Developmental Biology **238,** 133–144 (2001)
doi:10.1006/dbio.2001.0410, available online at http://www.idealibrary.com on IDEAL®

The *Gli2* Transcription Factor Is Required for Normal Mouse Mammary Gland Development

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The hedgehog signal transduction network performs critical roles in mediating cell–cell interactions during embryogenesis and organogenesis. Loss-of-function or misexpression mutation of hedgehog network components can cause birth defects, skin cancer, and other tumors. The *Gli* gene family (*Gli1, Gli2,* and *Gli3*) encodes zinc finger transcription factors that act as mediators of hedgehog signal transduction. In this study, we investigate the role of *Gli2* in mammary gland development. Mammary expression of *Gli2* is developmentally regulated in a tissue compartment-specific manner. Expression is exclusively stromal during virgin stages of development but becomes both epithelial and stromal during pregnancy and lactation. The null phenotype with respect to both ductal and alveolar development was examined by transplantation rescue of embryonic mammary glands into physiologically normal host females. Glands derived from both wild type and null embryo donors showed ductal outgrowths that developed to equivalent extents in virgin hosts. However, in null transplants, ducts were frequently distended or irregularly shaped and showed a range of histological alterations similar to micropapillary ductal hyperplasias in the human breast. Alveolar development during pregnancy was not overtly affected by loss of *Gli2* function. Ductal defects were not observed when homozygous null epithelium was transplanted into a wild type stromal background, indicating that *Gli2* function is required primarily in the stroma for proper ductal development. Δ*Gli2* heterozygotes also demonstrated an elevated frequency and severity of focal ductal dysplasia relative to that of wild type littermate- and age-matched control animals.

Key Words: **breast cancer; hedgehog signal transduction; organogenesis; tissue interactions; epithelial–stromal interactions; transplantation; ductal development; alveolar development.**

INTRODUCTION

Tissue interactions between epithelial and mesenchymal cells are critical for proper development and function of many organs. In the mammary gland, development and functional differentiation depend on interactions between an ectodermally derived epithelium and associated mesodermally derived mesenchyme (embryonic) or stroma (postnatal), and between epithelial cells themselves (Brisken *et al.,* 1998; Daniel and Silberstein, 1987; Sakakura, 1987). These interactions are dynamic, reciprocal, and tightly coordinated with the reproductive status of the animal.

The mouse mammary gland is established about Day 10 of embryonic development but consists of only a rudimentary ductal tree at birth. At puberty (about 4 weeks of age), ovarian hormones stimulate rapid and invasive ductal elongation driven by growth of the terminal end bud (TEB). The TEB consists of 4–6 layers of relatively undifferentiated "body cells" surrounded by a single layer of "cap cells." These two populations differentiate into lumenal epithelial cells (also consisting of multiple cell types) and myoepithelial cells, respectively, as the subtending duct is formed

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0012-1606/01 $35.00

(Chepko and Smith, 1999; Daniel and Silberstein, 1987). Upon reaching the limits of the fat pad at ductal maturity, ductal elongation ceases and TEBs regress to leave a branched system of differentiated ducts.

Hormonal changes during pregnancy initiate a cyclical phase of development in which there is a dramatic transition from a predominantly ductal to a predominantly lobuloalveolar gland morphology. Yet to be identified lobuloalveolar progenitor cells located within the ducts proliferate to form alveolar buds, which further differentiate to form the alveoli. After parturition, alveolar cells begin to secrete large quantities of milk. Upon weaning, milk secretion ceases and the gland undergoes involution, during which most alveolar cells undergo apoptosis while the remainder of the gland is extensively remodeled to resemble the prepregnant state.

Several hormone and growth factor signaling systems are known to participate in control of these developmental transitions, some of which have also been demonstrated to participate in mediating tissue interactions in the mammary gland. These signaling systems include those of the mammotropic hormones estrogen, progesterone, and prolactin as well as those of members of the TGF-β, Wnt, EGF, and FGF superfamilies. Recently, we reported that heterozygous disruption of the *Ptc1* hedgehog receptor subunit leads to developmental defects and cancer-like histological alterations in the mammary glands of virgin mice and parous mice after involution (Lewis *et al.*, 1999). These observations suggested a novel and important role for the hedgehog signal transduction network in regulation of histomorphology and the control of tissue interactions during mammary gland development.

In mammals, the genes encoding the hedgehog family of secreted signaling proteins [*Sonic Hedgehog* (*Shh*), *Indian Hedgehog* (*Ihh*), and *Desert Hedgehog* (*Dhh*)] and associated signaling network components are important regulators of cell identity, cell fate, proliferation, and pattern formation during embryogenesis and organogenesis (Hammerschmidt *et al.*, 1997; Ingham, 1998; Levin, 1997; Lewis *et al.*, 1999; Ruiz i Altaba, 1999a). Hedgehog proteins secreted by a given cell act as ligands for a receptor complex located on the membrane of nearby cells. The receptor complex consists of at least two transmembrane proteins, Smoothened (SMO) and either Patched-1 (PTC1) or Patched-2 (PTC2). In the absence of hedgehog ligand, PTC1 (and probably PTC2) acts as an inhibitor of the SMO subunit and prevents downstream signaling. Upon hedgehog binding, inhibition by PTC1 is relieved, thus allowing SMO to function. Ultimately, a largely uncharacterized series of downstream events leads to the activation of one or more members of the GLI family of transcription factors, GLI1, GLI2, and GLI3.

Strains of mice carrying loss-of-function alleles for each of the three known *Gli* genes exist. Targeted disruption of *Gli1* (Δ*Gli1*) in mice led to no discernible phenotype in homozygous null mice (Park *et al.*, 2000). In contrast, homozygous mutation of either the *Gli2* or *Gli3* gene results in perinatal lethality and a partially overlapping set of developmental defects (Ding *et al.*, 1998; Hughes *et al.*, 1997; Mo *et al.*, 1997; Motoyama *et al.*, 1998; Park *et al.*, 2000; Ruppert *et al.*, 1990; Walterhouse *et al.*, 1993). Current data suggest that *Gli2* plays a role primarily as a transcriptional activator, whereas the *Gli3* gene has been characterized primarily as a transcriptional repressor. However, recent work demonstrates that the activities of *Gli2* and *Gli3* are influenced by the presence of a repression domain in the N-terminus of each protein (Ruiz i Altaba, 1999b; Sasaki *et al.*, 1999). Together, these data suggest that *Gli2* and *Gli3* are the primary mediators of hedgehog signaling and that each may possess the same range of functional capabilities as *Ci* in *Drosophila*. Thus, the effect of disruption of *Gli2* or *Gli3* in a given tissue cannot be predicted *a priori*.

Given that homozygous disruption of *Gli1* did not lead to an overt mammary phenotype (e.g., lactational deficiency, tumor formation) (Park *et al.*, 2000) and that our ongoing analysis of the $Gli3^{xt}$ strain has yet to suggest any role in mammary gland development (M. T. Lewis, unpublished results cited in Lewis, 2001), we hypothesized that *Gli2* might act as the primary *Gli* gene that mediates hedgehog signaling during mammary gland development.

MATERIALS AND METHODS

Animals

The inbred mouse strain Balb/C is maintained in our laboratory. Athymic Balb/C *nu/nu* (*nude*) female mice were obtained from Simonson. Outbred CD1 female mice were obtained from Charles River Laboratories. B6D2F1 female mice were obtained from Taconic Farms.

Two breeding pairs of CD1 mice heterozygous for a disrupted *Gli2* gene (Δ*Gli2*) were used to initiate a breeding colony. For the majority of this study, the mutation was maintained in this outbred background by crossing male *Gli2* heterozygotes with CD1 female mice obtained periodically over approximately 2 years. The outbred background was maintained for these initial studies as a matter of choice because all other published phenotypes for *Gli2* disruption are manifest in this background and the effect of this mutation in other backgrounds has not yet been investigated. Genotyping was performed by PCR as described previously (Mo *et al.*, 1997).

For *Gli2* developmental expression studies (*in situ* hybridization) Balb/C animals were used to correlate results with expression of other genes in the hedgehog signaling network currently under study.

For investigation of *Gli2* function in alveolar development, the Δ*Gli2* allele was crossed into a B6D2F1 background for 7 generations prior to use in transplantation experiments.

Developmental Stages

Developmental stages examined were: 3, 5, 10, and 20 weeks postpartum virgin, early pregnant [5.5–9.5 days postcoitus (d.p.c.)], late pregnant (15.5–19.5 d.p.c.), lactating (Days 6–7), and involuting (Days 2, 10, and 14). For pregnancy, lactation, and involution

studies, mice were matured to 10 weeks of age prior to mating, to ensure complete filling of the mammary fat pad by a mature ductal tree. For involution, mice were allowed to lactate 10 days prior to pup removal to ensure that the dams were still actively feeding pups.

mRNA Isolation

Mammary glands (No. 4) of female Balb/C mice were used for RNA extractions. Lymph nodes were removed using forceps and the glands flash-frozen in liquid nitrogen immediately upon removal. Glands were stored at −80°C prior to use. Total RNA was isolated by column chromatography (Qiagen). Embryonic day 14 (E14) RNA was isolated in a similar fashion. RNA was used in preliminary Northern hybridizations and RT-PCR experiments to determine whether *Gli2* is expressed in the mouse mammary gland (data not shown).

In Situ Hybridization

The No. 2 and No. 3 mammary glands of Balb/C mice were used. Glands were fixed in ice-cold 4% paraformaldehyde:PBS for 3 h and processed for *in situ* hybridization (Friedmann and Daniel, 1996). *Gli2*-specific, digoxigenin-labeled riboprobes were prepared using T7 and SP6 RNA polymerases and hybridized essentially as described (Friedmann and Daniel, 1996). *In situ* hybridizations for *Ptc1* and *Ihh* were performed previously (Lewis *et al.*, 1999) and are presented again in this work to provide the context for interpreting *Gli2* expression patterns and phenotypes.

Whole Gland Morphological Analysis

Backcross-derived Δ*Gli2* heterozygotes and wild type littermate- or age-matched females were used. CD1 animals were also examined as controls for morphological variation in this outbred genetic background. At least four animals were examined at each stage for each genotype, except that only Δ*Gli2* heterozygotes and CD1 mice were examined after two pregnancies. Mammary glands 1–5 were harvested from one side of the animal at various developmental stages, fixed in ice-cold 4% paraformaldehyde:PBS, and hematoxylin stained as described (Daniel *et al.*, 1989). Each gland was examined for developmental abnormalities under a dissecting scope. Because they are more easily examined and photographed, only the No. 2 and No. 3 glands were scored for quantitative analyses (regardless of whether another gland in the same animal was affected).

Histological Analysis

The No. 2 or No. 3 mammary glands were used. At least three representative animals were examined for each strain at each developmental stage. Gland fragments were embedded in paraffin, sectioned at 7 μm, and hematoxylin/eosin stained.

Whole Gland Transplantation Rescue from Null Embryos

Mammary glands from homozygous null or wild type E18–E19.5 embryos were transplanted between the skin and body wall of 3-week-old female Balb/C *nu/nu* or B6D2F1 mice whose endogenous No. 4 mammary glands had been surgically removed. Transplanted mammary glands were allowed to regenerate ductal trees for 6–8 weeks. Glands were removed and processed for whole gland and histological analysis. To control for both genotype and sex, tails were removed from each of the donor embryos and both genotyped (as above) and sexed (by PCR amplification of the male SRY gene). No differences were detected in the degree or character of mammary outgrowths in glands from male or female donor embryos.

Epithelial Transplantation from Null Embryos

Fragments of mammary gland from wild type and Δ*Gli2*/Δ*Gli2* donor E18–E19.5 mice were transplanted into cleared No. 4 fat pads of 3-week-old Balb/C *nu/nu* mice. Subsequent outgrowths were examined 8 weeks posttransplantation as whole mounts and histological samples. To control for both genotype and sex, tails were removed from each of the embryos used and both genotyped and sexed (as above). No differences were detected in the degree or character of mammary outgrowths in glands from male or female donor embryos.

RESULTS

Gli2 Expression during Mammary Gland Development

Preliminary Northern hybridization and quantitative RT-PCR using total RNA demonstrated mammary expression of *Gli2* and suggested that *Gli2* transcription may be developmentally regulated (not shown). To obtain more specific data concerning spatial and temporal pattern of *Gli2* expression, we performed *in situ* hybridization using tissue at several phases of mammary gland development. At 5 weeks postpartum, *Gli2* was expressed exclusively in the periductal and fat pad stroma and was associated with condensing stroma around the neck of the end bud (Fig. 1A). Expression was elevated in condensed stroma relative to that in stroma that had not yet become associated with the subtending ducts at the neck of the terminal end bud. As published previously, *Ptc1* was expressed in both stromal and epithelial compartments at this phase (Fig. 1B). *Ihh* expression was low but detectable in terminal end bud and ductal epithelium but not in stroma (Fig. 1C). These expression patterns were maintained in ducts of mature animals (Figs. 1D–1F).

A fundamental transition occurred during pregnancy in which *Gli2* expression became both stromal and epithelial (Fig. 1G). Epithelial expression was primarily alveolar but was also observed in small ducts associated with developing lobuloalveolar structures (Fig. 1G). Expression in larger ducts remained low to undetectable (data not shown). Expression of *Ptc1* in epithelium of developing lobuloalveolar structures (Fig. 1H) and of *Ihh* in both small ducts and alveoli (Fig. 1I) also appeared elevated at this stage.

In late pregnancy, the spatial pattern of expression of all

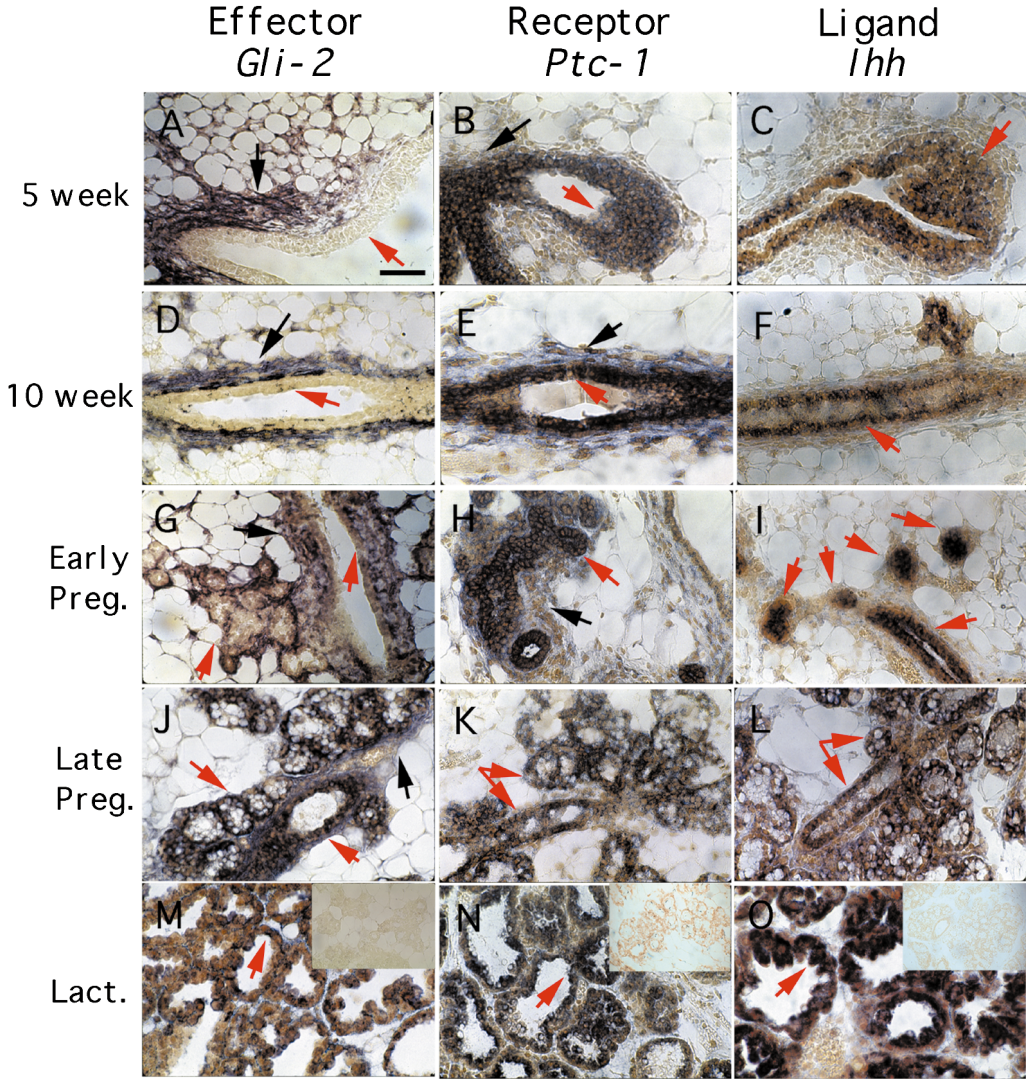

FIG. 1. *In situ* hybridization of *Gli2* during postnatal mammary gland development: *Gli2* expression is correlated with expression of other hedgehog network components as published previously. The gene from which the probe was designed is shown above the column to which it applies. Developmental stages examined are shown along the left side of the figure. Hybridization is detected by the accumulation of a blue-black precipitate in cells in which the gene is expressed. Epithelial expression is identified by red arrows; stromal expression is identified by black arrows. (A) *Gli2* expression appears graded in condensing and condensed periductal stroma at the neck of a terminal end bud. Expression is not detected in the epithelial compartment. (B) *Ptc1* expression in body cells of terminal end bud and periductal stroma. (C) *Ihh* expression in body cells of terminal end bud. (D) *Gli2* expression in the periductal stroma of a mature duct. Expression is not detected in the epithelial compartment. (E) *Ptc1* expression in both epithelial and stromal compartments. (F) *Ihh* expression is detectable only in the epithelial compartment. (G) *Gli2* expression in early pregnancy, demonstrating a transition to both stromal and epithelial localization, particularly in developing alveoli. (H) *Ptc1* expression in both stromal and epithelial compartments. (I) *Ihh* expression exclusively in the epithelial compartment with elevated expression in developing alveoli. (J) *Gli2* expression in the stromal and epithelial compartments at late pregnancy. Expression in alveoli appears to be elevated relative to that of early pregnancy. (K) *Ptc1* expression in late pregnancy. (L) *Ihh* expression in epithelial compartment in late pregnancy. (M–O) Apparently elevated expression of *Gli2*, *Ptc1*, and *Ihh* in the epithelial compartment during lactation. (M–O, insets) Sense strand control hybridizations showing no hybridization. Bar = 80 μm.

three genes remained consistent with those observed in early pregnancy. However, expression of each gene appeared elevated (Figs. 1J–1L). All appeared to be particularly highly expressed in epithelium during lactation (Figs. 1M–1O), as judged by more rapid accumulation of the blue-black precipitate in all *in situ* hybridization experiments. Sense strand control hybridizations showed no signal (Figs. 1M–1O, insets).

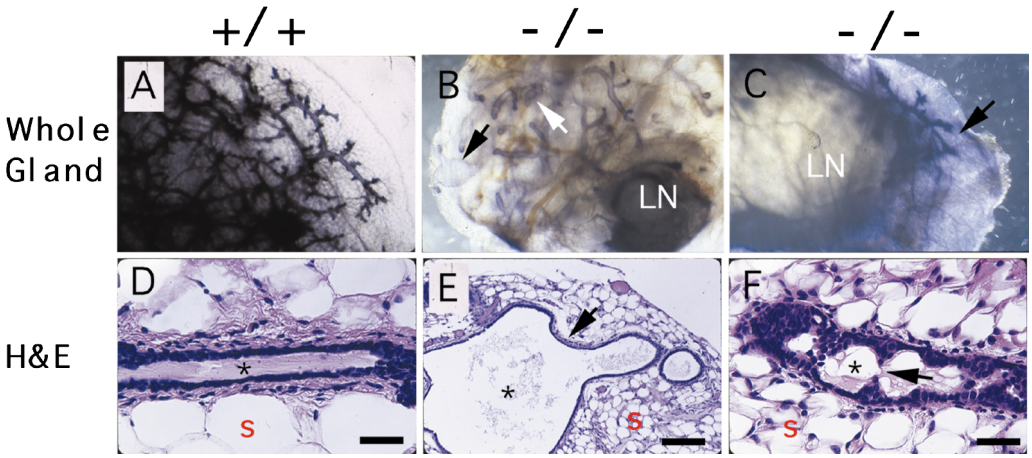

FIG. 2. Whole mammary gland transplantation rescue from nullizygous and wild type late-stage embryos into nude mouse hosts: ductal development. The genotype of the donor embryo from which the transplanted gland is derived is shown at the top of each column. Whole gland and corresponding histological analyses are presented. (A) Wild type donor transplant showing normal ductal patterning. (B) Nullizygous donor transplant showing the most prominent phenotype of distended ducts (black arrow) with regions of misshapen ducts (white arrow). A lymph node remnant is also present (LN). (C) Nullizygous donor transplant showing a severely affected gland that had only misshapen, stunted ducts and side branches (black arrow). A lymph node remnant is also present (LN). (D) Histological preparation of a wild type donor transplant showing normal ductal architecture. The ductal lumen is denoted by an asterisk. Bar = 80 μm. (E) Histological preparation of the nullizygous donor transplant depicted in B showing the organization of the highly distended duct terminus (B, black arrow). Misshapen ducts from this type of gland (B, white arrow) had the histoarchitecture depicted in F. The ductal lumen is denoted by an asterisk. Bar = 220 μm. (F) Nullizygous donor transplant showing representative micropapillary histoarchitecture (black arrow) of affected, misshapen ducts and termini. Histological defects of this type were observed in 100% of the nullizygous transplants examined. The ductal lumen is denoted by an asterisk. Bar = 80 μm.

The Null Phenotype for Ductal Development: Transplantation Rescue of Embryonic Mammary Glands

Δ*Gli2* is a homozygous perinatal lethal mutation, thus precluding analysis of the adult phenotype in intact animals. To circumvent this difficulty, we performed transplantation rescue experiments in which the entire intact embryonic (E18–E19.5) mammary glands (both epithelium and fat pad precursor mesenchyme) were removed and transplanted between the skin and body wall (their normal position) of 3-week-old virgin Balb/C *nu/nu* or B6D2F1 hosts whose endogenous No. 4 gland had been removed.

Transplants from wild type and nullizygous late-stage embryos (E18–E19.5) were examined 6–8 weeks posttransplantation. Transplanted mammary glands did not achieve full size but generally grew as disks about 0.5–1.5 cm in diameter. Transplants were usually adherent to, and vascularized from, the musculature of the body wall but could also be vascularized from the skin. As expected, mammary glands from wild type donor embryos grew with normal branching morphogenesis (Fig. 2A). Mammary glands from nullizygous embryos also showed ductal outgrowths. However, outgrowths showed a range of morphological disruptions. Many ducts appeared near normal; others were dysplastic, being either highly distended or undulating within the stroma (Fig. 2B). Rarely, transplants appeared severely altered with short ducts and stunted side branches (Fig. 2C).

In histological analysis, transplants from wild type donor embryos showed unperturbed histoarchitecture (Fig. 2D) with the ductal epithelium surrounded by a characteristic periductal stroma. Transplants from homozygous null embryos showed a range of histological disruptions. A minority of ducts appeared normal (not shown), whereas distended regions of ducts showed large lumena surrounded by a single thin layer of lumenal epithelial cells with very little periductal stroma (Fig. 2E). In all null transplants examined we detected extended regions of ductal dysplasia, consisting of micropapillary epithelial extensions or bridges that protruded into the lumen, in some cases appearing to occlude it, as determined by examination of serial sections through entire ducts (Fig. 2F). As with the distended regions, there was a reduced quantity of periductal stromal elements.

The Null Phenotype for Ductal Development: Epithelial Transplantation into Cleared Fat Pads of Wild Type Hosts

As an initial step in addressing the issue of tissue compartment-specific function(s) for *Gli2*, we wished to determine whether the Δ*Gli2*-induced ductal defects in glands derived from homozygous null donors reflected an intrinsic defect in the epithelium or stroma (or both). To address this question, wild type and homozygous null epithelium were transplanted contralaterally into both No.

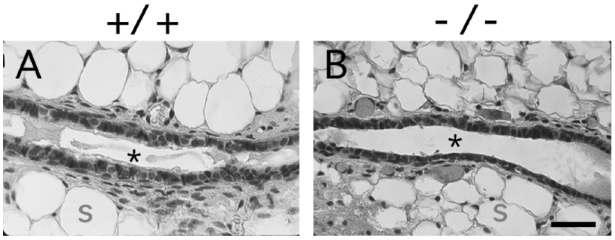

FIG. 3. Epithelial transplants into cleared fat pads of nude mouse hosts. The genotype of the donor animal from which the epithelium was derived is shown above the column. The ductal lumena are designated by asterisks. Adipose stroma is denoted by a letter "s." (A) Wild type epithelium showing normal ductal histoarchitecture. (B) Nullizygous epithelium showing normal ductal histoarchitecture. Bar = 27 μm.

4 epithelium-free (cleared) fat pads of Balb/c *nu/nu* mice and allowed to regenerated a ductal tree for 8 weeks.

As expected, transplants of wild type epithelium grew normally and completely filled the available fat pad. Null epithelial outgrowths also grew with normal branching morphology and completely filled the available fat pad. No defects were observed in any transplant at the level of whole gland analysis (data not shown). Histological analysis also demonstrated that the cellular architecture, unlike that in the whole gland transplants, was uniformly normal, regardless of whether the epithelium was genotypically wild type (Fig. 3A) or homozygous null (Fig. 3B). These results are consistent with a role for *Gli2* function in the periductal stroma during ductal development.

The Null Phenotype for Alveolar Development during Pregnancy: Transplantation Rescue of Embryonic Mammary Glands

The functional requirement for *Gli2* during ductal development coupled with the spatially and temporally regulated patterns of *Gli2* expression during pregnancy and lactation led to the hypothesis that *Gli2* function might be required for alveolar development. To test this hypothesis, we performed transplantation rescue experiments in which entire intact embryonic (E18–E19.5) mammary glands were removed and transplanted between the skin and body wall of 3-week-old virgin B6D2F1 hosts whose endogenous No. 4 gland had been removed. Transplanted glands were allowed to grow for 4 weeks and the host females impregnated. Transplants were harvested at 18 d.p.c. and examined.

In direct contradiction to the hypothesis, mammary glands derived from either wild type or homozygous null donors showed no overt differences in the extent of alveolar development or the degree to which the cells were prepared for lactation (Fig. 4). Alveoli in the null transplant (Fig. 4A) appeared indistinguishable from those in the wild type transplant (Fig. 4B) or from the host control glands for each transplant (Figs. 4C and 4D, respectively). Alveoli were morphologically and histologically equivalent in size and

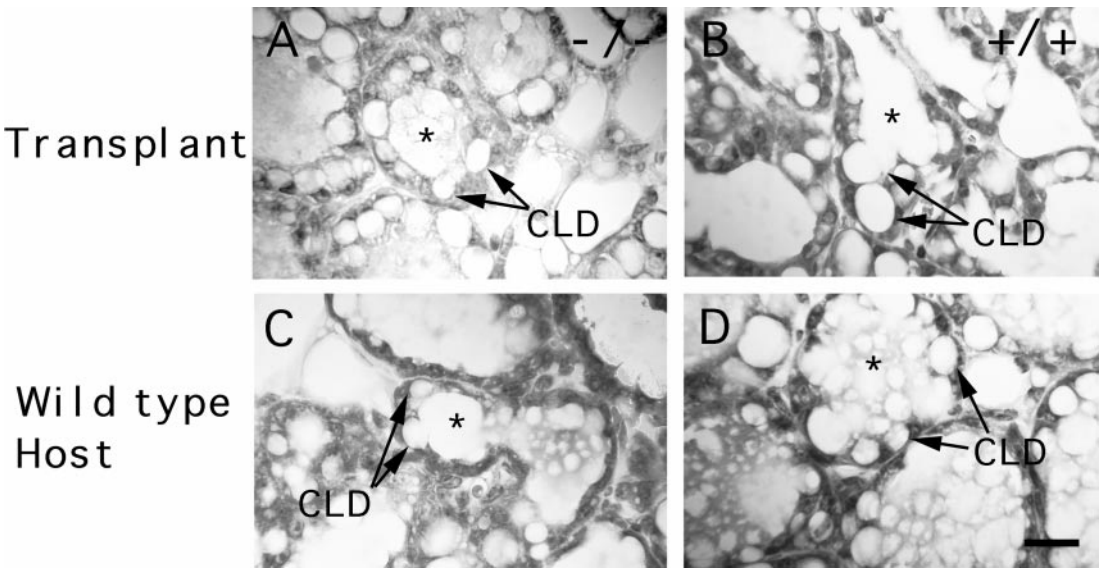

FIG. 4. Whole mammary gland transplantation rescue from nullizygous and wild type late-stage embryos into B6D2F1 mouse hosts: alveolar development. The genotype of the donor embryo from which the transplanted gland is derived is shown at the top of each column. Alveolar lumen is denoted by an asterisk. Cytoplasmic lipid droplets (CLD) characteristic of late pregnancy are denoted by arrows. (A) Nullizygous transplant. (B) Wild type transplant. (C) Wild type host for transplant shown in A. (D) Wild type host for transplant shown in B. Bar = 27 μm.

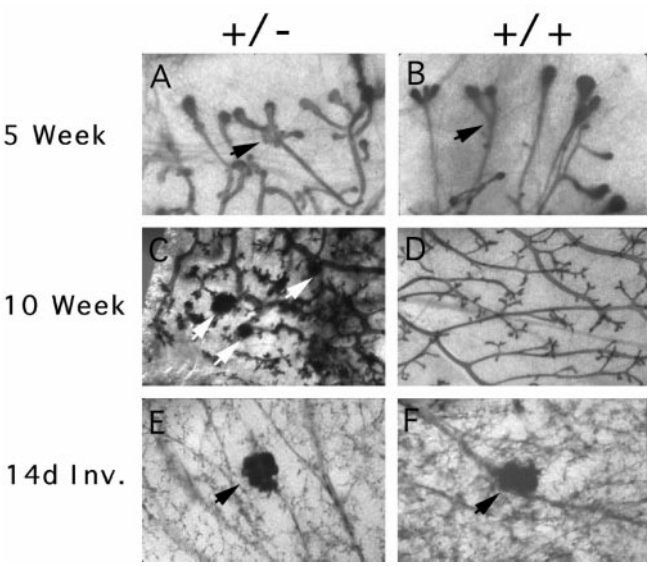

FIG. 5. Whole gland morphological analysis in virgin and parous animals. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column. (A) Radial bud (arrow) observed in heterozygotes. The buds appear to originate at a branchpoint behind otherwise normal-appearing terminal end buds. (B) Normal branchpoint in a wild type animal. (C) Multiple focal dysplasias (white arrows) in the most severely affected female. Duct morphology is within the normal range observed in control animals at this phase of development. (D) Normal ducts. Duct morphology is representative of the majority of animals at this phase of development. (E) Focal dysplasia (arrow) observed at elevated frequency in *Gli2* heterozygotes. (F) Focal dysplasia similar to that in E (arrow) observed in wild type animals at lower frequency.

showed a high proportion of the cells with large cytoplasmic lipid droplets, indicating that milk lipid synthesis was normal. It should be noted that, in several cases, development of the transplanted glands (both homozygous null and wild type controls) appeared slightly delayed relative to that of the host mammary glands.

The Heterozygous Phenotype: Ductal Morphogenesis

In whole-mount and histological analysis morphological defects were detected in heterozygotes as early as 5 weeks postpartum (Fig. 5A). Defects appeared as small rosettes of radial buds or branches emanating from what appeared to be a branchpoint. No such defects were observed in wild type control animals (Fig. 5B). At 10 and 20 weeks of age, defects in heterozygotes were generally larger and had the appearance of spherical staining densities in whole gland analysis (Fig. 5C). Again, no defects were detected in mammary glands of wild type control animals at these stages (Fig. 5D).

Parous heterozygous animals demonstrated the most dramatic defects (Fig. 5E). In each affected animal, staining densities were observed in whole gland analysis that bore superficial resemblance to precancerous hyperplastic alveolar nodules (HANs) observed in some strains of mice. Similar defects were detected in a small percentage of wild type control animals (both littermate and CD1 controls) after a single pregnancy (Fig. 5F).

The percentage of animals affected increased steadily in Δ*Gli2* heterozygotes from the earliest stages examined, such that after two pregnancies, 100% of heterozygotes showed defects (Fig. 6A). The percentage of wild type animals (either littermates or CD1 controls) affected remained low through the first pregnancy but increased after a second pregnancy, such that about 50% of the animals showed defects. The number of lesions observed per gland also increased in heterozygotes as a function of age and parity (Fig. 6B). Whereas the frequency remained low in wild type animals through two pregnancies, the frequency of defects per gland in Δ*Gli2* heterozygotes approached 1 in multiparous animals. Only a few glands displayed multiple focal dysplasias ($n \leq 3$).

Histological analysis of defects in 5-week-old virgin animals (Fig. 7A) demonstrated that the radial buds observed in Δ*Gli2* heterozygotes were similar to terminal end buds in having multiple layers of epithelial cells. However, these radial buds differed in many cases by having no clearly identifiable cap cell layer. In addition, approximately 25% of terminal end buds were disrupted in heterozygotes (Fig. 7B). Wild type glands showed no such defects (Fig. 7C).

At 10 and 20 weeks postpartum, glands from Δ*Gli2* heterozygotes showed a variety of histological phenotypes. Many showed an architecture similar to that of those observed at 5 weeks with multiple elongated radial ductules (Fig. 7D). Others were more severe (Fig. 7E), showing regions of densely packed, monomorphic epithelial cells abutting the adipose stroma and not surrounded by the usual fibrous elements of the periductal stroma. No histological defects were observed in wild type control glands at these stages (Fig. 7F).

Parous and multiparous animals showed an array of histological abnormalities. Defects detected after a single pregnancy were focal (Fig. 8A) but highly disorganized with loosely associated epithelial cells interspersed with stromal elements and eosinophilic regions. Ducts not immediately associated with focal defects appeared normal (Fig. 8B). Focal defects were also observed rarely in wild type animals after a single pregnancy that were histologically consistent with those in heterozygotes (Fig. 8C) but generally less severe.

A clear distinction between wild type and Δ*Gli2* heterozygous animals arose after a second pregnancy. In addition to dysplasias similar to those observed after a single pregnancy (Fig. 8D), alveolar hyperplasias were identified that consisted of multiple clusters of alveolar structures with a paucity of periepithelial stromal elements (Fig. 8E).

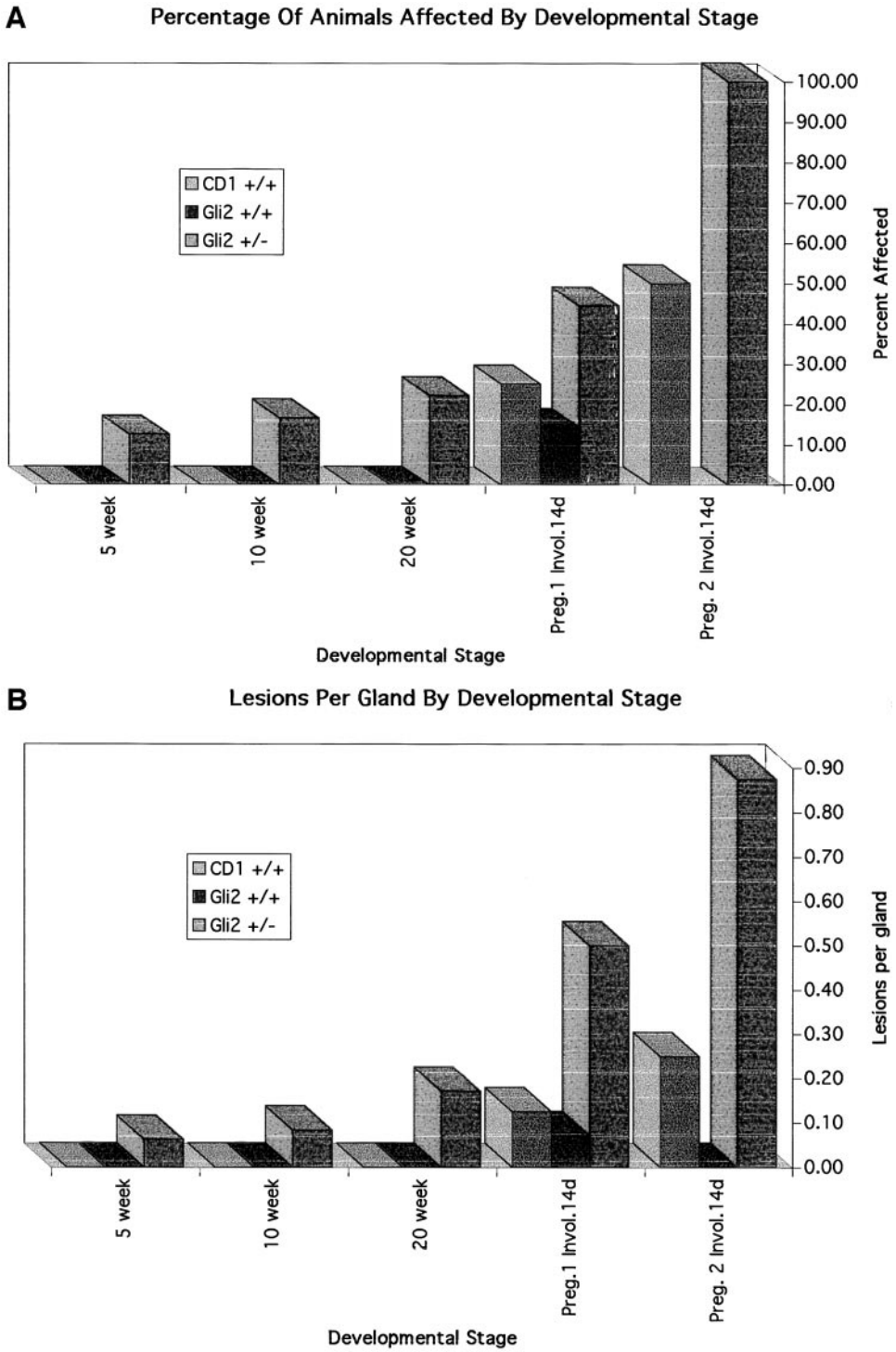

FIG. 6. Frequency of mammary defects detectable by whole gland morphological analysis at various stages of development. All defects were confirmed by histological evaluation. (A) Percentage of *Gli2* heterozygotes, littermate/age matched controls and wild type CD1 controls with developmental defects according to strain. (B) Number of defects observed per gland analyzed according to strain.

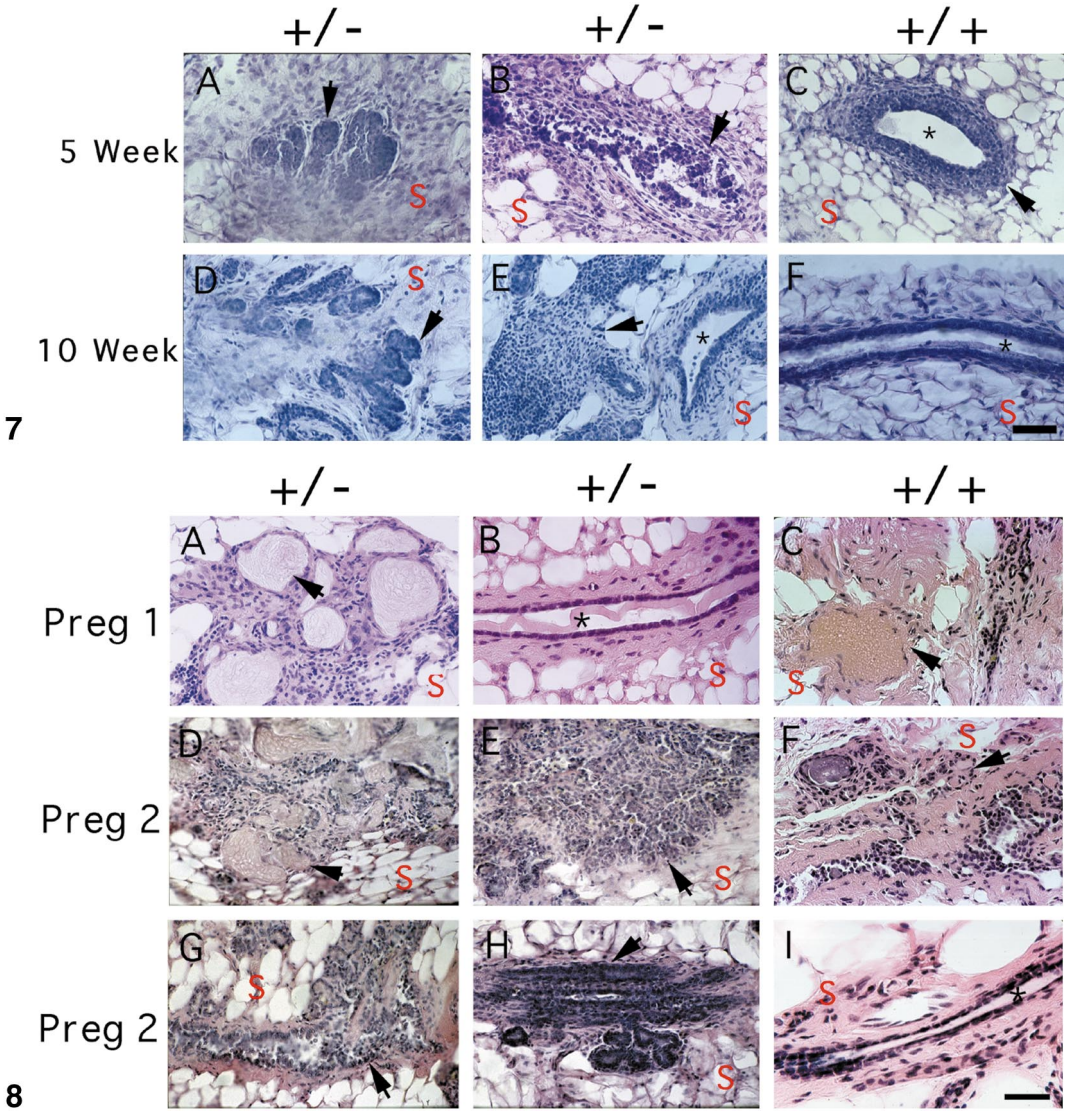

FIG. 7. Histological comparison of terminal end buds and ducts during virgin development. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column. Stroma is denoted by a red letter "s." Lumena are denoted by an asterisk. (A) Radial bud shown in Fig. 5A. Radial buds with end bud-like morphology are in close proximity to one another. (B) Affected terminal end bud. Cap cell and body cell layers are severely altered with respect to cell–cell contacts. Stromal condensation appears altered. (C) Normal terminal end bud. Note the ordered appearance of the cap and body cell layers and the small amount of stromal condensation at the neck of the terminal end bud. (D) Radial ductules. These structures likely originate as the radial buds shown in A. (E) Ductal dysplasia. Epithelial cells are prevalent and appear to occlude the duct. (F) Normal duct. Bar = 80 μm.

FIG. 8. Histological comparison of focal dysplasias in parous and multiparous animals. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column. (A) Focal dysplasia in a Δ*Gli2* heterozygote demonstrating disorganized epithelium with the inclusion of eosinophilic structures (arrow). Bar = 80 μm. (B) Normal duct representative of ducts both in heterozygotes and in wild type animals that were not associated with the focal dysplasias. (C) Focal dysplasia in a wild type animal showing similar histological character and eosinophilic structures (arrow) as those observed in Δ*Gli2* heterozygotes. Bar = 80 μm. (D) Focal dysplasia (arrow). Bar = 80 μm. (E) Alveolar hyperplasia (arrow) in heterozygous animal. Bar = 220 μm. (F) Focal dysplasia in a wild type animal similar to that in D. Bar = 200 μm. (G) Ductal dysplasia located in a region distant from the focal dysplasia depicted in D. Note the near occlusion of the ductal lumen by loosely associated epithelial cells and the unusual patterning of periductal stroma (arrow). Bar = 200 μm. (H) Ductal dysplasia showing an apparent "tube-within-a-tube-within-a-tube" arrangement of interdigitated epithelial and stromal cell layers (arrow). Bar = 200 μm. (I) Normal duct observed in wild type animals in regions distant from focal dysplasias. Bar = 200 μm.

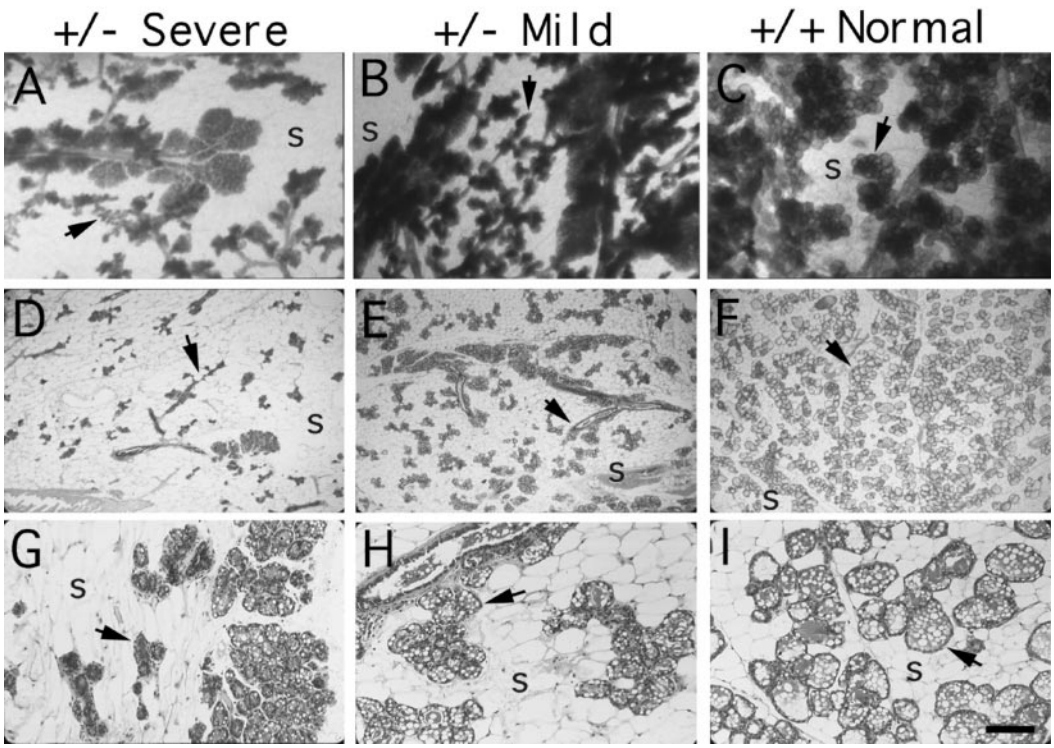

FIG. 9. Reduced alveolar development at late pregnancy (Day 18.5) in Δ*Gli2* heterozygotes. The genotype of the animal from which the gland is derived is shown above the column to which it applies. The severity of the phenotype is also shown. Representative alveolar structures are identified with black arrows. Adipose stroma is denoted by a letter "s." (A) Whole gland preparation of a severely affected heterozygote. Poorly developed alveoli are denoted by an arrow. (B) Whole gland preparation of a mildly affected heterozygote. Poorly developed alveoli are denoted by an arrow. (C) Whole gland preparation of a normal wild type animal. Note that alveoli are fully developed, showing the characteristic "grape cluster" morphology (arrow). (D) Histological preparation of the gland shown in A. Poorly developed alveoli are denoted by an arrow. Adipose stroma is inappropriately maintained. (E) Histological preparation of the gland shown in B. Alveolar development is stunted (arrow), again with inappropriate maintenance of adipose stroma. (F) Histological preparation of the gland shown in C. Alveoli are fully developed and enlarged (arrow) with little adipose stroma maintained. (G–I) Higher magnification views of D–F showing differences in cellular organization and a decreased number of cytoplasmic lipid droplets within alveolar cells of G and H relative to that of I. G–I, bar = 240 μm.

In wild type control animals, only the focal hyperplasias were observed (Fig. 8F). In addition, histological defects in heterozygotes were no longer confined to the focal lesions but were distributed in ducts not immediately associated with nodular defects (Figs. 8G and 8H). No defects were detected in multiparous wild type animals in ducts distant from focal dysplasias (Fig. 8I).

The Heterozygous Phenotype: Alveolar Morphogenesis

In contrast to the normal alveolar development observed in whole gland transplantation experiments using null mammary tissue, when heterozygous animals were examined in late pregnancy (P18.5–P19.0), approximately 37% of the animals (6/16) demonstrated marked hypoplasia of the alveolar epithelium (Figs. 9A and 9B) relative to that of wild type control animals (Fig. 9C). These observations were confirmed at the histological level at which affected heterozygotes showed reduced alveolar development and inappropriate retention of adipose stroma (Figs. 9D and 9E vs 9F).

At higher magnification, clear differences can be seen in both the extent of alveolar expansion and the degree to which alveolar cells are prepared for secretion (Figs. 9G and 9H vs 9I). In each of the affected heterozygotes, cytoplasmic lipid droplets are observed in the more highly developed regions of the gland but are rare in the underdeveloped portion. Wild type animals show uniform alveolar morphology and a high proportion of alveolar cells containing visible cytoplasmic lipid droplets. Despite these observations, no lactational defects or developmental abnormalities were detected when mammary glands were examined after 6 days of lactation, suggesting that alveolar development in late pregnant heterozygous animals was delayed but not entirely defective.

DISCUSSION

Gli2 Is a Key Gli Gene Active in the Mammary Gland

Previous analysis of *Ptc1* expression through mammary gland development showed regulated expression in both epithelium and stroma. In the same work, expression of *Ihh* was also shown to be developmentally regulated with a dramatic increase in developing alveoli during pregnancy (Lewis *et al.*, 1999). These observations predicted that expression (or function) of a key *Gli* gene active in the mammary gland should also be temporally or spatially regulated. In addition, some correlation might exist between *Gli* gene expression and the expression patterns of *Ptc1* and *Ihh*. If such a correlation were observed, these results might help explain the requirement for wild type levels of *Ptc1* function during ductal development and the phenotypic reversion observed in *Ptc1* heterozygotes during pregnancy.

Using *in situ* hybridization we have demonstrated that *Gli2* expression is spatially regulated, being restricted to the periductal stroma in virgin animals but becoming both stromal and epithelial during pregnancy and lactation. Expression levels also appeared to be temporally regulated in coordination with the reproductive status of the animal. The spatial and temporal pattern of *Gli2* expression is tightly correlated with enhanced expression of *Ihh* and *Ptc1* in the epithelium. Finally, the appearance of epithelial expression of *Gli2* correlated well with the reversion of defects in *Ptc1* heterozygotes in pregnancy and lactation (Lewis *et al.*, 1999).

The tissue compartment switch in *Gli2* expression between ductal and alveolar development is, to our knowledge, a unique observation. Whereas many genes show changes in expression levels from virgin to reproductive development and, indeed, can show altered distribution from one epithelial structure to another (e.g., ducts vs alveoli), expression remains in the same tissue compartment throughout postnatal development. As a general interpretation, we propose *Gli2* mRNA expression to be an indicator of active hedgehog signaling. If this interpretation is correct, hedgehog signaling status in the epithelial compartment changes from inactive during virgin development to active during pregnancy and lactation. *Gli2* expression may act as a unique molecular marker for this critical shift in the developmental and physiological state of lumenal epithelial cells.

With respect to *Gli2* function, our transplantation results demonstrate that disruption of *Gli2* leads to defects in ductal development in transplanted intact glands (the defects are intrinsic to the organ) but that loss of *Gli2* function solely in the epithelium is not sufficient to allow recapitulation of the null ductal phenotype. Consistent with the *in situ* hybridization results, the demonstration that mutant epithelium is phenotypically normal in the context of wild type stroma suggests that *Gli2* functions primarily in the stroma during ductal development. We have not yet determined whether mutant stroma can, in turn, direct defective development of wild type epithelium. Therefore, it remains formally possible that *Gli2* must be disrupted in both the stroma and the epithelium to recapitulate the null ductal phenotype.

Our ongoing analyses of the Δ*Gli1* and *Gli3*^{xt} mouse strains have thus far shown no demonstrable phenotype. The data presented here suggest that *Gli2* is a key *Gli* gene active in the mammary gland. Unfortunately, without detailed information concerning genes regulated directly by *Gli2*-mediated hedgehog signaling we cannot address whether *Gli2* is functioning as a transcriptional activator or as a transcriptional repressor in any given tissue compartment at any given phase of development.

Does Gli2 Have a Function in Alveolar Development?

Detection of regulated and enhanced *Gli2* expression in the epithelium during pregnancy and lactation suggested the hypothesis that *Gli2* functions in alveolar development or functional differentiation. This hypothesis was not supported by the whole gland transplantation assays or by epithelial transplantation assays (not shown) in which alveolar morphogenesis and differentiation appeared normal. However, the hypothesis was supported by the observation that approximately 37% of heterozygous female mice displayed hypoplastic development of alveoli during late pregnancy.

At present we are unable to reconcile these two conflicting results and, based on other unpublished data (cited in Lewis, 2001), are currently of the opinion that the alveolar hypoplasia observed in some *Gli2* heterozygotes is not artifactual. One possibility is that, in the null mammary gland, *Gli2* function is compensated by *Gli1*, *Gli3*, or both, but that this compensatory function is not permitted in the heterozygotes. Current data suggest that neither *Gli1* nor *Gli3* alone is essential for mammary gland development. However, the complex regulatory interactions known to exist among the *Gli* genes in other organs make this scenario plausible. Alternatively, hedgehog signaling required for alveolar development could be mediated by a *Gli*-independent mechanism that is influenced by *Gli2* in heterozygotes but not in the null mammary glands. Each of these hypotheses is currently being tested.

Does ΔGli2 Heterozygosity Contribute to Developmental Defects?

The observation that a low percentage of wild type animals displayed ductal alterations similar to those observed in parous Δ*Gli2* heterozygous animals is unusual. Our interpretation, in the absence of evidence to the contrary, is that heterozygosity of Δ*Gli2* is *causal* for defects in terminal end bud development and subsequent ductal dysplasias arising therefrom and that heterozygosity of Δ*Gli2* is

permissive for enhancement of an underlying propensity to form alveolar hyperplasias in the CD1 outbred population. Outcrossing of the Δ*Gli2* allele into the B6D2F1 background, which does not show an elevated frequency of spontaneous dysplasia in virgin or parous animals, should allow us to separate these two possibilities.

ACKNOWLEDGMENTS

This work was supported by a breast cancer research grant from the U.S. Department of the Army (DAMD 17-94-J-4230) and a Postdoctoral Research grant to M.T.L. from the University of California Breast Cancer Research Program (2FB-0047). The authors thank Dr. Margaret Neville for supporting completion of this work under Grant R37HD19547 and for helpful discussions.

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Received for publication October 19, 2000
Revised July 23, 2001
Accepted July 23, 2001
Published online August 29, 2001