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The overall aim of this project is to improve our understanding of genetic factors regulating the development, differentiation, function, and neoplastic progression of the breast. In the previous year we discovered the first clearly identified mammary phenotype in homebox genes, an engineered mutation in mouse Hoxd-10 that causes a deficiency in milk production. In 1998 we report the conclusion of this project. More important, we have carried out extensive functional and expression studies on the Hedgehog (Hh) pathway, not previously shown to be active in the breast. This pathway has been shown in several model systems to control many of the signaling pathways known to regulate mammary development, and thus has the potential to be considered a "master regulator." Using gene-targeted mice we have shown dramatic phenotypes associated with partial loss of receptor function and with loss of one of the transcription factors mediating Hh function. The discovery that Hh signaling is essential to mammary development has far-reaching implications for our understanding of both the normal and neoplastic breast.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>Report Documentation Page</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>TOC</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Narrative</td>
<td>5</td>
</tr>
<tr>
<td>Hoxd-10</td>
<td>5</td>
</tr>
<tr>
<td>Hedgehog Signalling: Overview</td>
<td>6</td>
</tr>
<tr>
<td>Patched-1</td>
<td>7</td>
</tr>
<tr>
<td>Gli-2</td>
<td>10</td>
</tr>
<tr>
<td>Conclusions</td>
<td>11</td>
</tr>
<tr>
<td>References</td>
<td>12</td>
</tr>
<tr>
<td>Publications</td>
<td>13</td>
</tr>
<tr>
<td>Personnel on Salary</td>
<td>13</td>
</tr>
<tr>
<td>Figure Legends</td>
<td>13</td>
</tr>
<tr>
<td>Figures</td>
<td>16</td>
</tr>
</tbody>
</table>
INTRODUCTION

1998 was an exciting research year in this project and we feel that the data generated reach far into the future, and may in fact provide a new paradigm for understanding the genetic regulation of breast development. As indicated in the 1997 progress report, we have continued our research on homeotic genes with the discovery of a mammary phenotype associated with a Hoxd-10 mutation. This work is now completed and a manuscript is in preparation. Interestingly, a recent publication in PNAS (Chen and Capecchi) reported a similar but weaker phenotype with a triple mutant of Hox-9 genes.

This year, as indicated in the revised statement of work, we have taken a much more focused approach and concentrated on identification in the mammary gland of a highly expressed upstream regulatory pathway with the potential to regulate these and other mammary-active genes, the Hedgehog (Hh) pathway (Task 3d in the revised SOW). Through study of a series of mutants, as well as expression studies, we have firmly established that this pathway is operative in the mammary gland and regulates mammary tissue structure. It has the potential of being considered a “master regulator” of mammary development.

The rational behind this Hh project has been to obtain a better and ultimately more clinically useful understanding of the genetic mechanisms underlying development of the normal breast and of the initiation, progression, and spread of breast cancer. The reasoning behind this question is as follows. The breast is a target organ for a variety of hormones. These, together with growth/differentiation factors, regulate the activities of the mammary cell. Unfortunately, this does not take us very far in understanding the biology of this interesting organ. Consider simply that other organs are also regulated by these same signaling molecules, but develop by quite a different pattern. The mammary gland itself varies enormously between species, between individuals, and of course in malignancy. How can this variation be accounted for, when the signals are the same? There must exist additional layers of genetic regulation that interpret these signals and give rise to particular patterns of development, or to neoplasia. How do we search for these developmental regulatory genes? In organisms such as Drosophila, where detailed genetic analysis is possible, mutations provide clues that have led geneticists to identify gene families that act as master regulators of cell fate. The discovery of these regulators has had an enormous impact on thinking in biology.

BODY OF NARRATIVE

HOXD-10: EXPRESSION AND FUNCTIONAL ANALYSIS (Task 4c in revised SOW).

Phenotype. In a recent report, Carpenter et al (1997) reported that targeted disruption of Hoxd-10 produces mice with hindlimb-specific defects in gait and adduction. To determine the underlying causes of this locomotor defect, mutant mice were examined for skeletal, muscular and neural abnormalities. Mutant mice exhibit alterations in the vertebral column and in the bones of the hindlimb. No major alterations in hindlimb musculature were observed, but defects in the nervous system were evident. There was a decrease in the number of spinal segments projecting nerve fibers through the sacral plexus to innervate the musculature of the hindlimb. Deletion of a hindlimb nerve was seen in some animals, and a shift was evident in the position of the lumbar lateral motor column. These observations suggest a role for the Hoxd-10 gene in establishing regional identity within the spinal cord.

Our initial phenotypic analysis of female mice homozygous for a disrupted HoxD10 gene identified a defect in lactation. Pups from early litters of mutant females died from a lack of milk, but survived if pups were fostered with lactating wild type females. Lactational failure appears to be most pronounced in the first litter, and becomes less severe in subsequent litters, such that multiparous breeders are able to nurse successfully. At least
three hypotheses could explain this defect: 1) glands are developmentally delayed, 2) glands are defective in lobule-alveolar differentiation, and 3) glands are defective in functional differentiation (lactogenesis) such that milk production and secretion is compromised.

We have completed a program to characterize this phenotype and obtain insights into the functional activity of Hoxd-10. A breeding program was been set up to permit examination of mice at all stages of mammary development and during the lactation cycle. Using these techniques, we have shown that the defect lies in failure to complete functional differentiation resulting in compromised lactational efficiency. This defect is not fully penetrant in the Hoxd-10 null mice, but results in a high incidence of malnourishment, lack of normal weight gain, and a high incidence of infant mortality. In the second or third pregnancy lactational performance improves. In the 1997 progress report we provided a detailed summary of the expression of this homeotic gene in the mammary gland. This, taken together with the functional data mentioned above, completes this project and provides the first functional understanding of the role of this homeotic gene in the breast.

HEDGEHOG SIGNALING: REGULATION OF MAMMOGENIC GENES BY A HIGH-ORDER UPSTREAM REGULATORY PATHWAYS (Task 3d in the revised SOW).

Introduction. This objective, added in the 1997 revised SOW, is a direct outcome of two of our recent findings. First is the discovery of a mammary role for Hoxd-10, and second is cloning and expression of the IRX gene family in the gland that has been previously reported. In model systems, all of these genes are regulated, at least in part, by the hedgehog signaling pathway. An investigation of this pathway in the mammary gland was a logical and interesting extension of this previous research. Although a great deal of attention has recently been focused on hedgehog signaling in various developmental systems (reviews: Altaba, 1997; Hammerschmidt et al, 1997), it has not been studied in the mammary gland to our knowledge, and certainly nothing has been published.

An outline of hedgehog signaling in Drosophila, where it was first discovered and investigated in detail, is shown in Fig. 1. A single hedgehog ligand (Hh) is implicated in both short-range and long-range signaling through its receptor patched (ptc), whose activity is modified by another membrane protein, Smo. Hedgehog signaling is mediated by cubitus interruptus (Ci), a putative transcription factor that regulates downstream homeotic genes such as members of the Iroquois family (Gomez-Skarmeta et al, 1996), decapentaplegic, wingless, and patched itself. In the fly the hedgehog pathway has many essential functions, is active in many locations, and is reactivated at various times in development, from early segmentation and axial patterning to development of structures such as the wing, leg, eye, in the larva.

In vertebrates the pathway is not only conserved, but new family members have been added with a resulting increase in complexity and developmental plasticity (Fig 1). In mammals and birds hedgehog has been expanded to include Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), whereas Ci has been expanded to include a family of three vertebrate transcription factors, the Gli genes, so named because of their initial identification and cloning from a glioblastoma (Rupperet et al, 1988). In addition to Gli, Ptc has recently been linked to both inherited and sporadic skin cancers, which include the basal cell carcinoma, the most common human cancer (Johnson et al, 1996). In spite of the expansion of family members, the signal transduction cascade appears to be remarkably conserved.

The multiple functions of the hedgehog pathway in vertebrate development are being studied in several laboratories and it is evident that, as in the fly, hedgehog regulation of developmental process occurs in many locations and in many developmental periods. One the most fully documented and elegant examples is in the developing limb, where Shh secretion regulates patterning of the anterior-posterior axis (Marigo et al, 1996). Numerous
developmental genes have been shown to be regulated by this pathway such as IRX, Hox, TGF-Beta, BMP, FGF, and even the recently discovered parathyroid-related-protein (PTRP), which in gene-targeting experiments has recently been shown to be essential for embryonic growth of the mammary gland. The above list reads like a litany of genes and cell products that are known to be important to mammary development and cancer. Thus our discovery that the hedgehog pathway is active in the mammary gland has far-reaching implications.

**Research Plan.** Our approach to studying the hedgehog pathway in the mammary gland involves three strategies. The first is expression, using appropriate combinations of molecular probes and antibodies as available. Second, we have obtained mutants of various components of the pathway and are well into a breeding program that will enable us to examine phenotypic changes in development, function, or neoplastic potential of the gland. These studies will make use of our nude colony, enabling us to examine mutant and control tissues in a uniform physiological environment.

**Patterns of expression of Hh signalling components.** In the 1997 Progress Report several of spatial and temporal expression patterns of Hh signaling components were described and illustrated in considerable detail. In 1998 this work was repeated and in some cases extended, but in no instance were contradictory results obtained. In order to summarize this very large amount of data we have prepared many of our results in graphic form (Fig. 2). In general most expression was robust, highly specific, and in all cases developmentally regulated. When considered in relation to the functional data summarized in the following sections, the expression is quite consistent with functional activity and again provides compelling evidence for the activity of this regulatory pathway.

**PATCHED-1:** This mutation in the primary receptor for the Hedgehog pathway creates defects in mouse mammary gland development caused by conditional haploinsufficiency (at press, *Development*)

**Summary.** We report investigation of the role of the *Patched-1 (Ptc1)* hedgehog receptor gene in mammary development and neoplasia. Haploinsufficiency at the *Ptc1* locus results in severe histological, but only minor morphological, defects in mammary glands of heterozygous postpubescent virgin animals. Defects are mainly ductal hyperplasias and dysplasias characterized by cellular impaction of ductal lumens. Haploinsufficiency is conditional in that lesions are reverted during late pregnancy and lactation but return upon involution and gland remodeling. Unlike most mouse mammary hyperplasias and tumors, *Ptc1*-induced lesions are not stable upon transplantation into an epithelium-free fat pad. This transplant behavior is similar to that of human basal cell carcinoma (BCC), which can be *Ptc1*-induced, when transplanted into athymic mice. Mammary expression of *Ptc1* mRNA is primarily epithelial and developmentally regulated. Data demonstrate a critical mammary role for at least one component of the hedgehog signaling network and suggest that *Ptc1* may act as a mammary tumor suppressor gene.

**Introduction.** A compelling reason to study the hedgehog signaling network in the mammary gland, in addition to its developmental interest, is the issue of breast cancer. With respect to a possible role in mammary tumorigenesis, several of the genes in the mammalian hedgehog signaling network have been identified as either protooncogenes or tumor suppressor genes. A number of these genes, including *Ptc1, Smo, Shh* and *Gli1*, contribute to the development of skin cancers, most notably basal cell carcinomas (BCC) (Dahmane et al., 1997; Fan et al., 1997; Ingham, 1998a; Oro et al., 1997; Reifenberger et al., 1998; Xie et al., 1998). In addition to skin lesions, *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). While *Ptc1* mutations have been
identified in a small fraction of human breast cancers (Xie et al., 1997), no general role for the gene has been established in the mammary gland.

Of the two known hedgehog receptors, Ptcl is most fully characterized. Animals homozygous for targeted disruption of Ptcl 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). The severity of these defects, even in heterozygotes, and the central role Ptcl plays in hedgehog network function made Ptcl a good candidate gene upon which to focus attention. We have investigated the role of the Patched-1 (Ptcl) hedgehog receptor gene in mammary gland development and neoplasia. Ptcl expression is both developmentally regulated and cell type specific. Wild type levels of Ptcl function are essential for proper mammary histogenesis, with heterozygous animals developing ductal hyperplasias resembling human ductal carcinoma in situ (DCIS). These lesions are reversible during pregnancy and lactation, allowing normal secretory function. Our finding that a central component of the hedgehog signaling network is active in the mammary gland and that its function is conditionally required for mammary histogenesis offers the possibility that this regulatory network will provide a genetic framework upon which to integrate many of the previously identified mammary developmental control genes and signaling pathways. In addition, altered hedgehog network function could provide novel insights into mammary cancer initiation and progression.

Materials and Methods. Two breeding pairs of mice heterozygous for a disrupted Ptcl gene were used to initiate a breeding colony and have been previously described (Goodrich et al., 1997). The original Ptcl mutation was maintained in a 129Sv:C57/B16 background with subsequent backcross to B6D2F1. In our laboratory, the mutation was likewise maintained in a B6D2F1 background by serial backcross but is still in a mixed background (as evidenced by segregation of coat color markers) which precluded transplants between animals (see below). Genotyping was performed by PCR as per Goodrich (1997). In all other respects, the materials and methods are as previously described in earlier reports.

Targeted disruption of the Ptcl gene results in defective tissue organization during virgin development. In situ hybridization demonstrated that Ptcl expression was both spatially and temporally regulated during mammary development, suggesting a functional role. To determine whether or not disruption of the Ptcl gene resulted in developmental defects in the mammary gland, glands were examined from several stages of development. No alterations were observed in overall patterning of the mammary tree at 3 weeks of age (data not shown). At 5 weeks of age, terminal end buds in wild type animals appeared normal in whole mount preparations whereas up to approximately 30% of terminal end buds in heterozygous animals appeared misshapen or disrupted. 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 in whole mount preparations could be made between wild type and heterozygous glands, leading us to think initially that the Ptc heterozygote had no mammary phenotype.

Upon histological analysis, however, we noted severe 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 (Figure 3A and 3B, respectively), severe histological abnormalities were observed at 5 weeks when compared with wild type controls (Figure 3C versus 3D). Normally, multilayered luminal epithelial cells (body cells) of the TEB thin to a monolayer as the subtending duct is established (Figure 3C). However, in some ducts of 5 week heterozygous animals, the luminal epithelium remained multilayered and, in some cases, the luminal space was completely occluded by epithelial cells (Figure 3D). 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 (Figure 3D). 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 (Figure 3E). By contrast in some endbuds of heterozygous animals, body cells were
disordered (Figure 3F) and the cap cell layer was visibly altered (Figure 3F).

Ductal impaction observed at 5 weeks of age is even more pronounced in glands
from 10 week old animals. Whereas wild type ducts show a clear lumen within a
monolayer of lumenal epithelial cells (Figure 3G), a majority of ducts in glands from
heterozygous are partially or completely impacted with cells (Figure 3H) as detected by
examination of serial sections through entire ducts. Interestingly, some ducts appear
relatively unaffected. Cells in impacted ducts are not monomorphic with respect to nuclear
morphology and can include large cells with round nuclei and clear cytoplasm suggesting
that multiple epithelial subtypes contribute to the lesions.

To begin to address which cell types contribute to ductal lesions and to further
characterize cells within the lesions, staining with propidium iodide (nuclear stain) and
phalloidin (actin stain) was performed to assay for alterations in actin localization in the
myoepithelial and epithelial cell layers. In wild type ducts (Figure 3I), actin staining clearly
identifies the myoepithelial cell layer as well as the terminal web and microvilli at the apical
(lumenal) surface of lumenal epithelial cells. Faint actin staining is also observed on the
lateral surfaces of lumenal cells. In affected ducts of heterozygous animals (Figure 3J),
myoepithelial cells do not appear to contribute to the cell population of the lesions but
remain associated with the basal lamina surrounding the impacted ducts. By contrast, actin
staining within the lesion is generally disorganized but can be observed at the apical cell
surface around microlumens formed by circular clusters of epithelial cells (Figure 3J). Data
suggest that only lumenal epithelial cells contribute to the lesions and that cells can become
polarized, albeit inappropriately, around microluminal spaces within the lesions.

Ptcl-induced lesions are reversible during pregnancy and lactation. Given the
severity of the mammary phenotype in virgin Ptcl heterozygotes, the question arises: why
does cellular impaction of ducts in mature animals not impair their ability to lactate? To
investigate, we examined glands at various stages of pregnancy, lactation and involution.
By histological analysis, many ducts in early pregnancy remain filled, or nearly filled, with
cells and are qualitatively similar to those of mature virgins (data not shown). However, by
late pregnancy and lactation 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, with only sporadic cellular
impaction of ducts remaining evident. Ducts in heterozygous animals remain open in early
stages of involution (data not shown) and in late involution as do wild type ducts but
elements of the impacted phenotype are re-established in some ducts by late involution (14
days) with an occasional observance of severe stromal overgrowth.

These results indicate that the hedgehog network is strongly influenced by
physiological changes that occur during pregnancy and maintained during lactation and
suggest that the network may interact with hormone- or growth factor-mediated signal
transduction networks. Since levels of several mammatropic hormones and growth factors
(e.g. estrogen, progesterone, prolactin, TGF-ß family members) are dramatically altered
during these stages, and disruption of each of these signaling networks independently
disrupts gland development and function, identification of the network(s) involved in
phenotypic reversion is likely to be complex.

Ptcl-induced lesions are not stable upon transplantation into cleared fat
pads of wild type recipients. With respect to a contributory role in breast cancer, an
important question concerning the nature of the Ptcl-1-induced lesions is whether or not the
lesions represent a preneoplastic or neoplastic state. To determine whether Ptcl-induced

9
lesions are stable or undergo neoplastic progression upon transplantation, wild type and heterozygous mammary epithelium were contralaterally transplanted into epithelium-free (cleared) fat pads of athymic mice and allowed to regenerate a ductal tree for 6 weeks to 8 months.

Donor epithelium from wild type animals was normal whereas heterozygous donor epithelium from the region surrounding the transplanted area showed mild-to-severe histological defects. Upon transplantation, wild type epithelium produced normal ductal outgrowths, as expected. Epithelium transplanted from affected heterozygous animals were also histologically normal even after 8 months posttransplantation. These data indicate that PtcI-induced lesions are not stable upon transplantation under these conditions and suggest that Ptc-1 lesions may represent an early or contributory step in neoplastic progression. Results further suggest that PtcI function may be required in the stroma (or in both epithelium and stroma) for transplanted heterozygous epithelium to recapitulate the mutant phenotype observed in virgin animals but do not rule out the possibility of hedgehog network interactions with systemic factors.

**PtcI is differentially expressed in mammary epithelial cell types.** To further investigate developmental regulation suggested by Northern analysis and to determine which cell types express PtcI, exhaustive *in situ* hybridization was performed against mammary tissue at various developmental stages. These data are summarized in Fig. 2.

**Conclusions.** By expression and functional analysis, we have shown that in gene-targeted mutants the PtcI hedgehog receptor is not only developmentally regulated at the mRNA level, but is also conditionally required for proper histogenesis during virgin development and late-stage involution. PtcI-induced lesions appear to be due primarily to failure of body cells of the terminal end bud to thin to a single cell layer in the subtending duct. This failure is compounded by progressive duct wall thickening with cellular impaction of the lumen in a majority of mammary ducts by 10 weeks of age. Cellular impaction is reversible during late pregnancy and lactation allowing successful milk secretion to occur. Mammary lesions are not stable on transplantation suggesting that PtcI function may also be required in the stroma. To our knowledge this is the first mutation in mice associated with tissue construction and maintenance.

An unusual aspect of the PtcI 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, overall gland architecture would be altered. The PtcI 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 PtcI 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.

**GLI-2:** Disruption leads to severe mammary defects in both the null and heterozygote

*Introduction* GLI-2 is one of three transcription factors in the vertebrates that mediate hedgehog signaling and regulate downstream targets. Very little is known about the specific roles of the mammalian GLI transcription factor genes (*Drosophila* Ci homologs) in hedgehog signaling. One recent model postulated that the GLI1 protein took on the transcriptional activation functions ascribed to *Drosophila* Ci\textsubscript{act} and that GLI3 protein took on the transcriptional repression function ascribed to Ci\textsubscript{rep} with GLI2 modulating the function of GLI1 and GLI3 in an unspecified manner [Dahmane, 1997 #1987]. Subsequent
mutational analysis of each of the three genes was not consistent with this model in that
disruption of Gli-1 shows no detectable defects, even in homozygotes, whereas disruptions
of Gli-2 and Gli-3 are both perinatal lethal and lead to overlapping, but distinct,
developmental defects (Table 1)[Matise, 1998 #49][Mo, 1997 #143]. These data suggest
that Gli-2 and Gli-3 can have overlapping and redundant function depending on the
structure being examined. By contrast, a competing model suggests the activity of GLI
proteins may be regulated by proteolytic processing similar to that observed for their
drosophila counterpart CI [Biesecker, 1997 #1514]. Under this model, GLI proteins may
act as both transcriptional activators or repressors depending on their cleavage state. This
model is supported by recent data that different two forms of GLI2 act as a transcriptional
activator and a transcriptional repressor, respectively [Tanimura, 1998 #1483]. Together,
these data suggest that Gli-2 and Gli-3 are the primary transcriptional regulatory genes that
mediate expression of target genes in mammals and that, in some tissues, these two genes
can act coordinately, either in the same or opposite directions, to regulate development.

**Materials and Methods.** Mice heterozygous for targeted inactivation of Gli-2,
generously provided by C.C.Hui. Unlike homozygous Ptc-1 mutants, which display very
early embryonic lethality, Gli-2 -/- mutants die shortly before birth. Although the
mammary glands of these null mice cannot be examined during postnatal development, we
successfully “rescued” the mammary glands by whole-gland transplants by removing the
embryonic gland and transplanting it between the skin and body wall of a host animal (in
this case, immunocompromised). The glands were allowed to develop in this wild type
hormonal background for several weeks. Although these transplants do not attain their full
normal size, they do undergo full organotypic development.

**Homozygous disruption leads to severe mammary defects.** Glands from wild
type and Gli-2 homozygous late-stage embryos were examined using this technique. Wild
type glands grew normally with unperturbed histoarchitecture. By contrast, glands from
Gli-2 homozygous donors showed multiple defects including hyperplastic or distended
ducts and severely altered histoarchitecture that closely resembles human micropapillary
ductal carcinoma in situ (Fig 4A). These transplant experiments indicate that the mammary
defects observed were intrinsic to the mammary gland and were not significantly effected
by environmental or systemic influences. In this respect, the gli-2 phenotype is simpler
than that of Ptc-1, in which the phenotype appears to reflect the interaction between the
local environment of the nude mouse and the mutated gene in the mammary gland. Another
conspicuous difference is that the gli-2 defects are even more pronounced and bizarre,
perhaps reflecting the homozygous null genotype.

**Heterozygous disruption of Gli-2 leads to focal mammary hyperplasia.**
Gli-2 heterozygotes have been examined at several developmental stages. Mammary
hyperplasias and dysplasias are detectable as early as 5 weeks postpartum and progress to
form easily identifiable focal lesions by 10 weeks postpartum. Histologically, advanced
lesions are highly disorganized with loosely adherent epithelial cells impacting the ductal
lumen similar to the phenotype in Ptc-1 heterozygotes. Interestingly, ducts removed from
the lesions can also show dramatic alteration of histoarchitecture while appearing normal in
whole mount preparations. Here again, as in the Ptc-1 mutant, there is a disconnection
between branching morphogenesis at the organ level, which is normal in appearance, and
histoarchitecture, which is severely disrupted. These lesions are illustrated in Fig. 4B.

**CONCLUSIONS.** Although there are distinct differences between the ptc-1 and the gli-2
phenotypes, there is a gratifying degree of overlap -- to be expected in mutations in
different components of the same signaling network. The more focal lesions in gli-2 must
now be examined for their transplantability and tumorigenic potential.
REFERENCES


**PUBLICATIONS**


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**FIGURE LEGENDS**

**Fig. 1.** (A) Hedgehog signaling in fruitfly (simplified). Known functions are depicted for several hedgehog signaling network proteins. Activating functions are noted with arrowheads; inhibitory interactions are noted with lines. Genes under transcriptional control of CI are noted in italics. (B) General model for the hedgehog signaling network in the vertebrates (simplified). Known functions are depicted for several hedgehog signaling network proteins. Activating functions are noted with arrowheads; inhibitory interactions are noted with lines. All network genes shown are expressed in the mammary gland.
Fig. 2. Summary of in situ hybridization expression data for Ptc-1, Ihh, and Gli-2 and correlation with phenotypic alterations in mutant strains through mammary development. Predominant morphological features present in mammary glands at different developmental stages are denoted by colored horizontal bars (upper section). Phenotypic alterations in Ptc-1 and Gli-2 knockout strains are noted by color for the altered mammary structure (middle section). Subjective evaluation of relative expression levels in predominant mammary tissue compartments and cell types are also shown by color (lower section).

Fig. 3. Histological comparison of glands 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 of panels. Panels A-H are stained with hematoxylin and eosin; panels I-J are stained with phalloidin (yellow-green, actin) and propidium iodide (red, nuclei). Ductal lumens are denoted by red asterisks (*); Adipose stroma is denoted by a red letter “s” 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 an well-defined lumen in the subtending duct (red arrow). 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 arrow) 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 arrow) appears unaffected. Clusters of epithelial cells which form microlumens within the ducts can be identified (white circles) with inappropriate actin localization at the microluminal surface. Bar = 80 μm.

Figure 4. (A) The Gli-2 null phenotype. A) Representative transplant gland from a Gli-2 homozygous donor. Ducts show altered branching and unusual termini. B) Representative transplant gland from a wild type donor showing normal branching and duct termini. C) Histology of ducts from Gli-2 null gland showing papillary epithelial structures. D) Histology of human micropapillary ductal carcinoma in situ. A and B hematoxylin only; C and D hematoxylin and eosin. (B). Histological analysis of representative mammary lesions from Gli2 heterozygotes and comparison with a similar human lesion. Hematoxylin and eosin staining (except A, hematoxylin only). Ductal lumens are noted by a blue letter L; Adipose stroma is noted by a blue letter S. A) Whole gland preparation showing a representative hyperplasia (red arrow) from a multiparous female and adjacent normal appearing ductal and alveolar structures (white arrow). B) Histological section of lesion in A. Note the epithelial and stromal proliferation and disorganization (arrow) and eosinophilic inclusions (asterisk) (probably keratin). C) Dysplastic duct adjacent to the cell mass in A. Duct walls appear multilayered with loosely associated cells in the lumen.
(arrow). D) Lobule-alveolar hyperplasia budding off duct with multiple layers of epithelial cells (arrow). E) Normal duct showing a single layer of epithelial cells lining the duct (arrow). Compare with B, C and D. F) Human ductal carcinoma in situ [Fechner, 1990 #2153]. Compare with C.
Hedgehog Proteins

Fig. 1A

Gli-mediated Transcriptional Regulation

Fig. 1B
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<tr>
<th>Developmental Stage</th>
<th>5 Week</th>
<th>10 Week</th>
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<th>Late Preg.</th>
<th>Lactation</th>
<th>Early Invol.</th>
<th>Late Invol.</th>
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- **Ducts**
- **Alveoli**
- **Periductal stroma**
- **Terminal end bud**

- ND: Not Determined