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Award Number: DAMD17-00-1-0477

TITLE: Hedgehog Signal Transduction Inhibitors in Breast Cancer Treatment and Prevention

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REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (15 Jul 00 - 14 Jul 01)

4. TITLE AND SUBTITLE

Hedgehog Signal Transduction Inhibitors in Breast Cancer Treatment and Prevention

5. FUNDING NUMBERS

DAMD17-00-1-0477

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Mutations in at least two hedgehog signal transduction network genes lead to defects in mammary gland development. These mutations can cause enhanced hedgehog signaling in some organs. If this mechanism is functioning in the mammary gland, defects should be inhibited or reverted by treatment with specific inhibitors of hedgehog signaling. One such inhibitor is called cyclopamine which causes birth defects in sheep when ingested during pregnancy. We are using slow-release pellets containing cyclopamine to test whether developmental defects can inhibit or revert defects caused by mutations in the hedgehog network. These mutations include null alleles for Ptc1 and Gli2 as well as a constitutively activated form of Smo. As controls we are also testing the in vivo effect of cyclopamine treatment on preneoplastic growths presumably caused by genetic alterations independent of hedgehog signaling. The effect of cyclopamine on normal mammary development and function is also being examined. Finally, we are testing the effect of cyclopamine on a series of human mammary epithelial cell lines for changes in their growth behavior. If cyclopamine can slow or prevent neoplastic growth, this class of inhibitors may be useful in breast cancer treatment or prevention

14. SUBJECT TERMS	15. NUMBER OF PAGES
mammary, breast, hedgehog, cancer, cyclopamine, veratramine	84
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The hedgehog signal transduction network mediates cell-cell communication during normal embryonic development. However, genetic mutation of hedgehog network genes can cause severe birth defects, basal cell carcinoma of the skin, and other tumors including medulloblastomas and glioblastomas of the brain.

Our recent work demonstrates a role for hedgehog signaling in mammary cancer and normal mammary gland development in the mouse. Loss-of-function mutations in two hedgehog network genes, *Patched-1 (Ptc-1)* and *Gli-2*, cause cancer-like lesions that closely resemble human ductal carcinoma *in situ* (DCIS. The lesions become invasive with age but, like basal cell carcinoma and medulloblastoma, are not stable upon transplantation.

The specific mechanism by which mutations in the hedgehog network lead to mammary lesions is not known. In basal cell carcinoma, loss of *Ptc-1* function or overproduction of either *Smoothened (Smo)* or one of the three hedgehog proteins, *Sonic hedgehog (Shh)*, leads to tumors. In frogs, skin tumor formation can also be fostered by inappropriate activation of the *Gli - 1* transcription factor gene. If the mechanics of the hedgehog signaling network are conserved between skin and mammary gland, a specialized skin derivative, these observations lead to the following hypothesis:

> *mutation of either the Ptc-1 or Gli-2 genes results in improper activation of the signaling network via inappropriate activity of either Smo or the hedgehog proteins themselves. This improper signaling leads to loss of normal growth control and mammary lesion formation.*

We will use two approaches to test this hypothesis. First, we will construct a transgenic mouse line that expresses a constitutively activated form of *Smo* that signals independently of hedgehog protein binding and cannot be inhibited by *Ptc-1*. When expressed in the skin, this form of *Smo* promotes skin tumors. We expect that altered *Smo* gene will promote tumor formation and will therefore identify *Smo* as a mammary oncogene.

Second, we will use specific inhibitors of hedgehog protein signaling and determine whether these agents can reverse *Ptc-1* - , *Gli-2* - or *Smo*-induced mammary lesions or lessen their severity. As controls we will examine the effects of these agents on well-characterized precancerous and cancerous lesions that arose, presumably, by mechanisms unrelated to the hedgehog network. Agents will be delivered via surgical implantation of slow-release plastic pellets within the gland. Mammary glands will be examined macroscopically and microscopically for changes in structure and effects on lesion growth. Glands will also be assayed for changes in cell division (DNA synthesis) and cell death (apoptosis) as well as in expression of hedgehog network genes in response to treatment.

In addition, we will test the effect of these specific inhibitors on a panel of human breast cancer cell lines. It is possible that these inhibitors may affect the growth characteristics of a subset of human cancers thereby implicating the hedgehog network as a contributory factor in breast cancer onset or progression.

If hedgehog activity is responsible for, or participates in lesion formation or progression, we anticipate that treatment will reverse the formation of lesions or slow their growth. Such findings would justify expanded pre-clinical and clinical investigation of related hedgehog signaling inhibitors for potential therapeutic value in the treatment or prevention of human breast cancers.

Summary of results

Task 1. To determine whether constituitive activation of hedgehog signaling leads to mammary lesions in transgenic mice using an activated form of *Smo* that signals independently of hedgehog protein binding and is unresponsive to *Ptc-1* inhibition.

a. Generation of *MMTV:Smo* transgenic mouse line

Three different transgene constructs have been developed that contain three different forms of the Smo cDNA under the control of the MMTV promoter:

1. Wild type Smo
2. Constitutively activated mutant SmoM1
3. Constitutively activated mutant SmoM2

Problem: Our original proposal requested funding for the generation of a single transgenic mouse line. The availability of three different forms of Smo offered the possibility of expanding this number. However, financial and housing constraints inherent in generating and maintaining three separate colonies of transgenic mice made it imperative that we decide on a single construct with which to generate the transgenic line. We elected to analyze the results of other ongoing work with each of these constructs in order to clarify our choice. As such, the construction of the MMTV:Smo transgenic line has been delayed slightly.

In an attempt to address this issue we have analyzed mammary glands from individual mice from three colonies, each expressing one of the three different forms of the Smo cDNA under control of the keratin 5 promoter. Expression of SmoM1 and SmoM2 constructs led to what appeared to be subtle focal alterations in ductal patterning in virgin animals. However, we could not demonstrate consistent alterations. Therefore, the experiments were ultimately uninformative. These experiments were initiated at Genentech and were supported by independent funding.

Fortunately, other recently completed *in vivo* studies in Xenopus (Zhang *et al.*, 2001) have demonstrated dramatic defects induced by expression of the SmoM2 cDNA. These defects include eye, skin and midgut differentiation failure. Given this result, we have decided that the MMTV:SmoM2 form will be used in transgenic analysis in this work. Strain construction is scheduled to begin August, 2001.

Task 2. To test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog network-induced lesions and the normal mammary gland.

The bulk of our progress has been made on this task. Analysis of implants has been completed for several stages of the proposed experiments. The results of these experiments are summarized below.

a. Implantation and analysis of inhibitors in normal mice

1. "Rangefinder" experiments in virgin mice.

As stated in the original proposal, use of Elvax implants requires a series of "rangefinder" experiments to determine the minimum effective dose and to

assess toxicity/adverse experimental outcomes (Daniel *et al.*, 1989; Silberstein and Daniel, 1982; Silberstein and Daniel, 1987).We have tested target delivery dosages per gland per day of 50μM, 30μM, 20μM and 10μM of both cyclopamine and veratramine. Implants were inserted contralaterally in the #3 mammary gland of 5 week and 10 week old virgin animals. Implants were maintained *in vivo* for 4 days after which implanted glands were harvested and compared to each other and to unimplanted host mammary glands.

We find that dosages of either cyclopamine or veratramine above the 20uM/day/gland target dosage lead to heavy reactive condensation of what appear to be fibroblasts, inflammatory cells or other connective tissue cells. These cells entirely encapsulate the implant. (Figure1A) Lower dosages do not typically cause cellular condensation (Figure 1B) but will, on occasion, do so.

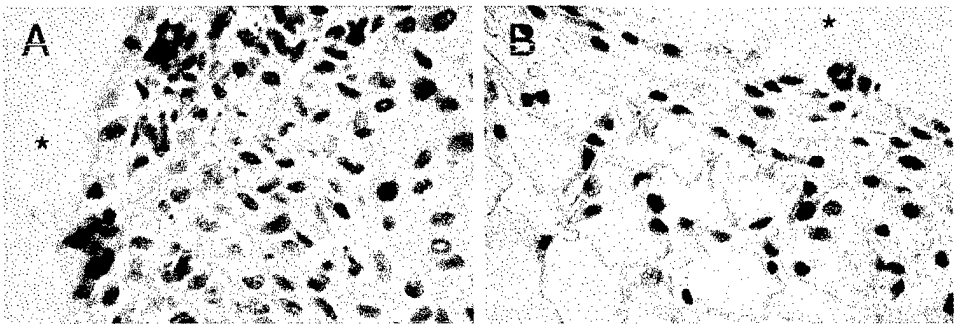

Figure 1. Cellular condensation around cyclopamine and veratramine implants. The location of the implant is denoted by an asterisk. A. Cellular condensation encapsulating a 50μM target dose of cyclopamine. B. Reduced cellular condensation adjacent to a 20μM target dose of cyclopamine. Results using veratramine at comparable dosages were indistinguishable from those shown.

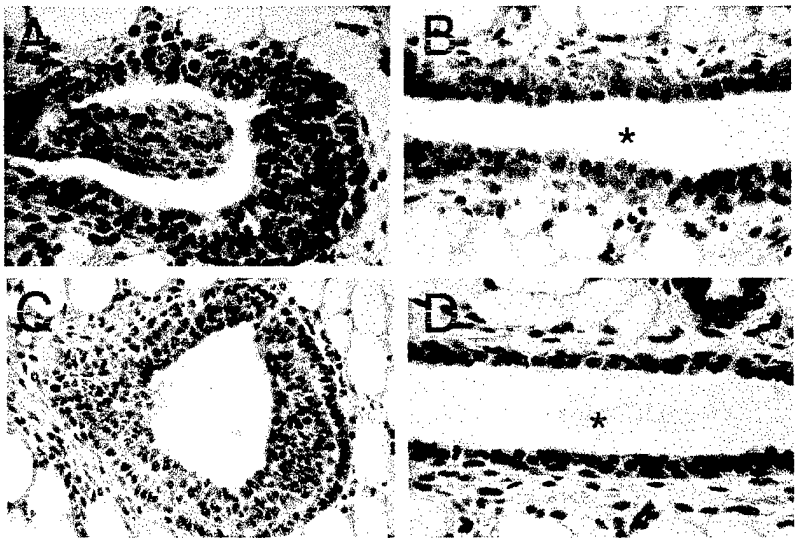

Figure 2. Cyclopamine implantation does not alter terminal end bud or ductal histoarchitecture. Ductal lumena are denoted by an asterisk. A. Terminal end bud adjacent to a cyclopamine implant. B. Mature duct adjacent to a cyclopamine implant. C. Terminal end bud adjacent to a veratramine implant. D. Mature duct adjacent to a veratramine implant.

Our working model (Lewis, 2001) predicted that hedgehog signaling should be active in the terminal end bud and in the periductal stroma.during virgin development. As such, we anticipated that inhibition of the signaling network by cyclopamine should have effects on terminal end bud growth or histoarchitecture.

Using the 20μM target dosage of cyclopamine and veratramine implanted contralaterally in the #3 mammary gland of virgin hosts, we do not observe alterations in terminal end bud architecture (Figure 2A and 2C, respectively), ductal growth or stromal condensation. (Figure 2B and 2D, respectively)

The results of these experiment suggested at least three plausible explanations for the lack of a detectable effect in mammary glands of virgin animals:

a. cyclopamine is not active in Elvax implants.

b. cyclopamine is active in Elvax implants but cellular condensation and normal stroma inhibit diffusion of the inhibitor such that it is not effective in affecting ductal growth or terminal end bud morphology.

c. cyclopamine is active in Elvax implants but virgin development is not sensitive to inhibition by cyclopamine.

The possibility that cyclopamine was inactive in elvax implants appeared unlikely given that implantation led to a biological response (albeit adverse) in the form of the heavy cellular condensation (Figure 2A).

To test whether cyclopamine was indeed active in Elvax implants, we examined the effect of implants during pregnancy and lactation, two additional phases of development predicted to be influenced by hedgehog signaling.

3. Implantation experiments in pregnant mice.

We have tested target delivery dosages of 10μM and 20μM of both cyclopamine and veratramine in late pregnant animals P17 and P18. Implants were inserted contralaterally and maintained in vivo until 2 days postpartum. Implanted glands were harvested and compared to each other and to unimplanted host mammary glands.

Implantation at P17 showed only slight evidence of an effect on the transition to lactation (both dosages) (not shown). These results were variable and we did not feel that they were consistent enough to perform meaningful molecular analyses or to make firm conclusions about the role of hedgehog signaling and its effect on downstream processes. However, when implanted at day 18 of pregnancy and examined at 2 days lactation, the 20μM dose cyclopamine implant inhibited the transition to lactation locally around the implant (Figure 3A and 3B) but had no measurable effect at sites away from the implant (Figure 3C and 3D). the veratramine implants showed no effect near the implant (Figure 3E and 3F) or at sites away from the implant (Figure 3G and 3H). Host control glands were uniformly unaffected (Figure 3I). These results were highly reproducible in three separate experiments using 3-4 mice in each experiment.

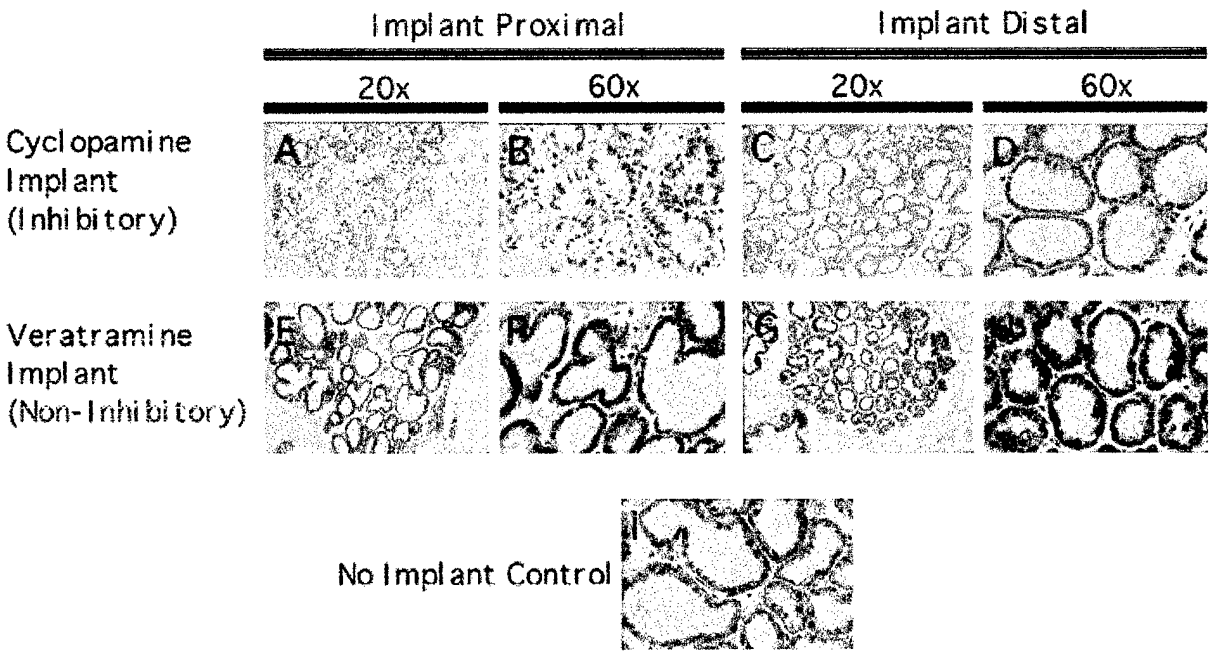

Figure 3. Effect of cyclopamine and veratramine treatment on the transition from pregnancy (P18) to lactation (2 days postpartum).

To begin to characterize the alterations observed using the cyclopamine implants we examined immunolocalization of ADRP (adipocyte differentiation related protein) which coats cytoplasmic lipid droplets in mammary secretory epithelium.

During normal pregnancy at day 18 (P18), cytoplasmic lipid droplets are large and are retained in the cytoplasm of alveolar epithelial cells (Figure 4, top left). At 2 days of lactation the cyctoplasmic lipid droplets are reduced in size and are secreted from the cell as a milk fat globule (Figure 4, bottom left). Implantation of cyclopamine inhibits the transition to lactation locally around the implant and appears to arrest the alveolar epithelial cells in the pregnant state (Figure 4, top right). Epithelial cells located away from the implant are not affected and complete the transition to the lactation state. (Figure 4, bottom right).

These results demonstrate that cyclopamine is indeed active in Elvax and suggest that treatment leads to developmental arrest. These results taken in the context of the results for implants in virgin mammary glands also suggest that some, but not all phases of development can be affected by cyclopamine. It remains unclear whether the normal stroma or the cellular condensation observed in some virgin glands in response to cyclopamine reduces it's effectiveness.

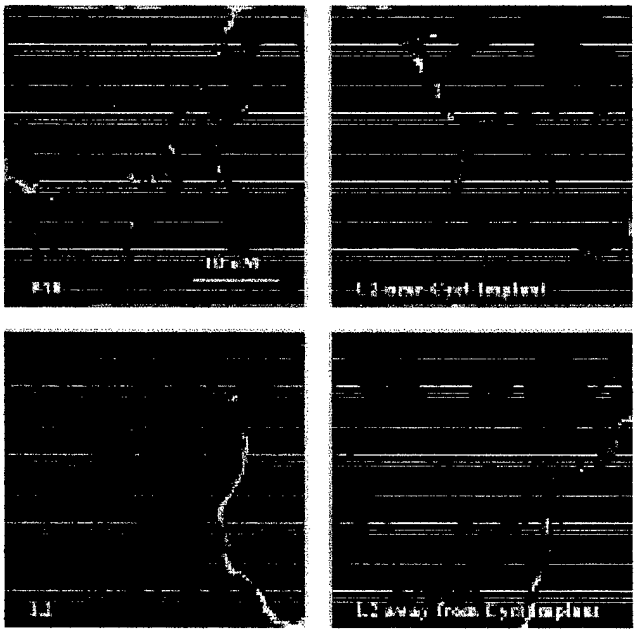

Figure 4. ADRP localization around cytoplasmic lipid droplets. ADRP surrounds cytoplasmic lipid droplets and is pseudocolored in red. Alveolar lumena are outlined in green with FITC-labeled wheat germ agglutinin. Nuclei were stained with DAPI and are presented in blue.

b. Implantation and analysis of inhibitors on *Patched-1* -induced mammary defects.

According to our working model, heterozygous disruption of Ptc1 would lead to enhanced hedgehog signaling that should be sensitive to inhibition by cyclopamine treatment (Lewis, 2001). Since heterozygous disruption of Ptc1 leads to ductal dysplasia (Lewis *et al.*, 1999), these defects were predicted to be reversible by cyclopamine treatment.

To determine whether or not cyclopamine treatment was capable of reversing Ptc1-induced ductal dysplasias, cyclopamine and veratramine implants (20 uM dose) were implanted contralaterally at mid-gland in 12 week old virgin heterozygous and wild type control mice. Glands were harvested and examined after 4 days. Results were difficult to interpret due to the intragland and interanimal variability of the Ptc1 phenotype. However, it is our opinion that cyclopamine treatment (Figure 5A) had no effect on the frequency or severity of Ptc1-induced ductal defects versus the veratramine control (Figure 5B).

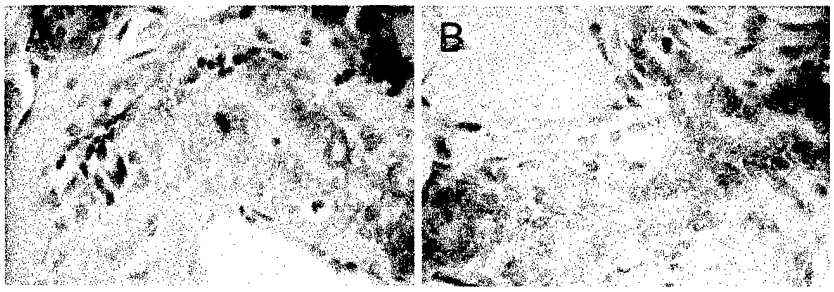

Figure 5. Cyclopamine does not reverse Ptc1-induced ductal defects.
A) Mature duct in a Ptc1 heterozygote adjacent to a cyclopamine implant. B) mature duct in a Ptc1 heterozygote adjacent to a veratramine implant. Both samples exhibit the characteristic cellular occlusion of the ductal lumen caused by disruption of Ptc1.

c. Implantation and analysis of inhibitors on *Gli-2* -induced mammary lesions (Lewis et. al., 2001, In press)

1. We have encountered technical difficulties due to the small size of transplanted glands and the failure of transplanted lesions to be recapitulated on transplantation as was anticipated.

Transplanted glands are generally small (~5mm diameter) with few glands reaching a maximum size of ~12 mm. The glands are also quite thin and are adherent to the musculature of the body wall. Such glands are difficult to implant without significant damage and, because of their size variability, it is difficult to maintain the same relative dosage as with an intact mammary gland (relatively uniform in size).

We are attempting to increase gland size and uniformity by modification of the transplantation methods. First, we are using alternative transplantation hosts (e.g. Rag1 mice, alternative athymic strains) to determine whether glands achieve larger size in one of these alternative hosts. We are also developing methods to use alternative transplantation material (skin grafts from embryonic sources). Thusfar, these methods have not proven themselves superior to the method originally developed for these studies.

Based on our model at the time of grant submission, we had expected that Gli2-induced lesions would be stable on transplantation to cleared fat pads of host mice. This expectation was not met, with Gli2-induced lesions growing with normal patterning and histoarchitecture. These data suggest that Gli2 function is required primarily in the stroma and not the epithelium for the gland to grow. Our current working model has been adjusted accordingly (Lewis, 2001).

d. Implantation and analysis of inhibitors on *Smo* -induced mammary lesions (expected from Task 1)

1. No progress.

Task 3. To test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog-independent lesions.

No Progress.

Task 4. To test the effect of hedgehog inhibitors on the growth and morphology of human breast cancer cell lines *in vitro*.

a. Acquire and establish human mammary epithelial cell lines.

Human mammary epithelial cell lines have been acquired from the ATCC. We are attempting to find a single culture condition that can be used for all lines such that results among the various cell lines can be compared directly. We have narrowed the conditions to two media formulations and hope to have a single condition identified within the next few weeks.

Cell lines established are:

1. MCF10A	"NORMAL"
2. MCF12A	"NORMAL"
3 MCF7	CANCER
4. T47D	CANCER
5. ZR75.1	CANCER
6. MCA231	CANCER
7. MDA468	CANCER
8. MDA330	CANCER

b. Test effect of inhibitors on growth and morphology of human cell lines

1. No progress.

Key Research Accomplishments

- Generation of three MMTV:Smo plasmid constructs for use in generating transgenic mice.

- Determination of biologically tolerated effective dosages of cyclopamine for use in Elvax implants in the mammary gland.

- Demonstration that hedgehog signaling is required for the functional differentiation of the mouse mammary gland at lactation.

- Possible identification of ductal elongation in virgins and alveolar differentiation in early pregnancy as cyclopamine-insensitive phases of mammary gland.

- Identification of lactation as a downstream target regulated by hedgehog signaling.

- Demonstration that cyclopamine treatment fails to reverse Ptc1-induced dysplasias. Result suggests the possibility that Ptc1 does not act through the conventional cyclopamine-sensitive mechanism during ductal development.

Reportable Outcomes

Publications and Manuscripts

1. Lewis, M.T. (2001) Hedgehog signaling in mouse mammary gland development and neoplasia. J. Mammary Gland Biol. Neoplasia. 6:53-66

2. Lewis, M.T., Ross, S., Strickland, P.A. Sugnet, C.W. Jimenez, E, Hui, C.C. and Daniel, C.W. (2001) The Gli2 transcription factor is required for normal mouse mammary gland development. Dev. Biol. (In press).

3. Lewis, M.T. and McManaman, J. Hedgehog signaling is required for lactation. (In preparation)

4. McManaman, J. and Lewis, M.T., Molecular and functional characterization of alternate cell fates in the mouse mammary gland. (In preparation).

Presentations

1. Lewis, M.T. University of Colorado Health Sciences Center (Multiple occasions)
2. Lewis, M.T. University of Colorado School of Dentistry (March 2000)
3. Lewis, M.T. Baylor College of Medicine (August 2000)
4. Lewis, M.T. Gordon Conference on Mammary Gland Biology (June 2001)

Employment received and research opportunities.

1. Promoted to Instructor at the University of Colorado Health Sciences Center
2. Appointed Director of the Animal Handling Core Facility for a Program Project Grant. . PO1-HD38129 (To Margaret Neville)
3. Co-PI (with Dr. Dean Edwards) for project 1 of Program Project Grant. PO1-HD38129 (To Margaret Neville)
4. Listed as Key Personnel for grant RO1-CA85736 (to Dr. Steven Anderson).
5. Appointed to the faculty of the Baylor College of Medicine Breast Center and the Department of Molecular and Cellular Biology at the rank of Assistant Professor.

Conclusions

1. Cyclopamine is effective and useful for inhibition of hedgehog signaling during lactation.

   Cyclopamine is well tolerated by the mouse mammary gland at dosages in the lower range of those examined and is effective in inhibiting hedgehog signaling in at least one phase of mammary gland development. The observation that cyclopamine can impair the transition to lactation at parturition (lactogenesis stage II) supports one portion of our working model for mammary hedgehog signaling. This observation also serves to indicate that milk protein gene expression and secretion are downstream targets (likely very indirect) of hedgehog signaling.

   Of possible significance is the finding that cyclopamine fails to alter ductal development during virgin phases. This observation is in direct opposition to our original hypothesis that hedgehog signaling would be active (and inhibitable) during ductal development. However, this observation is consistent with recently published studies demonstrating that suppression of hedgehog signaling promotes differentiation during the development of the chicken pancreas (Kim and Melton, 1998) and is required for development of the Xenopus midgut (Zhang *et al.*, 2001).

   Our studies funded under this grant are being informed continuously by other investigations in the lab. Among the projects underway is the study designed to determine the effects of overexpression of Ptc1 in transgenic animals (which suppresses hedgehog signaling). The results we have obtained examining mammary glands throughout normal development are entirely consistent with those using cyclopamine implants (Lewis and McManaman, 2001, In preparation).

   Another study investigating the function of the Indian hedgehog ligand is in it's early stages. Transplantation experiments conducted thus far indicate that signaling via Ihh is not required for duct formation or early alveolar development. However, our experiments demonstrate that signaling via Ihh is required for functional differentiation in late pregnancy and lactation.

   Given that these three different methods for inhibiting hedgehog signaling yield nearly identical results, we have growing confidence that the implant results reflect the true cyclopamine-sensitive period of hedgehog signaling in the gland.

2. Cyclopamine is not effective in reversing Ptc1 ductal dysplasia.

   Within the limits of our experiments, we find that cyclopamine fails to alter the development of ductal dysplasia in Ptc1 heterozygotes despite the fact that the cyclopamine is active in other in vivo assays.

   Why then does disruption of Ptc1 lead to altered ductal phenotypes? One intriguing possibility is that Ptc1 is functioning through an alternative mechanism to that which is sensitive to cyclopamine inhibition (i.e. through Smo, and Gli genes). It will be important for the interpretation of our results for us to attempt to address this possibility and assess the effect of ptc1 disruption on overall hedgehog signaling in

individual tissue compartments within the gland. In order to test this hypothesis in vivo, it now becomes critical to construct the constitutively activated Smo to assess whether the phenotype is similar to that of Ptc1 disruption. If not, Ptc1 is likely to be acting in a novel and unanticipated way during mammary development.

One other method to test our hypothesis is to stimulate hedgehog signaling during virgin development using Ihh implants. If hedgehog signaling is required to be inhibited (by Ptc1) during ductal development, treatment with Ihh should relieve this inhibition and yield a phenotype similar to Ptc1 disruption. If ectopic Ihh does not cause such phenotypic alterations, it would support the idea that Ptc1 may be acting through a novel mechanism other than the "classical" Smo-Gli axis.

References

Daniel, C. W., Silberstein, G. B., Van Horn, K., Strickland, P., and Robinson, S. (1989). TGF-beta 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev Biol* **135**, 20-30.

Kim, S. K., and Melton, D. A. (1998). Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. *Proc Natl Acad Sci U S A* **95**, 13036-41.

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Zhang, J., Rosenthal, A., de Sauvage, F. J., and Shivdasani, R. A. (2001). Downregulation of Hedgehog signaling is required for organogenesis of the small intestine in Xenopus. *Dev Biol* **229**, 188-202.

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Education:

College of William and Mary, Williamsburg, Virginia USA	B.S.	1982-1986	Biology
University of California Santa Cruz, California USA	Ph.D.	1989-1995	Biology
University of California Santa Cruz, California USA	Post-doc.	1995-1998	Biology
University of Colorado Denver, Colorado USA	Post-doc.	1999	Physiology and Biophysics

Research and professional experience:

10/86-7/88 **Biologist** - National Biomedical Research Foundation - Protein Information Resource (NBRF-PIR).3900 Reservoir Rd., N.W., Washington, D.C., 20007.
The NBRF-PIR Protein Sequence Database is an internationally used bioinformatics resource. Research directed toward evolutionary and functional characterization of proteins and development of advanced sequence analysis protocols.

7/88-8/89 **Research Scientist** - National Biomedical Research Foundation - Protein Information Resource (NBRF-PIR).

9/89-6/95 **Graduate Researcher** - University of California. Department of Biology. Santa Cruz, CA 95064. Laboratory of Dr. Jerry Feldman
Molecular genetics and evolution of circadian (daily) rhythms in the filamentous fungus *Neurospora crassa.*.

9/89-6/95 **Teaching Assistant** - University of California. Department of Biology. Santa Cruz, CA 95064.
In: Genetics (5 quarters), Howard Hughes Summer Institute for Molecular Biology (5 quarters), Genetics laboratory, Cell Biology, Neurobiology, and Virology.

7/95-12/98 **Post Graduate Researcher** - University of California, Santa Cruz CA 95064. Laboratory of Dr. Charles Daniel.
Molecular genetics of mammary gland development and breast cancer in mouse and human. Focus on the function of the *hedgehog* signal transduction network and homeobox genes.

1/99-6/99 **Postdoctoral Research Associate** - University of Colorado School of Medicine, Denver CO 80262. Department of Physiology and Biophysics. Laboratory of Dr. Peggy Neville.
Research description as above.

6/99-6/01 **Instructor** - University of Colorado School of Medicine, Denver CO 80262. Department of Physiology and Biophysics.
Research description as above.

7/01-present Assistant Professor - Baylor College of Medicine Breast Center and the Department of Molecular and Cellular Biology. Houston TX 77030
Research description as above.

Awards, Professional Activities and Honors:
Sigma Xi - Charter member, Santa Cruz chapter

Invited Talks: Genentech Inc. (7/98); Lawrence Berkeley National Laboratory (8/98); University of Miami School of Medicine (8/98); Gordon Research Conference on Mammary Gland Biology (6/99); National Cancer Institute (11/99); University of Colorado Health Sciences Center (3/00); Baylor Breast Center (8/00)

Completed and Ongoing Funded Research (within last 5 years):

ACTIVE

Lewis, M.T.

BC990641 - U.S. Department of the Army
Title: Hedgehog signal transduction inhibitors in breast cancer treatment and prevention.

Specific Aims:

1. To determine whether activation of hedgehog signaling can lead to mammary lesions in transgenic mice using an activated form of the *Smoothened (Smo)* receptor subunit.
2. To test the *in vivo* effect of hedgehog signaling inhibitors on hedgehog network caused mammary lesions.
3. To test the *in vivo* effect of hedgehog signaling inhibiors on independent lesions presumably not caused by mutation in hedgehog network genes.
4. To test the *in vitro* effect of hedgehog inhibitors on human breast cancer cell lines.

Neville, M.C.

NIH Proposal PO1-HD38129 (Ongoing)
Program Project Grant. Program Director M.C. Neville
Functional Development of the Mammary Gland

The major goals of this project are to determine the molecular and genetic mechanisms leading to functional differentiation of the mouse mammary gland during the transitions from pregnancy to lactation and from lactation to involution.

Steven Anderson

1-RO1-CA85736
National Cancer Institute
Induction of mammary cancer by signaling molecules

The major goals of this project are to determine whether constitutive activation of either the prolactin receptor or one of its downstream effectors (Akt) will contribute to neoplastic progression or developmental defects in the mouse mammary gland.

COMPLETED

Lewis, M.T.

2FB-0047 - University of California Breast Cancer Research Program Postdoctoral Fellowship (6/96-5/98)
Title: Homeobox Gene Expression in the Normal and Malignant Breast

Specific Aims:

1. Which homeobox genes are expressed in the human breast and in associated malignancies?
2. What is the pattern of expression of various homeogenes in the normal breast? Are breast cancers associated with altered levels of expression?
3. What cell types express homeogenes? What is the spatial pattern of expression in the normal and malignant breast?

4. Can associations be made between homeogene expression and parameters such as age, reproductive history, and endocrine status?

Daniel, C.W.

DAMD17-94-J-4230 - U.S. Department of the Army (1/95 - 12/98)
Title: Homeobox genes in the developing, preneoplastic and neoplastic Breast

Specific Aims:

1. Which homeobox genes are expressed in the normal and neoplastic mouse mammary gland?
2. Is human breast cancer associated with altered levels of expression?
3. Are homeobox genes regulated by mammogenic hormones and growth factors?
4. Does misexpression of homeobox genes lead to altered development or neoplasia?

RESEARCH ARTICLES:

Lewis, M.T., Ross, S., Strickland, P.A., Sugnet, C., Jimenez, E., Hui, C-c. and Daniel, C.W. (2001) The *Gli2* transcription factor is required for normal mouse mammary gland development. Dev. Biol. (In press)

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Appendices

Appendix 1. Curriculum vitae for Michael T. Lewis

Appendix 2. Curriculum vitae for Valerie S. Sawicki

Appendix 3. Lewis, M.T., Ross, S., Strickland, P.A., Sugnet, C.W., Jimenez, E., Scott, M.P. and Daniel, C.W. (1999) Defects in mouse mammary gland development caused by conditional haploinsufficiency of Patched-1. Development 126:5181-5193

Appendix 4. Lewis, M.T. (2001) Hedgehog signaling in mouse mammary gland development and neoplasia. J. Mammary Gland Biol. and Neoplasia 6:53-66

Appendix 5. Lewis, M.T., Ross, S., Strickland, P.A., Sugnet, C.W., Jimenez, E., Hui, C.C. and Daniel, C.W. (2001) The Gli2 transcription factor is required for normal mouse mammary gland development. Dev. Biol (In press).

With 9 figures.

Development 126, 5181-5193 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV4151

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

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Accepted 30 August; published on WWW 21 October 1999

SUMMARY

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

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

INTRODUCTION

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

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

Stimulated by ovarian hormones at puberty, the gland begins a proliferative phase of development, growing rapidly via the terminal end bud (TEB). The TEB is a bulb-like structure consisting of relatively undifferentiated epithelial cells at the tip

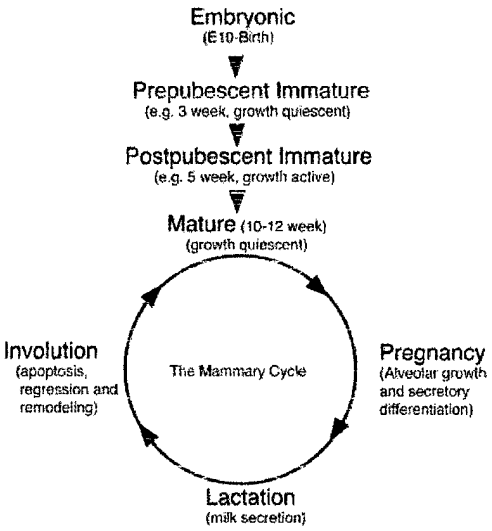

Fig. 1. Phases of mammary gland development. Proliferative development in virgin animals is represented by the linear portion of the diagram from embryonic day 10 (E10) through maturity. Cyclical development initiated by pregnancy is represented by the circular portion of the diagram.

of each growing duct, which invades and communicates with the fat pad stroma leaving differentiated ducts behind. In response to pregnancy, a cyclical phase of development is initiated in synchrony with the reproductive status of the animal. This cycle is characterized by growth and differentiation of secretory structures, lactation, and subsequent regression (involution) after weaning. At the end of involution, the morphology of the gland resembles that of the mature virgin animal.

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

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

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

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

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

Of the two known hedgehog receptors, *Ptc1* is most fully characterized. Animals homozygous for targeted disruption of *Ptc1* show early embryonic lethality (around embryonic day 9.5) with, among other alterations, severe defects in nervous system development accompanied by changes in neural cell

fates. Heterozygous animals can also show defects including skeletal abnormalities, failure of neural tube closure, medulloblastomas (brain tumors), rhabdomyosarcomas, and strain-dependent embryonic lethality (Goodrich et al., 1996; Hahn et al., 1998).

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

MATERIALS AND METHODS

Animals

The inbred mouse strains Balb/C and C57/Bl6 are maintained in our laboratory. C57/Bl6 × DBA2 F_1 (B6D2F1) female mice were obtained from Taconic. Athymic Balb/C *nu/nu (nude)* female mice were obtained from Simonson.

Two breeding pairs of mice heterozygous for a disrupted *Ptc1* gene were used to initiate a breeding colony and have been previously described (Goodrich et al., 1997). The original *Ptc1* mutation was maintained in a 129Sv:C57/Bl6 background with subsequent backcross to B6D2F1. In our laboratory, the mutation was likewise maintained in a B6D2F1 background by serial backcross but this background is still mixed (as evidenced by segregation of coat color markers) which precluded epithelial or whole mammary gland transplants between animals (see below). Genotyping was performed by PCR as per Goodrich (1997).

For expression studies (northern hybridization, and in situ hybridization) Balb/C animals were used to correlate results with expression of other genes in the hedgehog signaling network currently under study. In situ hybridizations for *Ptc-1* were replicated using C57/Bl6 mice to demonstrate consistency between strains (data not shown).

Developmental stages

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

mRNA isolation

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

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

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

Gene specific primers for *Dhh* (accession no. X76292), *Ihh* (accession no. U85610), *Shh* (accession no. X76290), *Ptc1* (accession no. AA080038) and *Ptc2* (accession no. AB000847) were designed to avoid highly conserved regions in either gene family. With the exception of those for *Ptc2*, primers were designed over introns to control for DNA contamination. The primer pairs used for this study are as follows: (*Dhh*) (sense) mDhhF1 5′-GACCTCGTA-CCCAACTACAACCCCG-3′, (antisense) mDhhR1 5′-ACG-TCGTTGACCAGCAGCGTCC-3′, (*Ihh*) (sense) mIhhF4 5′-CAAGCTCGTGCCTCTTGCCTACAAG-3′, (antisense) mIhhR3 5′-GCACATCACTGAAGGTGGGGGTCC-3′, (*Shh*) (sense) mShhF1 5′-TCCGAACGATTTAAGGAACTCACCC-3′, (antisense) mShhR1 5′-GGCTCCAGCGTCTCGATCACGTAG-3′, (*Ptc1*) (sense) m*Ptc1*F2 5′-GTCTTGGGGGTTCTCAATGGACTGG-3′, (antisense) m*Ptc1*R2 5′-ATGGCGGTGGACGTTGGGTTCC-3′, (*Ptc2*) (sense) mptc2F1 5′-GTGTGATCCTCACCCCGCTTGACTG-3′, (antisense) mptc2R1 5′-TCGCTCCAGCCGATGTCATGTGTC-3′

Specificity of the hedgehog family RT-PCR was confirmed by Southern hybridization of the reaction products according to standard techniques (Sambrook et al., 1989) using digoxigenin-11-dUTP-labeled plasmid-derived probes (Boehringer Mannheim, Genius System) for each of the cloned genes.

Northern hybridizations

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

In situ hybridization

The no. 2 and no. 3 mammary glands of Balb/C mice were used. Experiments using the *Ptc1* probes were also repeated using glands of C57/Bl6 mice to ensure consistency between strains. Glands were fixed in ice-cold 4% paraformaldehyde:PBS for 3 hours and processed for in situ hybridization (Friedmann and Daniel, 1996). Digoxigenin-labeled riboprobes for *Ptc1*, *Shh* and *Ihh* corresponded to the same cDNA fragments used in the Southern and northern hybridizations and were prepared using T7 and SP6 RNA polymerases and hybridized essentially as described (Friedmann and Daniel, 1996). The following stages were not examined: 3 week, 7 week.

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

Whole gland morphological analysis

Backcross-derived *Ptc1* heterozygotes and wild-type littermate or age matched females were used. B6D2F1 animals were also examined as controls. Mammary glands 1-5 were harvested at various developmental stages (at least 5 mice each stage), fixed in ice-cold 4% paraformaldehyde:PBS, and hematoxylin stained as described by

Daniel et al. (1989). Each gland was examined for developmental abnormalities under a dissecting scope.

Histological analysis

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

Hormone injection studies

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

Whole mammary gland transplantation studies

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

Epithelial transplantation studies

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

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

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

Since the genetic background of this strain is still mixed, reciprocal epithelial transplantaion between *Ptc-1* heterozygotes and wild-type littermates could not be performed due to histoincompatibility.

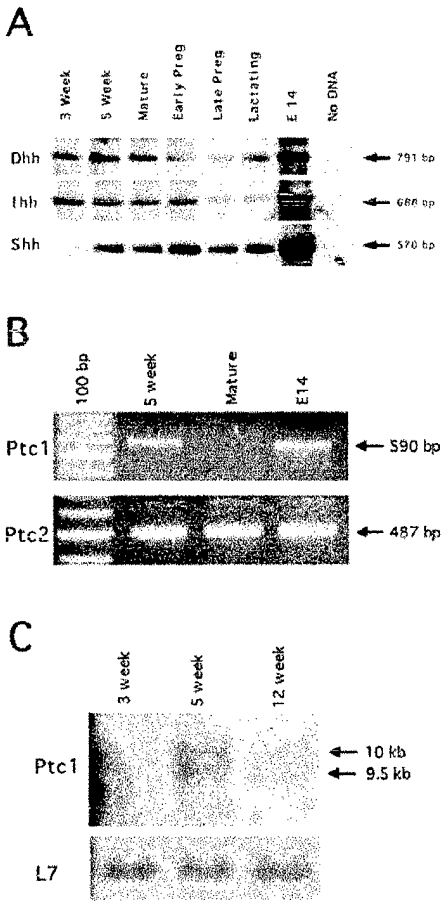

Fig. 2. Hedgehog network component expression – non-quantitative RT-PCR and northern blot hybridization. (A) Hedgehog gene expression detected by Southern blot hybridization of products from RT-PCR at different stages of mammary development. Each hybridizing band was of the expected size for each of the gene-specific primer pairs, as shown. (B) *Ptc1* and *Ptc2* gene expression detected by RT-PCR at selected stages of mammary development. Panels depict ethidium bromide-stained agarose gel separations of RT-PCR products. *Ptc1* products using RNA derived from mature animals were generally not observed but were detected in replicate experiments. Variable detection of an amplified band in 12-week samples is consistent with the reduction in *Ptc1* mRNA observed by northern hybridization relative to 5-week samples (below) but suggests that further optimization of the reaction conditions is required for consistent visible detection of this amplicon. (C) Northern blot hybridization for *Ptc1* expression during proliferative development. Transcript sizes are noted at the right side for *Ptc1*. Even loading of RNA for each sample was confirmed by hybridization with a probe for the L7 ribosomal protein mRNA.

RESULTS

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

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

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

mammary proliferative development, we performed developmental Northern hybridization (Fig. 2C). At least two transcripts of the expected sizes (9.5 kb and 10.0 kb) were readily detected in RNA from glands of 5-week old animals, a proliferative stage characterized by both rapid ductal growth and differentiation of epithelial and stromal elements. By contrast, expression was reduced in prepubescent glands of 3-week animals and mature glands of 12-week virgin animals. Data suggested that *Ptc1* expression is developmentally regulated.

Ptc1 is differentially expressed in mammary epithelial cell types

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

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

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

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

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

Ihh expression is enhanced during pregnancy and lactation

Ptc1 appears to be a universal target for transcriptional up-regulation in response to hedgehog signaling (Hammerschmidt et al., 1997). Enhanced expression of *Ptc-1* during pregnancy and lactation coupled with the timing of phenotypic reversion during these developmental stages (see below) suggested that there may be fundamental differences in hedgehog signaling status between virgin, pregnant and lactating states. To address this possibility, we performed in situ hybridization with probes for *Shh* and *Ihh* through mammary gland development.

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

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

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

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

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

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

In situ hybridization demonstrated that *Ptc1* expression was both spatially and temporally regulated during mammary development, suggesting a functional role. To determine whether or not disruption of the *Ptc1* gene resulted in developmental defects in the mammary gland, glands were examined at several stages of development. No alterations were observed in overall patterning of the mammary tree at 3 weeks

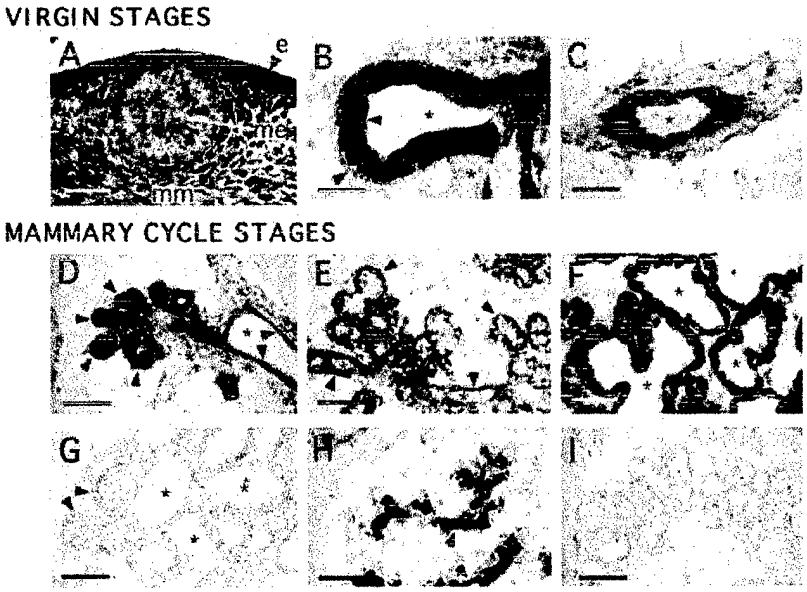

Fig. 3. In situ hybridization of *Ptc1* during embryonic and postnatal mammary development. Expression is detected by the accumulation of a blue-black precipitate. Selected lumenal spaces are denoted by red asterisks. (A) Embryonic day-14 mammary bud. Me, mammary epithelium; mm, mammary mesenchyme; e, epidermis. Bar, 80 μm. (B) 5-week terminal end bud. A red arrowhead indicates the body cell layer; a black arrowhead indicates the cap cell layer. A black asterisk indicates expression in periductal stroma. Bar, 240 μm. (C) Duct of 10-week mature gland. Lumenal epithelial cells stain darkly. A black asterisk indicates expression in periductal stroma. Bar, 100 μm. (D) Midpregnancy developing lobule. Expression in lumenal ductal epithelium (black arrowheads) is reduced relative to expression in developing lobule-alveolar structures (red arrowheads). Region of periductal expression is indicated by a black asterisk. Bar, 240 μm. (E) Late pregnancy lobule-alveolar structures and associated duct. Notations as for D. Bar, 200 μm. (F) Lactation. Expression is uniformly elevated in lumenal epithelial cells of alveoli. Bar, 80 μm. (G) Two days involution. Red arrowheads indicate selected alveoli. Bar, 200 μm. Note lack of staining. (H) 10 days involution. Partially remodeled ducts (black arrowheads) and alveolar structures regain *Ptc1* expression. Limited stromal expression can be detected at this stage but becomes readily detectable at 14 days involution (data not shown). Bar, 200 μm. (I) Sense control hybridization showing no hybridization signal. Late pregnancy. Panel is representative of control hybridizations at all stages of development. Bar, 200 μm.

of age (data not shown). At 5 weeks of age, terminal end buds in wild-type animals appeared normal in whole-mount preparations of glands (Fig. 5A), whereas up to approximately 30% of terminal end buds in heterozygous animals appeared misshapen or disrupted (Fig. 5B). These morphological changes do not lead to overt patterning defects, in that disruption of TEB at 5 weeks did not lead to alterations in ductal patterning in adult animals at 10 weeks of age. No morphological distinctions could be made between wild-type (Fig. 5C) and heterozygous (Fig. 5D) glands.

The small morphological changes belie dramatic changes in the properties of the tissues. Histological analysis revealed ductal dysplasias and hyperplasias in 100% of heterozygous animals by 5 weeks of age. While not apparent in glands taken from 3-week old wild-type and heterozygous animals (Fig. 6A and 6B, respectively), severe histological abnormalities were observed at 5-weeks of age when compared with wild-type controls (Fig. 6C versus 6D). In some ducts, the multilayered lumenal epithelial cells (body cells) of the TEB failed to thin to a monolayer as the subtending duct was established and, in

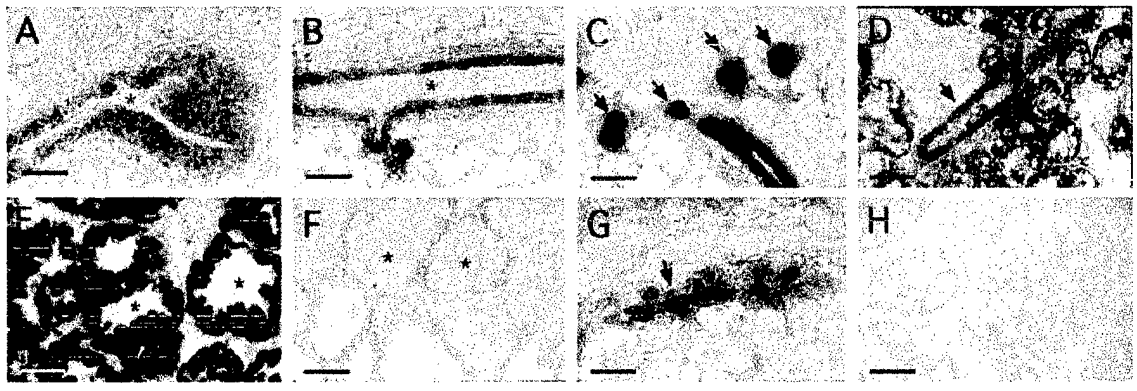

Fig. 4. In situ hybridization of *Ihh* during postnatal mammary development. Expression is detected by the accumulation of a blue-black precipitate. Red asterisks denote selected lumenal spaces. (A) 5-week terminal end bud showing body cell expression. (B) Duct of a 12-week mature gland with a sidebranch. Lumenal epithelial cells stain weakly. (C) Developing alveoli (black arrows) and associated duct during early pregnancy showing easily detected epithelial expression. (D) Lobule-alveolar structures and associated duct (black arrow) during late pregnancy showing uniformly enhanced expression of *Ihh* mRNA. (E) Lactation. expression is uniformly elevated in lumenal epithelial cells of alveoli. (F) Two days involution. *Ihh* expression is undetectable. (G) 14 days involution. Partially remodeled epithelium (black arrow) regains *Ihh* expression. (H) Sense control hybridization showing no hybridization signal. Late pregnancy. Panel is representative of control hybridizations at all stages of development. Bars, 80 μm.

many cases, the lumenal space was completely occluded by epithelial cells (Fig. 6D). Condensation of the periductal stroma around the neck of the TEB appeared altered in some cases such that adipocytes were included within the condensate and condensation appeared to occur at an unusual distance away from the duct (Fig. 6D). At higher magnification, body cells of wild-type end buds appear well ordered and cap cells form a distinct, organized layer as they differentiate into myoepithelial cells (Fig. 6E). By contrast in some endbuds of heterozygous animals, body cells were disordered (Fig. 6F) and the cap cell layer was visibly altered (Fig. 6F).

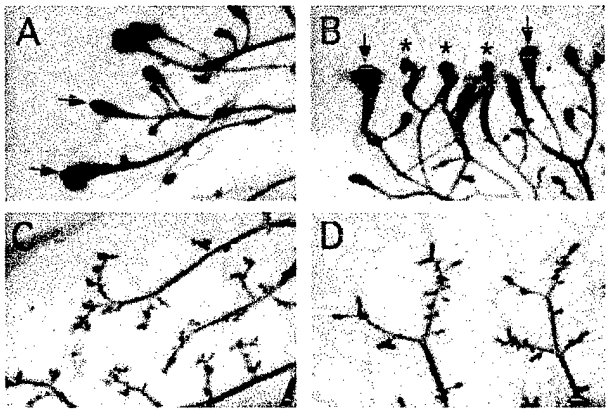

Fig. 5. Whole gland morphological analysis during proliferative development in virgin animals. (A) Wild-type, 5-weeks old. Terminal end buds (black arrows) and subtending ducts appear well formed. (B) *Ptc1* heterozygote, 5-weeks old. TEB are generally normal (black arrows) but a subset of TEBs are clearly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

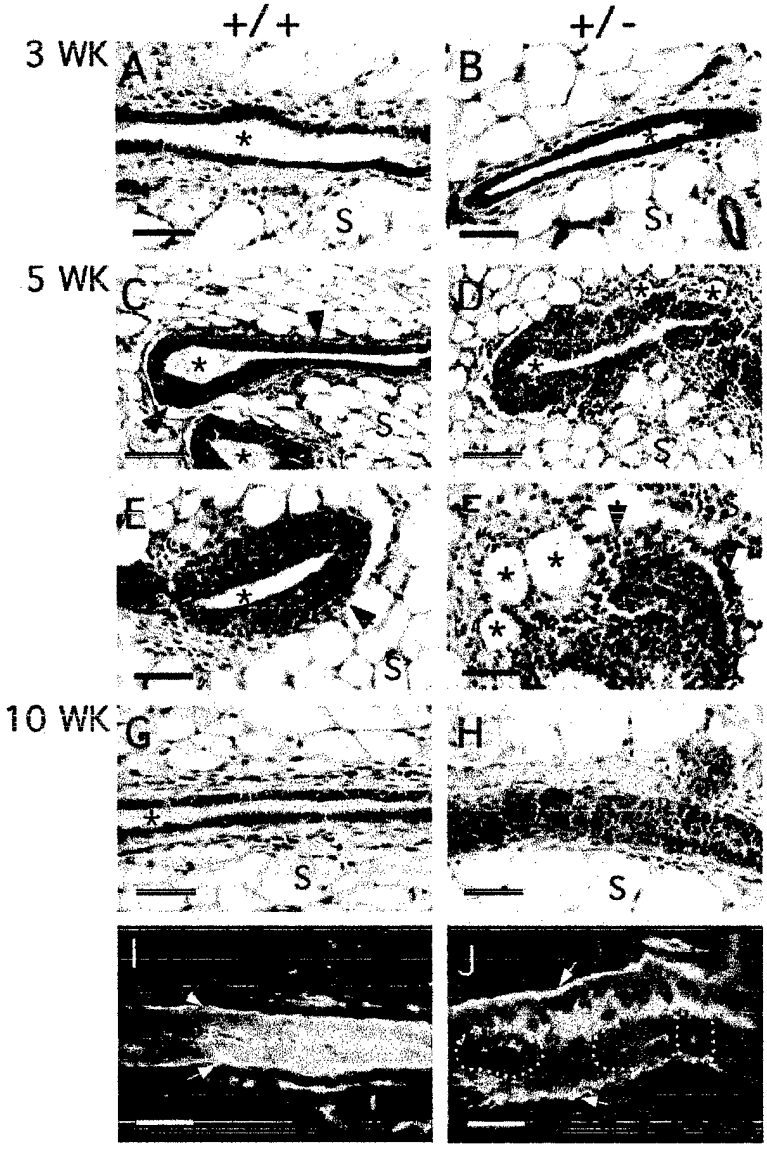

Fig. 6. Histological comparison of glands during development in virgin animals. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column. A-H are stained with hematoxylin and eosin; I-J are stained with phalloidin (yellow-green, actin) and propidium iodide (red, nuclei). Red asterisks denote ductal lumens; a red letter 's', adipose stroma. (A) Longitudinal section through a mammary duct. Lumenal epithelium is generally a monolayer of darkly staining cells surrounding the ductal lumen. Eosinophilic (pink) periductal stroma adjoins the duct and consists mainly of fibroblasts. Bar, 80 μm (B) Mammary duct which is indistinguishable from its normal counterpart. Bar, 80 μm. (C) Terminal end bud with characteristic body cell layer composed of 3-6 layers of epithelial cells thinning to a monolayer surrounding a well-defined lumen in the subtending duct (red arrowhead). A thin, uniform layer of condensing periductal stroma is shown at the neck of the TEB and along the duct. Bar, 200 μm. (D) Terminal end bud. Body cell layer fails to thin to a monolayer in the subtending duct (red arrowhead) resulting in ductal occlusion. Stromal condensation may occur at unusual distances from the TEB and can also appear disrupted with the inclusion of adipocytes within the condensate (black asterisks). Bar, 200 μm. (E) Terminal end bud at increased magnification. Body cell layer appears well ordered, surrounded by a well-defined monolayer of cap cells (black arrow). Bar, 80 μm. (F) Terminal end bud at increased magnification. Body cell layer appears less well organized with a clearly disrupted cap cell layer (black arrows). Note the unusual inclusion of adipocytes (black asterisks) within the condensed stroma at the tip of this end bud. Bar, 80 μm (G) Normal mammary duct. Bar, 80 μm. (H) Severely affected mammary duct showing complete occlusion by epithelial cells. Bar, 80 μm. (I) Normal mammary duct. Lumen is denoted by a white asterisk. A uniform layer of myoepithelial cells is identifiable (white arrows) as a line of yellow cells lining the outer surface of the duct. Bar, 80 μm. (J) Severely affected mammary duct showing complete occlusion by epithelial cells. The myoepithelial cell layer (white arrows) appears unaffected. Clusters of epithelial cells which form microlumens within the ducts can be identified (circled by white dots) with inappropriate actin localization at the microlumenal surface. Bar, 80 μm.

The ductal defects observed at 5 weeks of age become more pronounced when animals reach 10 weeks. Whereas wild-type ducts have a clear lumen within a monolayer of lumenal epithelial cells (Fig. 6G), a majority of ducts in glands from heterozygous animals are partially or completely filled with loosely associated epithelial cells, presumably arising by an alteration in cell-cell adhesion within the ductal wall (Fig. 6H). Examination of serial sections through entire ducts showed some areas appearing relatively unaffected (data not shown). Cells in occluded ducts are not uniform with respect to nuclear morphology and can include large cells with round nuclei and clear cytoplasm suggesting that multiple epithelial subtypes contribute to the dysplasias (Chepko and Smith, 1997; Smith, 1996).

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

Ptc1-induced dysplasias are reversible during pregancy and lactation

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

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

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

Ptc1-induced dysplasias reflect intrinsic defects in mammary gland development

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

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

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

We next wished to determine whether the *Ptc1*-induced dysplasias reflect an intrinsic defect in the epithelium or whether there may be a stromal function as well. In addition, with respect

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

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

DISCUSSION

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

Pattern formation is genetically separable from ductal morphogenesis

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

Novel aspects of the *Ptc1* phenotype

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

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

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

Possible mechanisms underlying the *Ptc-1* phenotype

Given that many genes under hedgehog network control in other organs are known to function in the mammary gland, it is possible that no single downstream alteration is solely responsible for the phenotypes observed and that they may,

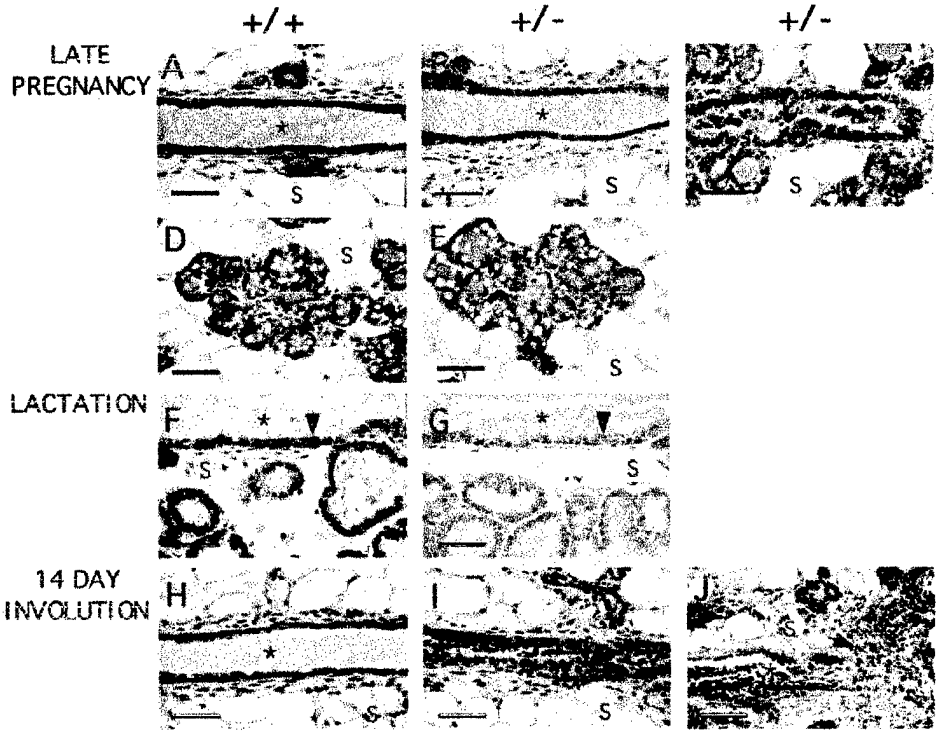

Fig. 7. Histological analysis of glands during the mammary cycle. Developmental stages are shown on the left. Genotype of the animal from which the glands are derived is shown at the top of each column of panels. Red asterisks denote ductal lumens; a red letter 's', adipose stroma. (A) Mammary duct.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

instead, be the cumulative result of relatively minor alterations in multiple cellular functions.

At least one mechanism underlying the *Ptc1* phenotype is suggested by the disorganization of the ductal cells. Proper ductal morphogenesis requires that cell-cell and cell-substrate adhesion systems be coordinated spatially and temporally with epithelial differentiation and apoptosis. Among many other candidates including integrins and laminins, P-cadherin emerges as a strong candidate for a cell adhesion molecule that may be influenced by *Ptc1* disruption. P-cadherin is primarily localized in the cap cell layer of the end bud and in differentiated myoepithelial cells in mature ducts. Disruption of P-cadherin function by antibodies delivered to the gland via slow-release plastic implants, caused disorganization of the end bud and impaction of the duct with dissociated cells, an effect reversible upon depletion of the antibody (Daniel et al., 1995). Further, a phenotype similar to *Ptc1* has been reported for animals homozygous for a targeted disruption of P-cadherin. In this mutant, loosely associated epithelial cells aggregating or floating within the lumen and excessive alveolar development have been observed (Radice et al., 1997). Preliminary immunohistochemical analysis of both E- and P-cadherin using wild-type and *Ptc1* heterozygous animals at 10 weeks postpartum thus far shows no consistent alterations in E-cadherin or P-cadherin expression (data not shown) but suggest that P-cadherin levels may be reduced in some animals. Since the *Ptc1* heterozygous phenotype is more severe than, and distinct from, that of P-cadherin loss-of-function, reduction in P-cadherin levels cannot fully account for the formation of *Ptc1*-induced dysplasias.

Impaired apoptosis or elevated frequency of cell division could also contribute to the *Ptc1* phenotype (Humphreys et al., 1996). Preliminary analyses of TUNEL apoptosis and BrdU incorporation assays using tissue derived from wild-type and *Ptc-1* heterozygous animals at 10 weeks postpartum do not show consistent alterations in labeling between the two genotypes.

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

In our experiments, severely affected epithelium from donor

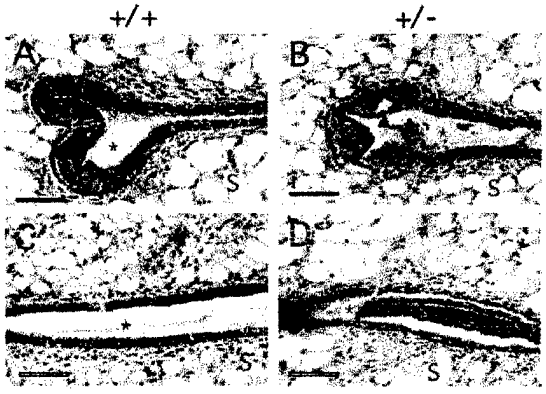

Fig. 8. Whole mammary gland transplant into nude mice. Genotype of the transplanted mammary gland is noted above the panel columns to which they refer. Red asterisks denote lumenal spaces; a red letter 's', adipose stroma. (A) Terminal end bud showing well-ordered cap and body cell layers. (B) Terminal end bud showing disrupted body cell layer with a prominent microlumen (black arrowhead). Cap cell layer disruption is also apparent. (C) Mammary duct showing a monolayer of lumenal epithelial cells along the duct wall and a clear lumen. (D) Mammary duct showing localized cellular impaction characteristic of intact *Ptc1* heterozygotes at comparable age. Bars, 80 μm.

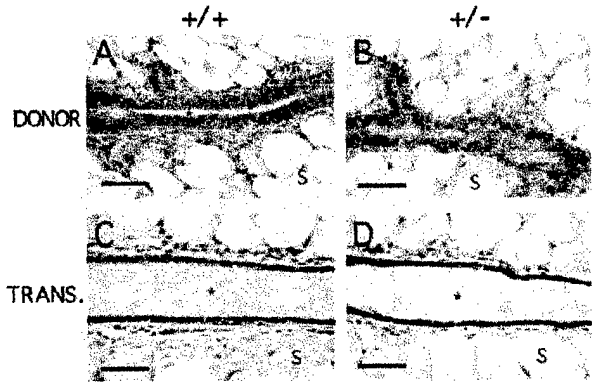

Fig. 9. Epithelial transplantation into cleared fat pads. Tissue source is noted on the left; genotype of the epithelium is noted above the panel columns to which they refer. Red asterisks denote lumenal spaces; a red letter 's', adipose stroma. (A) Mammary duct showing an open lumen and monolayer of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

animals underwent overtly normal differentiation and morphogenesis when it repopulated the recipient cleared fat pad. These results, coupled with the whole mammary gland transplant data showing partial recapitulation of the mutant phenotype, suggest that wild-type *Ptc1* function is required either in both the stroma and epithelium or in the stroma only during virgin stages of development. The possibility of both epithelial and stromal functions for *Ptc1* is consistent with its own expression pattern and with the expression patterns of both *Gli2* and *Ihh* which have developmentally regulated and cell type-specific mRNA expression in the mammary gland. *Gli2* is expressed exclusively in the periductal stroma through virgin stages of development but becomes both epithelial and stromal during pregnancy and lactation (M. T. L. and C. W. D., unpublished). In contrast, *Ihh* is expressed exclusively in the epithelium throughout development and is low during virgin stages but appears elevated during pregnancy and lactation (Fig. 4). Further, phenotypic and transplantation analysis of a targetted disruption strain of *Gli2* confirms that its function is also required for proper virgin mammary gland development (M. T. L. and C. W. D., unpublished). Together, these data support a general role for hedgehog signaling, and for *Ptc1*, *Ihh* and *Gli2* specifically, in mediating tissue interactions during mammary gland development.

A working model for hedgehog signaling in the mammary gland

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

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

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

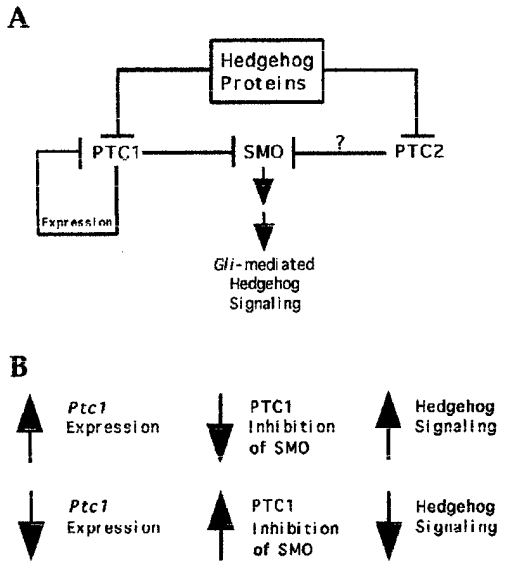

Fig. 10. Summary of a portion of the model for hedgehog signal transduction in mammals. (A) Schematic of functional interactions among the hedgehog proteins, PTC1, PTC2 and SMO leading to GLI-mediated control of target gene expression. (B) A generalized summary of *Ptc1* gene expression relative to protein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

Is *Ptc1* a mammary tumor suppressor gene?

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

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

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

These epithelial transplantation data could also serve as an in vivo correlate to in vitro observations in which tumor cells could be phenotypically reverted by altering their interaction with extracellular matrix components (Schmeichel et al., 1998; Sun et al., 1998; Weaver et al., 1997). In one set of experiments (Weaver et al., 1997) tumor cells were maintained in three-dimensional cultures to closely mimic the in vivo state and treated with function-blocking antibodies to β-1 integrin. Cells treated in such a way showed phenotypic reversion toward that of normal cells. These experiments further demonstrate the plasticity of mammary epithelium and that the microenvironment strongly influences the phenotypic behavior and is capable of overriding the genotype of the cell.

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

This work was supported by a breast cancer research grant from the US 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 Lisa Goodrich and Ljiljana Milenkovic of the Scott laboratory for generously supplying animals and for helpful discussions. We thank Dr Gary Silberstein, Kathy van Horn, Dr Eva Robinson and Dr Margaret Neville for critical reading of the manuscript and editorial assistance.

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Journal of Mammary Gland Biology and Neoplasia, Vol. 6, No. 1, 2001

Hedgehog Signaling in Mouse Mammary Gland Development and Neoplasia

Michael T. Lewis[1]

Genetic analyses of two hedgehog signal transduction network genes, *Patched-1* and *Gli2*, has demonstrated a critical role for hedgehog signaling in mediating epithelial-stromal tissue interactions during ductal development. Disruption of either gene leads to similar, yet distinct, defects in ductal morphogenesis. Defects are mainly ductal dysplasias that closely resemble some hyperplasias of the human breast. Phenotypic analyses have been coupled with *in situ* hybridization, transplantation and tissue recombination analyses to formulate a model for tissue compartment-specific control of mouse mammary gland development by hedgehog signaling. In addition, the similarities among hedgehog mutation-induced ductal dysplasias and human breast pathologies suggest a role for altered hedgehog signaling in the development of mammary cancer.

KEY WORDS: Tissue interactions; organogenesis; breast cancer, oncogene, tumor suppressor.

INTRODUCTION

Mammary gland development, like that of many organs, requires interactions between an epithelium and a surrounding mesenchyme (embryonic) or stroma (postnatal–including the extracellular matrix) (1–3) and between epithelial cells themselves (4). These tissue interactions are dynamic, reciprocal and tightly coordinated with the reproductive status of the animal in order to control growth, patterning, and gland function (5,6). In addition to these traditional "mammary" cell types, the concept of tissue interactions in the mammary gland can be extended to include "nonmammary" cell types such as those of the vascular and immune systems, both of which have been demonstrated to contribute to mammary gland development and function (7–13).

Several classes of genes have now been implicated in mediating mammary tissue interactions during normal development [for general reviews see (14–16)]. Among the gene classes identified thus far are those encoding growth factors, hormone receptors, proteinases and their inhibitors, cell adhesion proteins, and transcription factors. With the advent of the mouse as an efficient genetic model system, *in vivo* analyses of how individual genes within these classes function in the context of an intact mammary gland are now possible. The observation that many of these genes function in the stroma (or in both stroma and epithelium) to direct or modulate the behavior of lumenal mammary epithelial cells has highlighted the need to understand the full nature of tissue interactions in the gland and the need to determine how these interactions are coordinated to direct organotypic development (17–22).

In addition to the roles of epithelial-stromal tissue interactions in normal mammary gland development, there is growing recognition of a role for the mammary stroma in regulating the behavior of neoplastic epithelial cells in breast cancer progression (23–25). The recognition that these types of interactions exist is particularly important in light of the fact that many studies of breast cancer cells have been performed in cell culture in the absence of whatever epithelial-stromal interactions there might have been in the original tumor. Depending on the types

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1083-3021/01/0100-0053$19.50/0

of questions being asked concerning the behavior of the epithelial cells themselves, the possible influence of tissue interactions in their original environment *in vivo* must be taken into account.

Recently, the hedgehog signal transduction network was established as an important signaling system in mediation of epithelial-stromal interactions during normal mammary gland development (18). Genetic analyses of two hedgehog signal transduction network genes, *Patched-1* (*Ptc1*) and *Gli2* has shown that disruption of either gene leads to similar, yet distinct, defects in ductal morphogenesis. Because of the similarities noted between *Ptc1* or *Gli2*-induced dysplasias and some breast pathologies in humans, there is growing suspicion that the hedgehog network may also play a role in neoplastic progression.

AN OVERVIEW OF HEDGEHOG SIGNALING: FROM FLIES TO MICE

Genetic studies in the fruit fly *Drosophila melanogaster* first identified the hedgehog signal transduction network as a critical determinant of cell fate and cell identity. The network was shown to function by mediating cell-cell communication to establish and maintain, among others, anterior-posterior cell identity as well as to direct wing vein and bristle patterning. Shortly thereafter it was shown that the hedgehog signal transduction network was conserved and elaborated upon in mammals and other vertebrate species. During vertebrate embryogenesis and organogenesis, hedgehog network genes were often shown to be expressed in adjoining tissue compartments in organs and structures whose development requires inductive tissue interactions.

Determination of the genetic, molecular and biochemical organization of the hedgehog signal transduction network in any biological process is a work in progress. Similarly, the full range of target genes regulated by hedgehog signaling in a given process has yet to be determined. However, enough information is available from several different developmental model systems that detailed general models for hedgehog signaling are emerging. These models are complex but are generally consistent with one another and have been reviewed extensively (26–28).

Whereas the range of vertebrate developmental processes dependent on hedgehog signaling testifies to its critical importance, the mechanics of hedgehog signaling are best understood from genetic studies in the fruitfly *Drosophila melanogaster* (28,29). In flies, the signaling network consists of a single secreted hedgehog (HH)[2] protein which binds to a receptor subunit, patched (PTC), located in the membrane of nearby cells. In the absence of HH binding, PTC acts as a molecular brake to inhibit downstream signaling mediated by the smoothened (SMO) subunit of the hedgehog receptor also located in the membrane. Upon HH binding, PTC is inactivated allowing SMO to function. Exactly how SMO functions is unclear. However, a number of genes are known to be involved in regulating downstream events in the signaling process including *fused, suppressor of fused* (*Su(fu)*), *costal*-2, and *Slimb* (26). These events ultimately favor the conversion of a transcription factor, cubitus interruptus (CI) to a full-length activator form CI(act) over an alternative, cleaved repressor form CI(rep). These two different forms of CI, in turn, translocate from the cytoplasm to the nucleus to regulate expression of target genes that contribute to establishment of cell identity and to patterning of the fly body.

The mammalian hedgehog signal transduction network is considerably more elaborate with several of the *Drosophila* genes being duplicated to form multigene families (Fig. 1). Despite this increase in complexity, the mammalian network appears to act in a fashion similar to the system in flies. In general, one of three members of the hedgehog family of secreted signaling proteins (either *Sonic Hedgehog* (*Shh*), *Indian Hedgehog* (*Ihh*), and *Desert Hedgehog* (*Dhh*)) is produced by a given cell type in a given tissue compartment (the signaling cell). The hedgehog protein then acts as a ligand for a receptor complex located on the membrane of nearby cells, usually in a different tissue compartment (the responding cell). Availability of hedgehog ligands can be regulated by the activity of the *Hedgehog interacting protein* (*Hip*) gene which binds hedgehog proteins thereby preventing their interaction with the hedgehog receptor complex (30). As in *Drosophila*, it is also likely that hedgehog protein availability is modulated by the control of release from the signaling cell [via the activity of the *dispatched* (*disp*) gene] (31).

The mammalian hedgehog receptor complex consists of at least two transmembrane proteins, Smoothened (SMO) and either Patched-1 (PTC1) or Patched-2 (PTC2). As in flies, in the absence of a hedgehog ligand, PTC1 (and probably PTC2) acts as

[2] Abbreviations: Fibroblast growth factor (FGF); transforming growth factor-β (TGF-β); bone morphogenic protein (BMP); reverse transcriptase polymerase chain reaction (RT-PCR); hedgehog (HH); patched (PTC); smoothened (SMO); cubitus interruptus (CI).

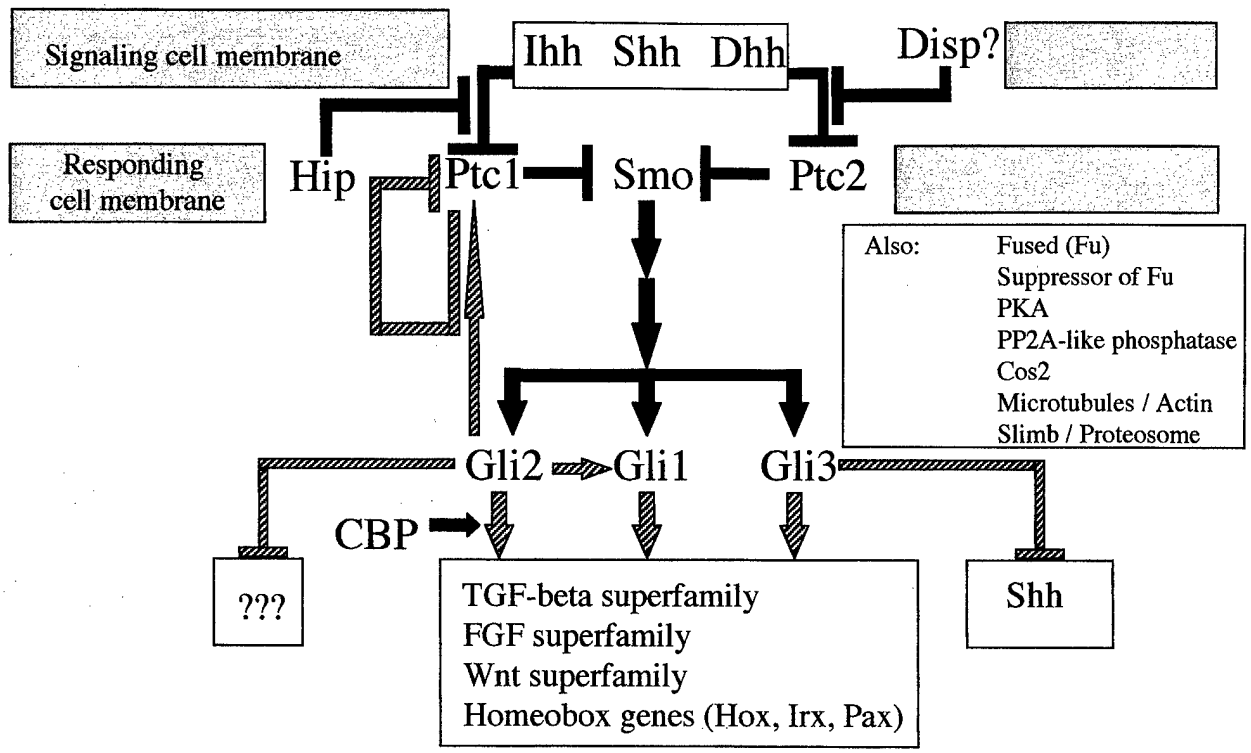

Fig. 1. Interactions among hedgehog signaling network components. Solid arrows and lines indicate protein activities; hatched arrows and lines indicate transcriptional regulatory activities. Additional proteins known to participate in modulating the hedgehog network are enclosed in boxes and are outlined by thin lines. Gene superfamilies known to be regulated by hedgehog signaling are shown below the *Gli* proteins.

an inhibitor of the SMO subunit and prevents downstream signaling. Upon hedgehog binding, inhibition by PTC1 is relieved allowing SMO to function. Ultimately, a series of downstream regulatory events similar to those observed in flies leads to the activation of one or more members of a family of transcription factors, *Gli1*, *Gli2* and *Gli3*, which are structurally and functionally related to the *Drosophila* CI transcription factor. While the full functional capabilities of GLI proteins remain unclear, upon hedgehog signaling, GLI proteins are modified and translocate from the cytoplasm to the nucleus to either activate or repress downstream target genes depending on the modified form of the GLI protein(s) produced (32–34).

Targeted disruption of *Gli1* (Δ*Gli1*) in mice led to no discernable phenotype in homozygous null mice (35). In contrast, homozygous mutation of either *Gli2* (Δ*Gli2*) (by targetted disruption) or *Gli3* (*Gli3*^{xt}) ("*extra toes*" allele; spontaneous mutation) led to perinatal lethality and a set of partially overlapping developmental defects (32–34). Current data suggest that the *Gli2* gene encodes a protein that acts primarily as a transcriptional activator while the *Gli3* gene encodes a protein that acts 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 (36,37). These data suggest that *Gli2* and *Gli3* are the primary mediators of hedgehog signaling and that each may encode proteins that possess the same range of functional capabilities as CI in *Drosophila*. (See Table I.)

WHY STUDY HEDGEHOG SIGNALING IN THE MAMMARY GLAND?

Several lines of evidence led Dr. Charles Daniel (University of California, Santa Cruz) to formulate the initial broad, but thoroughly testable, hypothesis that the hedgehog signal transduction network mediates tissue interactions during mammary gland development.

The four main lines of evidence are discussed as follows:

(i) Hedgehog signaling mediates tissue interactions during mammalian embryonic development and organogenesis.

Table I. Phenotype Analyses of Hedgehog Signaling Network Mutations in the Mouse Mammary Gland[a]

Gene	Mutation type	Mammary phenotype or project status	Mammary refs./General refs.
Shh	D	No overt mammary defects	G. Robinson, L. Hennighausen, personal communication see also (48,72–74)
Dhh	D	No overt mammary defects	(75)
Ihh	D	In progress	Lewis *et al.* (unpublished) see also (74,76)
Ptc1	D	Mammary ductal dysplasias Reversion of dysplasias in pregnancy and lactation	(18) See also (50)
	O	In progress	Lewis *et al.* (unpublished) see also (77,78)
Smo	O	In progress	Lewis *et al.* (unpublished) see also (45)
Gli1	D	No overt mammary defects detected	see (35)
Gli2	D	Mammary ductal dysplasias Delayed alveolar development	Lewis *et al.* (submitted) see also (79,80)
Gli3	D	In progress. No defects in mammary ductal development	Lewis *et al.* (unpublished) See also (81) and references therein.

[a] D: Disruption; O: Overexpression or activating mutation. Both mammary-specific and general references are provided.

(ii) In other mammalian organs, the hedgehog signaling network regulates, or is regulated by, genes with known functions in mammary gland development.
(iii) Hedgehog network genes act as oncogenes or tumor suppressor genes in several types of cancer.
(iv) In *Drosophila*, hedgehog signaling was shown to regulate expression and function of several homeobox genes. Mammalian homologs of some of these genes are known to regulate mammary gland development.

(i) Hedgehog signaling mediates tissue interactions during mammalian embryonic development and organogenesis. In mammals, the genes encoding the hedgehog family of secreted signaling proteins (*Sonic Hedgehog*, *Indian Hedgehog*, and *Desert Hedgehog* and associated signaling network components are important regulators of cellular identity, patterning, and tissue interactions during embryogenesis and organogenesis. As mentioned previously, these molecules are typically expressed in regions of inductive tissue interactions and are involved in diverse processes such as the development of skin, hair follicle, limbs, lung, eye, nervous system and tooth, the differentiation of cartilage and sperm, and the establishment of left-right asymmetry (Fig. 2) (28,29,38).

Given that the mammary gland requires tissue interactions similar to those required for the development of other organs, it was reasonable to suspect that the hedgehog signal transduction network might mediate such tissue interactions in the gland.

(ii) In other organs, the hedgehog signaling network regulates, or is regulated by, genes with known functions in mammary gland development. To exercise its control during vertebrate development, the hedgehog network regulates, or is a regulatory target of, a battery of gene families. Depending on the organ, these gene families include those encoding Fibroblast Growth Factors, WNT proteins (*Drosophila wingless* homologs), transforming growth factor-β (TGF-β) family members including TGF-β bone morphogenic proteins, activins and inhibins and (*Drosophila decapentaplegic* homologs), homeodomain transcription factors (including HOX, IRX, and PAX), and parathyroid hormone-related protein (PthRP) and its receptor PPR1 (Fig. 3) (26–28). Importantly, members of each of these gene families have known or suspected roles in mammary development or neoplastic progression (20,39–42).

At this point, it is unknown whether or not hedgehog network regulates, or is regulated by, any or all of these gene families in the mammary gland. However, given that the hedgehog network can interact with each of these mammotropic signaling networks, it is reasonable to predict that it does so in the mammary gland. Should this prediction be correct for at least some of the mammatropic signaling networks, it is possible that the hedgehog network could serve a type of signal integration function to direct organotypic responses.

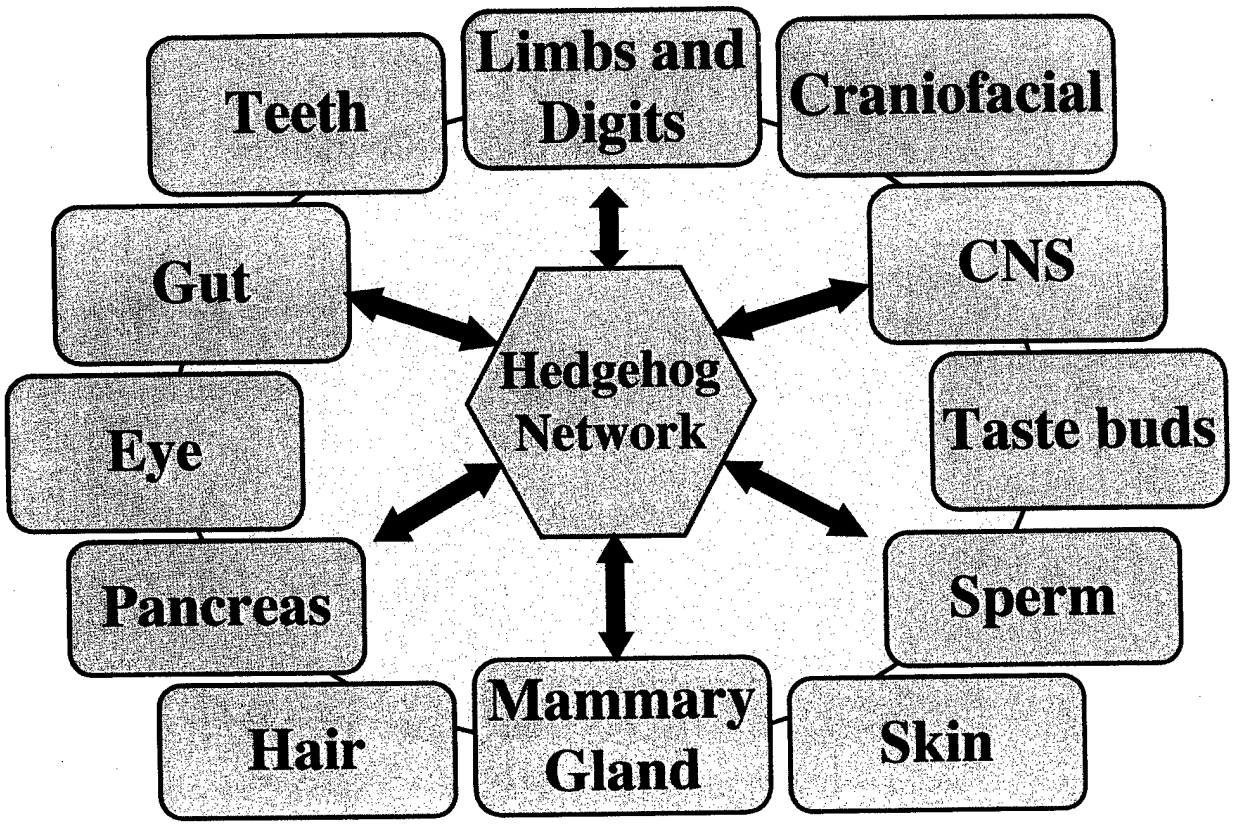

Fig. 2. Selected examples of organs and structures whose development is directed, in part, by hedgehog signaling.

(iii) Hedgehog network genes act as oncogenes or tumor suppressor genes in several types of cancer. Several of the genes in the mammalian hedgehog signaling network have been identified as either protooncogenes or tumor supressor genes. A number of these genes, including *Ptc1*, *Smo*, *Shh*, *Gli1*, and *Gli2* can contribute to the development of skin cancers, most notably basal cell carcinomas (43–49). *Ptc1* has also been causally implicated in the development of medulloblastomas (brain tumors) and other soft tissue tumors (50,51). *Gli1* was originally identified as an amplified gene in human glioblastomas (brain tumors) and amplification has since been observed in other tumor types (46,52,53).

Given that the mammary gland is a skin derivative, a connection between skin cancer and breast cancer was naturally suspected. Until recently, inquiry into the possible role for hedgehog signaling in breast cancer was limited to searches for known mutations in *Shh* and *Ptc1* that lead to basal cell caricinoma. No evidence was found for mutations in *Shh* in the breast tumor samples examined (54,55). However, in one small study, mutations in *Ptc1* were identified in 2 of 7 (~29%) human breast cancers (56). The significance of this finding was (and is) unclear since no general role for the hedgehog network had been established in the mammary gland, nor had the tumorigenic potential for altered network function in the mammary gland been explored.

(vi) In *Drosophila*, hedgehog signaling was shown to regulate expression and function of several homeobox genes. Mammalian homologs of some of these genes are known to regulate mammary gland development. Investigation of the role of homeobox genes in mammary gland development and neoplasia resulted in the identification and cloning of a novel family of homeobox genes that are expressed in the human breast (42). Regulated expression of one family member, later designated *IRX*-2, was demonstrated through human mammary gland development and evidence of misregulation was found in a subset of primary human breast cancers. This family of genes was

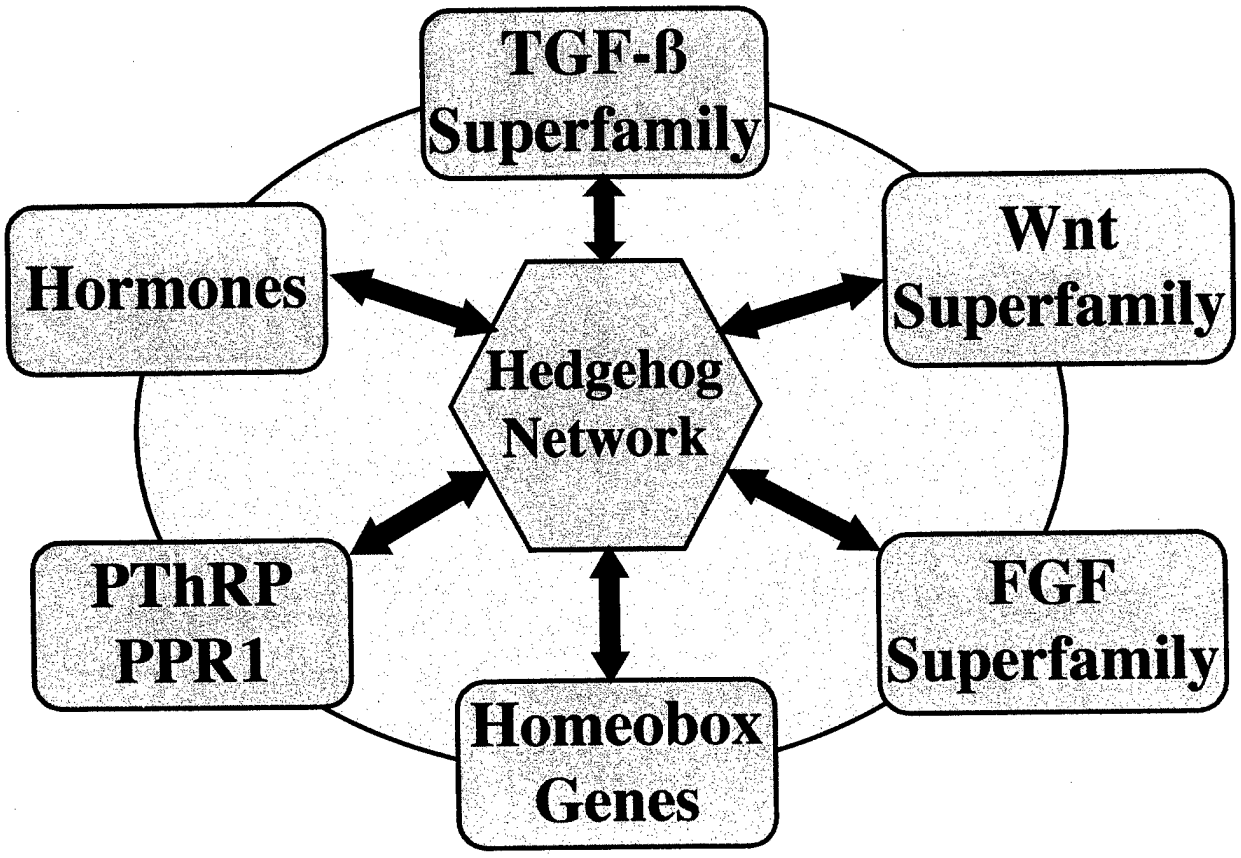

Fig. 3. Gene superfamilies and regulatory molecules active in other organs that either regulate, or are regulated by, the hedgehog signal transduction network. Each of the regulatory systems shown have known or suspected roles in mammary gland development.

designated *IRX* based on their most closely related homologs in *Drosophila*, the *Iroquois (Iro)* family.

In flies, the *Iroquois* family genes *araucan (ara), caupolican (caup)* and *mirror (mrr)* are important determinants of patterning and cell identity (57,58). It was shown by elegant genetic analyses, that spatial regulation of *ara* and *caup* expression in the wing imaginal disk was under control of *hedgehog* in conjunction with *decapentaplegic*, a *Drosophila* homolog of TGFβ.(57). These data suggest that the mammalian *IRX* genes might be under hedgehog control in the mammary gland.

In addition to this potential relationship, hedgehog signaling in flies and vertebrates is known to regulate the function of other homeobox genes, particularly the vertebrate *Hoxd* genes during limb development. Recently, two *Hoxd* genes have been shown to affect alveolar development and lactation in mouse knockout models (*Hoxd-9 and Hoxd-10*) (59,60). Again, the recognition that many developmental regulatory networks are, with modification, conserved between flies and vertebrates suggests that this regulatory heirarchy could be conserved in the mammary gland.

Tissue Interactions in Mouse Mammary Gland Development

Armed with the initial hypothesis that hedgehog signaling mediates tissue interactions in the mammary gland, the task at hand was to demonstrate whether or not the hypothesis is correct. The problem is, of course, that the mammary gland requires tissue interactions at virtually every phase of its development. It was therefore impossible to predict *a priori* where or when hedgehog signaling might be functioning in the gland.

The epithelial compartment of the mammary gland is derived from the embryonic ectoderm and

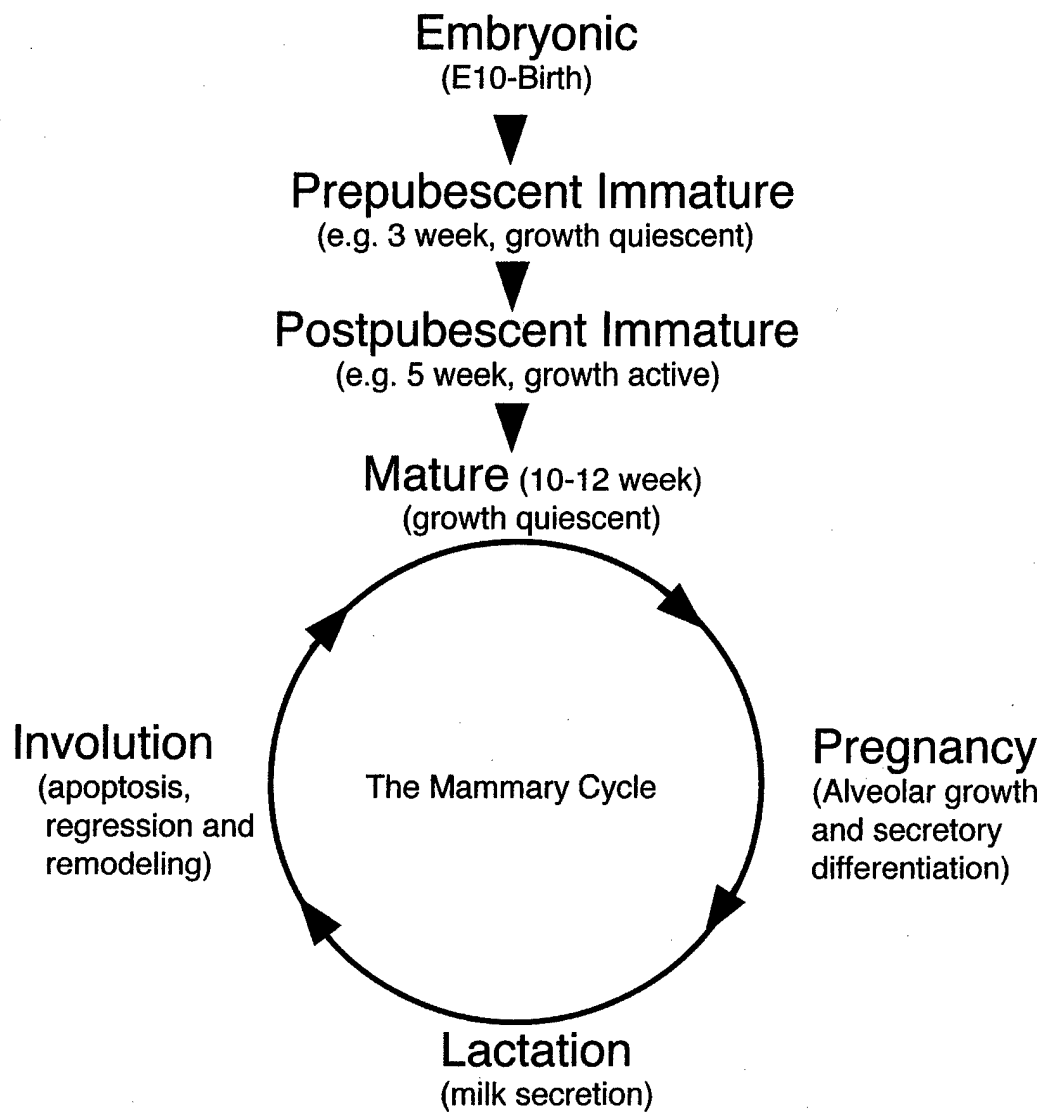

Fig. 4. Phases of mammary gland development. Proliferative development in virgin animals is represented by the linear portion of the diagram. Cyclical development initiated by pregnancy is represented by the circular portion of the diagram.

develops via reciprocal tissue interactions similar to those required for the development of other organs (e.g., tooth, lung, hair follicle). However, unlike most mammalian organs which develop primarily embryonically, development of the mammary gland is primarily post-pubertal and may be divided into both a linear and a cyclical phase (Fig. 4) [see (1–3) for detailed reviews]. These phases can be characterized further as a series of highly orchestrated transitions, or switches, in which critical developmental decisions are made concerning pattern formation, cell differentiation and cell function. Several of these transitions are known to be influenced by tissue interactions.

The mouse mammary gland is established about day 10 of embryonic development (E10) with the formation of two lines of thickened epithelium running anterior-to-posterior symetrically displaced off the ventral midline (the mammary streaks or milk line). This initial step in mammary patterning is followed closely by definition of the nipple region. Definition of the nipple region appears to occur via an inductive signal from the mesenchyme underlying the ectoderm and is characterized by condensation of the mesenchyme near the future location of each nipple (2,61). However, the molecular nature of this inductive signal, the mechanism of how the nipple region is

defined, and how mammary epithelial cell identity is established initially are not known.

Following establishment of the nipple region, the presumptive mammary epithelium interacts with two different mesenchymes. At about embryonic day 12, the mammary epithelium invades the underlying condensed mammary mesenchyme to establish a bulb of epithelial cells. After approximately embryonic day 16, the bulb elongates and invades a second type of mesenchyme, the mammary fat pad precursor mesenchyme. Tissue recombination studies have demonstrated that each of these two mesenchymes differentially affect mammary gland development but the significance of this difference and the mechanism by which these differences arise are unclear (2).

Once the fat pad precursor mesenchyme has been invaded, the gland then initiates a small amount of ductal growth and branching morphogenesis but consists only of a rudimentary ductal tree at birth. At puberty, ovarian hormones stimulate rapid and invasive ductal elongation driven by growth of the terminal end bud. The terminal end bud is a bulb-like structure consisting of 4–6 layers of relatively undifferentiated "body cells" and a surrounding 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 (3,62). As ducts form, they are ensheathed by a periductal stroma consisting mainly of fibroblasts and extracellular matrix material. These structures are further surrounded by adipose, vascular, and immune system cells within the confines of the mammary fat pad. Upon reaching the limits of the fat pad at ductal maturity, ductal elongation ceases and terminal end buds regress to leave a branched system of differentiated ducts. Virtually every aspect of ductal development, including ductal growth, branching morphogenesis, and tubulogenesis, are known to be influenced by epithelial-stromal interactions (63–66).

Hormonal changes during pregnancy inititiate a cyclical phase of development in which there is a dramatic transition from a predominantly ductal to a predominantly lobuloalveolar gland morphology. Lobuloalveolar progenitor cells located within the ducts proliferate to form alveolar buds which further differentiate to form alveoli. Near mid-pregnancy, the alveolar epithelium acquires the capacity to produce milk proteins (the stage I transition of lactogenesis) but secretory function is inhibited. At parturition, inhibition of secretory function is released and these cells begin to secrete large quantities of milk (the stage II transition of lactogenesis). These morphological changes in the epithelial compartment are mediated, in part, by epithelial-epithelial interactions (4) but are also accompanied by alterations in the stromal compartment such as the remodeling of the periductal stroma and the progressive depletion of lipid from adipocytes.

Upon weaning, milk secretion ceases and the gland involutes. During involution, most alveolar cells undergo apoptosis (programmed cell death) and are cleared from the gland by both macrophages and other mammary epithelial cells (67). The gland is then remodeled essentially to the pre-pregnant state to await the next pregnancy. This wholesale remodeling of the gland at involution is effected, in part, by the action of proteinases and their respective inhibitors, many of which are expressed in opposing tissue compartments (68–70).

A Genetic Approach to Hedgehog Function in the Mammary Gland

Given the success of the genetic approach in *Drosophila* and the availability of genetically modified mouse strains created previously for study of hedgehog function during embryogenesis, a genetic approach has been adopted for study of the mammary gland. This approach has been complemented by expression analysis, transplantation and tissue recombination experiments to develop a working model for hedgehog signaling status and function in the mouse mammary gland. While much of these data are as yet unpublished, the overall implication of the work is that hedgehog signaling plays a role in several stages of postnatal mammary gland development, including terminal end bud and ductal morphogenesis as well as alveolar development [(18) and M.T. Lewis *et al.* unpublished].

To date, mouse strains mutant for each of three hedgehog network genes have been examined in some detail for defects in mammary gland development. These genes are *Ptc1*, *Gli2*, and *Gli3*. Thus far, only *Ptc1* and *Gli2* have been demonstrated to function in mammary gland development. Other network genes have been shown to be expressed in the mammary gland by at least one detection method (either Reverse transcriptase polymerase chain reaction (RT-PCR), Northern hybridization or *in situ* hybridization). These genes include *Shh*, *Ihh*, *Dhh*, *Gli1*, *Ptc2* and *Smo* (S. Ross, Personal communation. Of these, only *Shh* and *Ihh* have been examined by *in situ* hybridization (18).

Patched-1 (Ptc1)

Of the two known Patched hedgehog receptor subunits, *Ptc1* has been most fully characterized. *Ptc1* mRNA is exprssed in both epithelial and stromal compartments and is developmentally regulated. Animals homozygous for targeted disruption of *Ptc1* show early embryonic lethality (around embryonic day 9.5) with, among other alterations, severe defects in nervous system development accompanied by changes in neural cell fates. Heterozygous animals can also show defects including skeletal abnormalities, failure of neural tube closure, medulloblastomas (brain tumors), rhabdomyosarcomas, and strain-dependent embryonic lethality (51,71)

In the mammary gland, haploinsufficiency at the *Ptc1* locus results in severe histological defects in ductal structure, and minor morphological changes in terminal end buds in heterozygous postpubescent virgin animals (18). Defects are mainly ductal hyperplasias and dysplasias characterized by multilayered ductal walls and dissociated cells impacting ductal lumens. This phenotype is 100% penetrant. Remarkably, defects are reverted during late pregnancy and lactation but return upon involution and gland remodeling. Whole mammary gland transplants into athymic mice demonstrate that the observed dysplasias reflect an intrinsic developmental defect within the gland. However, *Ptc1*-induced epithelial dysplasias are not recapitulated or maintained upon transplantation into a wild-type epithelium-free fat pad.

The observation that the phenotype is recapitulated in whole mammary gland transplantation (in which both epithelium and stroma are mutant) but is not recapitulated in epithelial tranplantation (in which only the epithelium is mutant), suggests that the primary function of *Ptc1* is in the stroma during ductal development. It has not yet been determined whether Δ*Ptc1* stroma can direct abnormal growth of wild type epithelium.

Gli2

By *in situ* hybridization, *Gli2* is expressed exclusively in the stromal compartment during virgin stages of mammary development. However, during pregnancy and lactation, *Gli2* expression becomes both epithelial and stromal. Δ*Gli2* heterozygotes demonstrate a low frequency of terminal end bud disruptions and focal ductal dysplasia. In addition, ~37% of Δ*Gli2* heterozygotes show delayed alveolar development in pregnancy.

The null phenotype with respect to ductal development was examined by transplantation rescue of intact embryonic mammary glands (both epithelium and fat pad mesenchyme) into immunocompromised host females. Glands derived from both wild type and null embryo donors showed ductal outgrowths that developed to equivalent extents. However, in null glands, ducts were frequently distended or irregularly shaped. Histological characterization demonstrated that misshapen ducts showed epithelial hyperplasia similar to micropapillary ductal hyperplasias in the human breast. As with Δ*Ptc1* heterozygous epithelium, morphological and histological defects were not observed when homozygous null epithelium was transplanted into a wild type stromal background suggesting that *Gli2* functions in the stroma during ductal development.

In addition to demonstrating a functional requirement during normal ductal development, these observations implicated *Gli2* as a candidate tumor suppressor gene. To investigate a possible tumor suppressor function for *Gli2*, mammary glands of female mice heterozygous for disruption of *Gli2* were reexamined. Heterozygotes demonstrated an elevated frequency of focal ductal dysplasia relative to wild type littermate and age-matched control animals at each stage examined. These defects continued to increase in frequency and severity with animal age and parity. Expression of *Gli2* in precancerous hyperplastic alveolar nodules and derivative tumors was also examined. *Gli2* was highly expressed in hyperplastic alveolar nodules but was undetectable in each of the derivative tumors. Data are consistent with a tumor suppressor function for *Gli2* and indicate that Δ*Gli2* should be examined genetically for synergistic interactions with known mammary oncogenes.

Ihh

Ptc1 appears to be a universal target for transcriptional up-regulation in response to hedgehog signaling (28). Enhanced expression of *Ptc1* during pregnancy and lactation coupled with phenotypic reversion in Δ*Ptc1* heterozygotes during these same developmental stages suggested that there may be fundamental differences in hedgehog signaling status between virgin, pregnant and lactating states. To begin to address this possibility, *in situ* hybridization was performed with probes for *Shh* and *Ihh* through mammary gland development.

Shh was not detectable by *in situ* hybridization at any stage of development nor was it detected by

A Model for Compartment-specific Hedgehog Signaling Status During Mammary Gland Development

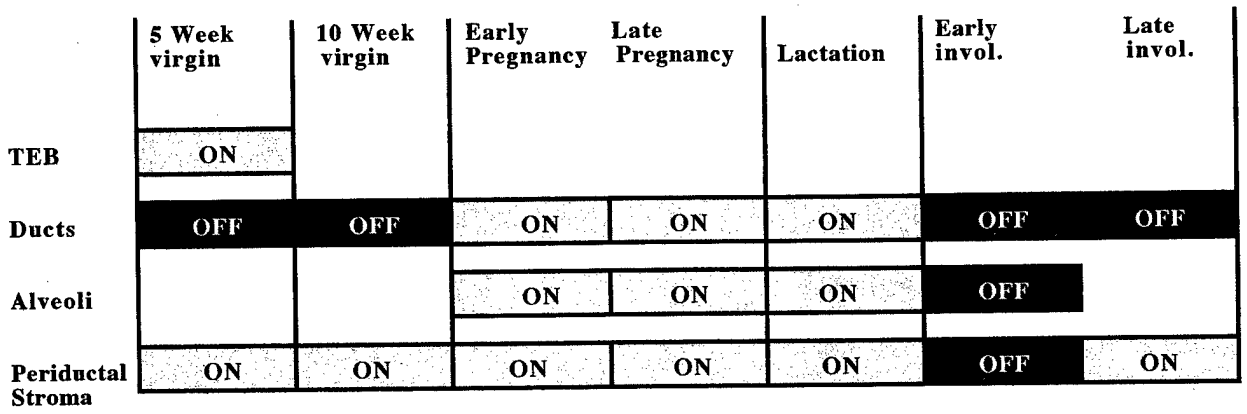

Fig. 5. Proposed status of hedgehog signaling by tissue compartment throughout mammary gland development and functional differentiation. Developmental stages for which the hypotheses applies are shown along the top of the figure. Epithelial structures present at various stages of mammary development are listed on the left side figure and include terminal end buds, mature ducts and alveoli. Periductal stroma is also listed on the left side of the figure. For simplification, hedgehog signaling status is shown as either "ON" or "OFF." However, it should be assumed that spatially and temporally graded signaling is likely to occur, particularly in the terminal end buds (spatial) and throughout the course of pregnancy and involution (temporal).

subsequent Northern hybridization (S. Ross and M.T. Lewis, unpublished). In contrast, *Ihh* expression was detectable by *in situ* hybridization and its expression was shown to be both epithelium-limited and developmentally regulated (18).

During virgin stages, *Ihh* expression was relatively low in body cells of the terminal end bud and low-to-undetectable in cap cells and differentiating myoepithelial cells at 5 weeks postpartum. Weak epithelial expression was maintained in ducts of mature animals at 12 weeks postpartum.

By contrast during both early and late pregancy, expression of *Ihh* appeared enhanced in both ducts and developing alveoli. As with *Ptc1*, *Ihh* expression appeared to be highest during lactation. Expression of *Ihh* during involution paralleled that of *Ptc1*, being undetectable by 2 days of involution and becoming detectable in remodeling epithelium at least as early as 14 days of involution.

A Model for Tissue Compartment-Specific Hedgehog Signaling Status and Control of Mammary Gland Development

Together, these observations have allowed development of a working model for hedgehog control of mammary gland development. In this model, compartment-specific control of hedgehog signaling status (Fig. 5) is required for normal development and is achieved by an interplay between the epithelium and the periductal stroma. Further, it is proposed that hedgehog signaling status must be tightly correlated with the reproductive state of the animal and that this coordination is critical for mammary gland development and functional differentiation.

Hedgehog Signaling in Terminal End Bud Development

During ductal growth, *Ihh* expression in the body cells of the terminal end bud acts as a short-range signal to other body cells ("hedgehog ON") to either support proliferation, maintain the undifferentiated state or to direct ductal differentiation. This interpretation for hedgehog signaling status in the end bud is tentative currently given the lack of demonstrated *Gli* gene expression in the body cells, but is consistent with apparent elevation of *Ptc1* mRNA levels in the terminal end bud relative to the immediately subtending duct (again, *Ptc1* is universally up-regulated in response to hedgehog signaling). At the same time, *Ihh* acts as an extended-range signal to uncondensed stromal cells in close proximity to the growing terminal end bud and directs, in part, subsequent condensation

and differention of these cells via the inactivation of *Ptc1* and activation of *Gli2* ("hedgehog ON").

Hedgehog Signaling in Ductal Development

As the terminal end bud grows through the stroma, *Ptc1* function becomes required in the epithelial compartment at the neck of the end bud and in the subtending duct to inactivate hedgehog signaling ("hedgehog OFF"). This hypothesis is consistent with a terminal end bud phenotype in Δ*Ptc1* heterozygotes in which body cells frequently fail to thin to a single layer in the subtending duct. In this case, haploinsufficiency of *Ptc1* in the neck of the end bud and subtending duct is proposed to result in failure to turn hedgehog signaling "OFF" thereby leading to the mutant phenotype. Similarly, loss of *Gli2* function would result in reduced hedgehog signal in the condensing stroma (where it is required) thereby resulting in abnormal stromal condensation around the terminal end bud. Abnormal terminal end bud architecture or stromal condensation would necessarily lead to changes in epithelial-epithelial and epithelial-stromal interactions and contribute to the mutant phenotypes.

In the mature gland, it is proposed that maintenance of the "OFF" status in ductal epithelium and "ON" status in periductal stroma is required to maintain duct integrity. Alteration of this relationship either by insufficient *Ptc1* activity in the epithelium or by insufficient *Gli2* activity in the stroma results in defective duct maintenance. This idea is consistent with the formation of alveolar-like clusters of cells in Δ*Ptc1* heterozygotes which can eventually burst out of the sides of ducts and with the increased frequency of focal dysplasias exhibited by Δ*Gli2* heterozygotes. This idea is also consistent with the observation that phenotypes in both mutants are progressive such that structures that appear generally well organized in younger animals can deteriorate with animal age and reproductive activity.

Hedgehog Signaling in Alveolar Development

With pregnancy and lactation, epithelial *Ihh* expression is enhanced and acts both as an extended range signal to the stroma and as a short-range signal in the epithelium itself. Under these conditions, stromal hedgehog signaling status is maintained in the "ON" state. However, the short-range epithelial signal results in the inactivation of *Ptc1* in the ducts (again consistent with elevated *Ptc1* transcript levels) and induction of *Gli2* expression and activity in the alveolar epithelium ("hedgehog ON"). This "OFF" to "ON" change in the hedgehog signaling status in the epithelial compartment represents a fundamental shift in the state of these cells and may be critical for the transition from a ductal to a lobuloalveolar gland morphology.

Again, this portion of the model is consistent with the lack of requirement for *Ptc1* function during pregnancy and lactation as demonstrated by the reversion of the *Ptc1* phenotype during these stages (18). This portion of the model is also consistent with an influence of *Gli2* function in the epithelium during alveolar development as supported by delayed alveolar development in some Δ*Gli2* heterozygotes during pregnancy (Lewis *et al.*, unpublished).

Hedgehog Signaling in Involution and Gland Remodeling

After weaning, *Ptc1* and *Ihh* expression is lost as early as two days involution. These observations suggest that the entire hedgehog signaling network is "OFF" in all mammary tissue compartments during early involution. Interestingly, with gland remodeling and the reestablishment of a near pre-pregnant ductal gland morphology, expression of *Ptc1* and *Ihh* gradually returns to the pre-pregnant state. The status of *Gli2* expression during involution has not yet been established.

In the case of both Δ*Ptc1* and Δ*Gli2* heterozygotes, there is no suggestion that early involution is altered. However, as evidenced by the reestablishment of ductal dysplasias in Δ*Ptc1* heterozygotes and the increased frequency and severity of focal dysplasias in Δ*Gli2* heterozygotes at 14 days of involution, it appears that hedgehog signaling is required for accurate gland remodeling later in involution. This proposal is supported further by the severe defects observed in multiparous Δ*Gli2* heterozygotes in which ducts throughout the gland can show dysplastic morphology.

SELECTED PREDICTIONS OF THE MODEL

The model presented here allows powerful predictions to be made with respect to tissue compartment-specific manipulation of hedgehog signaling status at virtually every stage of mammary gland development. For example, mutations that result in increased or ectopic hedgehog signaling in the ductal epithelium (e.g., overexpression of *Gli1*, *Gli2*,

Smo, or *Ihh* in the epithelium) would be expected to lead to phenotypes similar to those observed in *Ptc1* heterozygotes (18). It is noteworthy that this set of mutations is nearly identical with the set of mutations that can lead to basal cell carcinoma of the skin.

Though more difficult to accomplish technically, altered hedgehog signaling in the stromal compartment should also lead to predictable phenotypes. For example, mutations that result in reduced hedgehog signaling in the stroma (e.g., Δ*Ihh*, Δ*Smo* or *Ptc1* overexpression) should lead to terminal end bud and alveolar defects similar to those observed for the Δ*Gli2* mutation.

In a more general sense, the model also predicts that hedgehog signaling will be integrated, either directly or indirectly, will all other mammotropic regulatory networks including those of hormones, growth factors and other signaling molecules. Characterization of the nature and developmental timing of these interrelationships will be an active area of investigation in the near future.

CONCLUSIONS

While Nature has conserved the hedgehog signal transduction network from insects to mammals, She has implemented its use in different ways for a variety of developmental processes. No doubt the mammary gland will prove equally as interesting with respect to how the network is implemented and integrated with other mammotropic signaling networks to effect organotypic development. Fortunately, given the relatively high penetrance of the phenotypes observed thus far and the power of the technical repertoire available, the mammary gland experimental model offers a unique opportunity to dissect the mechanisms by which the hedgehog network influences tissue interactions in both normal mammary gland development and mammary cancer.

ACKNOWLEDGMENTS

The author thanks Dr. Charles Daniel (University of California, Santa Cruz) for providing the match, and Dr. Margaret Neville (University of Colorado Health Sciences Center) for keeping the fire burning. Preparation of this review was supported by Department of Defense grant BC990641 (to Michael Lewis) and NIH grant R37HD19547 (to Margaret Neville).

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Last modified: 6/18/01 6:49 PM

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

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Abstract

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 paper, 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. *ΔGli2* heterozygotes also demonstrated an elevated frequency and severity of focal ductal dysplasia relative to wild type littermate and age-matched control animals.

Keywords: 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, 1987a; 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 only of 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 (Chepko and Smith, 1999; Daniel and Silberstein, 1987b). 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-beta, Wnt, EGF and FGF superfamilies. Recently, we reported that heterozygous disruption of *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 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 (ΔGli1)* 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 while 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 *(ΔGli2)* 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 two years. The outbred background was maintained for these initial studies as a matter of choice since 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 in order 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 *ΔGli2* 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 post coitus (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. In transplantation studies, pregnancy and early lactation could not be examined due to inefficient impregnation of female Balb/C *nu/nu* hosts.

mRNA isolation: #4 mammary glands 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 -80C 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 or not *Gli2* is expressed in the mouse mammary gland (data not shown).

***In situ* hybridization:** The #2 and #3 mammary glands of Balb/C mice were used. Glands were fixed in ice-cold 4% paraformaldehyde:PBS for 3 hours 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* hybridization for *Ptc1* and *Ihh* were performed previously (Lewis *et al.*, 1999) and are presented again in this work to provide context for interpreting *Gli2* expression patterns and phenotypes.

Whole gland morphological analysis: Backcross-derived *ΔGli2* 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 #2 and #3 glands were scored for quantitative analyses (regardless of whether another gland in the same animal was affected).

Histological analysis: The #2 or #3 mammary glands were used. At least 3 representative animals were examined for each strain at each developmental stage. Gland fragments were embedded in paraffin, sectioned at 7μm and hematoxylin/eosin stained.

Whole gland transplantation rescue from null embryos: Mammary glands from homozygous null or wild type E18-19.5 embryos were transplanted between the skin and body wall of a three week old female Balb/C *nu/nu* or B6D2F1 mice whose endogenous #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 *ΔGli2/ΔGli2* donor E18-E19.5 mice were transplanted into cleared #4 fat pads of three 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 endbud (Figure 1A). Expression was elevated in condensed stroma relative to 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 (Figure 1B). *Ihh* expression was low but detectable in terminal end bud and ductal epithelium but not in stroma (Figure 1C). These expression patterns were maintained in ducts of mature animals (Figure 1D-F).

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

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

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 three week old virgin Balb/C *nu/nu* or B6D2F1 hosts whose endogenous #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 (Figure 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 (Figure 2B). Rarely, transplants appeared severely altered with short ducts and stunted sidebranches (Figure 2C).

In histological analysis, transplants from wild type donor embryos showed unperturbed histoarchitecture (Figure 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) while distended regions of ducts showed large lumena surrounded by a single thin layer of lumenal epithelial cells with very little periductal stroma (Figure 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 (Figure 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 #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 in the whole gland transplants, was uniformly normal regardless of whether the epithelium was genotypically wild type (Figure 3A) or homozygous null (Figure 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 the entire intact embryonic (E18-E19.5) mammary glands were removed and transplanted between the skin and body wall of three week old virgin B6D2F1 hosts whose endogenous #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 (Figure 4). Alveoli in the null transplant (Figure 4A) appeared indistinquishable from those in the wild type transplant (Figure 4B) or from the host control glands for each transplant (Figures 4C and 4D, respectively). Alveoli were morphologically and histologically equivalent size and 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 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 post partum (Figure 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 (Figure 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 (Figure 5C). Again, no defects were detected in mammary glands of wild type control animals at these stages (Figure 5D).

Parous heterozygous animals demonstrated the most dramatic defects (Figure 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 (Figure 5F).

The percentage of animals affected increased steadily in *ΔGli2* heterozygotes from the earliest stages examined such that after two pregnancies, 100% of heterozygotes showed defects (Figure 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 (Figure 6B). Whereas the frequency remained low in wild type animals through two pregnancies, the frequency of defects per gland in *ΔGli2* 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 (Figure 7A) demonstrated that the radial buds observed in *ΔGli2* 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 (Figure 7B). Wild type glands showed no such defects (Figure 7C)

At 10 and 20 weeks post partum, glands from *ΔGli2* heterozygotes showed a variety of histological phenotypes. Many showed similar architecture as those observed at 5 weeks with multiple elongated radial ductules (Figure 7D). Others were more severe (Figure 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 (Figure 7F).

Parous and multiparous animals showed an array of histological abnormalities. Defects detected after a single pregnancy were focal (Figure 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 (Figure 8B). Focal defects were also observed rarely in wild type animals after a single pregnancy that were histologically consistent with those in heterozygotes (Figure 8C) but generally less severe.

A clear distinction between wild type and *ΔGli2* heterozygous animals arose after a second pregnancy. In addition to dysplasias similar to those observed after a single pregnancy (Figure 8D), alveolar hyperplasias were identified that consisted of multiple clusters of alveolar structures with a

paucity of periepithelial stromal elements (Figure 8E). In wild type control animals, only the focal hyperplasias were observed (Figure 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 (Figure 8G and 8H). No defects were detected in multiparous wild type animals in ducts distant from focal dysplasias (Figure 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 (P18.5-19.0), approximately 37% of the animals (6/16) demonstrated marked hypoplasia of the alveolar epithelium (Figure 9A and 9B) relative to wild type control animals (Figure 9C). These observations were confirmed at the histological level in which affected heterozygotes showed reduced alveolar development and inappropriate retention of adipose stroma (Figure 9D and 9E versus 9F).

At higher magnification, clear differences can be seen both in the extent of alveolar expansion and the degree to which alveolar cells are prepared for secretion (Figure 9G and 9H versus 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 10 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 pregancy (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 pregancy 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 v. 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 *ΔGli1* 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 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 pregancy 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 obseration 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 are essential for mammary gland development. However, the complex regulatory interactions known to exist among the *Gli* genes in other organs makes 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 are 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 *ΔGli2* 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 *ΔGli2* 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.

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Figure Legends

Figure 1. *In situ* hybridization of *Gli2* during postnatal mammary gland development: *Gli2* expression is correlated with expression 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 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 pregancy. Expression in alveoli appears to be elevated relative to 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, 80μm.

Figure 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 which had only misshapen, stunted ducts and sidebranches (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, 80μm. (E) Histological preparation of the nullizygous donor transplant depicted in panel B showing the organization of the highly distended duct terminus (panel B, black arrow). Misshapen ducts from this type of gland (panel B, white arrow) had the histoarchitecture depicted in panel 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, 80μm.

Figure 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, 27μm.

Figure 4. Whole mammary gland transplantation rescue from nullizygous and wild type late-stage embryos into nude 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, 27μm.

Figure 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. Bar, 80μm.

Figure 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.

Figure 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 Figure 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, 80μm.

Figure 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 *ΔGli2* heterozygote demonstrating disorganized epithelium with the inclusion of eosinophilic structures (arrow). Bar, 80μm. B) Normal duct representative both of ducts 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 *ΔGli2* heterozygotes. Bar, 80μm. D) Focal dysplasia (arrow). Bar, 80μm. E) Alveolar hyperplasia (arrow) in heterozygous animal. Bar, 220μm. F) Focal dysplasia in a wild type animal similar to that in D. Bar, 200μm. 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, 200μm. H) Ductal dysplasia showing an apparent "tube-within-a-tube-within-a-tube" arrangement of interdigitated epithelial and stromal cell layers (arrow). Bar, 200μm. I) Normal duct observed in wild type animals in regions distant from focal dysplasias. Bar, 200μm.

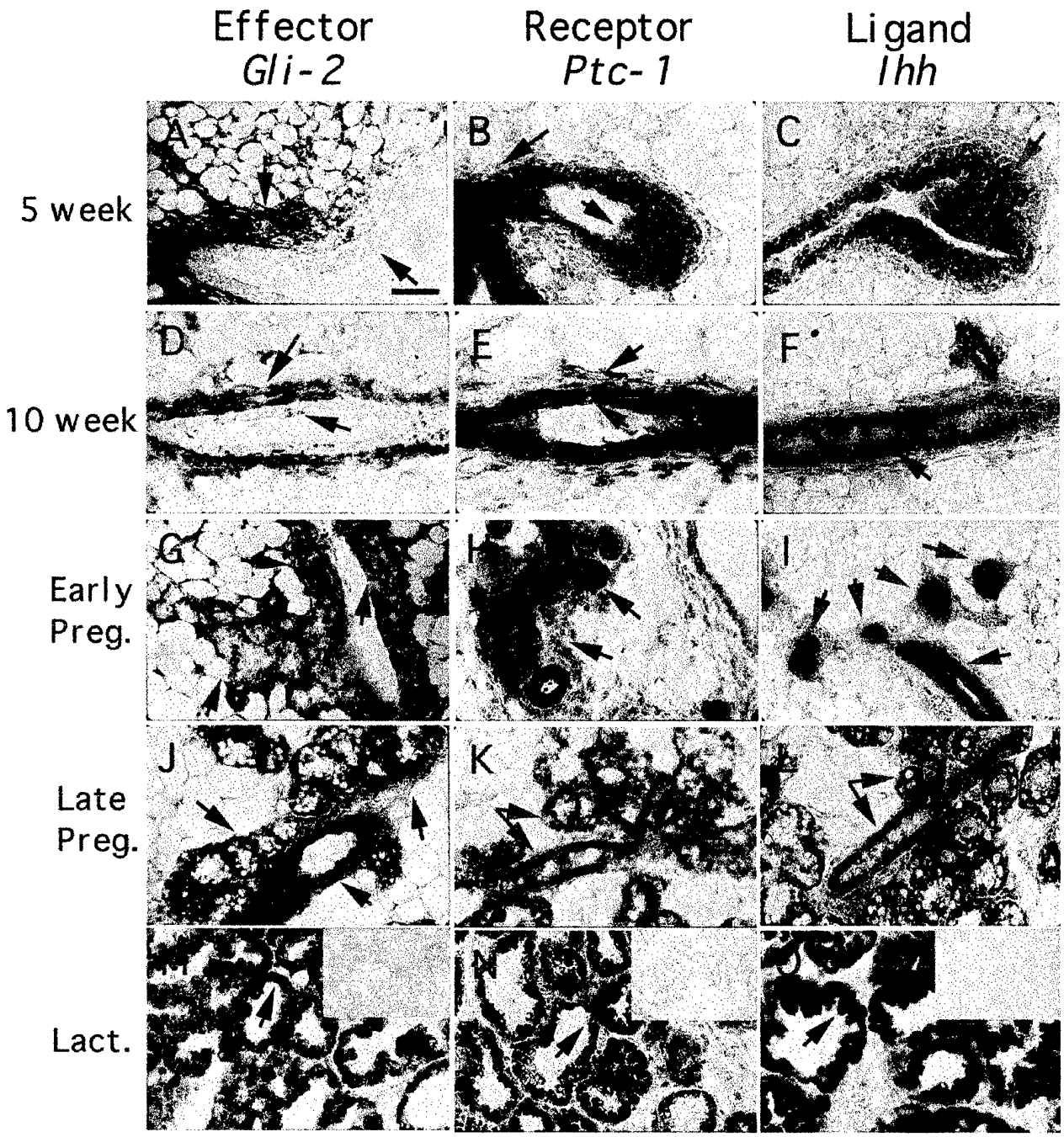
Figure 9. Reduced alveolar development at late pregnancy (day 18.5) in *ΔGli2* heterozygotes. The genotype of the animal from which the gland is derived is shown above the column to which it applies. The severity of the phenotype is also shown. Representative alveolar structures are identified with black 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 I. Panels G-I, Bar = 240μm.

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 wish to thank Dr. Margaret Neville for supporting completion of this work under grant R37HD19547 and for helpful discussions.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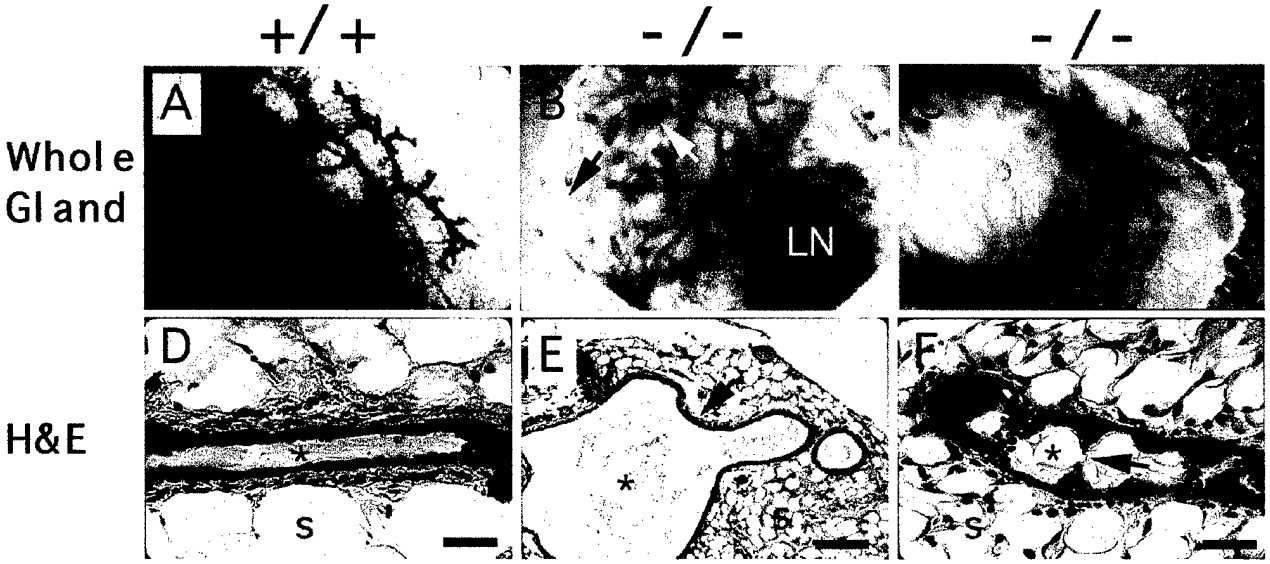
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LN
D
E
F
S
S
S

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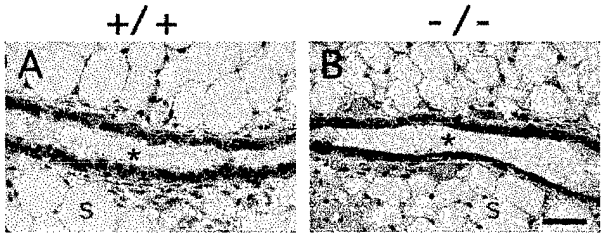
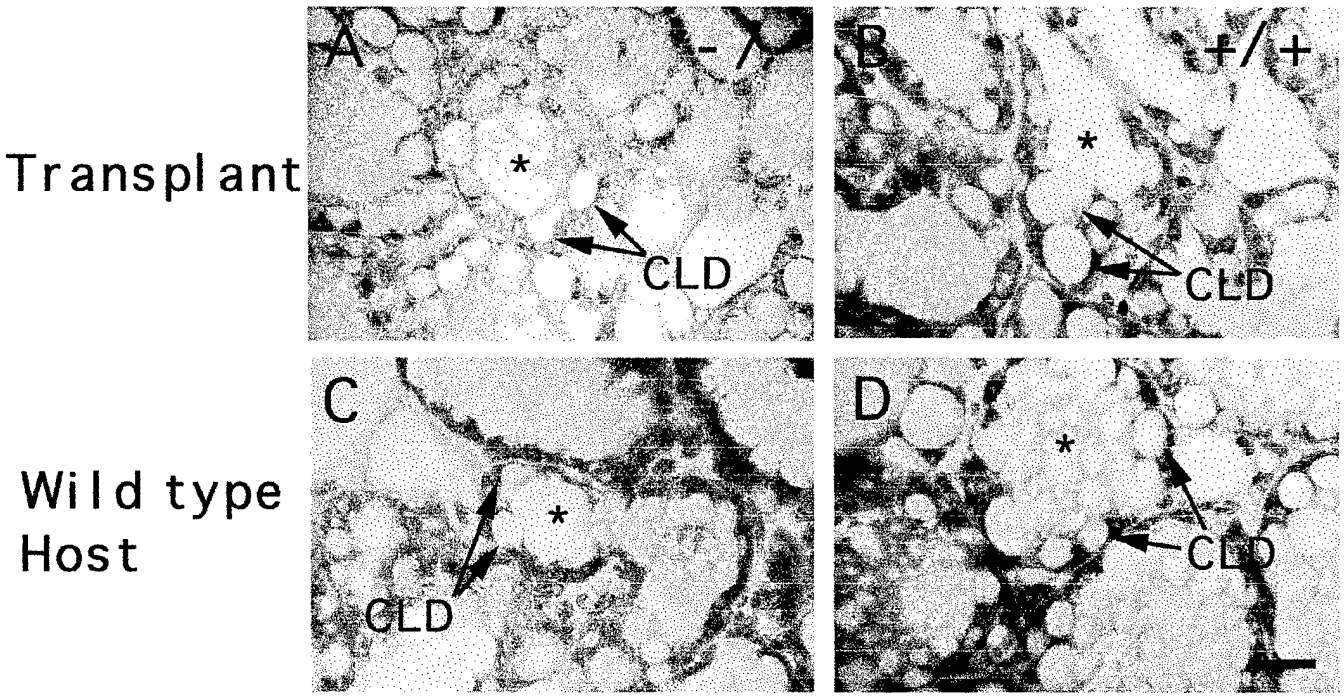
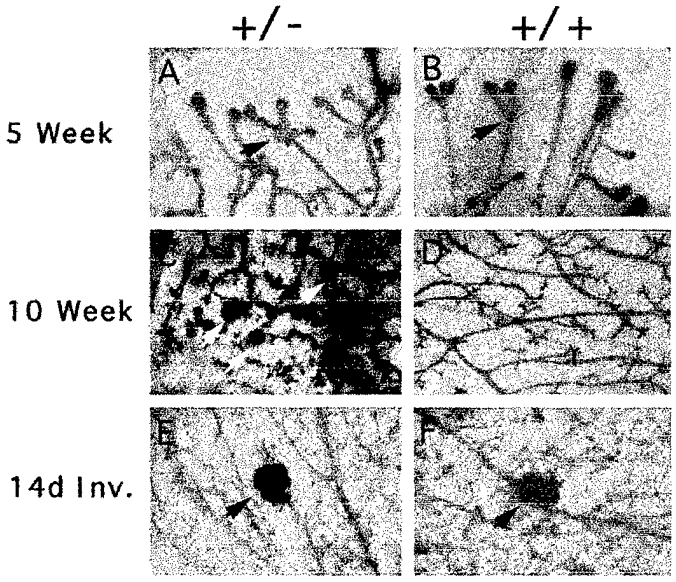
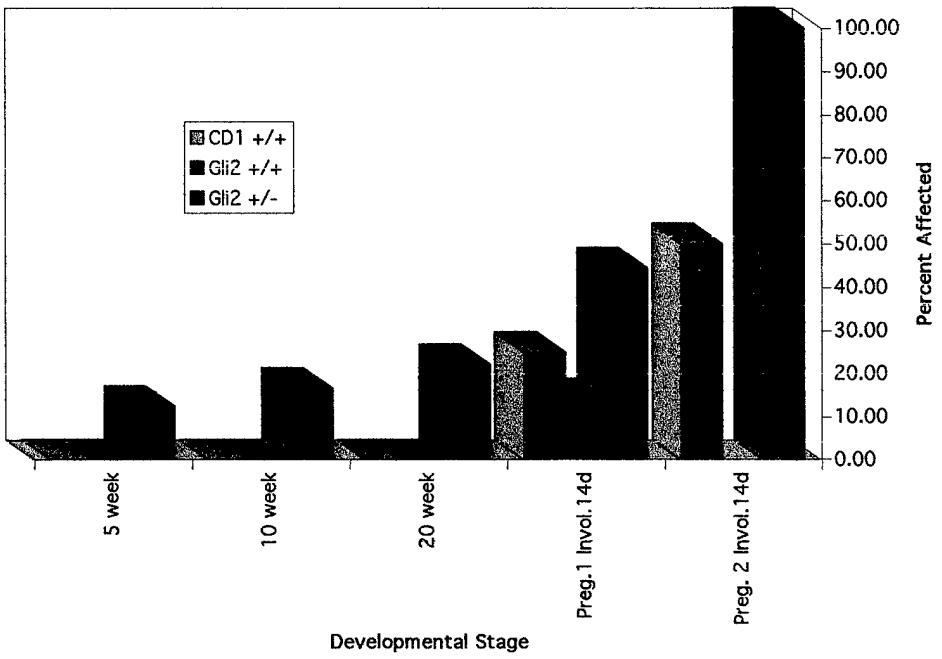
Figure 3




Transplant
Wild type
Host
A -/-
B +/+
C
D
CLD
CLD
CLD
CLD
*
*
*
*


+/-
+/+
5 Week
10 Week
14d Inv.
A
B
D
E
F

A


Percentage Of Animals Affected By Developmental Stage
CD1 +/+
Gli2 +/+
Gli2 +/-
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Percent Affected
5 week
10 week
20 week
Preg.1 Invol.14d
Preg. 2 Invol.14d
Developmental Stage

B

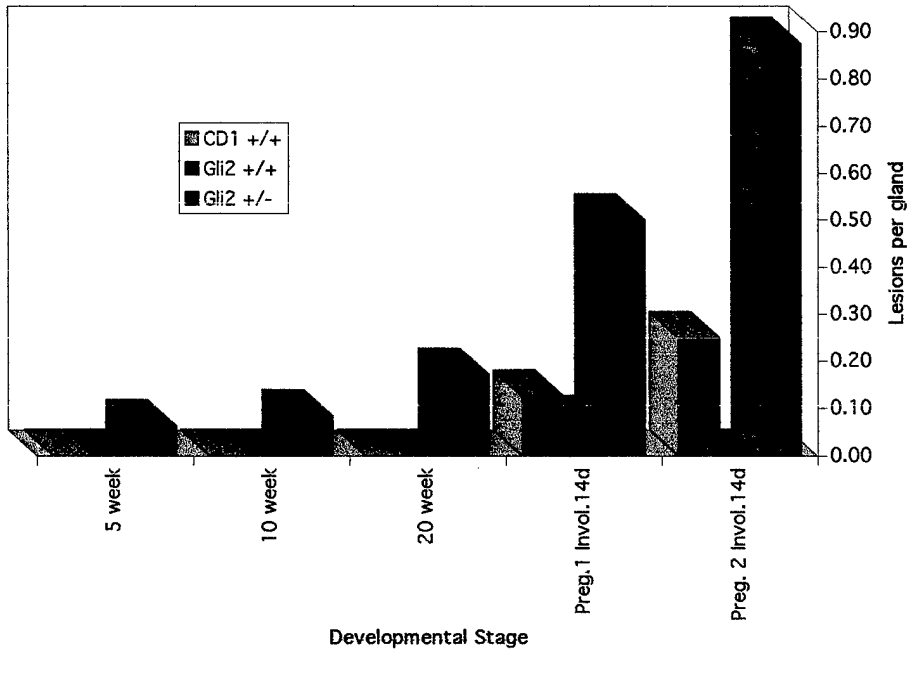

Lesions Per Gland By Developmental Stage
CD1 +/+
Gli2 +/+
Gli2 +/-
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Lesions per gland
5 week
10 week
20 week
Preg.1 Invol.14d
Preg. 2 Invol.14d
Developmental Stage

Figure 7

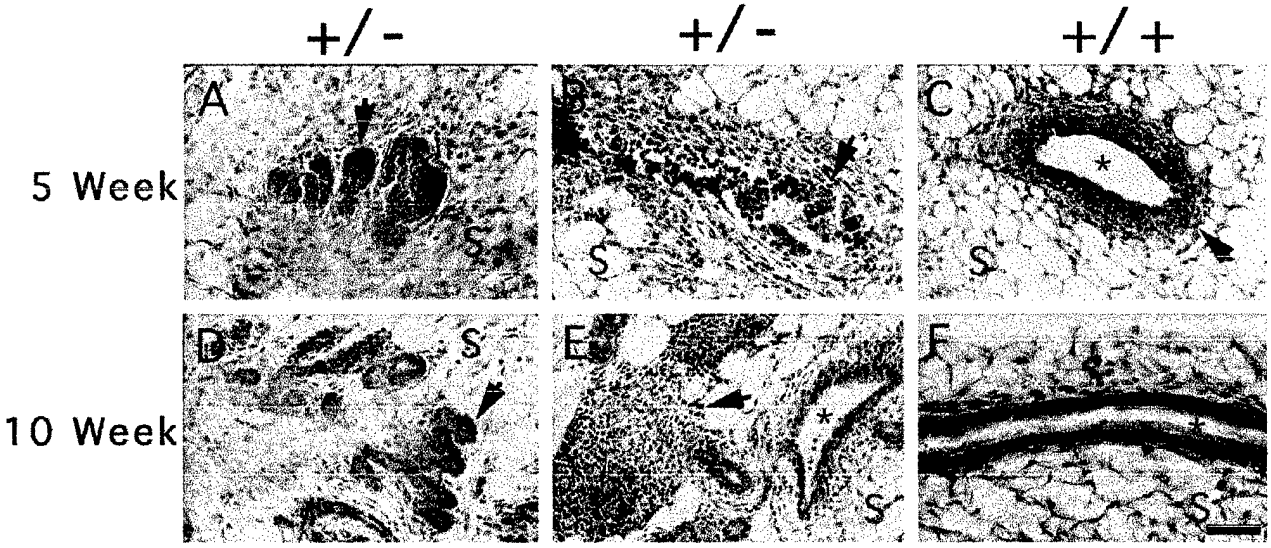


Figure 8

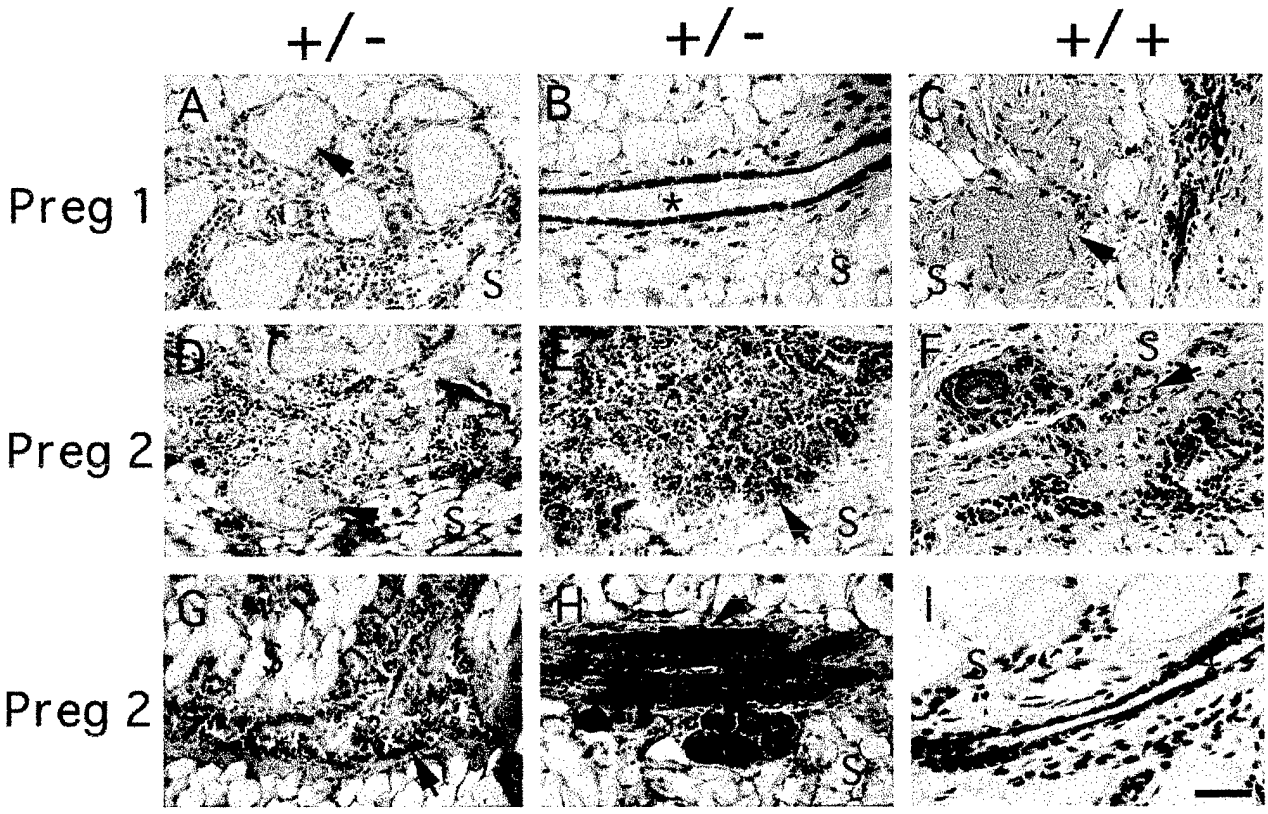


Figure 9