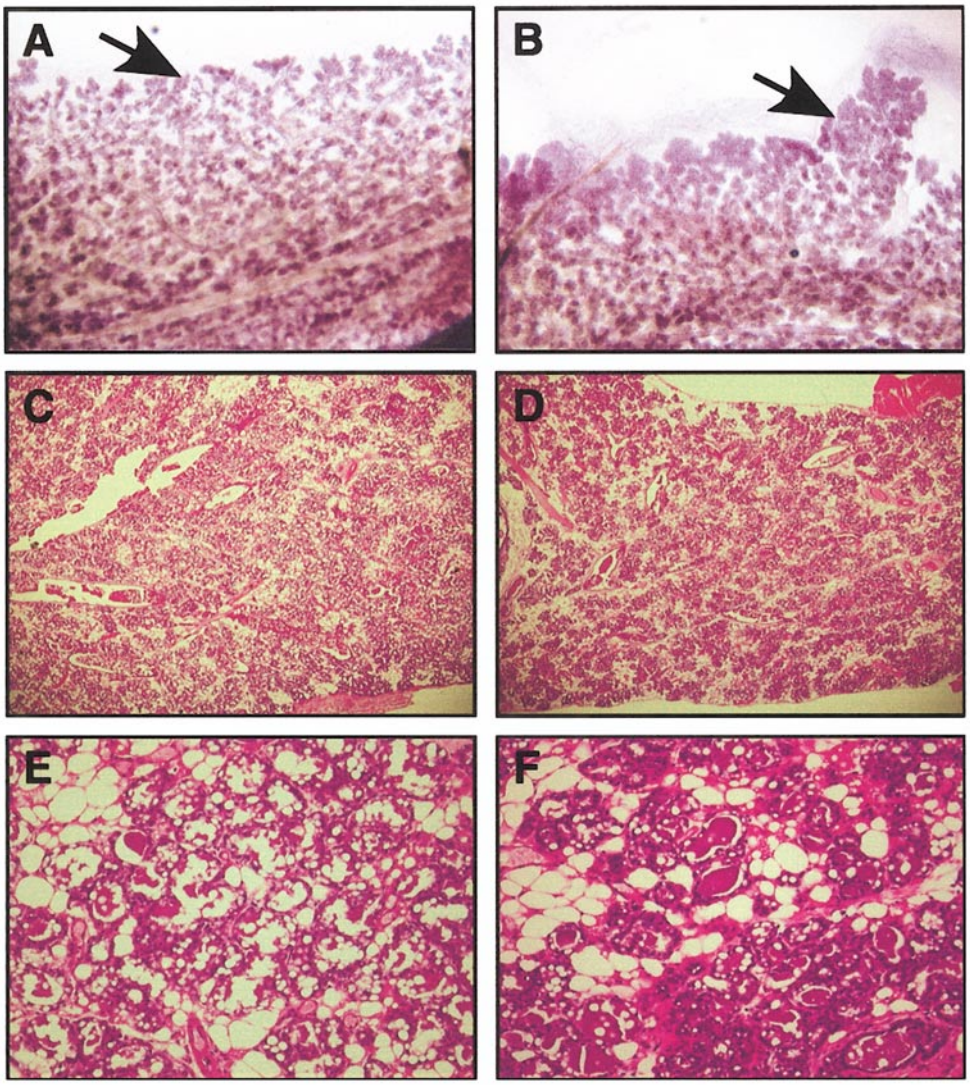

FIG. 5. Mammary development in Ihh-null transplants. Lactating samples were harvested within 12 h of parturition. Whole-mount (A, B) and histological analyses (C–F) of the transplants revealed no difference between Ihh-null (A, C, E) and wild type (B, D, F) epithelial transplants. The whole mounts were stained with carmine alum and photographed at 16×. The histological sections were H&E stained and photographed at 25× (C, D) and 200× (E, F). Arrows in (A) and (B) point to alveoli.

DISCUSSION

Morphogenesis of skin-derived organs, such as hair, teeth, whiskers, and the mammary gland depends on signaling between the mesenchyme and epithelium. Gene-deletion studies in mice have demonstrated that the hedgehog/patched pathway is required for the morphogenesis of hair, teeth, and whiskers (Chiang *et al.*, 1999; Dassule *et al.*, 2000). The three members of the Hh family, Shh, Ihh, and Dhh, bind to two receptors, Ptc1 and Ptc2, to govern developmental processes in mammals. Shh is expressed at sites of epithelial–mesenchymal interactions, which supports its function as mediator of tissue-inductive signals (Bitgood and McMahon, 1995). Deletion of the Shh gene results in multiple developmental defects and the fetuses die prior to birth (Chiang *et al.*, 1996). We show that Shh is expressed in mammary epithelium, and Ihh and Ptc1 are also located in the epithelium (Lewis *et al.*, 1999, 2001). Ptc1 expression is highest during pregnancy and lactation, suggesting that this pathway is critical for mammary gland differentiation. We have investigated whether Shh or Ihh are required for mammary gland development through the transplantation of Shh- and Ihh-null mammary epithelium into cleared fat pads of wild type hosts. Our results show

that ductal and alveolar development, as assessed by morphology and the expression of milk protein genes, is normal in the absence of Shh or Ihh. Although Shh is only expressed in the epithelial compartment of other skin appendages, it is conceivable that the action of Shh is non-cell-autonomous and that Shh from the wild type hosts could compensate for the loss of epithelial Shh in the transplants. We have addressed this issue through the transplantation of anlagen from Shh-null fetuses under the renal capsule of wild type hosts to derive a tissue that is entirely composed of Shh-null cell types. Development of this mammary tissue was normal while the hair follicles surrounding the anlagen did not develop, indicating that Shh requirements are different between this two skin appendages.

Shh-null fetuses contained mammary anlagen, which was expected as Shh is not required for the induction of skin-derived organs (Chiang *et al.*, 1999; St-Jacques *et al.*, 1998; reviewed in Chuong *et al.*, 2000). However, the finding that mammary gland development was normal in the absence of Shh was unexpected, as morphogenesis of all other skin-derived organs is impaired in the absence of Shh (Chiang *et al.*, 1999, Dassule *et al.*, 2000). It is possible that Ihh or Dhh, both of which are expressed in mammary tissue (Lewis *et al.*, 1999), could compensate for the absence of Shh. Dhh is not required for mammary development (Bitgood *et al.*, 1996) and the normal development of Ihh-null transplants demonstrates that, despite high levels of expression of Ihh in alveolar epithelial cells, Ihh is not required for epithelial development. However, our finding that the Shh expression pattern overlaps with Ihh expression (Lewis *et al.*, 1999) suggests that there may be functional redundancy between these two molecules. This is supported by the persistent expression of Ptc1 in Shh-null mammary transplants. Functional redundancy between Shh and Ihh has been observed (Ramalho-Santos *et al.*, 2000; Zhang *et al.*, 2001). The importance of Hh/Ptc signaling in the mammary gland is suggested by a transient defect in ductal development in mice from which one Ptc1 allele had been deleted (Lewis *et al.*, 1999). Haploinsufficiency in Ptc1 causes transient ductal hyperplasia and morphological defects in virgin mice. Ductal alterations were also detected in Gli2-null mammary glands (Lewis *et al.*, 2001). Gli proteins are zinc finger transcription factors that act as mediators of Hh signal transduction.

The mammary gland is a skin appendage, and consequently, some proteins that govern the morphogenesis and differentiation of skin-derived organs should also be necessary for mammary gland morphogenesis. This has been shown through gene-inactivation experiments for proteins required for the development of teeth and hair and the early stages of mammary gland morphogenesis. These include the transcription factors Lef-1 (van Genderen *et al.*, 1994), Msx1, Msx2 (Satokata *et al.*, 2000), and the secreted peptide PTHrP and its corresponding receptor (Dunbar *et al.*, 1999). However, the mammary gland has characteristics that distinguish it from other skin appendages. As an organ developed late in evolution, it has utilized existing signaling pathways from older organ systems and adapted them accordingly (Hennighausen and Robinson, 2001). Although the induction stages during fetal development resemble those of other skin appendages, subsequent development requires hormone-mediated cell proliferation and differentiation, which is executed by the Jak/Stat pathway (Liu *et al.*, 1997). The acquisition and adaptation of signaling molecules may have given the mammary gland a degree of functional redundancy that has not been observed in other skin-derived organs. For example, while Msx1-null mice present defects in tooth morphogenesis but not in the mammary gland, Msx2-null mice exhibit arrested mammogenesis at the embryonic gland stage. However, mice that have lost Msx1 and Msx2 function (Satokata *et al.*, 2000) completely lack mammary anlagen. This demonstrates overlapping roles of Msx1 and Msx2 in mammary gland morphogenesis. The presence of Stat5a and -5b in the mammary gland and their ability to partially compensate for each other constitutes another example of functional redundancy (Liu *et al.*, 1998). The overlapping expression of Ihh (Lewis *et al.*, 1999, 2001) and Shh (see Fig. 4F) in mammary epithelium during pregnancy suggests a functional redundancy of these pathways. Shh/Ihh-null double mutant mouse die prior to the formation of mammary anlagen (Ramalho-Santos *et al.*, 2000). Thus, it will be necessary to target these genes so that they can be deleted in specific cell types at different stages of development.

The Hh/Ptc signaling pathway has a central role in governing morphogenesis from flies to mammals. Our results demonstrating differences in this pathway between organs from the same developmental origin are of importance, as they will help us to dissect the nature of this signaling pathway to further comprehend the complexity of tissue interaction during morphogenesis of specific organs.

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