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**Journal**

Journal of Mammary Gland Biology and Neoplasia, 6(1)

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**Publication Date**

2001

**DOI**

10.1023/a:1009516515338

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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 © 2001 Plenum Publishing Corporation

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-$\beta$ (TGF-$\beta$); bone morphogenic protein (BMP); reverse transcriptase polymerase chain reaction (RT-PCR); hedgehog (HH); patched (PTC); smoothened (SMO); cubitus interruptus (CI).

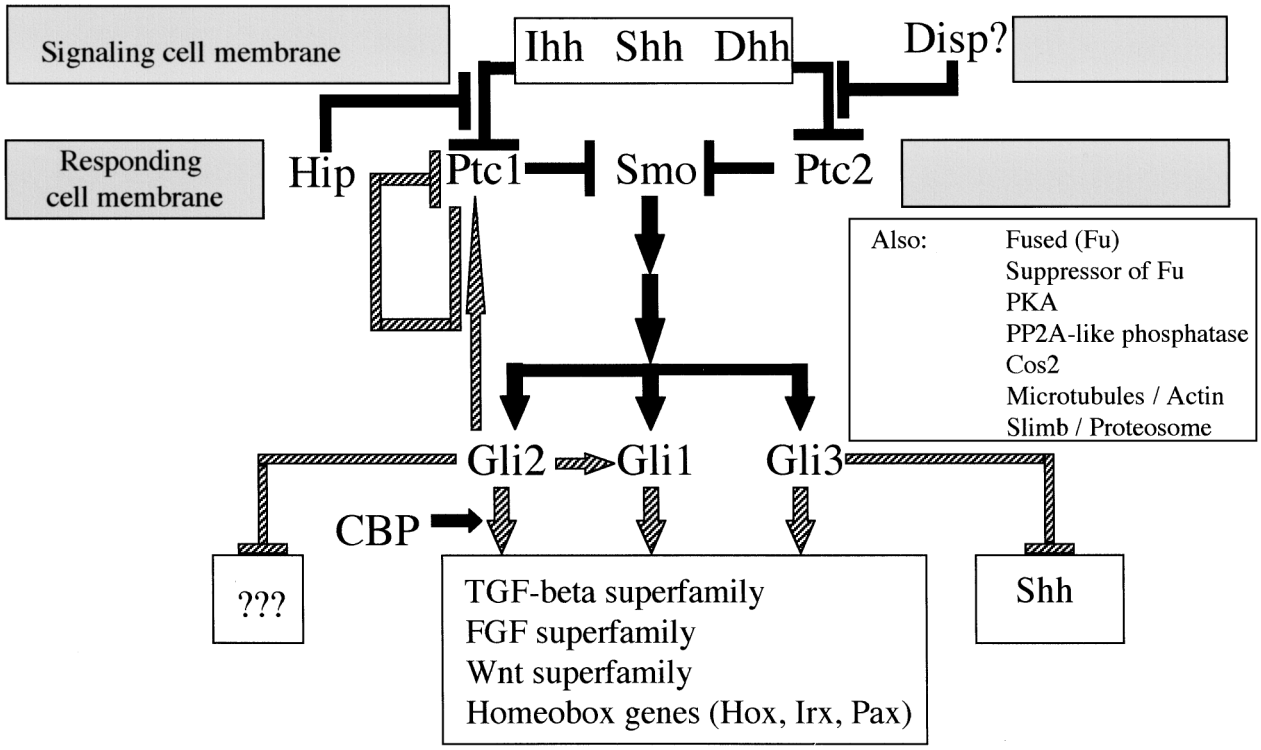


**Fig. 1.** Interactions among hedgehog signaling network components. Solid arrows and lines indicate protein activities; hatched arrows and lines indicate transcriptional regulatory activities. Additional proteins known to participate in modulating the hedgehog network are enclosed in boxes and are 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<br>see also (48,72–74) |
| *Dhh* | D | No overt mammary defects | (75) |
| *Ihh* | D | In progress | Lewis *et al*. (unpublished)<br>see also (74,76) |
| *Ptc1* | D | Mammary ductal dysplasias<br>Reversion of dysplasias in pregnancy and lactation | (18)<br>See also (50) |
| | O | In progress | Lewis *et al*. (unpublished)<br>see also (77,78) |
| *Smo* | O | In progress | Lewis *et al*. (unpublished)<br>see also (45) |
| *Gli1* | D | No overt mammary defects detected | see (35) |
| *Gli2* | D | Mammary ductal dysplasias<br>Delayed alveolar development | Lewis *et al*. (submitted)<br>see also (79,80) |
| *Gli3* | D | In progress. No defects in mammary ductal development | Lewis *et al*. (unpublished)<br>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-$\beta$ (TGF-$\beta$) family members including TGF-$\beta$ 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.

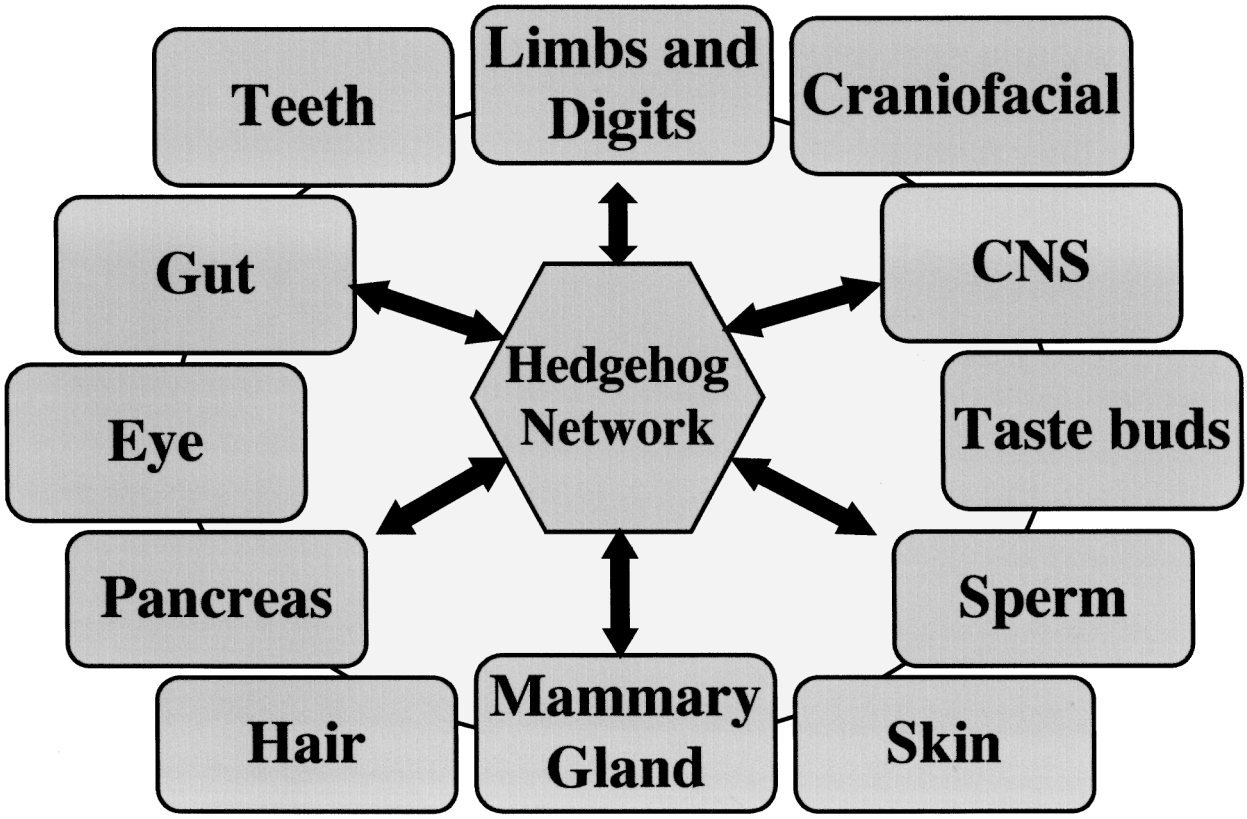


**Fig. 2.** Selected examples of organs and structures whose development is directed, in part, by hedgehog signaling.

**(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

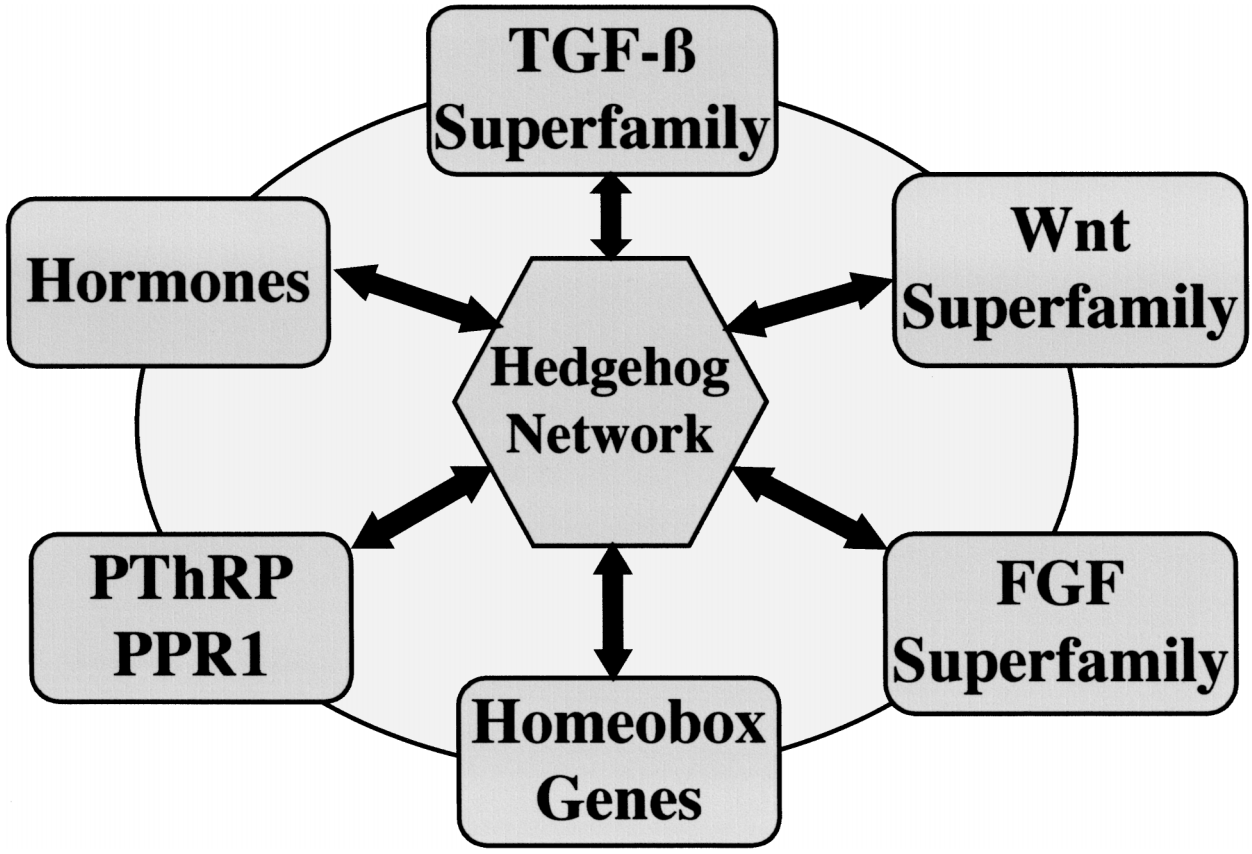


**Fig. 3.** Gene superfamilies and regulatory molecules active in other organs that either regulate, or are regulated by, the hedgehog signal transduction network. Each of the regulatory systems shown have known or suspected roles in mammary gland development.

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 TGF$\beta$.(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

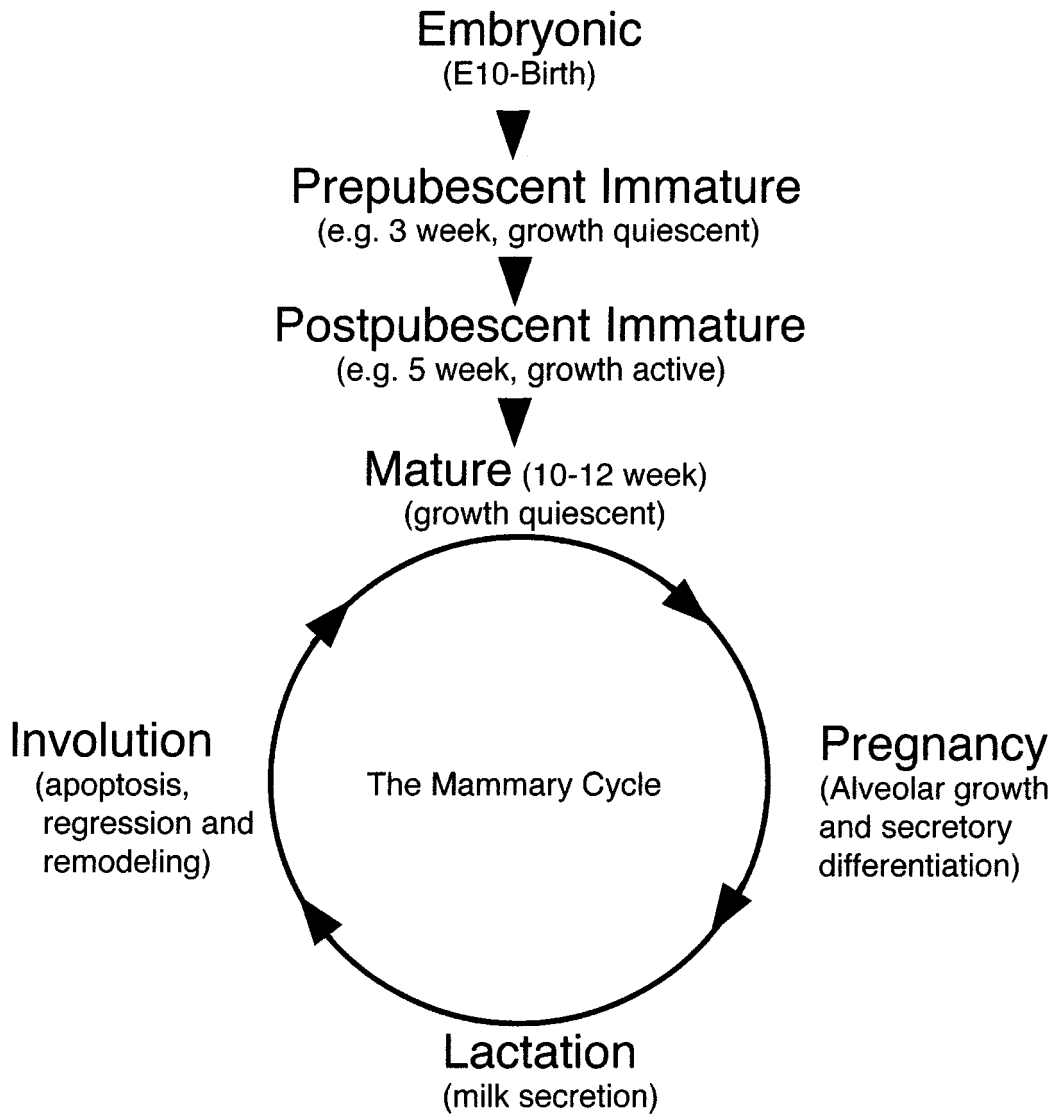


**Fig. 4.** Phases of mammary gland development. Proliferative development in virgin animals is represented by the linear portion of the diagram. Cyclical development initiated by pregnancy is represented by the circular portion of the diagram.

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

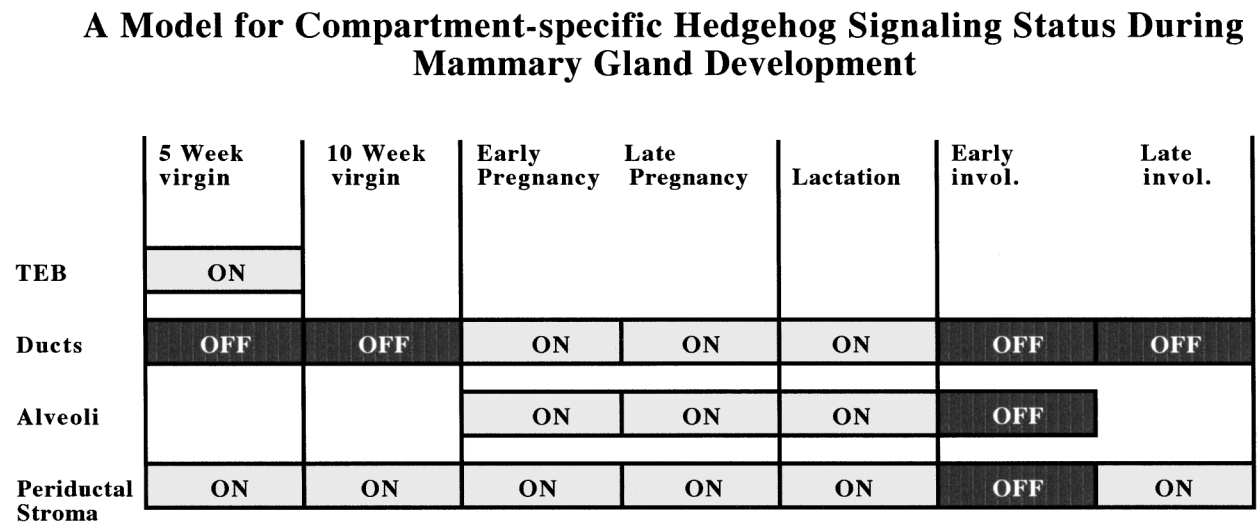


**Fig. 5.** Proposed status of hedgehog signaling by tissue compartment throughout mammary gland development and functional differentiation. Developmental stages for which the hypotheses applies are shown along the top of the figure. Epithelial structures present at various stages of mammary development 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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