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Shh expression is required for embryonic hair follicle but not mammary gland development

Kinga Michno,[a] Kata Boras-Granic,[a] Pleasantine Mill,[b] C.C. Hui,[b] and Paul A. Hamel[a,*]

[a] *Department of Laboratory Medicine, University of Toronto, Toronto, Ontario, Canada*
[b] *Hospital for Sick Children Research Institute, Toronto, Ontario, Canada*

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Abstract

The embryonic mammary gland and hair follicle are both derived from the ventral ectoderm, and their development depends on a number of common fundamental developmental pathways. While the Hedgehog (Hh) signaling pathway is required for hair follicle morphogenesis, the role of this pathway during embryonic mammary gland development remains undetermined. We demonstrate here that, unlike the hair follicle, both *Shh* and *Ihh* are expressed in the developing embryonic mouse mammary rudiment as early as E12.5. In $Shh^{-/-}$ embryos, hair follicle development becomes arrested at an early stage, while the mammary rudiment, which continues to express *Ihh*, develops in a manner indistinguishable from that of wild-type littermates. The five pairs of mammary buds in $Shh^{-/-}$ female embryos exhibit normal branching morphogenesis at E16.5, forming a rudimentary ductal structure identical to wild-type embryonic mammary glands. We further demonstrate that loss of Hh signaling causes altered cyclin D1 expression in the embryonic dermal mesenchyme. Specifically, cyclin D1 is expressed at E14.5 principally in the condensed mesenchymal cells of the presumptive hair follicles and in both mesenchymal and epithelial cells of the mammary rudiments in wild-type and *Shh*-deficient embryos. By E18.5, robust cyclin D1 expression is maintained in mammary rudiments of both wild-type and *Shh*-deficient embryos. In hair follicles of wild-type embryos by E18.5, cyclin D1 expression switches to follicular epithelial cells. In contrast, strong cyclin D1 expression is observed principally in the mesenchymal cells of arrested hair follicles in $Shh^{-/-}$ embryos at E18.5. These data reveal that, despite the common embryonic origin of hair follicles and mammary glands, distinct patterns of *Hh*-family expression occur in these two tissues. Furthermore, these data suggest that cyclin D1 expression in the embryonic hair follicle is mediated by both Hh-independent and Hh-dependent mechanisms.

Introduction

The mammary gland is an epidermal appendage which develops embryonically as a result of local reciprocal signaling interactions between mesenchymal cells underlying the epithelial cells of the ectoderm (for review, see Howlett and Bissell, 1993; Robinson et al., 1999). Although components of all the major signal transduction cascades have been identified in the postnatal mammary gland, considerably less is known concerning the roles of these pathways during embryonic phases of mammary gland development. The common origin of the embryonic mammary gland and ventral hair follicles suggests that similar signaling pathways may control their development. For example, both embryonic mammary gland and hair follicle development require the expression of the transcriptional mediator of the "canonical" Wnt pathway, *Lymphocyte enhancer factor-1* (*Lef-1;* van Genderen et al., 1994). In the absence of *Lef-1*, mammary gland and hair follicle development is arrested at primitive stages. Factors in other developmental pathways have also been shown to be expressed in analogous compartments of the postnatal mammary gland and hair follicles (for reviews, see Hennighausen and Robinson, 1998, 2001; Hennighausen et al., 1997a,b; Robinson et al., 2000), including receptor tyrosine kinases (Birchmeier and Gherardi, 1998; Matsumoto and Nakamura, 1996), bone morphogenetic proteins (Bitgood and McMahon, 1995; Thesleff et al., 1995), and Wnt-family proteins (Andl et al., 2002; Miyoshi et al., 2002).

* Corresponding author. Fax: +416-978-5959.
E-mail address: paul.hamel@utoronto.ca (P.A. Hamel).

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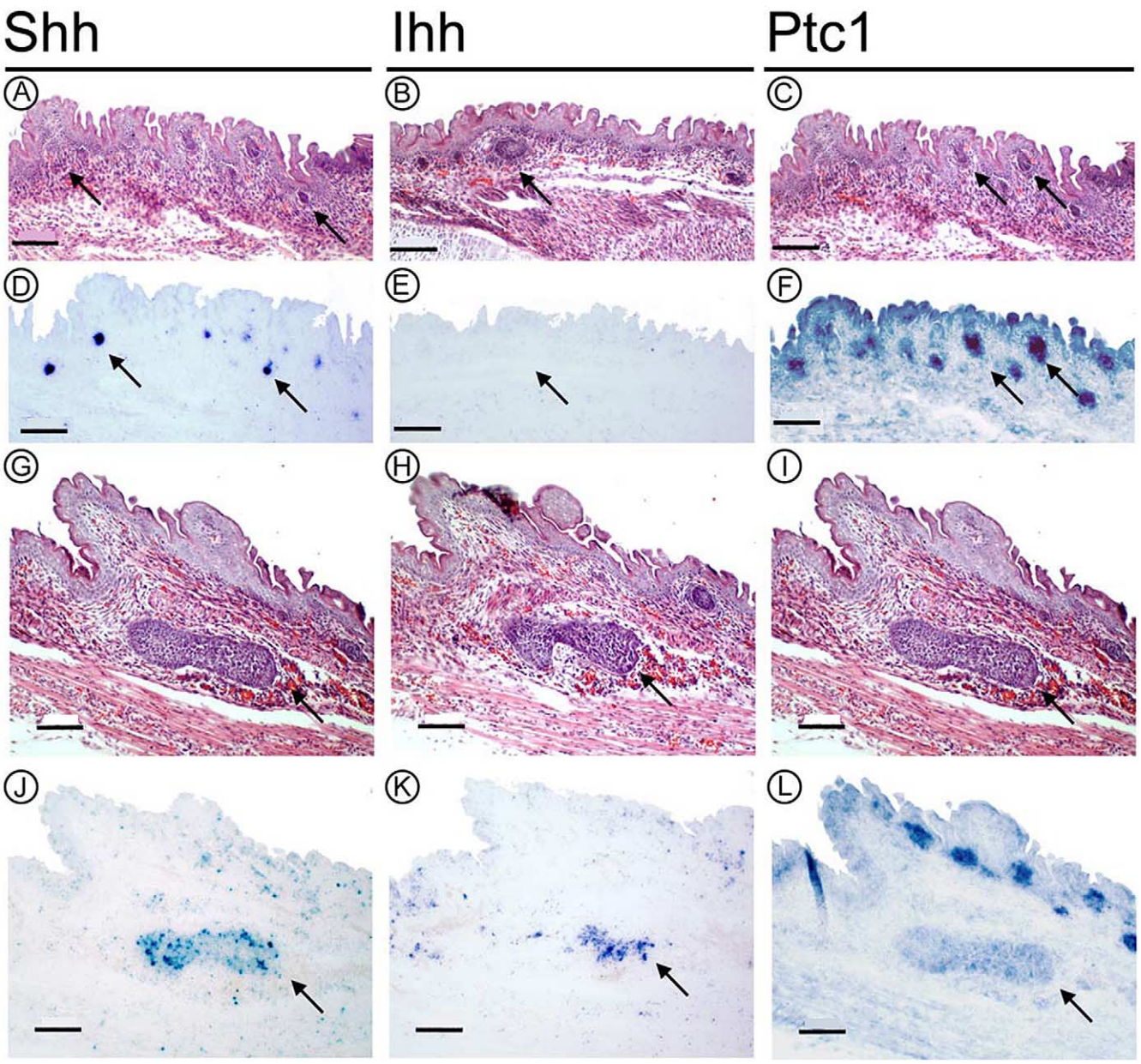

Fig. 1. Expression of *Shh*, *Ihh*, and *Ptc1* in E16.5 mammary gland and hair follicles. Hematoxylin and eosin staining depicts the orientation of the mammary rudiments (arrows in G–I) and hair follicles (black arrows in A–C). *In situ* hybridization in mammary glands (J–L) and hair follicles (D–F) using digoxygenin-labeled riboprobes against *Shh* (D and J), *Ihh* (E and K), and *Ptc1* (F and L). *Shh* (arrow in J) and *Ihh* (black arrow in K) were both detected only in the epithelial compartment of the developing mammary gland, while *Ptc1* (L) was expressed both within the epithelium (arrow in L) and mesenchyme. Note the characteristic pattern of *Shh* and *Ptc1* expression within the developing hair follicle (arrows in D and F, respectively) and the lack of *Ihh* signal. Bars, 100 μm.

The Hedgehog (Hh) signaling pathway is a fundamental pathway involved in regulating the development of many tissues. Identified originally in *Drosophila*, three homologues exists in mammals; *Sonic, Indian,* and *Desert Hedgehog (Shh, Ihh, Dhh,* respectively). These secreted molecules stimulate target cells expressing the Hh receptor, Patched-1 (Ptcl; Marigo et al., 1996) to undergo a variety of developmental responses, including cell fate decisions, proliferation, and differentiation. In the absence of Hh, Ptc1 negatively regulates the integral membrane factor, Smoothened (Smo; Murone et al., 1999). Binding of Hh to Ptc1 releases the inhibitory effects on the cascade and converts the Gli/Ci transcription factor family proteins into potent transcriptional activators (Sasaki et al., 1999). These, in turn, drive the expression of target genes mediating Hh-dependent responses. The Hh-family proteins are typically expressed during organogenesis in nonoverlapping tissues (Bitgood and McMahon, 1995; Marigo et al., 1995). This generally mutually exclusive expression pattern is reflected by tissue-specific defects exhibited in animals lacking individual Hh

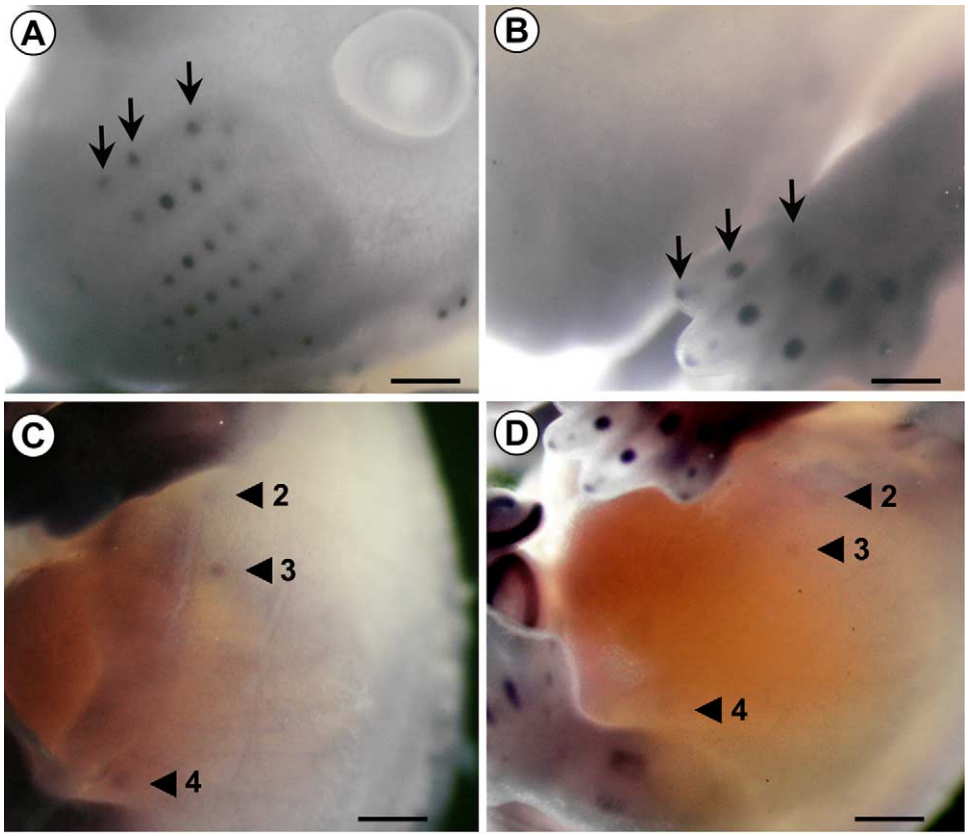

Fig. 2. Expression of *Shh* and *Ihh* in mammary placodes at E12.5. Expression of *Shh* (A and C) and *Ihh* (B and D) mRNA in E12.5 embryos was determined by whole-mount *in situ* hybridization. Weak expression of both *Shh* and *Ihh* was detected in mammary placodes 2, 3, and 4 (indicated by filled arrowheads in C and D) as well as in placodes 1 and 5 (not shown). Comparison of the signal for *Shh* in developing whiskers (arrows in A) with the signal in mammary placodes (arrowheads in C) in identical embryos indicated weaker *Shh* expression in the latter tissue. Likewise, *Ihh* expression in the mammary placodes is significantly weaker than in developing skeletal structures (arrows in D) in the same embryo.

proteins. For example, *Ihh* is expressed in the prehypertrophic chondrocytes in developing skeletal structures (Bitgood and McMahon, 1995; Koyama et al., 1996; Kronmiller and Nguyen, 1996; Marigo et al., 1995). Premature differentiation of chondrocytes occurs in mice lacking *Ihh*, giving rise to severe skeletal defects (St-Jacques et al., 1999). *Shh* is more widely expressed and gives rise to many distinct developmental defects when absent (Chiang et al., 1996; Roessler et al., 1996). In the developing skin, *Shh* is expressed in the epidermal layer and, following Lef-1-dependent signaling from the mesenchyme, is restricted to polarized epithelial cells of the presumptive hair follicle (Chiang et al., 1999; Morgan et al., 1998; St-Jacques et al., 1998). Shh expression is maintained in a specialized group of proliferating cells (matrix cells) at the distal tip of developing hair follicles (Gat et al., 1998). In the absence of *Shh*, hair follicle development is arrested at an early stage (stage 2) when polarization of the presumptive hair follicle and condensation of underlying mesenchymal cells can be observed.

While mammary gland development has been the subject of intense investigation, little is known about the role of Hh-signaling in this tissue. In the postnatal organ, *Shh*, *Ihh*, and *Dhh* have been detected by RT-PCR, although only *Ihh* expression has been detected by in situ analysis (Lewis et al., 1999a). *Ihh* is expressed throughout the postnatal period in epithelial cells predominantly near the terminal end buds and at points of ductal branching. The Hh-receptor, *Ptc1*, is expressed in both ductal epithelial cells and in fibroblasts in the surrounding mesenchyme. In contrast to *Ptc1*, the downstream effector of Hh signaling, the *Gli2* transcription factor, exhibits a dynamic expression pattern (Lewis et al., 2001). During puberty and early pregnancy, *Gli2* expression is restricted to mesenchymal cells surrounding the end buds, while in the later stages of pregnancy, it is also expressed in alveolar epithelial cells.

A role for Hh-signaling during mammary gland development has been shown using heterozygous knock-out animals and through transplantation studies. For example, heterozygous *Ptc1*-deficient animals exhibit ductal hyperplasia and dysplasia during puberty and in the adult virgin mammary gland (Lewis et al., 1999b). This phenotype is reversed during pregnancy where normal lobuloalveolar structures develop and proper lactation ensues. Transplantation of mammary epithelial cells from these $Ptc1^{+/-}$ haploinsufficient mice into the cleared fat pad of wild-type

recipient mice revealed that the defect in ductal morphogenesis was due to altered *Ptc1* expression in the mesenchymal cells since transplanted *Ptc1*$^{+/-}$ epithelial cells gave rise to normal mammary glands. Analogously, while *Gli2*-deficient mice die embryonically, transplantation of *Gli2*$^{-/-}$ epithelium also supported a requirement for Hh signaling in the mesenchymal compartment (Lewis et al., 2001). Thus, Hh signaling in at least the stromal compartment appears to be required for normal mammary gland development.

Given the common origin of ventral hair follicles and mammary glands during embryogenesis and their defective development in the absence of *Lef-1* (van Genderen et al., 1994), we hypothesized that expression of Hh-family proteins during embryonic mammary gland development would also be similar to that of the hair follicle. We demonstrate here that two Hh-family members, *Shh* and *Ihh*, are expressed in the epithelial compartment of the embryonic mammary gland. We show further that, in contrast to hair follicles, loss of *Shh* expression has no effect on embryonic mammary gland development. Furthermore, a target of Hh-signaling, cyclin D1 (Kenney and Rowitch, 2000; Long et al., 2001), exhibits an altered expression pattern in the arrested hair follicles in *Shh*-deficient animals relative to their wild-type counterparts, while its expression is maintained in the epithelial and stromal components of the mammary gland in these same embryos.

Materials and Methods

Animals

Heterozygous *Shh*$^{+/-}$ mutant mice, a gift from Dr. Chin Chiang, have been described previously (Chiang et al., 1996). The Shh colony is on a mixed CD1 background. The initial in situ analysis of *Shh*, *Ihh*, and *Ptc1* expression in the embryonic mammary was performed by using wild-type embryos generated from a separate CD1 colony. Otherwise, all histology and immunohistochemical analyses were performed utilizing wild-type littermates generated using breeding pairs from the *Shh*$^{+/-}$ colony. Presence of a vaginal plug was assessed in the morning and, when present, marked day 0.5 of pregnancy.

Histology, in situ, whole-mount, and immunohistochemical analyses

DIG in situ hybridization was performed as described previously (Mo et al., 1997; Motoyama et al., 1998). The riboprobes which specifically detect *Shh* (640 bp *Eco*RI fragment), *Ihh* (1.8 kb *Eco*RI fragment), and *Ptcl* (841 bp *Eco*RI fragment) have been described previously (Mo et al., 1997; Motoyama et al., 1998a,b). Embryos were decapitated and fixed overnight in 4% PFA, dehydrated, and embedded in paraffin. Then, 6- to 7-μm sections were generated, dried overnight at 40°C, and used for histology, mRNA expression, and/or protein expression analysis. For whole mounts, embryos were dehydrated in methanol and stored at −20°C until use. Whole mounts were performed as previously described (Motoyama et al., 1998). For DIG in situ, riboprobes were added to sections and incubated overnight at 55°C, as described in Mo et al. (1997). After standard posthybridization washes, sections were blocked with 1% Blocking Reagent (Roche) for 1 h, followed by 1 h room temperature incubation with 1:2000 α-DIG-alkaline phosphatase (Roche). Color reaction with BM Purple (Roche) reagent was allowed to proceed overnight at room temperature. All immunohistochemical analysis was performed according to the instructions provided in the R&D HRP-AEC immunostaining kits, with the following amendments. Antigen retrieval was performed in a pressure cooker containing a 10 μM sodium citrate solution, pH 6, preheated to 50−60°C, and microwaved at 50% power for two 10-min intervals. Following cooling and blocking steps, primary antibodies were added. The rabbit α-Lef-1 antisera (1:1500 dilution; a kind gift from Dr. R Grosschedl) and mouse α-cyclin D1 monoclonal antibody (Santa Cruz Biotech. #sc-450; 1:200 dilution) were incubated overnight at 4°C. All sections were counterstained with hematoxylin (Vector; H-3404) and images were taken by using a Nikon DXM 1200 digital camera driven by Nikon ACT-1 imaging software.

Results

Both Shh and Ihh are expressed in the developing embryonic mammary gland

To date, the expression of *Hh*-family members and the role of the Hh-signaling pathway have been only assessed in the postnatal mammary gland (Gallego et al., 2002; Lewis et al., 1999b). However, the common requirement of a number of signal transduction pathways for development of both mammary glands and hair follicles and the essential role of Hh signaling during folliculogenesis predict that one or more *Hh*-family members may also be expressed during the embryonic phases of mammary gland development. We tested this prediction by probing for *Shh*, *Ihh*, and *Ptc1* in E16.5 female embryos, a stage in development where the transition from a spherical mammary placode to the rudimentary branched embryonic mammary gland occurs (Fig. 1). Both *Shh* and *Ihh* are expressed exclusively in the mammary epithelium (Fig. 1J and K). In contrast, adjacent hair follicles, which are derived from the same ventral ectoderm, express only *Shh* in the distal epithelial cells of the hair germ (Fig. 1D and E) as described previously (Chiang et al., 1999). *Ptc1* is expressed in the mammary epithelium and mesenchyme (Fig. 1L) analogous to its expression in the embryonic hair follicle (Fig. 1F). Expression of *Shh* and *Ihh* is detected during earlier stages of mammary bud formation (Fig. 2). At E12.5, both *Ihh* (Fig. 2D) and *Shh* (Fig. 2C) are

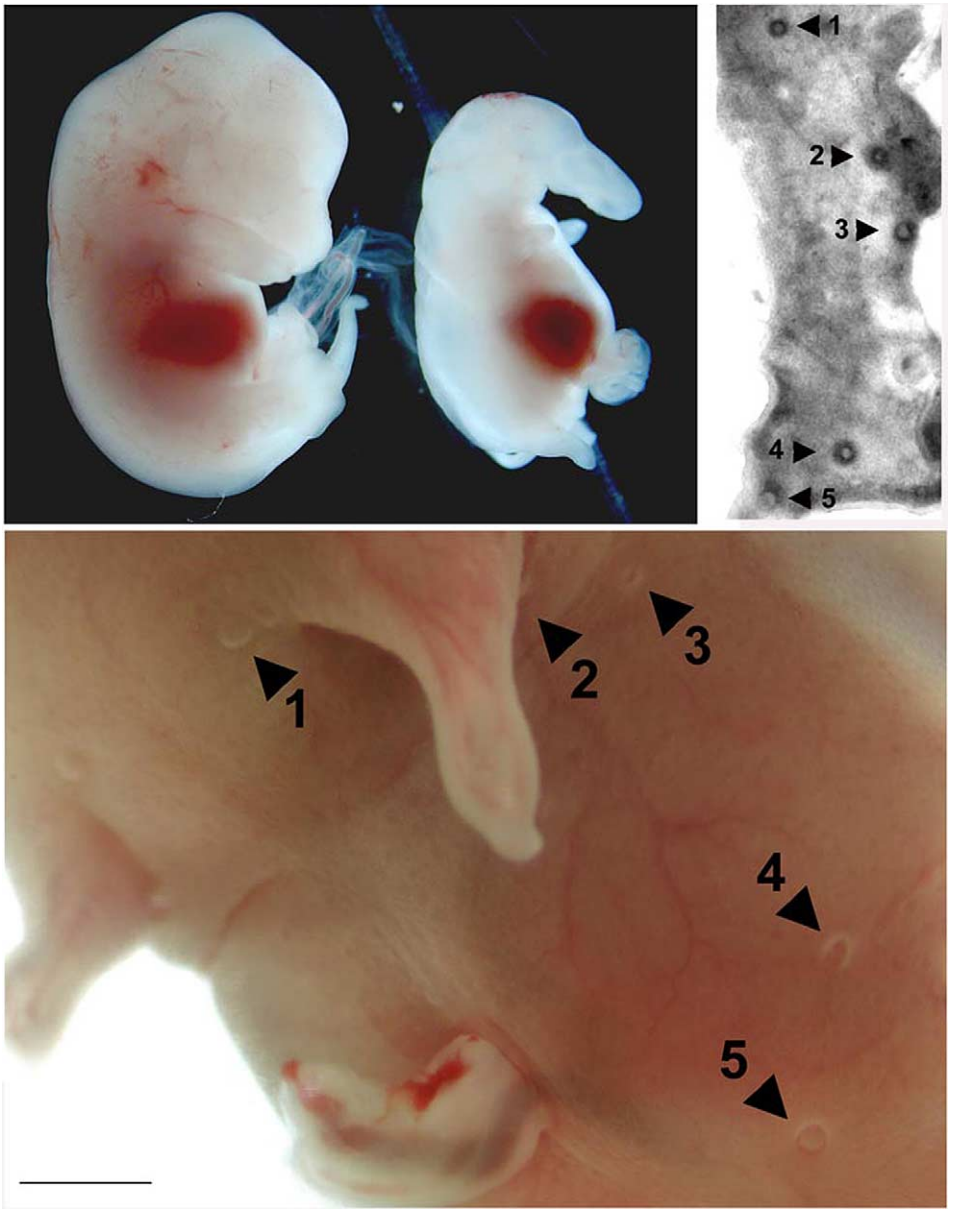

Fig. 3. Mammary bud formation in *Shh*$^{-/-}$ embryos. *Shh*-deficient embryos (right embryo, top left panel) exhibit various defects, including holoprocencephaly and arrested limb development compared with wild-type littermates (left embryo, top left panel). However, isolated ventral dermis from E14.5 *Shh*$^{-/-}$ embryos (top right panel) reveal the formation of five pairs of mammary buds (black arrows) in the correct spatial orientation. The bottom panel illustrates that the mammary placodes (arrows) are clearly visible on the ventral surface of E16.5 *Shh*$^{-/-}$ embryos.

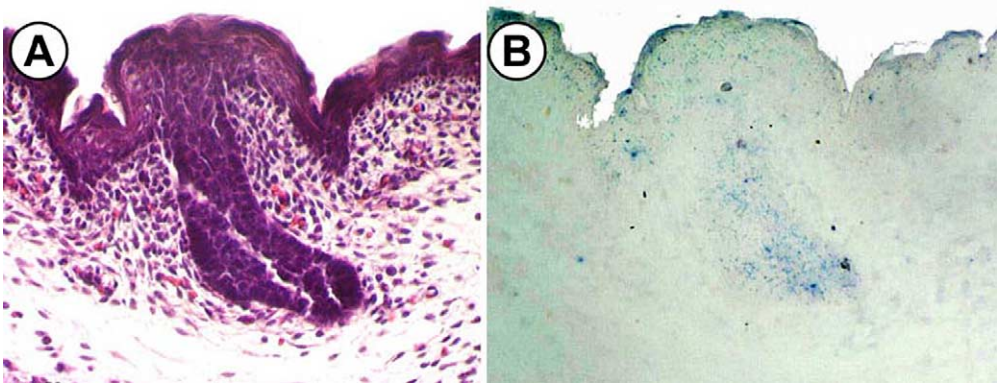

Fig. 4. *Ihh* expression in the *Shh*$^{-/-}$ mammary rudiment. (A) Hematoxylin and eosin staining depicts the histology of the *Shh*$^{-/-}$ mammary rudiment. (B) Serial sections probed for *Ihh* mRNA. *Ihh* expression is maintained in the epithelial compartment of the mammary placode in *Shh*$^{-/-}$ embryos. Bars, 50 μm.

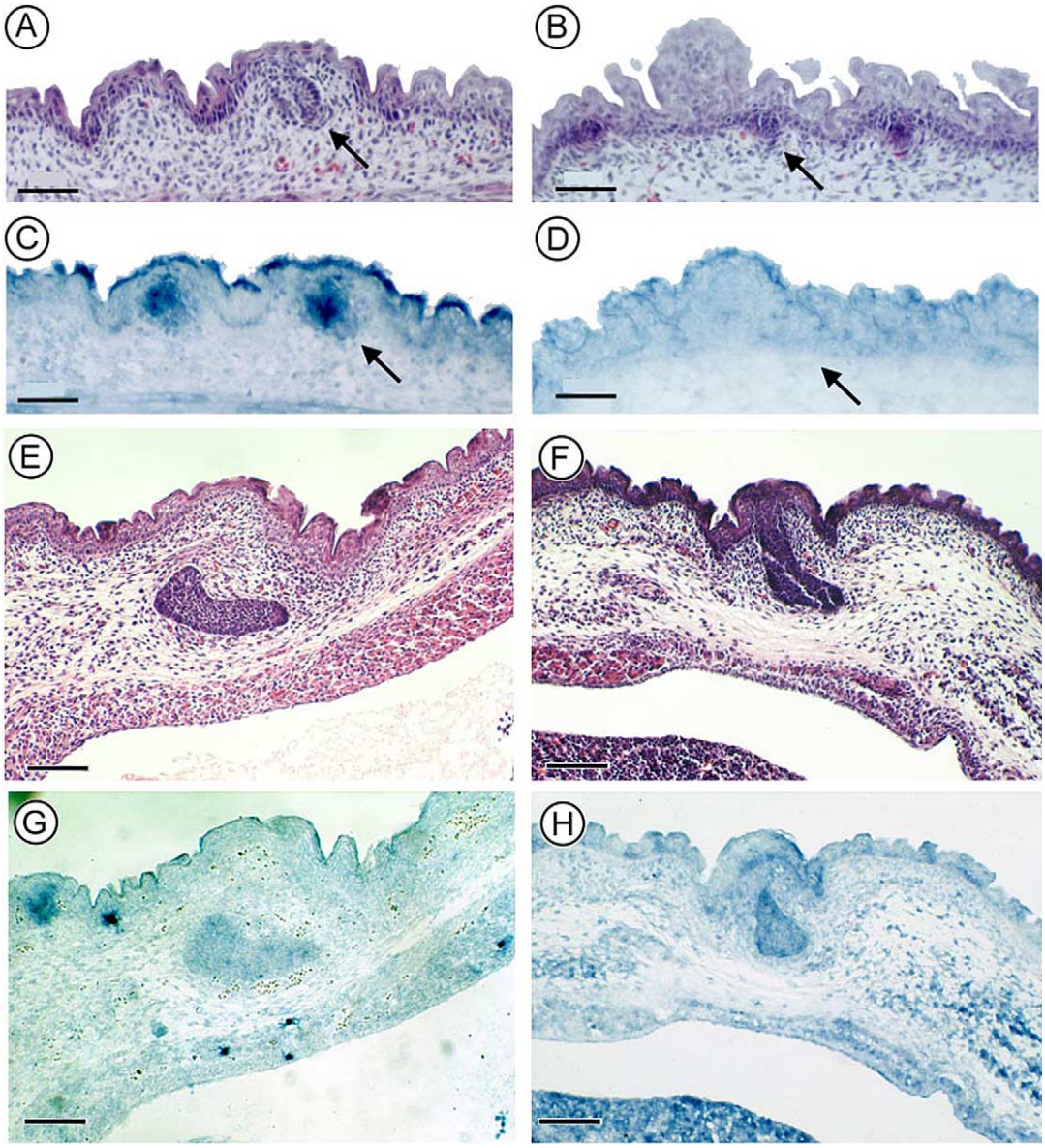

Fig. 5. *Ptc1* expression in wild-type and *Shh*$^{-/-}$ E16.5 embryos. Hematoxylin and eosin staining (A–B, E–F) and *Ptc1* expression (C–D, G–H) in wild-type hair follicles (A and C), wild-type mammary rudiments (E and G), *Shh*-deficient hair follicles (B and D), and *Shh*-deficient mammary rudiments (F–H) at E16.5. In *Shh*-deficient embryos, *Ptc1* is clearly detected in the mammary rudiment, whereas only background expression is detected in the arrested hair follicle (D). Arrows in (A) and (C) as well as (B) and (D), respectively, indicate identical hair follicles in serial sections. Bars, 50 μm in (A–D). Bars, 100 μm in (E–H).

detected in all five pairs of mammary placodes (mammary placodes 1 and 5 not shown). Expression of *Shh* and *Ihh* in the mammary placodes at this stage appeared weaker relative to the signals for *Shh* in whisker follicles (arrows, Fig. 2A) and *Ihh* in developing skeletal structures (arrows, Fig. 2B). Furthermore, the signal for both *Shh* and *Ihh* in placode #3 was typically stronger than for other placodes (e.g., compare with placode #2 or #4). Expression of neither *Shh* nor *Ihh* was detected in embryonic mammary glands earlier than E12.5 (data not shown). Thus, while hair follicle and mammary gland development are dependent on a number of common pathways and cell precursors, the embryonic mammary gland uniquely expresses both *Ihh* and *Shh*. Furthermore, expression of *Shh* and *Ihh* appear coincidently, albeit weakly, in the murine mammary gland by E12.5.

Shh$^{-/-}$ *mice have normal embryonic mammary glands*

Given that hair follicle and mammary gland morphogenesis are dependent on common developmental processes and that folliculogenesis in mice which lack *Shh* becomes arrested, we determined whether the loss of *Shh* expression also effected embryonic mammary gland development. As Fig. 3 illustrates, five pairs of mammary placodes (three

thoracic and two inguinal) are apparent in female *Shh*$^{-/-}$ embryos. Furthermore, although the body structure of *Shh*$^{-/-}$ animals displays severe abnormalities exhibiting, for example, holoprosencephaly and arrested limb development, the distribution of the mammary placodes, relative to one another, is normal. Specifically, placodes number 1 and 5 are closest to the midline, whereas number 3 is farthest. Mammary buds are not apparent for male *Shh*$^{-/-}$ embryos at E16.5, suggesting that regression of the male mammary apparatus also occurs normally (data not shown).

Since all five pairs of rudimentary mammary glands are apparent on the intact *Shh*$^{-/-}$ embryo, we predicted that *Ihh* would continue to be expressed in this tissue. Indeed, significant expression of *Ihh* is seen at E16.5 in *Shh*-deficient embryos (Fig. 4B). Consistent with the expression of *Ihh* in the mammary rudiment of *Shh*-deficient animals, *Ptc1* also continues to be expressed (compare Fig. 5G and H). For both *Ihh* and *Ptc1* expression, localization to specific cells is not apparent, the signal for both appearing uniform throughout the tissue. In contrast to the mammary rudiment, in the developing hair follicles, which depends exclusively on Shh expression, *Ptc1* is undetectable (compare Fig. 5C and D) in the same *Shh*-deficient embryos. We conclude that, in *Shh*$^{-/-}$ animals, the redundant activity of Ihh in the mammary gland maintains Hh-signaling

Cyclin D1 expression is maintained in Shh-deficient embryonic mammary glands

At E14.5 in wild-type embryos, the rudimentary mammary apparatus is comprised of a bulb-shaped epithelial bud surrounded by a closely associated, condensed mesenchyme (Fig. 6C–D, G–H). At this stage, the hair germ consists of a lenticular epithelial bud closely associated with a dermal condensate, which constitutes the primordial dermal papilla (Fig. 6A and B). More primitive presumptive hair follicles, where only polarized epithelial cells associated with an underlying condensation of mesenchymal cells, can also be observed at this stage (Fig. 6E and F). The histology of both the mammary anlagen and presumptive hair follicle appears normal in *Shh*$^{-/-}$ mice relative to wild-type littermates at this stage.

The developmental progression of both the mammary placode and the rudimentary hair follicles at E14.5 in wild-type and *Shh*-deficient animals were verified by examining expression of factors involved in the development of both tissues. We specifically determined the expression pattern for Lef-1, which is required for both hair follicle and mammary gland development (van Genderen et al., 1994), as well as for cyclin D1, which is required for normal mammary gland development (Sicinski et al., 1995). Cyclin D1 is a known target of both Lef-1 activity (Shtutman et al., 1999) and Hh-signaling (Kenney and Rowitch, 2000; Long et al., 2001). At E14.5, the expression of Lef-1, the earliest known marker for mammary bud formation (Mailleux et al., 2002), is unperturbed in the absence of *Shh* (Fig. 6B and D) relative to wild-type littermates (Fig. 6A and C) in both hair follicles and mammary placodes. Likewise, cyclin D1 expression is indistinguishable between wild-type (Fig. 6E and G) and *Shh*$^{-/-}$ embryos (Fig. 6F and H) at this stage. Specifically, cyclin D1 is expressed in many cells of the mammary placode and the surrounding mesenchyme in both *Shh*$^{-/-}$ and wild-type embryos (Fig. 6G and H). Only the layer of closely associated fibroblasts fail to express detectable levels of cyclin D1 protein. In hair follicles, the condensed mesenchymal cells exhibit high levels of cyclin D1 in both wild-type (Fig. 6E) and *Shh*$^{-/-}$ (Fig. 6F) embryos.

By E16.5, significant differences in cyclin D1 expression between wild-type and *Shh*-deficient littermates are apparent in developing hair follicles, but not in the mammary placode. In both wild-type (Fig. 7C) and *Shh*$^{-/-}$ embryos (Fig. 7D), cyclin D1 continues to be expressed in the mammary epithelium and surrounding mesenchyme. As Fig. 7E and F illustrates, we also determined that, for both wild-type and *Shh*$^{-/-}$ embryos, expansion of the mammary epithelium into the underlying dermis and the onset of branching morphogenesis appear normal. In contrast to the mammary placode, expression of cyclin D1 is significantly different in hair follicles of *Shh*-deficient embryos relative to wild-type littermates. Cyclin D1 expression in hair follicles of wild-type embryos switches from being principally in the mesenchyme destined to become the dermal papilla to epithelial cells adjacent the condensed mesenchyme. At this point, hair follicles at several distinct stages are observed in these E16.5 embryos. Hair follicles which reach at least stage 2 exhibit this epithelial expression pattern, while those at earlier stages retain cyclin D1 expression primarily in the condensed mesenchyme. In contrast, cyclin D1 expression is still present in the condensed mesenchyme in Shh$^{-/-}$ littermates. We also observe cyclin D1 expression in epithelial cells in the arrested hair follicles. However, epithelial cells which do express cyclin D1 are randomly distributed and generally stain more weakly than the epithelial cells adjacent to the condensed mesenchyme in wild-type embryos (Fig. 7B).

In the rudimentary ductal structure of the mammary gland at E18.5, cyclin D1 expression is identical in wild-type and *Shh*$^{-/-}$ embryos (Fig. 8C and D). Here, cyclin D1 protein is expressed primarilly in epithelial cells. A lumen is also evident in these ductal structures, suggesting that the overall three-dimensional structure of the mammary rudiment is normal. We also determined that the nipple sheath develops normally in the *Shh*$^{-/-}$ embryos (see Fig. 4A) in a manner indistinguishable from wild-type embryos (Fig. 8C), indicating that developmental pathways involved in other aspects of mammary gland morphogenesis are also unaltered. In contrast to the mammary gland, a distinct expression pattern for cyclin D1 in hair follicles of *Shh*$^{-/-}$ mice is apparent at E18.5 (Fig. 8). In wild-type embryos (Fig. 8A), robust cyclin D1 expression occurs in epithelial

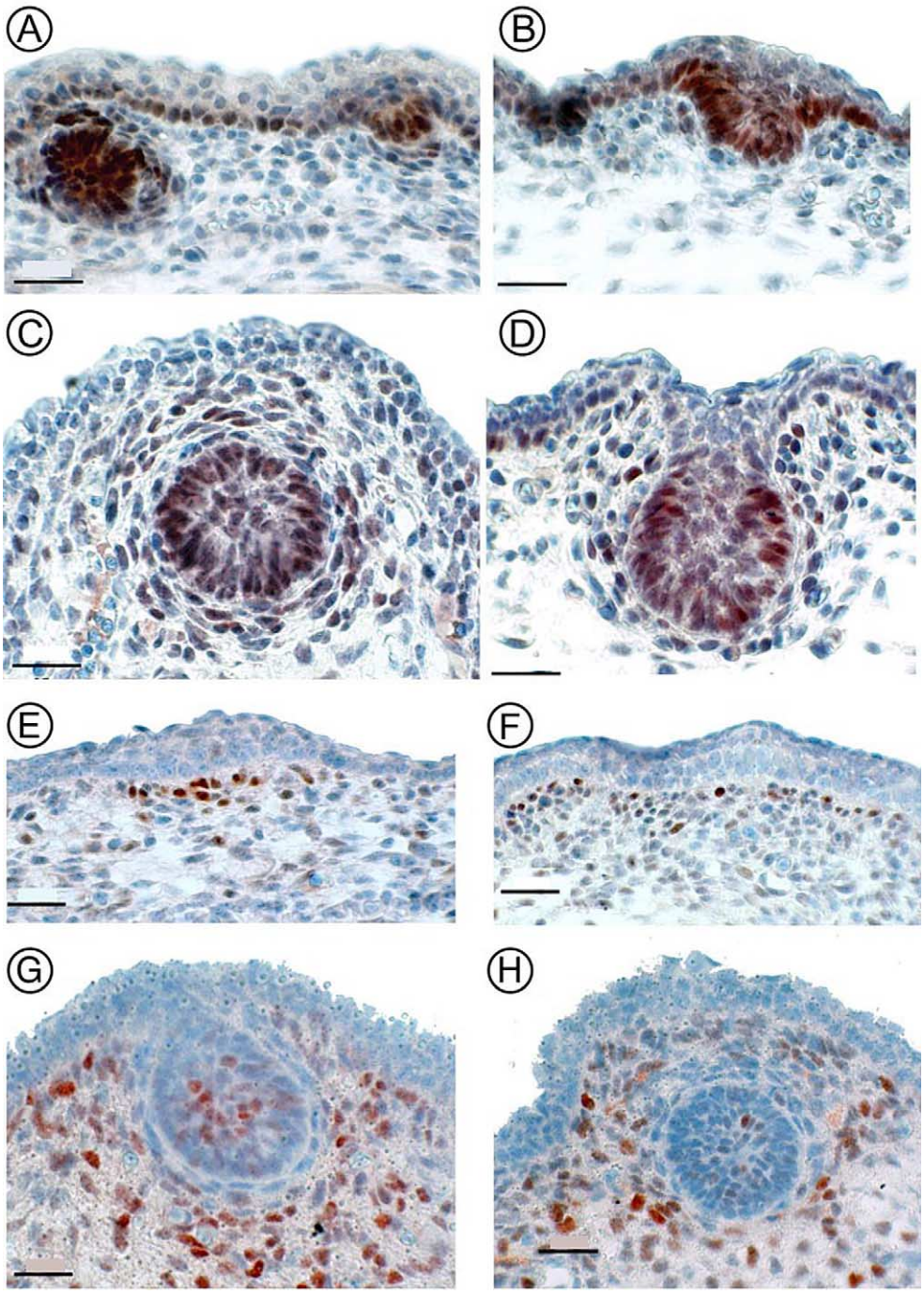

Fig. 6. Unaltered cyclin D1 and Lef-1 expression in *Shh*$^{-/-}$ embryos at E14.5. Sections from E14.5 wild-type (A, C, E, G) or *Shh*$^{-/-}$ (B, D, F, H) littermates were immunostained for Lef-1 (A–D) or cyclin D1 (E–H) and counterstained with hematoxylin. Lef-1 expression is prominent in the nuclei of most epithelial cells and associated (condensed) mesenchymal cells in both wild-type and *Shh*-deficient hair follicles and mammary placodes. For cyclin D1, expression is confined principally to the condensed mesenchymal cells underlying the presumptive hair follicle and in both epithelial and mesenchymal cells of the mammary placode in wild-type and *Shh*$^{-/-}$ littermates. Bars, 25 μm.

cells adjacent to the dermal papilla as well as in epithelial cells of the developing outer root sheath of the hair follicle. However, in *Shh*$^{-/-}$ embryos, cyclin D1 is expressed primarily in the condensed mesenchymal cells in the arrested follicle; its expression has failed to be maintained in the epithelial cells (weak staining for cyclin D1 is apparent in the occasional epithelial cell; data not shown). While cyclin D1 expression is altered in the *Shh*$^{-/-}$ embryos, other factors important in hair follicle or dermis development are expressed in correct cells types. Lef-1 expression in hair follicles in both wild-type and *Shh*$^{-/-}$ mice is detected in most epithelial and mesenchymal cells (Fig. 9A–B). Inter-

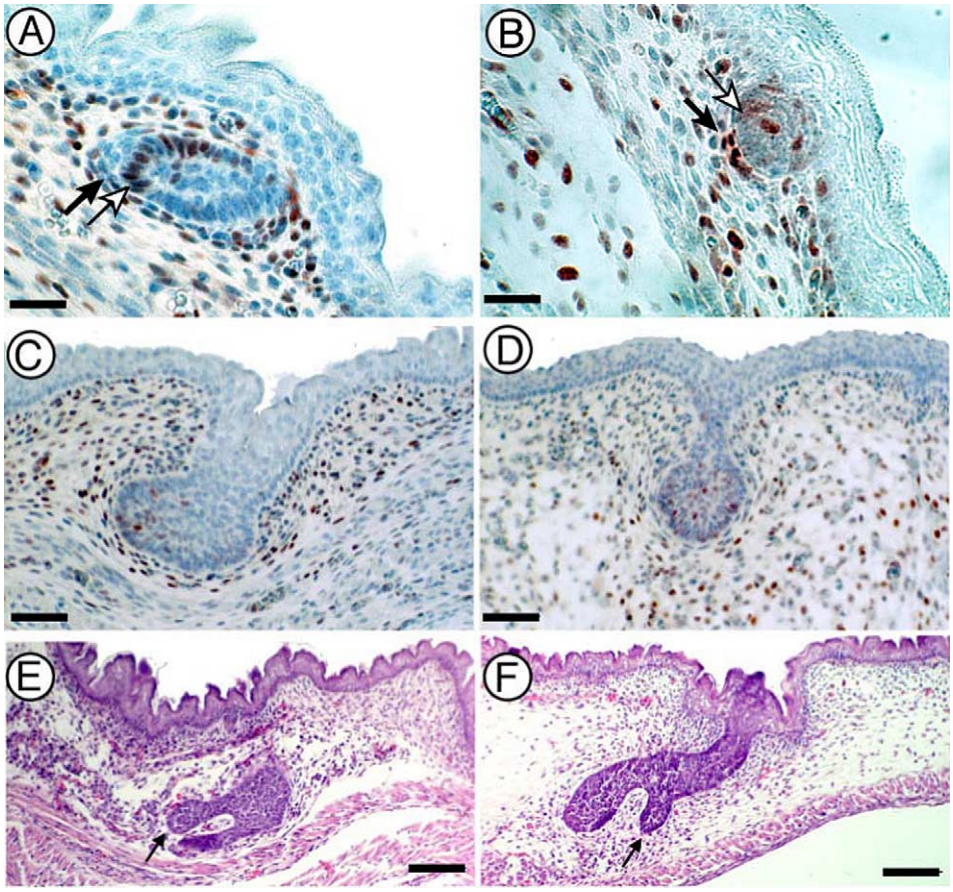

Fig. 7. Cyclin D1 expression in the mammary and hair rudiment at E16.5. Immunohistochemical analysis of the expression of cyclin D1 protein at E16.5 revealed that wild-type hair follicles (A) express cyclin D1 predominantly in epithelial cells (open arrow) adjacent to the condensed mesenchymal cells (closed arrow). In $Shh^{-/-}$ animals (B), cyclin D1 is expressed predominantly in mesenchymal cells and randomly in epithelial cells. In the mammary gland in both wild-type (C) and $Shh^{-/-}$ (D) embryos, cyclin D1 is detected in both the epithelial and mesenchymal compartments. Hematoxylin and eosin staining of the mammary rudiment in wild-type (E) and $Shh^{-/-}$ (F) littermates also reveals that elongation and rudimentary branching (arrows in E–F) appears to proceed normally in the absence of *Shh*. Bars, 25 μm in (A–B). Bars, 50 μm in (C–D). Bars, 100 μm in (E–F).

estingly, in the mammary rudiment at E18.5, significant cytoplasmic staining for Lef-1 is also evident in epithelial cells of the ductal structure, while cells in the surrounding stroma exhibit staining for Lef-1 exclusively in the nucleus in both wild-type and $Shh^{-/-}$ animals (Fig. 9C and D). Normal expression of other factors, such as A1x4 (restricted to condensed mesenchyme; Hudson et al., 1998), keratin 5, and keratin-14, were also determined to be identical in the mammary rudiment and in hair follicles of wild-type and $Shh^{-/-}$ littermates (data not shown).

Thus, these data demonstrate that, despite the common origin of epithelial cells of the embryonic mammary gland and hair follicle, distinct expression patterns of Hh-family members exists. Our data further demonstrate that, at early stages, cyclin D1 expression in the hair follicle is regulated by distinct genetic pathways. Specifically, in mesenchymal cells destined to become the dermal papilla, cyclin D1 expression occurs in an Hh-independent manner. However, maintenance of cyclin D1 in specific epithelial cells of hair follicles after stage 2 of development requires Hh activity.

Discussion

Despite the obvious differences in the structure and function between hair follicles and mammary glands, embryonically their development is very similar (Gat et al., 1998; Hudson et al., 1998; Satokata et al., 2000; van Genderen et al., 1994; Wysolmerski et al., 1994, 1995). Both hair follicles and mammary glands are dependent on common developmental processes, beginning with the elongation of localized epidermal cells, proceeded by condensation of the underlying mesenchyme, epithelial bud formation, and progressive dermal downgrowth. Interestingly, mammary glands in the lowest order of Mammals, marsupials and monotremes, are derived from a primary hair placode, thus implying an evolutionary relationship between these two epidermal appendages (Raynaud, 1961). In fact, in adult monotremes, a stiff hair protrudes from the mammary apparatus and recapitulates the function of the nipple since milk is delivered to the neonate along the hair shaft.

One pathway critical for hair follicle development, but as

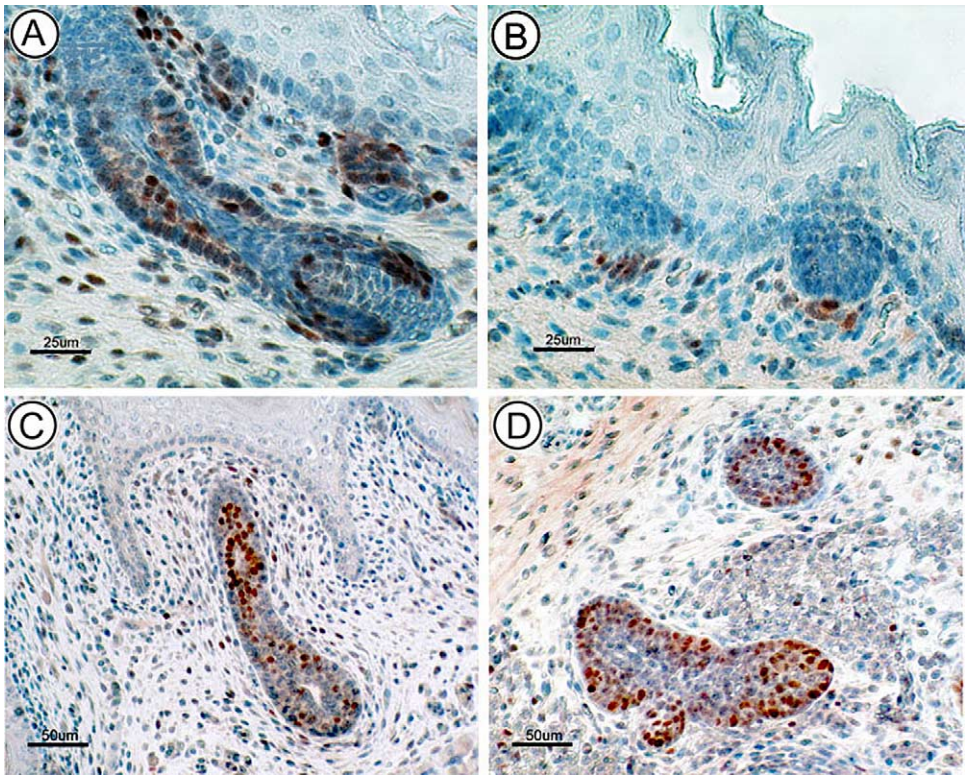

Fig. 8. Cyclin D1 expression in the mammary and hair rudiment at E18.5. Immunohistochemical analysis of cyclin D1 expression in hair follicles (A–B) and mammary rudiments (C–D) of wild-type (A and C) and $Shh^{-/-}$ (B and D) E18.5 littermates. In mammary glands, robust cyclin D1 expression is evident in epithelial cells of the rudiments, luminal spaces also being evident at this stage. In hair follicles of wild-type embryos (A), cyclin D1 is strongly expressed in matrix cells as well as in specific epithelial cells further up the developing hair shaft. In contrast, cyclin D1 remains restricted to the condensed mesenchymal cells of arrested hair follicles in $Shh^{-/-}$ littermates (B). Bars, 25 μm in (A–B). Bars, 50 μm in (C–D).

yet not studied in the context of embryonic mammary gland development, is the Hh-signaling cascade. Loss of *Shh* results in arrested folliculogenesis at rudimentary stages (St-Jacques et al., 1998). In the postnatal mammary gland, all three *Hh*-family members have been detected by RT-PCR, although only *Ihh* was detected by in situ analysis (Lewis et al., 1999b). Based on these findings, it was inferred that Ihh may be the only relevant Hh-family member during mammary development. However, the essential role that Shh signaling plays during embryonic hair follicle development suggested to us that this Hh-family member may also be required for normal mammary development during embryogenesis. Indeed, recent mammary transplantation studies suggest that loss of either *Ihh* or *Shh* individually is not sufficient to impair postnatal mammary gland development (Gallego et al., 2002). In our study, we determined that both *Shh* and *Ihh* are expressed in epithelial cells of the embryonic mammary rudiment.

Expression of *Shh* in the embryonic mammary gland and hair follicle is consistent with their common embryonic origin (ventral ectoderm). However, the expression of *Ihh* in the mammary rudiment is the first indication that embryonic mammary gland and hair follicle development have divergent requirements for Hh signaling; *Ihh* is not detected in the developing hair follicle. Other than coexpression of two Hh-family members, other components of the Hh-signaling pathway appear to be expressed in analogous domains of the mammary placode and hair follicle. *Ptc1*, for example, is detected within both epithelial and mesenchymal compartments of the embryonic mammary and hair follicle rudiments. The expression of *Ptc1* in both compartments suggests further that, during embryonic mammary morphogenesis, Hh signaling may participate in intraepithelial as well as epithelial–mesenchymal interactions. Our preliminary in situ analysis revealed that, at E16.5, *Gli1* is expressed exclusively in the contiguous mammary mesenchyme suggesting there may be differential requirements for Gli activity between the mammary epithelium and mesenchyme (K.M., unpublished observation).

Embryonic mammary gland development is not dependent on Shh signaling

Mammary gland morphogenesis in *Shh*-deficient mice proceeds unperturbed at all embryonic stages of development, including placode formation, elongation, and during branching morphogenesis. The normal histoarchitecture at all stages analyzed was complemented by normal epithelial differentiation, as determined by K5 and K14 expression. We suspected that since *Ihh* is also expressed in the mam-

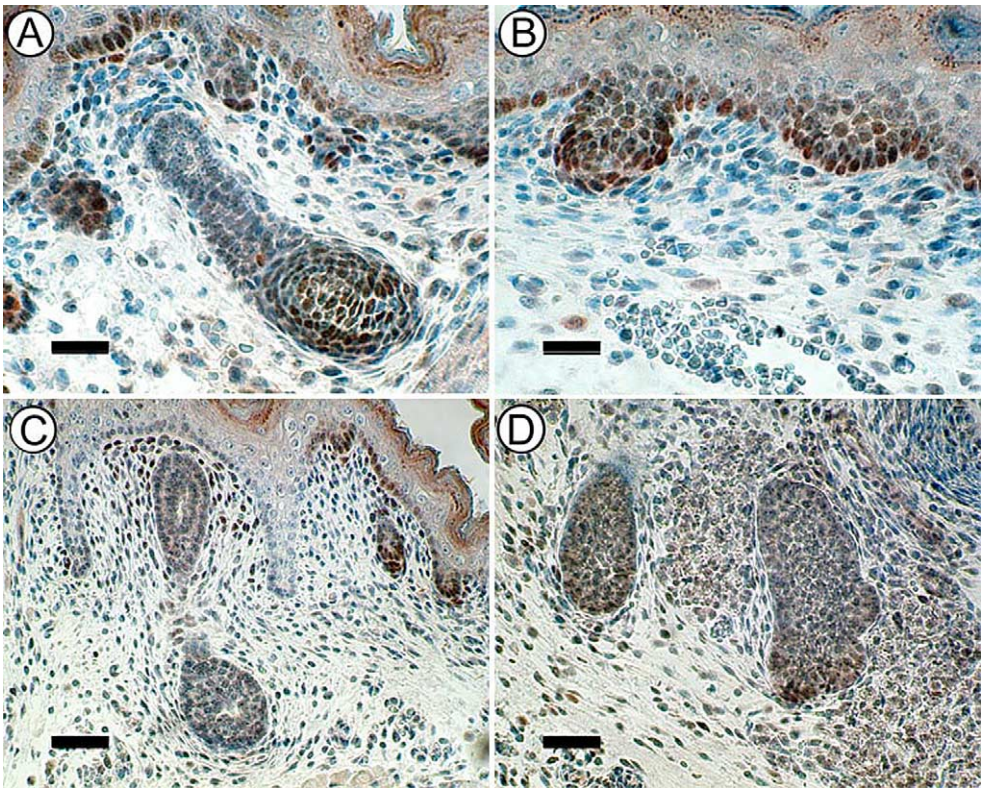

Fig. 9. Lef-1 expression in the mammary rudiment and hair follicle at E18.5. Immunohistochemical analysis of Lef-1 expression in hair follicles (A–B) and mammary rudiments (C–D) of wild-type (A and C) and *Shh*$^{-/-}$ (B and D) E18.5 littermates. In mammary rudiments, Lef-1 expression is evident in the cytoplasmic compartment of epithelial cells and in the nucleus of cells in the surrounding stroma in wild-type and *Shh*$^{-/-}$ littermates. In the case of hair follicles, Lef-1 is expressed exclusively in the nucleus in both epithelial cells and mesenchymal cells in wild-type and *Shh*$^{-/-}$ embryos. Bars, 25 μm in (A–B). Bars, 50 μm in (C–D).

mary rudiment, its activity may be sufficient to compensate for the loss of *Shh*. Indeed, the Hh-signaling target, *Ptc1,* is detected in the mammary apparatus of the *Shh*$^{-/-}$ embryos.

Cyclin D1 expression in hair and mammary rudiments of Shh mutants

Our analysis of cyclin D1 expression revealed that the expression of cyclin D1 is maintained at all stages of embryonic mammary gland morphogenesis in wild-type and *Shh*$^{-/-}$ embryos. However, cyclin D1 expression is confined primarily to the condensed mesenchymal cells adjacent to the polarized epithelial cells of the presumptive hair follicle in *Shh*$^{-/-}$ embryos. It should be noted, however, that despite the expression of cyclin D1 in these mesenchymal cells, these cells do not appear to be cycling, as has recently been demonstrated (Mill et al., 2003). The expression pattern for cyclin D1 in *Shh*$^{-/-}$ embryos is distinct from hair follicles which have developed past stage 2 in wild-type littermates. After this stage, cyclin D1 expression switches from the mesenchymal cells destined to become the dermal papilla to epithelial cells adjacent to these mesenchymal cells. Thus, the expression of cyclin D1 in the condensed mesenchyme of arrested hair follicles in *Shh*-deficient embryos is consistent with the notion that development is blocked at an early stage (stage 2) in the absence of *Shh*.

These data suggest further that the expression of cyclin D1 in the hair follicle is regulated in both a Hh-dependent and Hh-independent manner. Specifically, robust cyclin D1 expression in the condensed mesenchymal cells in *Shh*$^{-/-}$ embryos indicates that its expression is Shh-independent in these cells. The very weak, baseline level of *Ptc1* expression in these same cells (see also Mill et al., 2003) further argues against induction by a different Hh-family member. In wild-type mice, cyclin D1 expression switches to the epithelial compartment as the hair follicle develops, whereas it fails to do so in *Shh*-deficient embryos. Thus, at later stages of hair follicle development, maintenance of cyclin D1 expression in the epithelial cells is Shh-dependent. Cyclin D1 is a direct target of Lef-1 activity (Shtutman et al., 1999). We hypothesize that the Hh-dependent expression of cyclin D1 may occur indirectly through establishment of a reciprocal signaling cascade with Wnt-family members. We are currently investigating the genetic relationship between the Hh and Wnt-signaling pathways in the induction of cyclin D1 expression during embryogenesis.

Thus, our data provide the first demonstration of over-

lapping expression of *Ihh* and *Shh* in the embryonic mammary gland. Unlike the hair follicle in which *Shh* is exclusively expressed, mammary gland development proceeds normally in mice deficient for *Shh* likely through the compensatory function of *Ihh*. We are currently determining whether blocking all Hh-signaling simultaneously in the embryonic mammary gland prevents the development of this tissue.

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