Gli3-mediated repression of Hedgehog targets is required for normal mammary development

Sarah J. Hatsell1,2 and Pamela Cowin1,2,*

The Hedgehog pathway is vital for the development of many epidermal appendages, but its role in mammary development has been unclear. Here, we show that although Gli2 and Gli3 are expressed during embryonic mammary development, transcriptional reporters of positive Hedgehog signaling are absent. Nevertheless, Gli3 mutant embryos show aberrant early mammary marker expression and lack two pairs of mammary buds, demonstrating that Gli3 is essential for mammary bud formation and preceding patterning events. Misactivation of the Hedgehog pathway by targeted expression of the constitutive activator Gli1, from the Gli2 promoter in Gli3 heterozygous mice, also induces mammary bud loss. Moreover, loss of Gli3 expression induces Gli1 misexpression in mammary mesenchyme. These results establish that the essential function of Gli3 during embryonic mammary development is to repress Hedgehog/Gli1-inducible targets. During postnatal mammary development, Gli2 and Gli3 are expressed in stromal and myoepithelial cells, and Gli3 is also found within the luminal epithelium. Again, transcriptional reporters of positive Hedgehog signaling are absent from these cell types, yet are expressed robustly within mammary lymphatics. Thus, positive Hedgehog signaling is absent throughout mammary development, distinguishing the mammary gland from other epidermal appendages, such as hair follicles, which require Hedgehog pathway activity.

KEY WORDS: Mammary, Breast, Gli, Hedgehog, Wnt

INTRODUCTION

The Hedgehog (Hh) family of secreted morphogens controls the patterning, growth, morphogenesis and homeostasis of many tissues, including vertebrate and invertebrate epidermal appendages (Ingham and McMahon, 2001). The Hh pathway was elucidated and is best understood in the development of cuticular denticles belts of the fruitfly, Drosophila melanogaster (Hammerschmidt et al., 1997). Ptc association relieves the seven-pass transmembrane receptor, on neighboring cells. Hh-Ptc association relieves the seven-pass transmembrane protein, Smoothened (Smo), from Ptc-mediated repression. Smo then promotes phosphorylation of Cubitus interruptus (Ci), a microtubule-bound transcription factor, inhibiting its proteolysis into a transcriptional repressor, CiR, and converting it into a full-length transcriptional activator, CiA (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998). Thus, the Hh morphogen gradient is translated into position-specific gene expression by modulating the CiA:CiR ratio (Aza-Blanc and Kornberg, 1999).

In mammals, the Hh pathway is far more complicated. Mammals express three Hh genes [sonic (Shh), Indian (Ihh) and desert (Dhh) hedgehog] and two patched genes (Ptc1 and Ptc2) (Echelard et al., 1993; Goodrich et al., 1997; Lewis et al., 1999a; Motoyama et al., 1998; Pathi et al., 2001; Pearse et al., 2001). Moreover, the transcriptional activator and repressor roles of Ci have been subdivided in complex ways among three homologues: Gli1, Gli2 and Gli3 (Hui et al., 1994). Gli2 is expressed in the absence of Hh signals and, in this situation, is either inactive or functions as a weak transcriptional repressor (Aza-Blanc et al., 2000; Bai and Joyner, 2001; Sasaki et al., 1999; Sheng et al., 2002). Hh signals activate Gli2 initiating transcription of Hh target genes, including Ptc1 and Gli1 (Bai et al., 2002). Gli1 is strictly dependent on Hh signals for its expression and thus is an excellent reporter of positive Hh signaling (Bai et al., 2002; Bai et al., 2004). It lacks a transcriptional repressor domain and is a strong activator of Hh target genes, including itself. It can effectively substitute for Gli2 and antagonize Gli3, yet it appears to be dispensable for Hh signaling as demonstrated by the normal phenotype of Gli1−/− mice (Bai et al., 2002; Bai and Joyner, 2001; Dai et al., 1999; Hynes et al., 1997; Lee et al., 1997; Park et al., 2000). Gli3 can be expressed in the absence of Hh signals. However, Shh signaling suppresses both Gli3 transcription and the N-terminal proteolytic processing that produces the Gli3 repressor (Gli3R) (Aza-Blanc et al., 2000; Li et al., 2004; Marigo et al., 1996; Wang et al., 2000). Gli3 can function as a transcriptional activator or repressor of Gli1 and other target genes depending upon the cell context (Bai et al., 2004; Wang et al., 2000). In the simplest model, Gli2 acts at the top of the pathway to induce expression of the amplifier Gli1, which antagonizes the repressor activity of Gli3. However, cell context-specific roles of Gli2 and Gli3 mean that activator and repressor functions cannot be assumed for these proteins but must be determined empirically.

The Shh pathway plays a central role in the formation of many vertebrate epidermal appendages (sweat, sebaceous, lachrymal and salivary glands, hair, whiskers, feathers, scales, teeth and nails) that arise as a result of epithelial-mesenchymal interactions (Chuang et al., 2000; Cobourne and Sharpe, 2005; Dassule et al., 2000; Gallego et al., 2002; Michno et al., 2003; Pispas and Thesleff, 2003; Ting-Bereth and Chuong, 1996). The requirement for Shh during embryonic and adult hair follicle development and downward growth has been particularly well documented (Chiang et al., 1999; Mill et al., 2003; St-Jacques et al., 1998). Hair follicles and mammary glands co-evolved and share many local inductive pathways (Wnt, Fgf, Bmp and Pthlh) (Andl et al., 2002; Chu et al., 2004; Hens and Wyssolmerski, 2005; Mailleux et al., 2002; Oftedal, 2002; Wyssolmerski, 2002; Wyssolmerski et al., 1998). Many similarities exist between the cyclical development of the mammary

1Department of Cell Biology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA. 2Department of Dermatology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA.
*Author for correspondence (e-mail: cowinp01@med.nyu.edu)

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gland during pregnancy, lactation and involution, and the rounds of hair follicle growth (anagen), regression (catagen) and resting (telogen). Mammary glands first form around embryonic day 10 (E10.5) as bilateral ectodermal thickenings between the fore and hindlimbs, known as milk lines (Velmaat et al., 2003). At about E11, the lines fragment and cells coalesce into five pairs of mammary placodes that, within 1 day, form elevated mammary buds. Between E13-E14 the mammary buds invaginate, forming a bulb below the surface of the epithelium. This structure induces the underlying stroma to condense and differentiate into mammary mesenchyme. In males, fetal androgens stimulate mammary mesenchymal fibroblasts to constrict around the epidermal buds, choking further development (Dunbar et al., 1999). In females, epithelial buds elongate at E16, forming the mammary sprout, which penetrates the underlying fat pad precursor and branches to form 5-6 primary ductules by E18. Further development does not occur until puberty, when estrogen and growth hormone induce stromal IGF secretion, stimulating proliferation of cells at the tips of each duct within terminal end buds, leading to ductal elongation (Marshman and Streuli, 2002). During each cycle of pregnancy, progesterone and prolactin stimulate several local paracrine pathways that promote extensive ductal side-branching and alveologenesis (Brisken et al., 2000; Brisken et al., 1998; Henninghausen and Robinson, 1998; Robinson et al., 2000). At the end of lactation, the mammary gland involutes by a sequential process involving epithelial apoptosis, extensive matrix remodeling and a wave of adipogenesis, which replenishes the mammary fat pad (Lund et al., 1996).

Both Shh and Ihh mRNA have been detected within embryonic mammary bud epithelium by in situ hybridization, but elimination of either gene has no effect on bud development (Gallego et al., 2002; Michno et al., 2003). Thus, the function of Hh signaling within the mammary gland is obscure, and questions remain as to whether signaling by Shh and Ihh is redundant or dispensable for embryonic mammary gland development. To explore the role of the Hh pathway further, we determined the expression of the three downstream transcription factors, Gli1, Gli2 and Gli3, and examined the effects of altering the Gli activator/repressor ratio during mammary development. Our results demonstrate that, contrary to previous suggestion, and, in contrast to other epidermal appendages, positive Hh signaling is absent throughout mammary development. Furthermore, we show that Gli3-mediated transcriptional repression is essential for the formation of two pairs of mammary buds, and misactivation of the Hh pathway, by targeted expression of Gli1, induces bud loss.

MATERIALS AND METHODS

Mice

The following mice, maintained on an outbred background, were a kind gift from Dr Alexandra Joyner, Skirball Institute, NYU School of Medicine. Gli1lzki/+ mice were constructed as described (Bai et al., 2002). Mice carrying Gli1 or lacZ knocked into the Gli2 locus (Gli21zki/ and Gli22zki/) were constructed as described (Bai and Joyner, 2001). Gli2a+/+ PtcF5/4 and TOP-Gal mice were obtained from Jackson Laboratories (Bar Harbor, ME). Ptc1zki/+ mice were as described (Goodrich et al., 1997).

Whole-mount analysis

For detection of lacZ expression, mammary glands or embryos were fixed in 4% paraformaldehyde (PFA) diluted in phosphate-buffered saline (PBS) for 1 hour, followed by three 1-hour washes in rinse buffer (2 mM MgCl₂, 0.1% sodium deoxycholate, 0.2% NP-40 in PBS). X-gal staining was carried out overnight in staining buffer (50 μg/ml X-gal in rinse buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide). Mammary glands were washed in PBS, post-fixed for 1 hour in 4% PFA, dehydrated through a graded series of ethanol, cleared for ~30 minutes in Citrisolve (Fisher Scientific, Pittsburgh, PA) and mounted in Securemount (Fisher Scientific) and viewed under a Leica dissecting microscope (Bannockburn, IL).

Histology

For histological analysis, mammary glands and embryos were stained as above with X-gal then post-fixed with 4% PFA overnight at 4°C. They were embedded in paraffin and sectioned and in some cases processed further for immunohistochemistry as described below.

Immunohistochemistry

Sections (4 μm) were deparaffinized with xylene and rehydrated through a graded series of ethanol. Citric acid antigen retrieval was performed for all antibodies by placing slides in 1.92 g/l of sodium citrate (pH 6.0) and microwaving for 20 minutes. Rabbit anti-Keratin 14 (K14) (Covance, Berkeley, CA) (1:400) primary antibody was detected by using the DAKO EnVision Kit comprising horse-radish peroxidase (HRP) coupled anti-rabbit IgG followed by diaminobenzidine (DAB) following the manufacturer’s protocol (DAKO, Carpinteria, CA). Mouse anti-p63 (Neomarkers, Freemont, CA) (1:500) was detected using biotin labeled goat anti-mouse IgG (Vector Labs, Burlingame, CA) and rabbit anti-Gli3 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100) was detected using biotin labeled goat anti-rabbit IgG (Vector Labs) (1:1000) followed by streptavidin-HRP, which was detected using DAB.

Whole-mount in situ hybridization

Embryos were fixed overnight in 4% PFA diluted in PBS, dehydrated in methanol and stored at ~20°C. Before hybridization embryos were rehydrated, bleached by incubating for 30 minutes in 6% H₂O₂, treated with 6 μg/ml proteinase K for 10 minutes, washed in 2 mg/ml glycine, then fixed in 4% PFA for 20 minutes. All solutions were made up in PBS-T (PBS, 1% Tween-20) and three 5 minute PBS-T washes followed each step. Embryos were prehybridized for 2-3 hours in 50% formamide 5× SSC, 50 μg/ml tRNA, 1% SDS, 50 μg/ml heparin then hybridized overnight at 70°C in the same buffer containing 2 μg/ml of digoxigenin (DIG)-labeled Gli3 probe.

Following several washes, DIG was detected by overnight incubation at 4°C in alkaline phosphatase (AP) labeled anti-DIG Fab’ fragments (Roche Indianapolis IN). Color was developed with BM-purple AP substrate (Roche). Further protocol details are available at http://saturn.med.nyu.edu/research/dg/joynerlab/protocols.html

Section in situ hybridization

Sections were dewaxed in xylene and rehydrated, fixed with 4% PFA, treated with 1 μg/ml proteinase K for 15 minutes at 37°C, post-fixed in 4% PFA and dehydrated. Sections were hybridized overnight at 55°C in 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 300 mM NaCl, 0.02 M Tris-HCl pH 8.0, 5 mM EDTA, 0.01% sarkosyl, 250 μg/ml yeast tRNA containing 1 μg/ml DIG labeled Gli3 probe. DIG was detected as above.

Northern analysis

Total RNA was isolated from mammary gland using the ToTALLY RNA kit (Ambion, Austin, Texas) (Imbert et al., 2001). mRNA was purified from 30 μg of total RNA using the Poly(A)Pure kit (Ambion). Northern analysis was carried out on these mRNA samples using the NorthernMax-Gly kit (Ambion). The Gli3 cDNA probe was obtained from Dr Alexandra Joyner and the K18 cDNA probe was obtained from Caroline Alexander (University of Wisconsin, Madison, WI).

RESULTS

Gli2-lacZ is expressed in the dermal mesenchyme and the basal epithelium and surrounding condensed stroma of embryonic mammary buds

Gli2 is converted into a transcriptional activator by Hh signals and functions as the primary Hh transducer in many tissues (Aza-Blanc et al., 2000; Bai et al., 2002; Sasaki et al., 1999; Sheng et al., 2002). To determine the activity of the Gli2 promoter during embryonic mammary development, we examined expression of a lacZ reporter knocked into the Gli2 locus (Gli21zki/) (Bai and Joyner, 2001). In the
region of the developing mammary line. E11 Gli2lacZ embryo whole mounts and sections showed mesenchymal lacZ expression in a diffuse arc between the fore and hindlimbs and in the underlying somites (Fig. 1A). By E14, strong expression was observed within all five pairs of mammary buds as well as in hair follicles and whisker pads (Fig. 1B). Cross-sections through Gli2-lacZ embryos stained with X-gal. Staining is seen within the basal epithelial layer (epi) and surrounding stroma (str) of E14.5 mammary buds (D) and E16.5 mammary sprout (E). Epithelial Gli2-lacZ expression is restricted to a basal subset of p63- (F) and K14- (G) positive cells.

Gli3 is expressed in embryonic mammary bud epithelium and mesenchyme
Gli3 is a strong transcriptional repressor of Hh target genes during lung and limb development but is able to weakly activate Hh target genes during ventral spinal cord patterning (Bai et al., 2004). A reporter allele of Gli3 has not been described. Therefore, we examined expression of Gli3 mRNA by in situ hybridization. Gli3 mRNA was detected in E11 embryo whole mounts in a diffuse arc between the fore and hind limb and the underlying somites, a pattern similar to that observed for Gli2-lacZ (Fig. 2A). This pattern is consistent with recent reports of somitic and weaker dermal mesenchymal Gli3 mRNA expression (Veltmaat et al., 2006). E13.5 embryo wholemounts showed weak Gli3 expression in the vicinity of all mammary buds with stronger expression in pair number 3 (Fig. 2B). Gli3 protein was localized by immunohistochemistry within the nuclei of mammary bud epithelial cells and surrounding stromal cells (Fig. 2C,D), in a similar expression pattern to that of Gli2-lacZ.

Lack of Hh target gene expression distinguishes mammary buds from other embryonic epidermal appendages
Gli1 is a direct transcriptional target of positive Hh signaling, and its expression is strictly dependent on Hh signals transduced by either Gli2 or Gli3 activators. Thus, reporters of Gli1 promoter activity provide reliable indicators of positive Hh pathway activation (Bai et al., 2002; Bai et al., 2004). Gli1-lacZ expression in Gli1lzki/+ mice was absent throughout embryonic mammary development yet was prominent in embryonic hair follicles and whisker pads (Fig. 3A,B). The absence of Gli1-lacZ reporter expression in mammary buds and robust activity in hair follicles strongly suggests that Hh pathway activation and Gli-dependent gene expression diverges among distinct types of epidermal appendages. To test this hypothesis further, we examined the activity of a second Hh target gene, Ptc1 (Goodrich et al., 1997). Hh signals upregulate Ptc1 expression as part of a negative feedback mechanism. Although Ptc1 provides a receptor for Hh proteins, at high levels it suppresses Hh activity by sequestering Smo (Casali and Struhl, 2004). Ptc1-lacZ expression
was observed in hair follicles but was absent throughout embryonic mammary development (Fig. 3C,D). These results confirm that the Hh pathway is active in epidermal appendages, such as hair follicles, but is either inactive or repressed throughout embryonic mammary development.

**Postnatal mammary Gli2-lacZ expression is observed continuously in stromal cells and cyclically in myoepithelial cells**

At birth Gli2-lacZ expression was found in both basal epithelial and stromal layers of the mammary gland (Fig. 4A,B). At the onset of puberty, it was lost from the epithelial cells but was expressed in spikes along the entire ductal system (Fig. 4C,E). Gli2-lacZ was also prominently expressed in mammary lymphatics (Fig. 4E, asterisk). Histological sections showed that Gli2-lacZ periductal spikes comprise groups of tightly adherent stromal cells that triangulate between adipocytes and the myoepithelium (Fig. 4D,F). Immunohistochemical analysis confirmed that in virgin (Fig. 4G,H) and early pregnant (P8) mice all Gli2-lacZ-positive cells lay beneath the K14/p63-positive myoepithelial layer and thus were stromal. P14 glands, however, showed additional myoepithelial expression surrounding alveoli as revealed by Gli2-lacZ colocalization with K14 and p63 (Fig. 5A-D). This Gli2-lacZ-positive myoepithelial population persisted during lactation but was lost during involution (data not shown).

**Postnatal Gli3 is expressed in mammary ducts and alveoli and surrounding stroma**

Northern analysis detected Gli3 mRNA expression in mammary glands from virgin and pregnant mice (Fig. 6A). In situ hybridization showed diffuse Gli3 mRNA expression throughout the mammary gland, with stronger expression in mammary ducts and alveolar clusters (Fig. 6B,D). Immunohistochemistry confirmed prominent nuclear expression of Gli3 within lumenal and myoepithelial cell types (Fig. 6F,G) and also detected Gli3 expression in stromal cells and adipocytes (Fig. 6E,G, arrows). Thus, Gli2 and Gli3 expression overlap in stromal and myoepithelial compartments, but only Gli3 is expressed in luminal epithelial cells, suggesting that regulation of the Hh pathway differs in these distinct cell types.

**Postnatal mammary expression of Hh target genes, Gli1-lacZ and Ptc1-lacZ, is restricted to lymphatics**

In contrast to the pattern of Gli2-lacZ and Gli3 expression, mammary glands from Gli1lacZ/+/ mice showed no Gli1-lacZ reporter expression within epithelial, stromal, myoepithelial or adipocyte cell-types at any stage of development. Yet, in all cases a subset of mammary vessels stained intensely (Fig. 7A,B). Ptc1-lacZ was also expressed exclusively within the same subset of mammary vessels (data not shown). Many of these vessels had a large diameter and extended from the periphery of the fatpad towards the lymph node. Inspection at higher magnification showed that Gli1-lacZ and Ptc1-lacZ were expressed only in vessels lacking red blood cells (Fig. 7B). Similar
vessels were observed in many other tissues, including the surface of the heart and omentum (Fig. 7C, D). Gli1-lacZ and Ptc1-lacZ-positive vessels stained with anti-LYVE, a specific marker of lymphatics (data not shown). The absence of Hh target gene expression within the adult mammary tree again shows that positive Hh signaling is absent or repressed, challenging previous reports that suggest positive Hh signaling is active in postnatal mammary gland development (Lewis et al., 2001; Lewis et al., 1999b).

Gli3xt/xt mice show aberrant placode development and lack two pairs of mammary buds

After establishing the expression patterns of the three Gli genes, we tested the consequences of removing their function on embryonic mammary development. In keeping with its lack of expression within the mammary tree, loss of Gli1 (Gli1lzki/lzki) had no effect on mammary development: all ten mammary glands were present in newborn Gli1lzki/lzki mice and showed normal function, exemplified by the ability of adults to raise normal size litters. Mice lacking Gli2 (Gli2lzki/lzki) die perinatally but form all ten buds at E13, and no obvious mammary phenotype is observed in histological sections (Table 1). The spontaneously occurring extra-toes4 mouse mutant (Gli3xt/xt) carries an intragenic deletion in Gli3 resulting in loss of Gli3 expression (Hui and Joyner, 1993). Gli3xt/xt mice showed normal embryonic development of all ten mammary glands and, as adults, could successfully feed their litters (Table 1). By contrast, all Gli3xt/xt embryo whole-mounts lacked bud pair number 5 and the majority lacked bud number 3. A small percentage showed reduction and/or misplacement of bud number 3 (Fig. 8B, D; Table 1). To analyze which stage of mammary bud formation is affected by loss of Gli3 function, we examined the embryos of Gli3xt/+ mice crossed to Gli3xt/xt;TOP-Gal mice harboring the TOP-Gal transgenic reporter of Lef/Tcf transcriptional activity (DasGupta and Fuchs, 1999). TOP-Gal is expressed during the earliest stages of mammary line formation and later in the mammary placodes and buds (Chu et al., 2004). At E11 TOP-Gal expression was seen in the developing placode pairs number 3 and number 4 in all wild-type and Gli3xt/+ embryos (Fig. 8A). By contrast, 80% of Gli3xt/+ embryos lacked TOP-Gal expression from the placode 3 region of the mammary line (Fig. 8B; Table 1). Fourteen percent of embryos showed normal marker expression on one side and absence of marker expression on the contralateral side. Similar results were observed by in situ

Table 1. Embryonic mammary phenotype of Gli2 and Gli3 mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number buds analyzed</th>
<th>Number of buds with phenotype</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>90</td>
<td>All normal</td>
</tr>
<tr>
<td>Gli2lzki/lzki</td>
<td>14</td>
<td>All normal</td>
</tr>
<tr>
<td>Gli3xt/xt</td>
<td>80</td>
<td>Fifty-eight missing bud 3, 13 hypoplastic/misplaced bud 3, 80 missing bud 5</td>
</tr>
<tr>
<td>Gli3as/Gli3as</td>
<td>6</td>
<td>Seven missing bud 3, one hypoplastic bud 3, eight missing bud 5</td>
</tr>
<tr>
<td>Gli3xt/xt;TOP-Gal</td>
<td>18</td>
<td>All normal</td>
</tr>
<tr>
<td>Gli3xt/xt;TOP-Gal</td>
<td>16</td>
<td>All normal</td>
</tr>
<tr>
<td>Gli3xt/xt;Gli3xt/xt</td>
<td>14</td>
<td>Thirteen missing bud 3, one hypoplastic bud 3, 12 missing bud 5, two hypoplastic bud 5</td>
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hybridization for Wnt10b, another early marker of the mammary line and placodes (Veltmaat et al., 2004) (data not shown). These data demonstrate that early patterning of the mammary line and placode formation is compromised in Gli3\textsuperscript{xt/xt} embryos. By E12.5, TOP-Gal was expressed in all five pairs of mammary buds in wild-type and Gli3\textsuperscript{xt/xt} embryos (Table 1; Fig. 8C). TOP-Gal staining in Gli3\textsuperscript{xt/xt} embryos revealed that bud pair number 5 was always missing and bud number 3 was absent 83% of the time or was small and/or misplaced in the remaining 17% (Fig. 8D; Table 1). Thus, Gli3 function is essential for the normal complement and positioning of mammary buds. A similar percentage of absence of bud pairs number 3 and 5 was observed in E14.5 Gli3\textsuperscript{xt/xt} embryos. Histological sections of Gli3\textsuperscript{xt/xt} embryos showed that the remaining bud pairs are normal at E14.5 (Fig. 2D).

**Misactivation of Hh signaling results in selective mammary bud loss**

In several developmental processes, Gli3 is a strong transcriptional repressor of Hh target genes in the absence of Hh but has recently been shown to activate Hh target genes weakly in ventral spinal cord (Bai et al., 2004). To investigate whether the repressor or activator function of Gli3 is essential for formation of mammary bud pairs number 3 and number 5, we examined a series of double mutant mice (Table 1). Gli2 requires positive hedgehog signaling for activation and, in the absence of signaling, Gli2 is present in an inactive or weakly repressive state. Gli1, however, lacks a repressor domain and is a strong amplifier of the pathway (Dai et al., 1999; Park et al., 2000). Thus, driving expression of the constitutive Gli1 activator under the control of the Gli2 promoter (Gli2\textsuperscript{1nki/+} and Gli2\textsuperscript{1nki/1nki}) tests the effect of progressively increasing the activator to repressor ratio within the Gli2 field of expression (embryonic mammary bud and mesenchyme). In similar experiments, substitution of Gli2 by Gli1 has been shown to exacerbate the Gli3\textsuperscript{xt/xt} limb phenotype (Bai and Joyner, 2001). In the E13.5 mammary gland, substituting one or even two copies of Gli2 with Gli1 (Gli2\textsuperscript{1nki/+} and Gli2\textsuperscript{1nki/1nki}) had no effect on mammary development (Fig. 8E-G; Table 1). Likewise, replacing a single copy of Gli2 by Gli1 and at the same time lowering Gli3 expression (Gli2\textsuperscript{1nki/+};Gli3\textsuperscript{1nki/1nki}) failed to produce a mammary phenotype. However, when both Gli2 copies were replaced by Gli1, and Gli3 was simultaneously reduced (Gli2\textsuperscript{1nki/1nki};Gli3\textsuperscript{1nki/1nki}), mammary bud pairs number 3 and number 5 were lost in the majority of embryos (Fig. 8H-J; Table 1). Thus, in the mammary gland, two copies of the Gli1 activator expressed from the Gli2 promoter are sufficient to antagonize Gli3 repressor expressed from a single copy of Gli3. This establishes that the function of Gli3, within the embryonic mammary gland, is to repress Hh target genes. Our results further demonstrate that the Gli activator/repressor ratio of hedgehog signaling is crucial for correct mammary gland patterning and that buds 3 and 5 are particularly susceptible to changes in this ratio.

**Analysis of Gli2 function in double mutants**

The lack of phenotype in Gli2\textsuperscript{1nki/1nki} mice suggests that Gli2 is either inactive, functions redundantly with other mammary Gli proteins (Gli3) or is completely antagonized by Gli3. We used double

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**Fig. 8. Mammary bud formation is impaired in Gli3\textsuperscript{xt/xt} mice and after mis-activation of the hedgehog pathway.** TOP-Gal expression is seen in the developing mammary placodes number 3 and number 4 in wild-type E11 embryos (arrows) (A). TOP-Gal expression is absent from the region of bud 3 in E11 Gli3\textsuperscript{xt/xt} embryos (white arrow) but is present in the developing bud 4 (black arrow) (B). In E12.5 wild-type embryos, TOP-Gal expression reveals the development of five pairs of mammary buds (C, arrows), whereas Gli3\textsuperscript{xt/xt} embryos lack bud pairs 3 and 5 (D, predicted position of missing buds is marked by white arrows). E13.5 Gli2\textsuperscript{1nki/1nki} embryos (E) show normal development of five pairs of mammary bud, but Gli2\textsuperscript{1nki/1nki};Gli3\textsuperscript{1nki/1nki} embryos (H) show loss of buds 3 and 5 (white arrows). Higher magnification of the thoracic buds (F) and inguinal buds (G, J) is seen in Gli3\textsuperscript{xt/xt} embryos. A hypoplastic bud 3 (arrowhead) is seen in some Gli2\textsuperscript{1nki/1nki};Gli3\textsuperscript{1nki/1nki} embryos (K). Misactivation of Gli1-lacZ is seen in the stroma (str) surrounding buds 1 and 4 (arrow) in Gli3\textsuperscript{xt/xt} embryo wholemounts (L, M) and sections (N).
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mammary buds seen in one another, then Gli2 reduction or loss could rescue the loss of bud number 3 was seen in one out of eight mammary development. The presence of positive Hh activity remain silent throughout mammary development; mice lacking expression of Hh activators, Gli1 or Gli2, of Hh signaling in mammary development. These studies detected reporter mice to Gli3xt/+ embryos and examined the effect of loss of Gli3 expression on the Gli1-lacZ reporter. At E14.5 Gli1-lacZ was detected in the stroma surrounding bud pairs 1 and 4 of Gli3wt/+ embryos, n=4 (Fig. 8L-N) but not wild-type embryos (Fig. 3A,B). As Gli1 expression is dependent on Gli2-mediated transcriptional activation, we conclude that Gli2 is present in an activator form but is fully antagonized by Gli3R in normal embryos. These experiments further demonstrate that, in addition to affecting early patterning events, which govern placode formation, Gli3R continues to repress Hh target genes within the stroma surrounding mammary buds after their formation.

DISCUSSION

Our key findings are that Gli3-mediated repression is essential for the normal complement of mammary buds and that contrary to previous suggestion Hh target gene expression is absent throughout embryonic and postnatal mammary development. Repression of Hh signaling distinguishes mammary glands from other epidermal appendages, which require Hh pathway activation. These conclusions are based on the following results: lacZ reporters of positive Hh activity remain silent throughout mammary development; mice lacking expression of Hh activators, Gli1 or Gli2, show normal mammary development; Gli3A/−/− mutants lack two pairs of mammary placodes and buds; and misactivation of the pathway by targeted expression of Gli1 to the Gli2 locus in Gli3wt/+ mice phenocopies complete loss of Gli3 function.

Positive Hh signaling is absent during embryonic mammary development

The Hh pathway is crucial for the patterning and growth of many epidermal appendages (Chuong et al., 2000). An absolute requirement for its activity has been documented in the development of hair, teeth and feathers (Hardcastle et al., 1998; St-Jacques et al., 1998; Ting-Bereth and Chuong, 1996). The fact that epidermal appendages have a common origin and share many developmental pathways has prompted several recent investigations into the role of Hh signaling in mammary development. These studies detected Shh and Ihh mRNA within mammary bud epithelium, but, nevertheless, showed that Shh−/− and Ihh−/− embryos develop ten mammary buds, which undergo normal postnatal development if transplanted into adult mice (Gallego et al., 2002; Michno et al., 2003). These data suggest that Hh signaling is either redundant or dispensable for mammary development. The presence of Ptc1 mRNA within Shh−/− mammary glands was interpreted as evidence for Hh redundancy (Michno et al., 2003). However, in comparison with the hair follicle, where the Hh pathway is unquestionably active, mammary Ptc1 mRNA levels are barely detectable. Such basal levels are consistent with absence of Hh signaling resulting in repression of Ptc1 transcription. Consistent with the viewpoint that Shh and Ihh are dispensable for mammary bud development, our results show that two well-characterized and sensitive reporters of positive Hh signaling, Gli1-lacZ and Ptc1-lacZ, are absent during embryonic mammary development. Further challenging the concept that positive Hh signaling is required for mammary development, our analyses show that Gli1 and Gli2 mutants have no obvious defects in mammary bud formation.

Gli3 acts as a repressor during embryonic mammary development

In stark contrast to the lack of mammary phenotypes in Gli1lkDlkDlk and Gli2lkDlkDlk embryos, the majority of Gli3wt/+ mutants lack mammary placodes number 3 and 5, and the remainder show hypoplastic, asymmetric or lateral displacement of placode number 3. Marker analysis indicates that Gli3 is essential for the earliest stages of embryonic mammary development, affecting the positioning and formation of mammary placodes. Gli3 is present in the absence of Hh signaling and thus can have Hh-independent functions. However, Hh signals downregulate Gli3 transcription and inhibit Gli3 proteolysis into Gli3A, a process referred to as negative Hh signaling. Although Gli3 often acts as a potent repressor of the Hh pathway, several studies have shown that in certain contexts it functions as a weak activator (Gli3A). For example, Gli3 is essential for the correct patterning of the ventral spinal cord where its activation of Gli1 is involved in the development of motoneuromuscular systems (Bai et al., 2004). Additional examples of Gli3A function are found in the development of the glandular epithelium of the embryonic stomach and skeletal muscle (Kim et al., 2005; McDermott et al., 2005). To determine whether Gli3 functions as an activator or repressor of Hh regulated genes within the embryonic mammary gland, we conducted double Gli mutant experiments. Targeted replacement of Gli2 by Gli1 within Gli3A/−/− mice resulted in loss of mammary bud pairs number 3 and number 5. The ability of the constitutive Gli1 activator to antagonize Gli3 reveals that Gli3 functions as a repressor in this developmental context. Whether Gli3A functions independently of Hh signals or is modulated by them during embryonic mammary development remains to be determined. However, the induction of the Hh target gene Gli1 in the stroma of the remaining Gli3A/−/− E14.5 mammary buds suggests that, in normal embryos, Gli3 is actively repressing Hh signaling at this stage.

Mammary placodes arise in a distinct order: number 3, number 4, number 1 + number 5, number 2. Pairs 3 and 4 form at the anterior and posterior ends of the mammary line. Pairs 1 and 5 form from independent streaks of cells that encircle the fore- and hindlimbs (Veltmaat et al., 2004). Pair 2 develops last from streaks of Wnt10b-positive cells extending from placodes 1 and 3. Wnt/catenin signaling is the earliest known marker of embryonic mammary development, and mice misexpressing the Wnt inhibitor Dkk within the epidermis fail to form any mammary placodes (Andl et al., 2002; Chu et al., 2004). Lack of expression of the Wnt signaling reporter TOP-Gal in the central region of the mammary line in E11 Gli3/−/− embryos demonstrates that Gli3 repression is required prior to these early patterning events that precede mammary placode formation. It has been proposed that cells migrate along the mammary line and peri- and limbial streaks and coalesce to form placodes (Veltmaat et al., 2004). Intriguingly, genetic interaction has been reported between Gli3 and Twist, a regulator of epithelial-mesenchymal transitions that are critical for cell migratory processes (O’Rourke et al., 2002). The loss of only two pairs of mammary buds upon misactivation of the Hh pathway in Gli3A/−/− mice reinforces previous data showing that specific combinations of molecular cues govern the formation...
of different pairs of placodes (Velthuis et al., 2003). Analysis of inbred mouse strains and, more recently, of scaramanga (Ska) mice implicates variable susceptibility of specific bud pairs to both loss and supernumerary formation (Howard et al., 2005; Little and McDonald, 1945; Velthuis et al., 2003). In these studies, correct regulation of morphogenic interactions involved in mammary line and placode formation appears to be most crucial for the formation of bud pair 3 and least crucial for that of bud pair 4. Further examples are found in studies on loss of Tbx3, which in humans produces mammary hypoplasia and nipple loss, as well as in studies on Lef1 and Fgf pathways. Mice lacking Tbx3 show loss of mammary buds but occasional retention of bud number 2 (Bamshad et al., 1997; Davenport et al., 2003; Eblaghie et al., 2004). Lef1–/– mice form small mammary placodes that degenerate and occasionally retain bud 4 (van Genderen et al., 1994). Mice that lack Fgf10, which is expressed within the ventrolateral portion of somites, or its receptor Fgfr2b, which is expressed in the mammary placodes, fail to develop mammary buds 1, 2, 3, and 5, yet retain bud number 4 (Mailleux et al., 2002). Recently, a genetic requirement for Gli3 in the ventral somitic expression of Fgf10 has been described (Velthuis et al., 2006). Our demonstration that Gli3 acts as a transcriptional repressor now shows that this Gli3-mediated induction of Fgf10 expression must be indirect.

The studies described above suggest that different placodes vary in their susceptibility to a crucial developmental threshold during the earliest stages of mammary development (Velthuis et al., 2003). Our experiments reveal that the Gli1/Gli2 ratio provides such a crucial developmental threshold for buds number 3 and number 5. Yet there are no reports of mammary bud loss in mouse models or human syndromes (basal cell nevus syndrome) where the Hh pathway is aberrantly activated (Johnson et al., 1996; Nilsson et al., 2000; Sheng et al., 2002). However, this is not surprising because, in mice, mammary bud loss occurs only under conditions that result in embryonic lethality (complete loss of Gli3 or partial loss of Gli3 in conjunction with pathway misactivation) and are likely to have the same outcome in humans. Nevertheless, there are occasional reports of rare human syndromes with features suggestive of pathway misactivation, such as polydactyly associated with hypoplastnic nipples (Teebi and Druker, 2001).

Epidermal appendages show distinct requirements and proliferative responses to Hh signaling: repression of hedgehog signaling is essential for mammary development

The results of this study show that, despite their common origin, mammary glands differ from other epidermal appendages in their requirement for Hh pathway repression rather than activation. Hh-positive activity is essential for tooth and hair follicle development (Cobourne et al., 2001; Dassule et al., 2000; Gritli-Linde et al., 2002; Johnson et al., 1996; Nilsson et al., 2000; Sheng et al., 2002). However, this is not surprising because, in mice, mammary bud loss occurs only under conditions that result in embryonic lethality (complete loss of Gli3 or partial loss of Gli3 in conjunction with pathway misactivation) and are likely to have the same outcome in humans. Nevertheless, there are occasional reports of rare human syndromes with features suggestive of pathway misactivation, such as polydactyly associated with hypoplastnic nipples (Teebi and Druker, 2001).

Hh target gene expression is absent during development of the adult mammary tree but is robust within lymphatics

In addition to defining the roles of Hh signaling in embryonic mammary development, our results provide new insight into the role of the Hh pathway during adolescent and adult mammary gland development. Gli2-lacZ expression within the stroma encasing virgin mammary ducts is entirely consistent with a previous report of Gli2 mRNA expression (Lewis et al., 2001). However, the greater sensitivity and clarity afforded by the lacZ reporter, combined with immunohistochemical marker analysis, allows us to define that Gli2 expression occurs cyclically in mid-pregnant mice within myoepithelial cells and not within all epithelial cells as reported previously (Lewis et al., 2001). Furthermore, we show that although Gli3 is co-expressed with Gli2 in stroma and myoepithelia, it is the only Gli found within the luminal epithelium of the adult gland. Lack of expression of Gli1-lacZ and Ptc1-lacZ within mammary epithelia, myoepithelial, stroma or adipocytes leads us to conclude that Hh signaling is absent or stifled within the parenchyma of the adult gland yet is robust within lymphatics. This finding is at odds with a previous report of Ptc1 mRNA expression in the epithelial layers of virgin ducts and a haploinsufficient phenotype in Ptc1+/– mice involving minor changes in terminal end bud clefting and transient ductal hyperplasia that rectifies during pregnancy (Lewis et al., 1999b). Of note, our examination of Ptc1-lacZ expression employs the same mouse, albeit on an outbred background. We have looked extensively and have seen no evidence of these phenotypic aberrations. Moreover, our observations are entirely consistent with other studies reporting lack of Gli1 expression in normal human breast tissue (Kubota et al., 2004). Intriguingly, these studies reported Gli1 protein upregulation in 52/52 epithelial breast cancers and 4/6 epithelial breast tumor cell lines probably resulting from epigenetic regulatory events, as mutations in Hh pathway components are infrequent in breast tumors (Chang-Claude et al., 2003; Kubo et al., 2004; Vorechovsky et al., 1999; Wicking et al., 1998; Xie et al., 1997). Our results show that Gli3 is the only Gli expressed in normal luminal epithelial cells. Whether Gli1 misexpression in breast tumors results from loss of Gli3 activity is an important issue for future investigation.


DEVELOPMENT


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