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PRINCIPAL INVESTIGATOR: Charles W. Daniel, Ph.D.

CONTRACTING ORGANIZATION: The Regents of the University of California
Santa Cruz, California 95064

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Charles W. Daniel, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Regents of the University of California
Santa Cruz, California 95064 E*Mail: daniel@darwin.ucsc.edu

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13. ABSTRACT (Maximum 200 words)
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. The homeotic (hox; homeobox) genes are candidates for "master regulators" of cell fate and tissue interactions. Early in the project we showed that this large family is widely expressed in the mouse and human mammary gland and their tumors, as well as precancerous lesions. Expression was hormonally and developmentally regulated. Hoxa-1 was expressed in tumors, but not in normal or preneoplastic tissues. The large numbers of expressed genes and their complex patterns precluded investigation of all, giving rise to a revised and more focused Statement of work (SOW) following the second annual report. This led to investigation of an engineered mutation in Hoxd-10, which produced a differentiation failure and lactational deficit. We discovered and cloned several members of the Iroquois family of homeotic genes, that we found to be expressed in the normal and neoplastic human breast. Because of the extreme complexity of the expression pattern of hox genes, we turned to the hedgehog (Hh) signaling pathway, not previously shown to be active in the mammary gland, and which has the potential to regulate homeotic genes as well as many other mammary-active pathways. Using engineered mutations in both a Hh receptor and a downstream transcription factor, we demonstrated striking phenotypes characterized by disruption of normal morphogenesis and histogenesis. The discovery of Hh activity in the mammary gland has far reaching implication for both development and cancer.

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1. Two Tables (pp. 18-19)
2. Six Figures (pp. 21-27
3. Eight Reprints (Appendix)

STATEMENT OF THE PROBLEM STUDIED

The general aim of this 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 the observation that other organs are also regulated by these same signaling molecules, but develop by quite a different spatial and temporal 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 almost universally shared? 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, determining for example, whether a wing or an antenna will develop at a particular location. The discovery of these regulators has had an enormous impact on thinking in biology. In this project, we have used genetic clues from model systems, primarily *Drosophila* and the mouse, to determine whether these regulatory pathways may determine the course of mammary development, function, and neoplasia, acting perhaps through well-studied signaling pathways in the gland.

SUMMARY OF THE MOST IMPORTANT RESULTS

Note: for published studies reprints are provided, and for experimental details and literature the reader is referred to these. For studies not yet published, additional detail is provided)

1. Hox Genes: Mouse: See (Friedmann *et al.*, 1994) for details.

One intensively studied group of regulatory genes is the homeobox family, which act as transcription factors, switching on or off groups of genes that specify the details of developmental processes. One of the triumphs of molecular biology during the past decade has been the discovery that these "homeobox" genes are not limited to the fly, but are ubiquitously distributed and remarkably conserved. In the mammals, including the human, not only do they occur in the genome, but their numbers and types are greatly amplified. It is now well established by gene targeting in the mouse that these genes are essential for determining many aspects of early embryogenesis, and more recently, it seems that they may be active in the development of tissues and organs, and that their malfunctions may contribute to cancer.

Do homeobox genes influence the breast, and if so, could they contribute to cancer? This was the original question underlying our research, and in the early stages of this project we discovered that many of these homeobox genes are active during the growth and development of both the mouse mammary gland and human breast. Their patterns of expression are frequently altered in cancer, some overexpressed, but much more often, underexpressed or not expressed at all (Table 1). This reduced expression in cancers is consistent with the role of Hox genes in determining cell fate and promoting differentiation in embryonic development of various organs and tissues. Because these genes serves such central regulatory roles, these discoveries create optimism that new insights into mammary development will be a product of these studies, and that an entirely new class of mammary oncogenes or tumor suppresser genes may be shown to exist. In general, it was found that expression was present, in either the epithelium or stroma (or occasionally both) during the periods of rapid growth, morphogenesis, and functional differentiation. Expression generally declined during lactation. These experiments and observations are not described in detail here, but are available in the attached reprint (Friedmann *et al.*, 1994).

2. The cancer-related Hoxa-1 and Hoxa-2 genes

As described (Friedmann and Daniel, 1996) the expression of Hoxa-1 is of particular interest, and to it we devoted considerable study. Hoxa-1 is not expressed in normal mouse gland or in precancerous hyperplastic nodules. It is, however, expressed at high levels in mammary tumors. This suggests that it may be associated with later rather than earlier stages of cancer progression, a particularly interesting finding in that these represent in the human the most clinically dangerous stages, when invasion and metastasis commence.

An obvious experiment was to ectopically express Hoxa-1 in normal mammary epithelial cells and attempt to detect precancerous or malignant changes. We chose to use the *ex vivo* method (Bradbury *et al.*, 1991; Edwards *et al.*, 1992), in which mammary epithelial cells are cultured by established methods and infected with a retroviral vector containing Hoxa-1 driven by a CMV promoter. Although we were successful in obtaining expression of a fraction of cultured cells, these cells did poorly upon transplantation and no Hoxa-1 expressing outgrowths were obtained, despite considerable effort. It is noted in retrospect that others have encountered similar difficulties with this method, and it appears

to have fallen out of favor. Consequently the interesting question as to whether Hoxa-1 is an oncogene remains open.

We have also examined the expression of the contiguous gene in this linkage group, Hoxa-2. Expression is not detectable in normal gland, but substantial levels of transcripts were seen in two of three tumors examined. Thus, the 3' genes of the Hoxa cluster may prove to be particularly important to cancer initiation and progression, though it is considered likely that they may act in a combinatorial manner and their effects may be extremely complex.

3. Hormonal effects: Msx genes: see (Friedmann and Daniel, 1996) for details

Not all homeobox-containing genes are contained within the four mammalian Hox clusters. . In *Drosophila, msh* is mainly expressed in the central nervous system and in segmented striated muscles of the body wall. In the mouse there appear to be three distinct msh-like genes, named *Msx-1, Msx-2* and *Msx-3*, which are found at separate loci and are not clustered The murine homeobox genes Msx-1 and Msx-2 are related to the Drosophila msh gene and are expressed in a variety of tissues during mouse embryogenesis. We found the developmentally regulated expression of Msx-1 and Msx-2 in the mouse mammary gland and show that their expression patterns point toward significant functional roles. Msx-1 and Msx-2 transcripts were present in glands of virgin mice and in glands of mice in early pregnancy, but transcripts decreased dramatically during late pregnancy. Low levels of Msx-1 transcripts were detected in glands from lactating animals and during the first days of involution, whereas Msx-2 expression was not detected during lactation or early involution. Expression of both genes increased gradually as involution progressed. Msx-2 but not Msx-1 expression was decreased following ovariectomy or following exposure to anti-estrogen implanted directly into the gland. Hormonal regulation of Msx-2 expression was confirmed when transcripts returned to normal levels after estrogen was administered to ovariectomized animals. In situ molecular hybridization for Msx-1 showed transcripts localized to the mammary epithelium, whereas Msx-2 expression was confined to the periductal stroma. Mammary stroma from which mammary epithelium had been removed did not transcribe detectable amounts of Msx-2, showing that expression is regulated by contiguous mammary epithelium, and indicating a role for these homeobox genes in mesenchymal-epithelial interactions during mammary development. These finding are published and details may be found there (Friedmann and Daniel, 1996). Interestingly, Msx-1 and Msx-2 have been found in gene targeting studies to be necessary for mammary development in early embryonic stages (Maas, personal communication).

4. The Extracellular Matrix (ECM) as Mediator of Hox Expression: see (Srebrow *et al.*, 1998) for details.

The stroma and ECM are known to influence morphological and functional development of the gland, and we inquired whether the expression of certain Hox genes, expressed postnatally and in cell lines, would be modulated by ECM. We showed the expression of five Hox genes in cultured cells, but focused on Hoxa-1 and Hoxb-7. It was found that culturing CIC-9 cells on basement membrane served to up-regulate Hoxb-7, whereas a change in cell shape brought about by culturing cells on an inert substrate caused down-regulation of Hoxa-1. It was also interesting that the expression pattern of Hoxa-1 did not precisely match that seen in the animal, indicating an influence of cultural conditions. These studies should permit further analysis of the molecular mechanism by which ECM

signaling and homeobox genes may interact to influence tissue organization (Srebrow *et al.*, 1998).

5. Analysis of Hoxd-10 (Lewis et al, in preparation)

Targeted disruption of Hoxd-10 produces mice with hindlimb-specific defects in gait and adduction (Carpenter *et al.*, 1997; de la Cruz *et al.*, 1999). 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. In addition, Dr. Carpenter described an apparent mammary defect in these mutants and proposed a collaboration. We obtained breeding pairs of Hoxd-10 heterozygotes and began an extensive breeding program. In addition, we have developed a small colony of athymic mice to be used as hosts in transplantation studies of these and other engineered mice. A germ-free surgical facility for these immunologically challenged mice has also been established in our animal facility and is in routine use .

Hoxd-10 Expression: Temporal Pattern. The discovery of a *Hoxd-10* mammary phenotype indicated an need for detailed expression data. The data indicate robust expression levels of the gene in the mammary gland. We investigated the normal expression pattern of the *Hoxd-10* gene by both Northern blot analysis and in situ hybridization. We have examined gland development in homozygous mutant and age-matched wild type control animals at many critical stages. These include: immature (5 week), mature (12-13 weeks), early pregnant (7.5 d.p.c.), late pregnant (17.5-19.5 d.p.c.), lactation (6 hours and 6 days), involution (days 2, 10 and 14), and lactation in a second pregnancy. Significantly, the highest levels are found in late pregnancy and lactation. This is an unusual pattern, since expression patterns of other Hox genes generally show high levels during ductal development and early pregnancy, but low to undetectable amounts in lactation (Table 1).

Hoxd-10 Expression: Spatial Pattern.. Expression during lactation shown by northern analysis (Table 1) must be evaluated in light of the dilution effect resulting from large amounts of milk protein transcripts. In our experience and that of others, even moderate levels detected during milk secretion indicates very high steady-state transcript levels. We have Therefore used hybridization *in situ* with gene-specific *Hoxd-10* probes to detect expression patterns in various stages of mammary development. In 5 week animals, *Hoxd-10* expression is concentrated in body cells of terminal end buds and is slightly reduced in cap cells (Fig. 1A). Considerable expression is also observed in the periductal stroma of the subtending duct. This expression pattern is maintained in mature glands which also show reduced expression in myoepithelial cells relative to lumenal epithelial cells (Fig. 1B). During pregnancy, lobule-alveolar cells also express *Hoxd-10* (Fig. 1C and D). In late pregnancy, alveolar epithelium begins to express at higher levels than in ducts (Fig. 1D). Highest levels of *Hoxd-10* expression are observed during lactation, in which 100% of alveolar epithelial cells stain darkly early in the color development (Fig. 1E). During

involution (data not shown) expression is undetectable at 2 days and returns as early as 10 days. At 14 days involution, the pattern of expression is comparable to that observed in the mature virgin. Sense strand control hybridizations show no staining, indicating specificity of antisense probe binding. These *in situ* hybridization results are generally consistent with the northern hybridization results, but indicate much higher levels during lactation.

Hox d-10 Phenotype. Our phenotypic analysis of female mice homozygous for a disrupted *HoxD10* gene verified a conspicuous defect in lactation. Pups from early litters of mutant females died from a lack of milk after several days of suckling, but survived if pups were fostered with lactating wild type females (Fig. 2). 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/or secretion is compromised.

A long-term breeding program has been used to permit examination of mice at all stages of mammary development and during the lactation cycle. In addition, we have transplanted gland from mutant females (-/-) into nude hosts, such that one inguinal cleared fat pad is transplanted with mutant tissue and the contralateral fat pad with control gland from littermates. This permits comparison of growth, differentiation, and lactation against a uniform hormonal and physiological environment. This powerful approach also permitted us to investigate the role of Hoxd-10 in mammary epithelium vs the peri-glandular stroma, since the glands resulting from these transplants are chimeric, in which the stromal component is always +/+.

Histological analysis of the hundreds of glands derived from this study is only recently completed. The results clearly indicate that at the morphological and histological level, structural development of the gland proceeds normally, as does the development of secretory lobules. However, the mammary glands from a null mouse in early lactation morphologically resembles the glands from late pregnant mice, with normally developed alveoli and ducts. The accumulation of milk in the lumena is dramatically reduced. This can be seen both from gross observation of the gland and from histological section (data not shown). The phenotype is not 100% penetrant, with some regions more normal in appearance than others.

Our interpretation of this large amount of data is that the null mouse fails to successfully complete the transition into lactation. To what extent this is attributable to changes is transcription, translation, vectorial secretion, or even suckling efficiency is not known and would be the subject of another subject. A significant contribution of this study is that it represents the most clearly defined and dramatic phenotype yet attributable to Hox mutations in the mammary gland. In a recent publication, (Chen and Capecchi, 1999) it was reported that the paralogous genes Hoxa-9, b-9, and d-9 resulted in defective lactation in triple mutants. Single genes had no apparent phenotype. In addition, the authors were unable to furnish in situ hybridization data to establish spatial patterns of organization. Hoxd-10 therefore shows a stronger phenotype. In addition, the results described above now represent the most fully documented functional analysis of a Hox phenotype in the mouse mammary gland (Lewis et al, in preparation).

6. The IRX Complex: a large new family of human homeotic genes expressed in the breast and in breast cancer: See (Lewis *et al.*, 1999a) for details.

To expand our studies into the area of human breast development and breast cancer, we undertook a search for homeobox-containing genes expressed in the breast. In addition to the expression of a daunting multitude of Hox and other homeobox genes, we identified five members of a new family of human Iroquois-class homeobox-containing genes (IRX genes) we cloned from a human breast cDNA library. We designated these genes IRX after the most closely related class of homeobox genes cloned thus far from Drosophila, the Iroquois complex (IROC) genes araucan (ara) and caupolican (caup) (Gomez-Skarmeta *et al.*, 1996). We cloned a full-length 1.8 kb cDNA derived from one of these genes, IRX-2, and have characterized its expression by *in situ* hybridization and Northern analysis in several human normal and tumor samples. In Northern blot analysis, IRX-2 gave rise to at least two transcripts, one 2.6 kb transcript that contains the homeobox and one 3.9 kb transcript that does not. By *in situ* hybridization using a gene-specific probe that recognizes both transcripts, we showed that IRX-2 is expressed almost exclusively in lobule-alveolar and lumenal ductal epithelium of the normal breast and not in myoepithelium or periductal stroma. We also showed that expression of IRX-2 is maintained in three different tumor types: infiltrating ductal carcinomas (IDC), infiltrating lobular carcinomas (ILC) and fibroadenomas (FA). *In situ* hybridization using a probe that recognizes all closely related homeodomain-containing transcripts suggests that all of these genes are likely to be expressed predominantly in breast epithelial cells. Together, these data indicate that IRX-2 and the related Iroquois-class genes may play a role in establishing and/or maintaining epithelial cell identity in normal and neoplastic breast. Importantly, continued robust expression and/or misexpression of IRX-2 in mammary tumors and hyperplasias also suggests a possible role in cell proliferation and cancer progression. Please see the enclosed reprint for details (Lewis *et al.*, 1999a).

7. A role for Hedgehog signaling in the mammary gland: Regulation of homeobox and other mammogenic genes by upstream regulatory pathways.

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. In model systems such as Drosophila and the early development of the mouse, 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 (review (Hammerschmidt *et al.*, 1997), it has not been studied in the mammary gland to our knowledge, and certainly nothing had previously been published before the landmark paper of Lewis and colleagues (Lewis *et al.*, 1999b).

In Drosophila, where it was first discovered and investigated in detail, 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 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 3). 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. 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. In spite of the expansion of family members, the basic plan of 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 (Wysolmerski *et al.*, 1998). The above list reads like a litany of genes and cell products that are known to be important to mammary development and cancer. If it is eventually found that the hedgehog pathway influences only a fraction of these in the mammary gland, that will be a discovery of considerable significance.

7a. Ptc-1: Function and expression pattern: see (Lewis *et al.*, 1999b) for details.

This paper investigates 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. This phenotype differs from most mutations affecting the mammary gland, in that much of the phenotype is associated with defects in histogenesis, rather than grossly observable alterations in morphogenesis and ductal patterning. This suggests that some of the downstream mediators of hedgehog signaling are cell-cell and cell-ECM adhesion mechanisms. 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. These 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. Demonstrating the requirement for hedgehog signaling in the mammary gland establishes a new paradigm for high-order genetic regulation of mammary development, function and tumorigenesis (Lewis *et al.*, 1999b).

7b. Gli-2: Function and expression patterns (Lewis et al: In manuscript).

Expression of Hedgehog components using RT-PCR.

Expression studies of Gli-2 and Ptc-1 were extended to include all three Hh mammalian ligands, to provide a more comprehensive overall picture of the temporal pattern of expression of all these Hh components. Only Indian hedgehog (Ihh) was expressed at significant levels, although low levels of Desert hedgehog (Dhh) sere also found. These data suggest but do not prove that Ihh is the functional ligand in the mammary gland. Indian hedgehog was shown to be expressed in wild-type mammary gland at stages 3 week, 5 week, mature, early pregnant, late pregnant, and 4 day involuting mammary tissue and in the 14 day embryo positive control . The round one RT-PCR products were of the expected size (565 bp) and hybridized to an *Ihh* probe in a Southern Blot of the RT-PCR products (not shown).This strong expression of Ihh is surprising in light of the much more frequently demonstrated role in the embryo for Sonic hedgehog (Shh).In the mammary gland, only traces of Shh were occasionally observed. Our RT-PCR studies have been combined into a single chart, intended to give a semi-quantitative overview (Fig. 4). Although the pattern is complex and not easily summarized, it is apparent that highest levels of ligand (Ihh), receptor (Ptc-2), and transcription factor (Gli-2), are found in lactation.

Spatial expression of gli-2

Gli2 was expressed exclusively in the stromal compartment during virgin stages of mammary development and was particularly associated with actively condensing stroma at the neck of growing terminal end buds. However, during pregnancy and lactation, Gli2 expression was both epithelial and stromal suggesting differential function during reproductive development. This tissue compartment expression switch correlates well with increased expression of Indian hedgehog during these stages as well as with the known period of functional requirement of ptc1 during ductal, but not alveolar, development (Fig 5).

Disruption of gli-2 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* homologues) in mediating hedgehog signaling. One recent model postulated that the GLI1 protein took on the transcriptional activation functions ascribed to *Drosophila* Ci_{act} and that Gli-3 protein took on the transcriptional repression function ascribed to Ci_{rep} with GLI2 modulating the function of GLI1 and GLI3 in an unspecified manner (Dahmane *et al.*, 1997). 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. 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. 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, (Tanimura *et al.*, 1998). 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.

Mice heterozygous for targeted inactivation of *Gli-2.* 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. These transplants undergo 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.5). 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 were 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. Histological characterization demonstrated that misshapen ducts showed epithelial hyperplasia similar to micropapillary ductal hyperplasias in the human breast. Interestingly, these morphological and histological defects were not observed in homozygous null epithelium when transplanted into a wild type stromal background. These observations suggest a stromal (or both epithelial and stromal) function for Gli2 during ductal development.

Conclusions. Although there are 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* (Fig. 5A) must now be examined for their transplantability and tumorigenic potential.

LIST OF PUBLICATIONS

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PARTICIPATING SCIENTIFIC PERSONNEL

Charles W. Daniel, P.I.
Dr. Michael Lewis, Postdoctoral Fellow
Dr. Yael Friedmann: *Ph.D. obtained*
Dr. Gary Silberstein, Staff Research Scientist
Sarajane Ross, Graduate Student: *MS obtained*
Phyllis Strickland, Staff Research Associate
Charles Sugnet, Staff Research Associate
Elsa Jiminez, Staff Research Associate

REPORT OF INVENTIONS

None

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TABLE 1

Table 1. Northern blot (polyA RNA) using a gene-specific probe to Hoxd-10. Band densities were standardized against the ribosomal protein L7.

	5 wk	mature	EP	LP	Lact*	2 day Invol	10 day Invol	14 day Invol
HoxD10	+	++	+++	++	++	ND	ND	ND

*Expression during lactation must be evaluated in light of the dilution effect resulting from large amounts of milk protein transcripts. In our experience and that of others, even moderate levels detected during milk secretion indicates very high steady-state transcript levels.

TABLE 2. Summary of homeobox gene expression in mouse mammary gland[1,2] (Northern Hybridization)

	5 weeks old	8-9 weeks old	16 weeks old	5-8 days pregnant	15-18 days pregnant	lactating[3]	ovariectomized at 5 weeks	ovariectomized at 12 weeks	tumor D1a	tumor D2a	tumor D2d	myc tumor
Hoxd-3	–	–	–	–	–	–	–	–	+	–	–	–
Hoxd-4	++	++	++	++	+	–	+++	++	–	–	–	–
Hoxd-8	+++	+++	+++	+++	+	+	+++	+++	–	–	–	–
Hoxd-9	++	++	++	+++	+	+	++	++	–	–	–	–
Hoxd-10	+	+	++	+++	++	++	++	++	–	–	–	–
Hoxd-11	–	–	–	–	–	–	–	–	–	–	–	–
Hoxd-12	–	–	–	–	–	–	–	–	–	–	–	+++
Hoxc-6	++	++	++	+	–	–	+++	+++	–	–	–	–
Hoxc-8	++	++	++	+	–	–	+++	+++	–	–	–	–
Hoxb-6	++	++	++	+	–	–	++	++	–	–	–	–
Hoxb-7	++	++	++	+	–	–	++	++	–	–	–	–
Hoxa-1	–	–	–	–	–	–	–	–	+++	+	+++	–
En-1	++	++	++	++	–	–	+++	++	+	+	+	+
En-2	–	–	–	–	–	–	–	–	–	–	–	–
Msx-1	++	+++	+++	+++	++	+	+++	+++	++	+	+/–	++
Msx-2	+++	+++	+++	++	–	–	+	+	–	–	–	–

1. Levels are compared for each gene individually and cannot be compared between different genes. (–) no expression detected; (+/–) very low expression; (+) low expression; (++) moderate expression; (+++) abundant expression.
2. Levels are adjusted according to L7 loading control.

FIGURE LEGENDS

Figure 1. Hybridization *in situ* with gene-specific *Hoxd-10* probes. (A) Terminal end bud and subtending duct. (B) Adult duct in virgin. (C) Early pregnancy and (D) late pregnancy. (E) Lactating gland. (F) Sense strand control.

Figure 2. Weight gain of pups from early litters of Hoxd-10 mutant females. Pups suckled on mutant mothers died from a lack of milk after several days of suckling, but survived if pups were fostered with lactating wild type females.

Figure 3. (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.

Figure 4. 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 develop developmental stages are denoted by colored horizontal bars (upper section). Phenotypic alterations in Ptc-1 and Gli-2knockout strains are noted by color for the altered mammary structure(middle section). Relative expression levels in predominant mammary tissue compartments and cell types are also shown by color (lower section).

FIGURE 5. Spatial localization of Gli-2, Ihh, and Ptc-1 transcripts shown by in situ hybridization using a gene-specific probes. The various adult developmental stages are indicated, with the exception of involution.

Figure 6. (Upper) 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. **(Lower).** Histological analysis of representative mammary lesions from *Gli2* heterozygotes and comparison with a similar human lesion. Ductal lumens are noted by a blue letter L; Adipose stroma is noted by a blue letter S. A) Whole gland preparation showning 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. Compare with C.

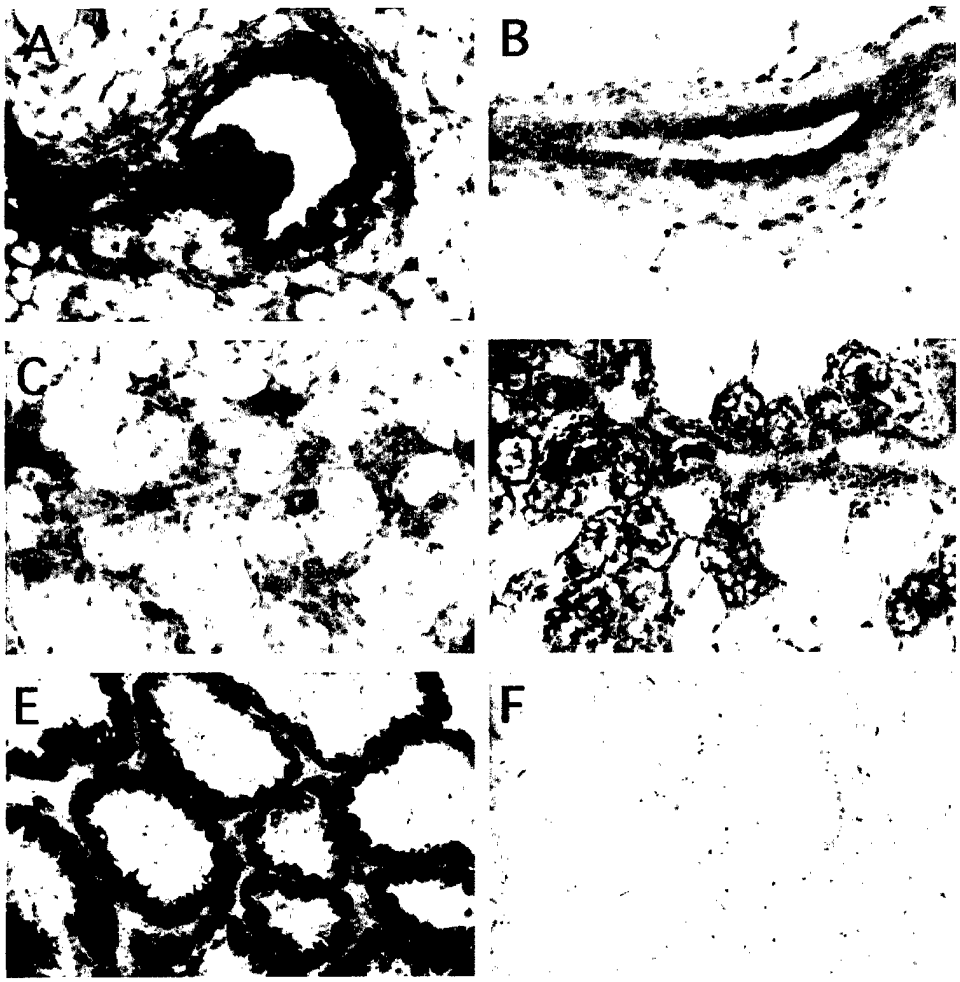

FIGURE 1

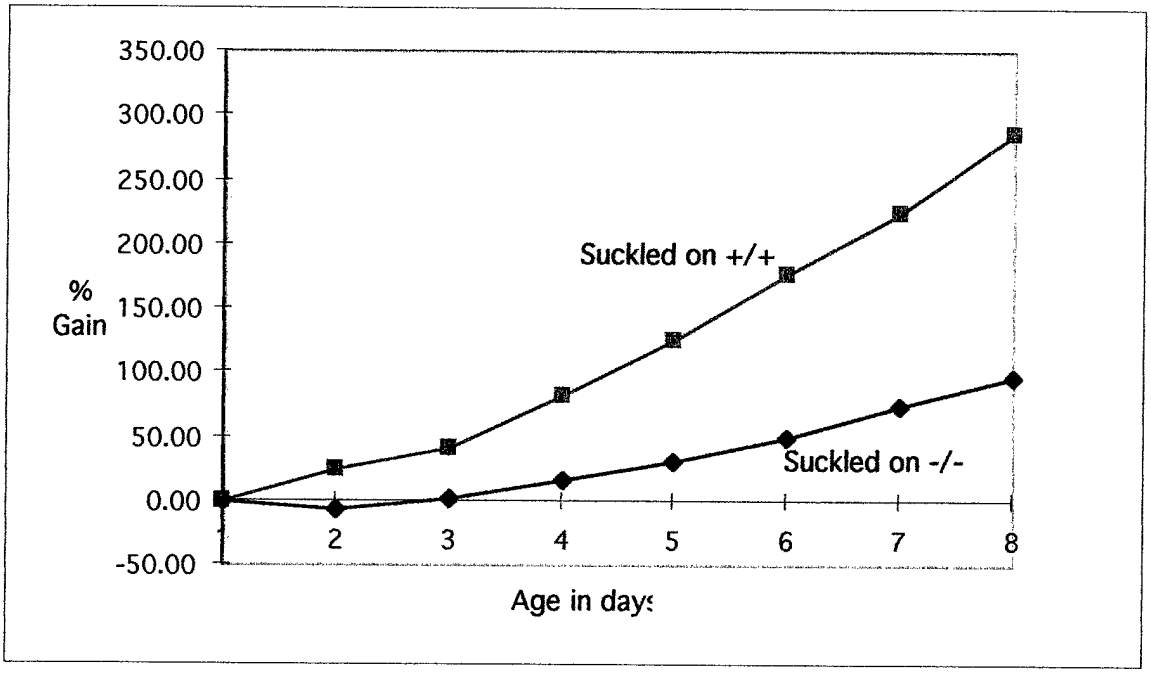
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
-50.00
%
Gain
Suckled on +/+
Suckled on -/-
2
3
4
5
6
7
8
Age in days

FIGURE 2

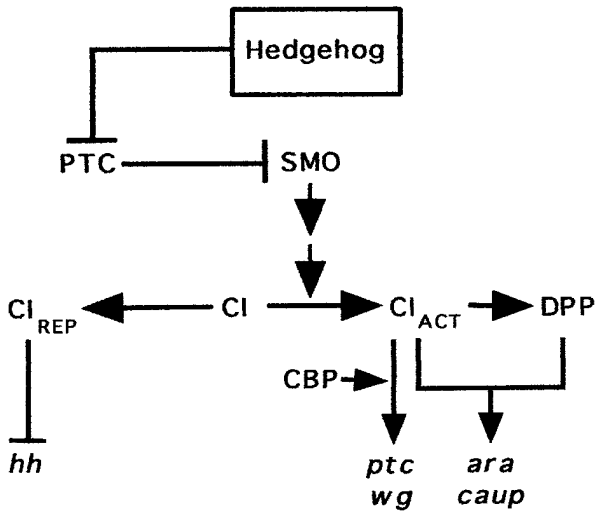

FIGURE 3 **A**

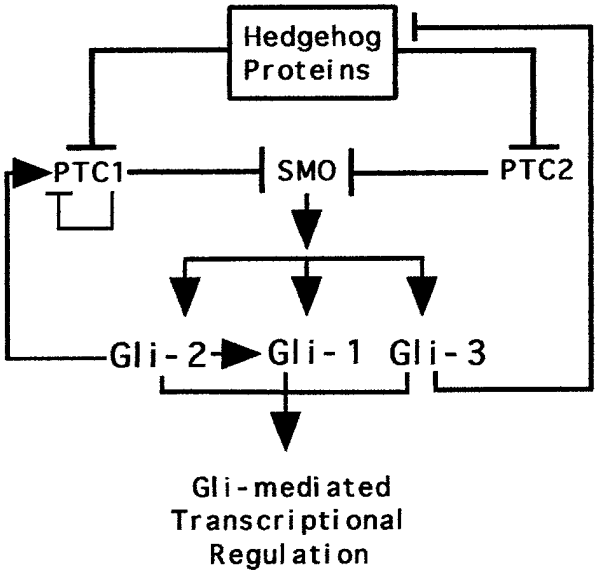

FIGURE 3 **B**

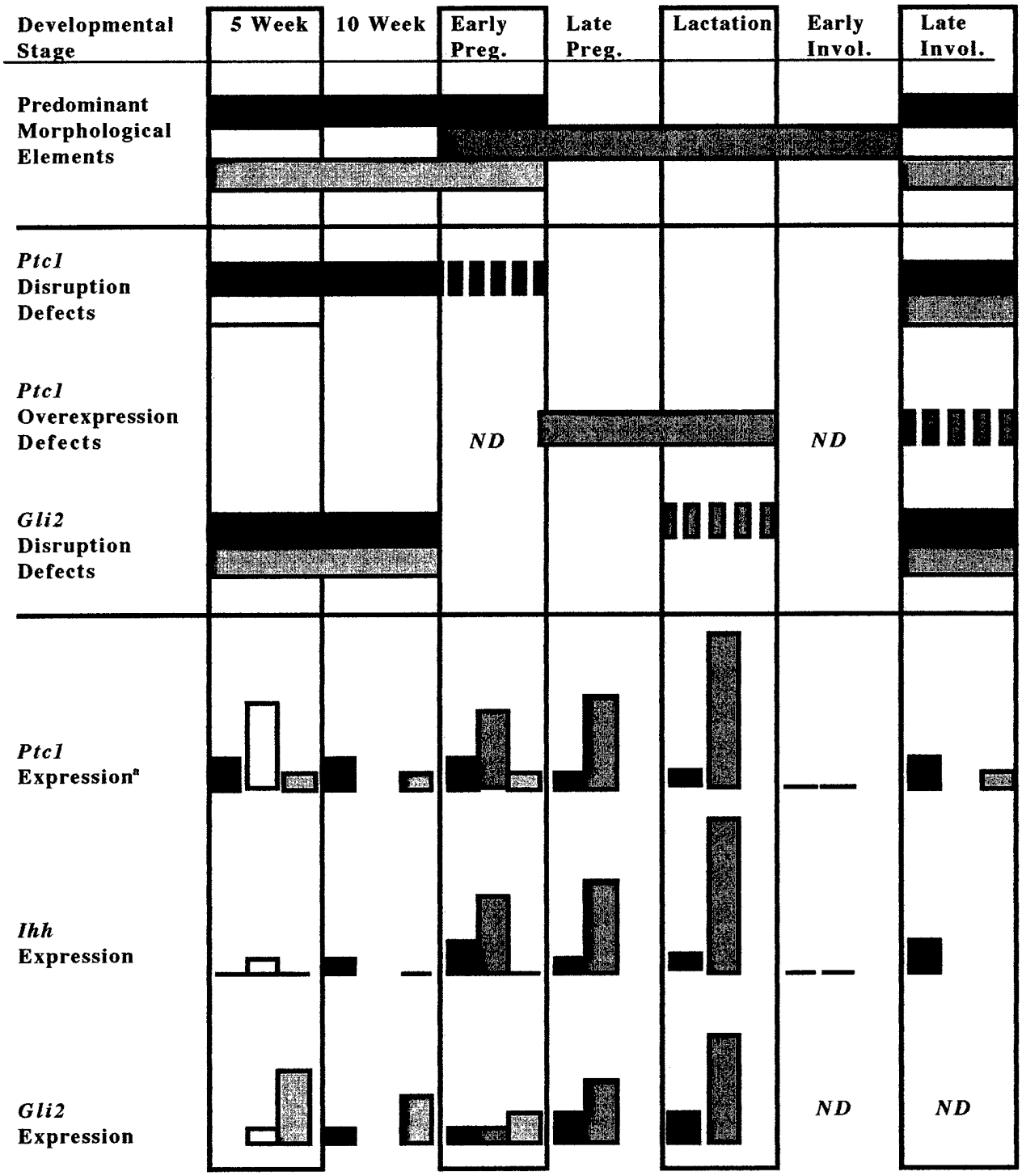
Developmental Stage
5 Week
10 Week
Early Preg.
Late Preg.
Lactation
Early Involution
Late Involution
Predominant Morphological Elements
Ptc1 Disruption Defects
Ptc1 Overexpression Defects
ND
ND
Gli2 Disruption Defects
Ptc1 Expression[a]
Ihh Expression
Gli2 Expression
ND
ND
Ducts
Periductal stroma
Alveoli
Terminal end bud

FIGURE 4

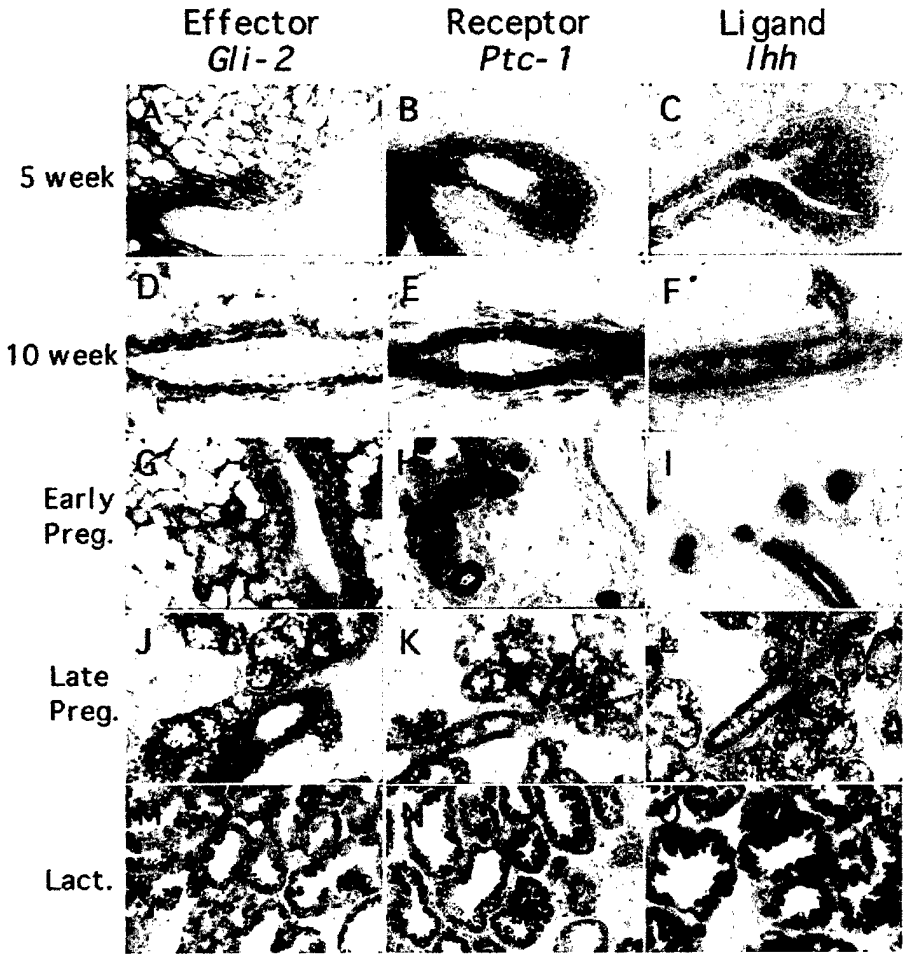
Effector
Gli-2
Receptor
Ptc-1
Ligand
Ihh
5 week
10 week
Early
Preg.
Late
Preg.
Lact.
A
B
C
D
E
F
G
H
I
J
K
L

FIGURE 5

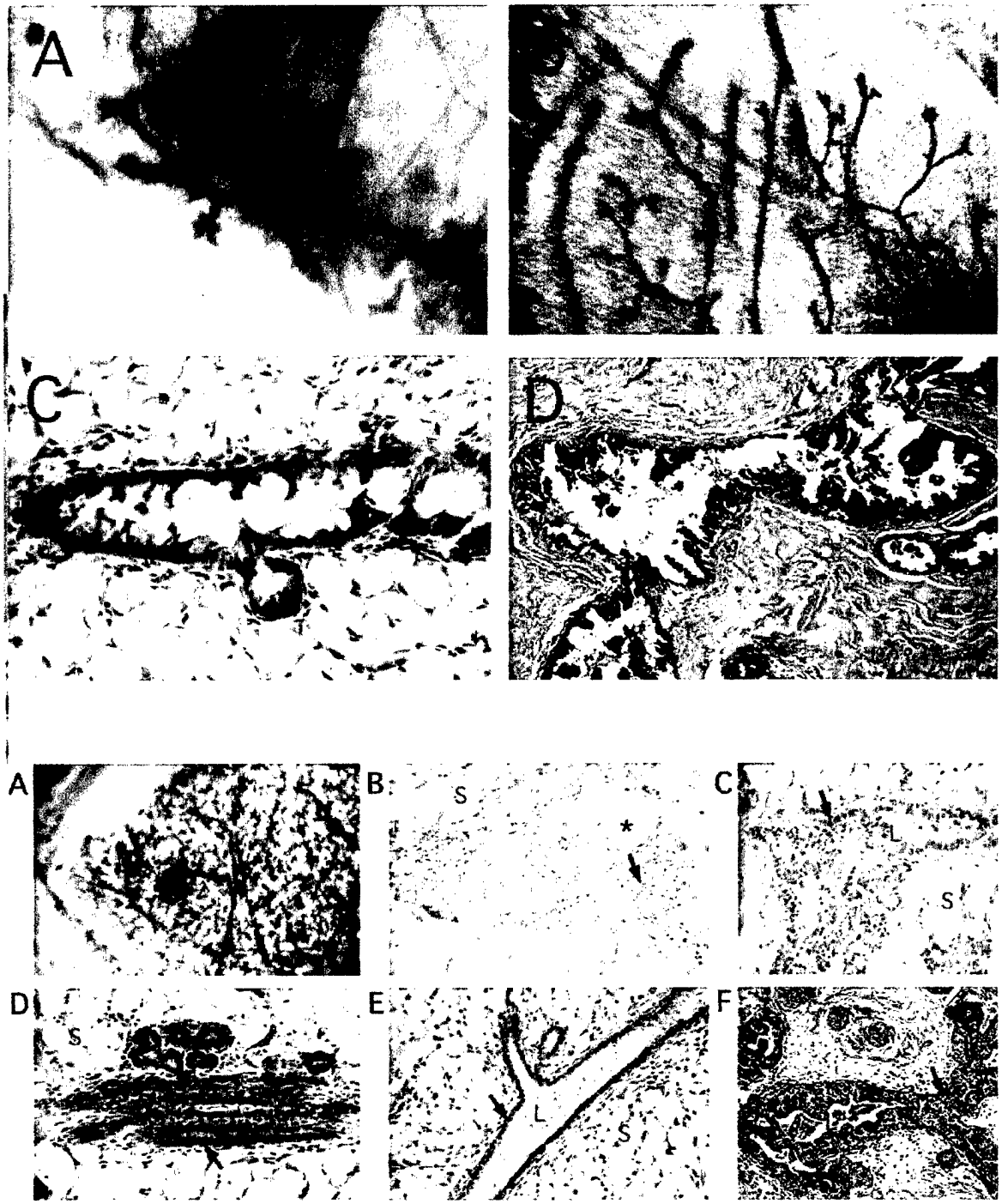

FIGURE 6

APPENDIX (REPRINTS)

[CANCER RESEARCH 54, 5981–5985, November 15, 1994]

Hox Genes in Normal and Neoplastic Mouse Mammary Gland[1]

Yael Friedmann,[2] Carla A. Daniel, Phyllis Strickland and Charles W. Daniel

Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

ABSTRACT

Homeobox-containing genes regulate embryonic developmental programs and are expressed in certain adult tissues and cancers. There has been no report of expression in the breast. We amplified homeobox complementary DNA from mouse mammary gland and found expression of members from each of the four major *Hox* gene clusters. The regulation of expression of two *Hox* genes was examined in greater depth. *Hoxc-6* transcripts were present in the glands of pubescent and mature mice and decreased during pregnancy. Levels were increased substantially following ovariectomy, indicating possible negative regulation by steroid hormones. *Hox* expression was studied in mammary adenocarcinomas and in transplant lines of the benign, precancerous tissues from which the cancers arose. *Hoxc-6* was expressed at low levels in the precancerous tissue but was not expressed in cancers. In contrast, *Hoxa-1* was expressed only in cancers, not in normal gland or in precancerous mammary tissues, suggesting that *Hox* genes may play a role in a late stage in the stepwise development of mammary malignancies.

INTRODUCTION

Homeobox-containing genes (homeogenes) comprise a large collection of developmental regulators that have aroused interest due to their role in specifying developmental pathways in embryos. Mutations in certain of these genes may result in incorrect specification of body parts in organisms ranging from invertebrates to mammals (1–3). Recently, interest in possible roles for homeobox genes in adult animals has been sparked by the observation that a number of homeobox genes are transcribed by more mature tissues, including kidney (4) and hemopoietic cell lineages (5). Of particular interest is the observation that certain cancers display altered homeobox gene expression (6–8); misexpression of homeobox genes can transform cells *in vitro* and produce tumors in mice on transplantation (9, 10). Thus, homeobox-containing genes may constitute a new class of proto-oncogenes (11).

All homeobox genes have a common structural characteristic of a box motif of about 183 bases, coding the 61 amino acid homeodomain. Thirty-eight murine genes containing an Antennapedia-like homeobox have been characterized and comprise the four *Hox* gene clusters. Recent work carried out with *Drosophila* genes encoding comparable homeodomain sequences suggests that homeoproteins can bind to DNA by a helix-turn-helix structure and thus probably play a regulatory function by influencing the expression of downstream genes (12–14). Although it is likely that these homeoproteins are involved in important developmental processes in vertebrates, the full extent of their influence, particularly in adults, remains unclear. One way to gain insight into this question is to examine the pattern of homeogene expression under normal and altered conditions, thus identifying some of the levels at which these genes may exert effects.

The mouse mammary gland represents the most fully developed system for the analysis of the normal and neoplastic breast, making it an attractive model for studying the regulation of *Hox* gene expression. The mammary gland is unique in that most of its growth and morphogenesis takes place in the subadult or adult mammal, where it is experimentally accessible. During puberty, a ductal tree is generated by epithelial growth and branching, resulting from embryonic-like inductive interactions between mammary epithelium and mammary stroma (15). The differentiative pathway of ectodermal epithelial cells depends on their position within the growth buds; they may become cells destined for ductal walls, milk synthesis, and secretion, or contractile myoepithelium (16).

The development of mouse mammary tumors is a multistage process that occurs through definable stages: normal; preneoplastic; and neoplastic. These cell populations have biologically distinct growth properties. Normal mouse mammary cells have a finite life span *in vivo* and after five or six serial transplant generations show a diminished growth potential (17). The preneoplastic stage is represented by the HAN[3] (18, 19), which represents a benign, immortalized cell population that is morphologically similar to differentiated alveolar cells normally found in glands of pregnant mice but does not require lactogenic hormones for maintenance. HANs can be grown indefinitely *in vivo* as tissue lines when serially transplanted into mammary gland-free fat pads. Because HANs have been shown to exhibit a far greater probability for tumor formation than the normal alveoli found in pregnant mice (20, 19), they have been termed "preneoplastic" and may represent an obligatory intermediate in the conversion to malignancy. The availability of large amounts of premalignant tissue and of tumors derived from it permits the simultaneous investigation of different stages of tumor progression within a well defined cell population.

The mammary gland appears to be a candidate target for homeobox gene action. It represents a variety of developmental interactions and pathway decisions as it undergoes its complex developmental cycle of branching morphogenesis, functional differentiation, secretion, and involution, in which master regulatory genes are likely to play a role. The stepwise progression from normal mammary gland to malignancy is more fully documented and is experimentally more accessible than other animal tumors.

In this paper we report expression of *Hox* genes in the mammary gland and show it to be independently regulated among the genes examined and developmental stage specific. We further demonstrate that expression of two *Hox* genes in the mammary gland responds to hormonal secretions from the ovaries and is altered in cancer, suggesting a role for homeogenes in mammary gland development and neoplasia.

MATERIALS AND METHODS

Animals. C57BL/crl and BALB/c mice were used for collection of the inguinal mammary glands in all RNA preparations, with the exception of the HANs and the tumors which were derived from them, which are carried only in BALB/c mice. Virgin mice were chosen randomly from multiple cages to minimize the chances of selecting animals in a particular stage of estrus. Thoracic glands of virgin and ovariectomized C57BL/crl mice and HANs carried in inguinal glands of BALB/c mice were used for whole mount histology. Tumors carried in inguinal glands of BALB/c mice were used for section histology. Stage of estrus was determined by vaginal smears.

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[1] This work was supported by PHS Grant HD 27845.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: HAN, hyperplastic alveolar nodules; poly(A)$^+$ RNA, polyadenylated RNA; PCR, polymerase chain reaction.

Surgery. Ovariectomy was carried out at about 5 weeks of age (animal weight, 16–17 g). The inguinal fat pads were cleared of epithelium at 3 weeks of age by removing the portion of the gland containing epithelium and cauterizing the necessary blood vessels and the nipple area as described (20).

Transplantation. A small piece of HAN or tumor tissue from a donor animal was cut out and inserted into the cleared fat pad of host mice. Tissue lines were left at least 2 months before the animals were used for tissue harvest or as donors for extending the lines.

RNA Preparation and Northern Hybridization. Inguinal mammary glands were frozen in liquid nitrogen immediately after removal, and total RNA was prepared by the guanidine isothiocynate (4 M)-cesium chloride (5.7 M) method (21). Total RNA from the glands (number and age of animals used for each experiment is given in figure legends) was isolated. In several cases poly(A)$^+$ RNA was purified by oligodeoxythymidylic acid-cellulose chromatography as described (22). Five μg of poly(A)$^+$ enriched RNA or 20 μg of total RNA was electrophoresed in 1.0% agarose containing 2.3 M formaldehyde in 0.2 M morpholinopropanesulphonic acid-50 mM sodium acetate-5.0 mM EDTA, pH 7.0. RNA was transferred to a Nylon transfer membrane (0.45 μm Magna NT; Micron Separation, Inc.) by the established procedure of Maniatis *et al.* (23). Northern hybridizations were carried out under high stringency conditions using ^{32}P random primed labeled ($1–10 \times 10^9$ cpm/ μg) murine *Hoxa-1* and *Hoxc-6* and human L7 cDNAs. *Hoxa-1* fragment is ~770 base pairs, derived from the 3′-trailer sequence and does not include the polyadenylic acid tail or the homeobox. The *Hoxc-6* fragment is ~270 base pairs, derived from coding sequence 5′ of the homeobox. Washes after hybridizations were in 0.1× saline-sodium phosphate-EDTA-0.1% sodium dodecyl sulfate at 65°C.

PCR Amplification, Cloning of Homeobox Sequences, and Sequence Analysis. RNA was extracted from glands from subadult and pregnant mice as described and used to make cDNA by reverse transcriptase, using oligodeoxythymidylic acid as a primer. cDNA was purified by centrifuging through Ultrafree-MC 30,000 NMWL filter unit (Millipore) and served as a template for PCR amplification using 40 μg/ml of degenerate oligonucleotide primers. The reaction conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 3 min, and a final incubation of 72°C for 20 min. The sequences of the degenerate primers were (International Union of Pure and Applied Chemistry code used): (I/L/V)YPWM, 5′-CGCGGATCCNTNTAYCCNTGGATG-3′; ELEKEF, 5′-CGCGGATCCGARYTNGARAARGARTT-3′; KIWFQN, 5′-CCCAAGCTTRTTYTGRAACCADATYTT-3′.

Similar sequences were used by Mackem and Mahon (24). PCR products were cloned into pGEM1 and sequenced using the dideoxy sequencing method of Sanger *et al.* (25)

Histology. For whole mount preparations, glands were fixed overnight in Tellyesnicky's fixative. They were defatted in three changes of acetone, hydrated through graded alcohols, stained for 2 h with hematoxylin, dehydrated through graded alcohols to xylene, and photographed. For histological sections, glands were treated as described for whole mount preparations, embedded in paraffin, sectioned at 5 μm, and mounted on slides. Sections were deparaffinized through three changes of xylene, hydrated through graded alcohols, stained with hematoxylin and eosin, dehydrated through graded alcohols to xylene, and coverslipped.

RESULTS

Detection of Candidate Homeobox Genes Expressed in the Mouse Mammary Gland. To identify homeobox genes that are expressed during mammary gland development, several degenerate oligonucleotide primers were constructed from highly conserved domains within and just upstream of the homeobox, and used to amplify cDNA from mammary glands of subadult and pregnant animals. The PCR products yielded predominantly DNA fragments of the expected size, which were cloned, and the DNA sequences of several individual clones were determined. Fig. 1 shows the deduced amino acid sequences for representatives of *Hox* genes from the four clusters [*Hoxa-7*, *Hoxb-6*, *Hoxc-6*, and *Hoxd-12*; in accordance with nomenclature suggested by Scott (26)]. Two *Hox* genes were chosen for further study, *Hoxc-6* and *Hoxa-1*.

Expression of *Hoxa-1* and *Hoxc-6* Is Independently Regulated and *Hoxc-6* Transcript Levels Are Developmentally Regulated. Total cellular RNA was extracted from mammary glands of mice at the onset of puberty (about 5 weeks after birth), 2 time points during adulthood of virgin animals (8–9 weeks and 16 weeks after birth), early pregnancy (5–8 days postcoitus), late pregnancy (16–18 days postcoitus), and lactation. The RNA was poly(A)$^+$ enriched and Northern blots of these RNAs were hybridized consecutively with *Hoxc-6*, *Hoxa-1*, and L7 probes. L7 RNA was used as a control for amount of RNA and its integrity. Fig. 2 shows the pattern of expression of the RNAs during mammary development. *Hoxc-6* transcripts (~1.8 kilobases; Fig. 2*A*) were readily detected in glands from pubescent and mature virgin animals. Transcript levels decreased in glands from animals that were in early stages of pregnancy and could not be detected in a later stage of pregnancy or during lactation (the same pattern was seen also in the BALB/c strain; data not shown). During lactation, high levels of transcripts for milk proteins may dilute other mRNAs, as can be seen for the L7 loading control. *Hoxc-6* may therefore be expressed during lactation at higher levels than indicated. *Hoxa-1* was not detected in any of these stages of development, even after prolonged autoradiographic exposure (Fig. 2*B*), nor was it detected in normal developmental stages of the BALB/c strain (data not shown). These different expression patterns indicate that the two *Hox* genes are independently regulated and that *Hoxc-6* transcript levels are developmentally regulated during the gland cycle, declining in pregnancy.

***Hoxc-6* Expression in Glands from Ovariectomized Animals and during the Estrus Cycle.** Mammary gland development is stimulated by the ovarian hormones estrogen and progesterone (27–29). These act directly on the mammary gland (30), probably by regulating growth factors (31), and may also act indirectly through mediation of pituitary secretions (32). In response to ovariectomy, mammary ducts become much smaller in diameter and simpler in pattern of branching (Fig. 3, *C* and *D*). If homeogenes have a significant role in the

```
Hoxa-7:
        FYPWMRSSGPDSKRGRQAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQN
Hoxb-6:
LYPWMQRMNSCNSSSFGPSGRRGRQTYTRYQTLELEKEFHYNRYLTRRRRMEIAHALCLTERQIKIWFQN
Hoxc-6:
LYPWMQRMNSHSGVGYGADRRRGRQIYSRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQN
Hoxd-12:
                                ELEKEFLVNEFINRQKRKELSNSLNLSDQQVKIWFQN
```

Fig. 1. Deduced amino acid sequences of homeobox domains from the four *Hox* clusters as amplified by PCR from cDNA from mammary glands of mature and pregnant mice. The primer-derived sequences are underlined.

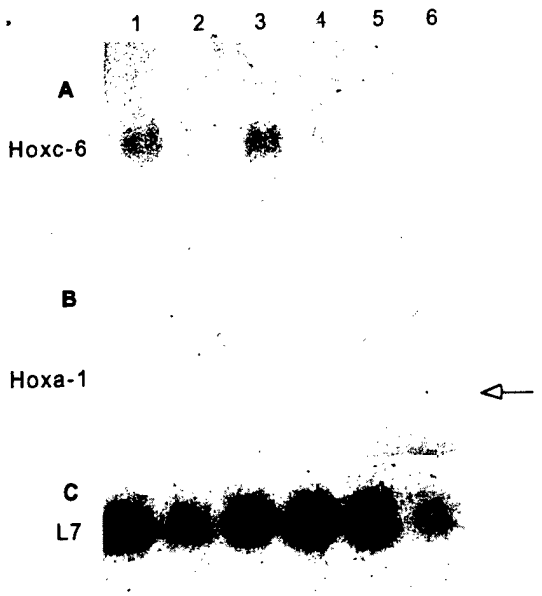

Fig. 2. RNA expression of *Hoxc-6* and *Hoxa-1* during different developmental stages of the mouse mammary gland. Poly(A)$^+$ RNAs were extracted from different stages of the mammary gland development. *Lanes 1–3*, 13–25 virgin mice were taken; *Lanes 4–5*, 5–7 timed-pregnant mice were taken; *Lane 6*, 3 lactating animals were taken. The Northern analysis was performed as described in "Materials and Methods." *Lane 1*, 5-week-old animals; *Lane 2*, 8–9-week-old animals; *Lane 3*, 16-week-old animals; *Lane 4*, 5–8 days pregnant; *Lane 5*, 15–18 days pregnant; *Lane 6*, 3–4 days lactating. Each lane contains 5 μg of poly(A)$^+$ RNA. *A*, *Hoxc-6*; *B*, *Hoxa-1*; *C*, L7 RNA. *Arrow*, ~2.5 kilobases, where *Hoxa-1* transcripts were detected in Fig. 4*B*.

mammary gland development, it is likely that a linkage between them and the mammogenic hormones that are required for growth, morphogenesis, and functional activity would be discovered.

To determine whether *Hoxc-6* transcript levels are regulated by ovarian secretions, we isolated RNA from glands of animals that had been ovariectomized at 5 weeks of age and allowed to mature for 4 weeks after ovariectomy, a time period that was determined adequate for the ovarian steroids to be depleted from the tissues. Fig. 3*A* shows the result of a Northern blot hybridization with a *Hoxc-6* probe, followed by L7 loading control (Fig. 3*B*). In glands from ovariectomized animals (Fig. 3, *Lane 1*) *Hoxc-6* transcript levels are substantially elevated compared to glands from intact virgin mice (Fig. 3, *Lanes 2* and *3*).

We also compared glands from animals that were in diestrus (low steroid) and early estrus (high steroid). *Hoxc-6* appeared to be expressed at slightly higher levels in glands from animals in diestrus (Fig. 3, *Lane 2*) than in glands from animals in early estrus (Fig. 3, *Lane 3*). The difference was small (13–22% as determined by densitometry measurements) but reproducible. *Hoxa-1* transcripts were not detected in glands from ovariectomized animals (not shown). These results suggest that *Hoxc-6* transcripts are down-regulated by ovarian secretions, the candidate hormones being estrogen, progesterone, and possibly prolactin, which is secreted by the pituitary and is regulated by estrogen (33).

***Hoxa-1* and *Hoxc-6* Expression in Preneoplastic and Neoplastic Mammary Glands.** Steroid hormones are implicated in breast cancer (34), and our observed regulation of *Hoxc-6* expression by the ovaries suggested a possible role for these genes in mammary cancer. The availability of precancerous tissues, and of several mammary carcinomas derived from them (Fig. 4, *D* and *E*), made it possible to examine *Hox* expression patterns in two stages of tumor progression.

Total cellular RNA was extracted from HAN lines D1 and D2 (18). We chose three tumors that arose spontaneously from these precancerous tissues. One tumor arose from nodule D1 and was designated D1a. Two tumors arose from nodule D2 and were designated D2a and D2d, respectively. Total cellular RNA was extracted from generation 1 of tumor D1a, generation 3 of tumor D2a, and generation 10 of tumor D2d. RNAs from HAN transplant lines and from their tumors were subjected to Northern blot hybridization analysis using *Hoxc-6*, *Hoxa-1*, and L7 probes, consecutively. *Hoxc-6* showed very low levels of expression in the two HANs and was not detected in any of the tumors, even after prolonged autoradiographic exposure (Fig. 4*A*). In contrast, *Hoxa-1* transcripts (~2.5 kilobases) were present in high levels in all three tumors but were not detected in glands from virgin or pregnant animals or in either of the HANs from which the tumors arose (Fig. 4*B*).

DISCUSSION

This paper describes the expression of several *Hox* gene members in the mouse mammary gland; it is likely that other *Hox* genes, as well as homeogenes outside the *Hox* clusters, are transcribed in one or more of the mammary developmental stages. In this initial report,

Fig. 3. RNA expression of *Hoxc-6* in the mammary gland in response to different levels of steroids. Northern analysis was performed as described in "Materials and Methods." *Lane 1*, mammary gland RNA from ovariectomized mice. Sixteen mice were ovariectomized at 5 week of age and allowed to mature to 9 weeks prior to glands collection. *Lanes 2–3*, the stage of estrus of 5-week-old mice was determined by vaginal smears; *Lane 2*, RNA from glands of 3 animals in diestrus (low steroid); *Lane 3*, RNA from glands of 6 animals in early estrus (high steroid). Each lane contains 20 μg of total RNA. *A*, *Hoxc-6*; *B*, L7; *C* and *D*, whole mount preparations of glands from an ovariectomized C57/Bl mouse (*C*) and an intact mature virgin C57/Bl mouse (*D*) *Arrows*, epithelial duct showing a decrease in ductal diameter and a simplification of ductal patterning in response to ovariectomy. *Bar*, 1 mm.

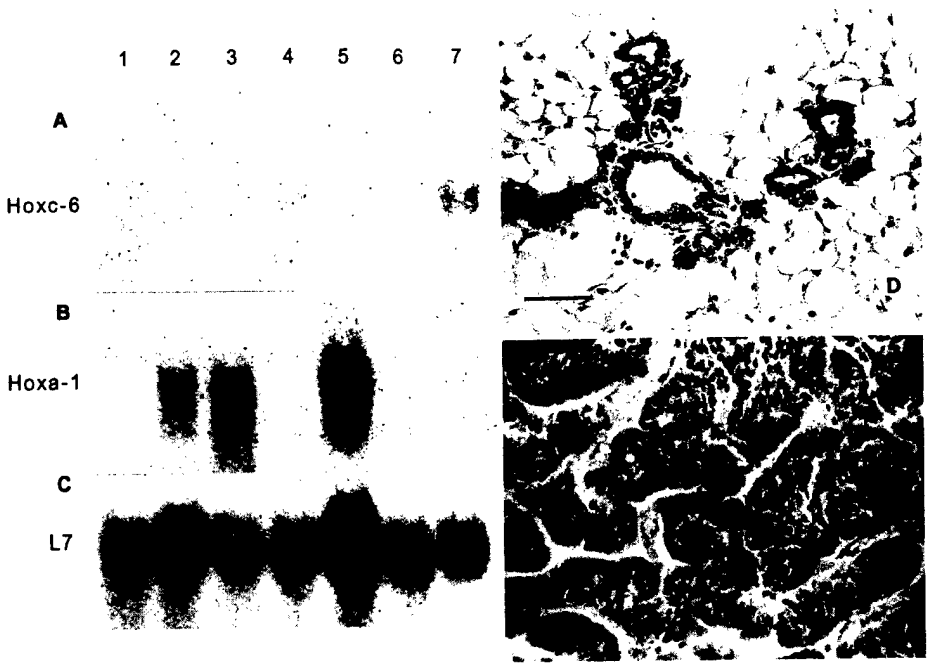

Fig. 4. *Hoxa-1* and *Hoxc-6* expression in preneoplastic and neoplastic mammary outgrowths. RNA was isolated from hyperplastic alveolar nodules (18) that were serially transplanted in BALB/c gland-free fat pads and from spontaneous carcinomas that arose from these nodules and were subsequently transplanted. *Lane 1*, HAN D2; *Lane 2*, tumor D2a; *Lane 3*, tumor D2d; *Lane 4*, HAN D1. *Lane 5*, tumor D1a; *Lane 6*, mammary gland RNA from 15–18 days pregnant animals; *Lane 7*, mammary gland RNA from mature virgin animals. Northern analysis was performed as described in "Materials and Methods." Each lane contains 20 μg of total RNA. (A) *Hoxc-6*. (*B*) *Hoxa-1*. (*C*) L7. (*D*) 5-μm section of HAN D1. (*E*) 5-μm section of tumor D1a. Like the C57/Bl strain, expression of *Hoxa-1* could not be detected in normal developmental stages of the BALB/c gland (data not shown). *Bars*, 45 μm.

rather than carry out a large scale survey of the 38 known *Hox* genes, we chose to examine two genes from different clusters, *Hoxa-1* and *Hoxc-6*, in sufficient depth to determine whether their expression patterns might suggest a functional role in mammary development or neoplasia. The criteria we chose were expression patterns that were: (*a*) regulated by developmental stage; (*b*) responsive to mammogenic hormones, or (*c*) altered in preneoplastic or neoplastic tissues.

Hoxa-1 is not expressed in the normal gland, while *Hoxc-6* is expressed during mammary development. Expression of *Hoxc-6* is related to the developmental stage of the gland, with declining transcript levels observed in response to pregnancy. The data show that homeogene expression is not simply uniform or constitutive. Although homeobox gene expression has not been reported previously in the mammary gland, *Wnt* proto-oncogene expression in the mammary gland has been documented (35). The *Drosophila* homologue of *Wnt-1* is the segment polarity gene *wingless*, which is known to regulate the homeobox-containing gene *engrailed* (36).

The mammary gland is an endocrine target organ of considerable complexity, in which hormones of reproduction drive the gland through its cycles of growth, secretory differentiation, lactation, and postweaning involution. Primary among these hormones are the ovarian steroids. Genes regulating tissue-specific responses to these hormones must be downstream of the primary steroid response elements, and it is reasonable to suppose that if *Hox* genes play a role in the mammary gland, their activity will be linked to this primary endocrine regulation. This was tested by examining glands from ovariectomized animals and glands from animals at different time points during the estrus cycle. *Hoxc-6* expression was greatly increased following ovariectomy. Transcript levels appeared to be slightly reduced during early estrus, a small but reproducible effect that supports the ovariectomy results by indicating an inverse relationship between ovarian steroids and *Hoxc-6* transcript levels.

At least two essential alterations occur during the development of mammary cancers in mice, the acquisition of immortality at the preneoplastic (HAN) stage, and the acquisition of cellular autonomy at the neoplastic stage (18). Like normal cells, preneoplastic HANs are growth-inhibited by the presence of nearby normal mammary epithelium and remain as discrete nodules. They are also unable to grow normally in ectopic sites, requiring mammary adipose tissue. Tumor cells, on the other hand, are able to overgrow normal mammary tissues and have escaped growth dependence on mammary stroma. Our results show that with respect to the expression of *Hoxc-6* and *Hoxa-1*, the preneoplastic stage of mammary tumor progression is similar to the normal mammary gland, whereas tumors derived from these tissues show elevated expression of *Hoxa-1* but not *Hoxc-6*.

Hox gene expression, in general, affects cell growth, differentiation, and fate (37). In vertebrate embryos, *Hox* genes are associated with maintenance of the proliferative state in the developing limb, while overexpression in cultured myoblasts induced the transformed phenotype (10). It has been shown that overexpression of *Hoxa-1* in NIH-3T3 or 208-F cells leads to transformation, enabling the cells to grow and form foci in soft agar (11). On injection of cells overexpressing *Hoxa-1* cells into athymic mice, the mice developed tumors as early as 12 days postinjection. More examples showing that deregulated homeogene expression leads to aberrant cell proliferation are recently available (38, 7). These and other correlative studies make the case that certain homeobox genes may constitute a new family of oncogenes (11). Our results are in accord with these data and extend the possible roles of homeobox genes to regulation of the mammary gland in the subadult and mature animal.

The perturbation of cell fate-determining switches may reasonably be expected to play a role in the acquisition of cell autonomy in the progression of cells to malignancy. Many of the known oncogenes are thought to alter early stages in the malignant progression, affecting the cell cycle or participating in the immortalization process. Our data show misexpression of *Hoxa-1* in malignant but not benign immortalized precancerous tissues, suggesting that *Hoxa-1* and perhaps other homeobox genes may play a role in the latter stage of tumor progression as cells acquire the ability to invade ectopic sites and establish dispersed metastatic colonies.

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ARTICLES

Expression of *Hoxa-1* and *Hoxb-7* Is Regulated by Extracellular Matrix-Dependent Signals in Mammary Epithelial Cells

Anabella Srebrow,[1] Yael Friedmann,[2] Ali Ravanpay,[1] Charles W. Daniel,[2] and Mina J. Bissell[1]*

[1]Lawrence Berkeley National Laboratory, Berkeley, California 94720
[2]Department of Biology, University of California, Santa Cruz, California 95064

Abstract Homeobox-containing genes encode transcriptional regulators involved in cell fate and pattern formation during embryogenesis. Recently, it has become clear that their expression in continuously developing adult tissues, as well as in tumorigenesis, may be of equal importance. In the mouse mammary gland, expression patterns of several homeobox genes suggest a role in epithelial–stromal interactions. Because the stroma and the extracellular matrix (ECM) are known to influence both functional and morphological development of the mammary gland, we asked whether these genes would be expressed postnatally in the gland and also in cell lines in culture and whether they could be modulated by ECM. Using a polymerase chain reaction-base strategy five members of the *Hox* gene clusters *a* and *b* were shown to be expressed in cultured mouse mammary cells. *Hoxa-1* and *Hoxb-7* were chosen for further analysis. *Hoxb-7* was chosen because it had not been described previously in the mammary gland and was modulated at different stages of gland development. *Hoxa-1* was chosen because it was reported previously to be expressed only in mammary tumors, and not in normal glands. We showed that culturing the mammary epithelial cell lines SCp2 and CID-9 on a basement membrane (BM) that was previously shown to induce a lactational phenotype was necessary to turn off *Hoxb-7,* but a change in cell shape, brought about by culturing the cells on an inert substratum such as polyHEMA, was sufficient to downregulate *Hoxa-1*. This is the first report of modulation of homeobox genes by ECM. The results provide a rationale for the differential pattern of expression in vivo of *Hoxa-1* and *Hoxb-7* during different stages of development. The culture model should permit further in-depth analysis of the molecular mechanisms involved in how ECM signaling and homeobox genes may interact to bring about tissue organization. J. Cell. Biochem. 69:377–391, 1998.

Key words: homeobox; mammary gland; morphogenesis; basement membrane; gene expression

Extracellular matrix (ECM) profoundly influences cellular form and function during embryogenesis, as well as in the adult organism [Adams and Watt, 1993; Martins-Green and Bissell, 1995; Ashkenas et al., 1996]. This is particularly well documented for the mouse mammary gland, in which epithelial cells and the surrounding ECM interact dynamically throughout development [Bissell and Hall, 1987]. Unlike other organs, the mammary gland undergoes most of its growth and morphogenesis in the subadult and adult animal, and certain stages of the postnatal gland exhibit embryonic-like features [Sakakura et al., 1979; Cunha et al., 1992]. Regulation of pattern formation, cell differentiation, and epithelial–stromal interactions takes place after completion of embryogenesis, and recurs during each cycle of pregnancy, lactation, and involution. During branching morphogenesis in virgin animals, the ECM at the edge of the stroma influences end-bud development and ductal branching [Silberstein et al., 1992]. An intact basement membrane (BM) is required for epithelial morphology and functional differentiation during pregnancy and lac-

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Anabella Srebrow is currently at the Laboratorio de Fisiologia y Biologia Molecular, Departamento de Ciencias Biologicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellon II (1428) Buenos Aires, Argentina.

*Correspondence to: Mina J. Bissell, Life Sciences Division, Lawrence Berkeley National Laboratory, One Cyclotron Rd., MS 83-101, Berkeley, CA 94720. E-mail: mjbissell@lbl.gov

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tation [Aggeler et al., 1991; Sympson et al., 1994]. Finally, after weaning, involution is completed only if the BM is degraded by matrix metalloproteinases (MMPs) [Talhouk et al., 1992], an event that triggers programmed cell death, allowing the gland to return to its resting state [Strange et al., 1992; Boudreau et al., 1995].

A continuing, consistent body of work has demonstrated that the ECM plays a key role in mammary epithelial cell differentiation in culture as well [for review, see Roskelley et al., 1995]. Primary mouse mammary cultures, as well as various cell lines established from the glands of pregnant animals [Danielson et al., 1984; Schmidhauser et al., 1990; Desprez et al., 1993], when placed on a reconstituted BM, undergo a morphogenic process that leads to the formation of polarized three-dimensional structures similar to the alveoli observed in vivo [Aggeler et al., 1991]. These spheroids are able to synthesize and vectorially secrete large amounts of milk proteins in the presence of lactogenic hormones [Li et al., 1987; Barcellos-Hoff et al., 1989].

From *Drosophila* to mammals, homeobox-containing genes have been identified as master regulators of cell fate and pattern formation during development [McGinnis and Krumlauf, 1992; Kenyon, 1994; Krumlauf, 1994; Lawrence and Morata, 1994]. These genes are characterized by a highly conserved 183-bp sequence encoding a 61-amino acid domain (homeodomain), which includes a helix-turn-helix DNA-binding motif [Gehring et al., 1994]. Despite extensive literature on their role in embryogenesis, their expression in the adult has been reported only recently in a few tissues, including kidney, intestine, testis, and the mammary gland [Wolgemuth et al., 1987; James and Kazenwadel, 1991; Wolf et al., 1991; Friedmann et al., 1994]. Furthermore, their regulation and function in these tissues is poorly understood.

In the mouse mammary gland, the expression of *Hoxc-6* is differentially regulated during postnatal development and is influenced by mammogenic hormones. *Hoxc-6* transcripts are present in normal glands and absent in mammary neoplasia. By contrast, *Hoxa-1* is detected only in mammary tumors of epithelial origin, but not in the normal gland or in precancerous outgrowths [Friedmann et al., 1994].

We hypothesized that a regulatory crosstalk may exist between some homeobox genes and the ECM and that this interaction may play a role in different stages of mammary gland development. To test this hypothesis, we used degenerate primers and reverse transcription-polymerase chain reaction (RT-PCR) and identified several *Hox* genes that are expressed in two cell lines isolated from a functional mouse mammary cell strain that are ECM responsive [Schmidhauser et al., 1990; Desprez et al., 1993]. Here, we focus our studies on the expression and regulation of *Hoxa-1* and *Hoxb-7* for the reasons described in the summary. The former was previously identified only in mammary adenocarcinomas [Friedmann et al., 1994] and the latter has not been described previously in the mammary gland. This report shows that functional nontumorigenic mammary cells cultured on tissue culture plastic express these genes, but a reconstituted BM suppresses the expression of both genes. The mechanism of suppression, however, differs between *Hoxa-1* and *Hoxb-7* such that their mode of expression in vivo in normal gland and in tumors may be explained by ECM-dependent signals.

MATERIALS AND METHODS

Cell Culture

CID-9 cells [Schmidhauser et al., 1990] were derived from the mouse mammary cell strain COMMA-1-D [Danielson et al., 1984] by an enrichment of the epithelial subpopulation. As a result, the percentage of cells able to produce β-casein in response to ECM and lactogenic hormones was increased from 8 to more than 43. Homogeneous epithelial cells, SCp2, were isolated from the heterogeneous CID-9 cell line by limiting dilution cloning. SCp2 do not deposit their own BM, but more than 90% of these cells are able to produce β-casein in response to an exogenous BM-like ECM or purified laminin and lactogenic hormones [Desprez et al., 1993].

Each cell line was routinely grown in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (F12) (1:1) (GIBCO-BRL, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO); insulin, 5 μg/ml (Sigma); and gentamicin, 10 μg/ml (UC, San Francisco). At the onset of the experiments, 5×10^4 cells/cm^2 were plated on different substrata in DMEM–F12 medium supplemented with insulin, 5 μg/ml (Sigma), and the lactogenic hormones hydrocortisone, 1 μg/ml (Sigma) and prolactin, 3 μg/ml (National Institute of Diabetes

and Digestive and Kidney Disease, Bethesda, MD), as well as 0.25–2% FBS. After 24 h, the medium was replaced with serum-free DMEM–F12 plus insulin and lactogenic hormones, and the cells were maintained under these conditions for the times indicated for each experiment.

For studies on the influence of cell proliferation on *Hoxa-1* expression, the medium was changed to serum-free DMEM–F12 plus hydrocortisone and prolactin (as indicated above), but with increasing amounts of insulin, 0, 0.25, 1.25, or 5 μg/ml, 24 h after plating. Cells were maintained for another 24 h. This included a 12-h labeling period with 5-bromo-2′-deoxyuridine (BrdU), as described below.

Cell Proliferation Assay

DNA synthesis was measured by BrdU incorporation using a BrdU Labeling and Detection Kit (Boehringer Mannheim, Germany). Cells were incubated with 10 μM BrdU for 12 h, and labeled nuclei were detected according to the manufacturer's instructions with the addition of a blocking step before the incubation with anti-BrdU antibody. Blocking was performed by incubating with 0.1% bovine serum albumin (BSA), 10% goat serum in 1× phosphate-buffered saline (PBS) for 1–2 h. Nuclear labeling indices were determined by counting at least 300 cells from randomly selected visual fields and calculating the percentage of cells with labeled nuclei.

Substrata

When indicated, 100-mm tissue culture plates were precoated with 1–2 ml of reconstituted BM-like ECM purified from the mouse Engelbreth-Holm-Swarm tumor (EHS matrix). EHS or "factor-free" EHS was prepared as described by Kleinman et al. [1986] or was obtained from Collaborative Biomedical Products (Matrigel) (Collaborative Research, Waltham, MA).

For studies on the influence of cell shape and of cytoskeleton, tissue culture plates were coated with the nonadhesive substratum poly(2-hydroxyethylmethacrylate) (polyHEMA) (Sigma) as described by Folkman and Moscona [1978] and Roskelley et al. [1994], using an initial concentration of 10 mg/ml in 95% ethanol before drying.

Animals

For RNA preparation, CD-1 mice were used as a source of normal mammary glands. Hyperplastic glands and tumors were collected from transgenic animals made in a CD-1 background. For involuting glands, pups were weaned at 9 days after birth; the next day was counted as day 1 of involution. C57Bl/crl mice were also used in parallel with CD-1 mice and yield similar results as the CD-1 mice. Thoracic glands of virgin and pregnant C57BL/crl mice were used for in situ hybridization.

RNA Isolation, cDNA Synthesis, and PCR Amplification

Mouse mammary glands were frozen in liquid nitrogen immediately after removal. Total RNA was prepared using TRIzol reagent (GIBCO-BRL) according to the manufacturer's instructions. Total RNA from cultured cells was prepared as described by Chomczynski and Sacchi [1987].

Poly A^+ mRNA was purified from 1 mg total RNA from cultured cells by chromatography on oligo(dT)-cellulose column as described by Maniatis et al. [1989]. cDNA was synthesized from 5 μg poly A^+ RNA using cDNA Synthesis System Plus (Amersham, Cleveland, OH), following the manufacturer's instructions. One-tenth of the cDNA obtained was heated for 10 min at 95°C and then combined with a set of degenerate oligonucleotide primers at a concentration of 1 μM each, 10 μl of 10× PCR buffer II (Perkin Elmer), 2 mM $MgCl_2$, 200 μM each dNTP, and 2.5 units of Taq DNA polymerase (Perkin Elmer) in a total volume of 100 μl and amplified by PCR. The reaction was carried out for 35 cycles with the following profile: 95°C, 1 min, 40°C, 1 min, and 70°C, 30 s.

The following degenerate primers were used (the code is used according to the International Union of Pure and Applied Chemistry): ELEKEF: 5′-CGCGGATCCGARYTNGARAARGARTT-3′ or 5′-GGAATTCCGARCTNGARAARGARTT-3′; KIWFQN: 5′-CCCAAGCTTR-TTYTGRAACCA-DATYTT-3′; WFQNRR: 5′-CGGGATCCCGNC-GRTTYTGRAACCA-3′.

Similar sequences were used to amplify *Hox* genes by Friedmann et al. [1994], Levine and Schechter [1993], Frohman et al. [1990], and others. Restriction endonuclease sites were added at the 5′ ends of the primers to facilitate cloning of PCR products.

Cloning and Identification of PCR Products

The PCR product (~130 bp) was digested with the corresponding restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII; from NEB Biolabs, Beverly, MA), size purified from an ethidium bromide-agarose gel with QIAEX kit (Qiagen, Chatsworth, CA) and ligated into digested-vector pBluescript KS II (Stratagene, La Jolla, CA). Miniprep DNA was prepared from individual colonies, checked for the presence of the 130-bp insert, and sequenced using the Sequenase version 2, DNA Sequencing Kit (U.S. Biochemicals, Cleveland, OH) according to the manufacturer's instructions. Sequences were compared with nucleotide databanks employing various programs of the GCG (Genetics Computer Group, Madison, WI) sequence analysis software package.

Northern Blot Analysis

Aliquots of 20–25 μg total RNA purified as described above were resolved on 1% agarose/2.3 M formaldehyde gels by electrophoresis. RNA was then transferred by capillary blot onto nylon membranes (Hybond-N, Amersham or Magna NT, MSI, Westboro, MA) as indicated by Maniatis et al. [1989]. Hybridization of the membranes was carried out in 50% formamide, 5× Denhardt's reagent, 4× SSPE, 20 mM KPO_4 (pH 6.5), 1% sodium dodecyl sulfate (SDS) and 10% dextran sulfate. *Hoxa-1* and *Hoxb-7* mouse cDNA fragments were labeled with ^{32}P-dCTP by random primed (REDIPRIME Kit from Amersham). *Hoxa-1* fragment was ~770 bp, derived from the 3′ untranslated region (a gift from Dr. Chris Wright, Vanderbilt School of Medicine, Nashville, TN). *Hoxb-7* fragment was ~550 bp, derived from coding sequence 3′ of the homeobox plus 3′ untranslated region. This fragment was generated by PCR using the following primers: direct primer 5′-AACCTCAGGACCGGGAACCACCG-3′ and reverse primer 5′-GAGGCTCGTGAATAGGACCTAG-3′. None of these probes includes either the homeobox or the polyadenylic tail. Final post-hybridization washes were done in 0.1× SSPE–0.1% SDS at 65°C. The filters underwent subsequent hybridization with a probe for 28S ribosomal RNA, for internal standardization. Bands on autoradiographs were scanned and analyzed by densitometry with Image 1.44 software from the National Institutes of Health (NIH).

Tissue Sections and In Situ Hybridization

Mammary glands from virgin and pregnant mice were fixed for 3 h in 4% paraformaldehyde/PBS, dehydrated through graded series of ethanol to xylene, and then embedded in paraffin wax; 7-μm sections were cut and mounted on siliconized glass slides. In situ hybridization was performed as described by Friedmann and Daniel [1996]. A ~640 bp fragment corresponding to 269-bp 5′ noncoding plus 390 bp coding sequence of *Hoxb-7* cDNA (lacking the homeobox) was amplified by PCR and cloned into the *Eco*RI site of pBluescript KSII multiple cloning site (Stratagene). Two independent clones, carrying the fragment in the sense or antisense orientation with respect to the T7 promoter, were selected and used for the generation of *Hoxb-7* digoxigenin-labeled RNA probes. Transcription reactions were done with DIG RNA labeling Kit (Genius 4, Boheringer Mannheim) according to the manufacturer's instructions.

The primers used for PCR amplification were 1896: 5′-GGAATTCCCAAT-CCGCAGAGCTCG-3′; and 2535: 5′-GGAATTCCAAGTTACTCTCG-GCCGCCA-3′. Assigned numbers correspond to nucleotide position on the mRNA (accession number X06762). Restriction sites were added to facilitate cloning.

RESULTS

Homeobox Gene Expression in Cultured Mouse Mammary Cells

In our initial search for homeobox-containing genes in breast cells, we chose a functional but heterogeneous mouse mammary cell line, CID-9 [Schmidhauser et al., 1990, 1992], which is composed of both epithelial and stromal-like populations. This was because the expression of certain homeobox-containing genes (e.g., *Msx-1* and *Msx-2*) has been shown to depend on epithelial–mesenchymal interactions [Jowett et al., 1993; Pavlova et al., 1994; Friedmann and Daniel, 1996]. CID-9 cells undergo morphological and functional differentiation in response to a laminin-rich BM and lactogenic hormones. The process can be evaluated by assessing the formation of three-dimensional alveolar-like structures and the synthesis and secretion of milk proteins by a large fraction of the epithelial cells.

To identify which *Hox* genes may be expressed in CID-9 cells, degenerate oligonucleotide primers were used in PCR to amplify cDNA from cells that were either proliferating or undergoing differentiation. The primers were designed based on the amino acid sequence of two highly conserved regions within the homeodomain of different members of the *Hox* family. The same strategy has been used successfully in other systems such as mouse intestinal epithelium [James and Kazenwadel, 1991], goldfish retina [Levine et al., 1993], erythroleukemia cells [Takeshita et al., 1993], pancreatic cells [Rudnick et al., 1994], and mouse mammary gland [Friedmann et al., 1994], as well as in the identification of known and new members from other gene families, such as protein kinases [Blaschke et al., 1991] and pou-proteins [He et al., 1989].

Electrophoretic analysis of PCR products showed a band of the expected size (~130 bp) which was cloned, and several individual colonies were randomly chosen and sequenced. Using this approach, we were able to identify five known members of the *Hox* gene family expressed in mouse mammary cells in culture: *Hoxa-1, Hoxa-5, Hoxb-7, Hoxb-8,* and *Hoxb-9,* all belonging to clusters *a* and *b* respectively, according to the nomenclature suggested by Scott [1992] (Table I).

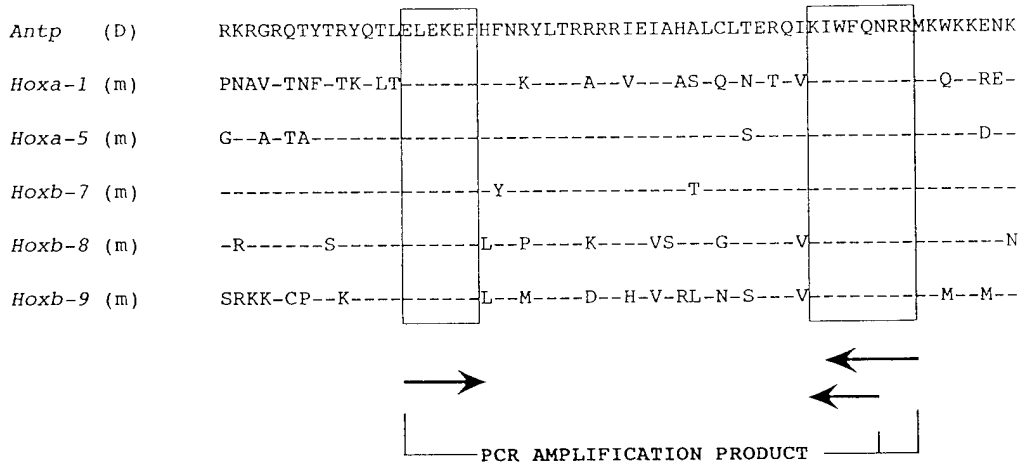
Hoxa-1 and *Hoxb-7* were chosen for further characterization for the reasons described. Total RNA was extracted from subconfluent cultures of CID-9 cells grown on tissue culture plastic and the expression of *Hoxa-1* and *Hoxb-7* was confirmed by Northern blots. We detected one *Hoxa-1* transcript of approximately 2.5 kb and two *Hoxb-7* transcripts of 1.6 and 1.4 kb (Fig. 1A). These are in agreement with the sizes described in previous reports [Friedmann et al., 1994; Meijlink et al., 1987].

Expression and Localization of *Hoxa-1* and *Hoxb-7* In Vivo

The expression of *Hoxa-1* in the mammary gland was studied previously by Friedmann et al. [1994]. Whereas no expression was detected in the normal gland and hyperplastic alveolar nodules (HANs), this gene was found to be expressed in mouse mammary adenocarcinomas generated from serially transplanted HANs. Thus, the expression of *Hoxa-1* in functionally normal cells in culture was surprising and suggested either the absence of regulatory suppressors or the presence of inducers in culture.

Expression of *Hoxb-7* in mammary tissue had not been studied previously. We carried out Northern blot analysis of different stages of mouse mammary gland development in normal mice. Total RNA was isolated from mammary glands of several mature virgin (70 days of age); midpregnant (13 days postcoitus); lactating (2, 5, and 9 days) and involuting (2, 5, and 9 days) animals. Only one transcript, of approximately

TABLE I. Alignment of Amino Acid Homeodomain Sequences Corresponding to *Drosophila* Anntenapedia Gene (D), and Mouse *Hox* Genes (m) Detected in Cultured Mammary Cells by RT-PCR*



*Boxed areas correspond to conserved regions among members of the *Hox* complex; arrows, degenerate oligonucleotide primers used in the amplification reactions.

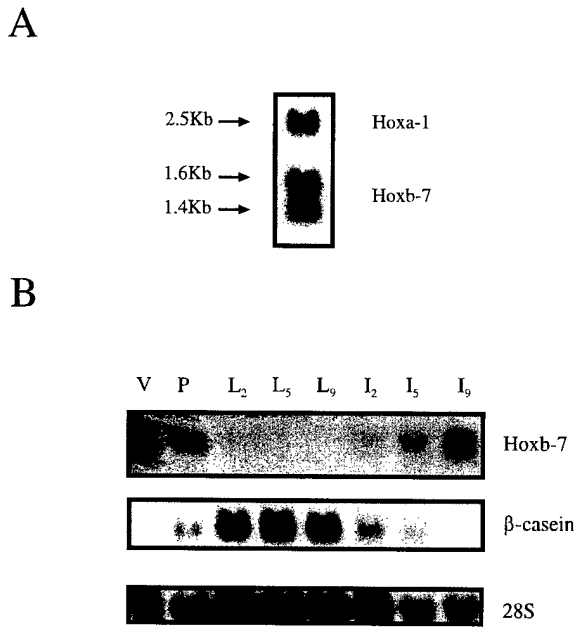


Fig. 1. Expression of *Hoxa-1* and *Hoxb-7* in culture and of *Hoxb-7* during mammary gland development. **A:** Northern blot analysis of total RNA from subconfluent monolayers of mouse mammary cells (CID-9) consecutively probed with ^{32}P-labeled *Hoxa-1* and *Hoxb-7* cDNA fragments. Arrows, observed transcripts. Approximate sizes are indicated (in kilobases) on the left side of the panel. **B:** Northern blot analysis of *Hoxb-7* mRNA in the mammary gland. Northern blot analysis of total RNA from glands of 70 days virgin (V) mice; 13 days pregnant (P); 2, 5, and 9 days lactating (L) mice; and, 2, 5, and 9 days involuting (I) mice. The same blot was sequentially hybridized with *Hoxb-7*, β-casein and 28S rRNA probes. Please note that the expression of *Hoxa-1* was shown previously to be absent in vivo [Friedman and Daniel, 1994]; see also Figure 5.

1.6 kb, was detected throughout development with higher expression in glands of virgin animals, decreasing during pregnancy, practically absent during lactation, but increasing again up to day 9 of involution by which time the gland had reached a resting state (Fig. 1B). Thus, the expression of *Hoxb-7* and full differentiation of the mammary gland, achieved during lactation, are inversely correlated.

To examine which cell type is responsible for the expression of *Hoxb-7* in this tissue, we performed in situ hybridization with antisense and sense *Hoxb-7* RNA probes. By this technique, *Hoxb-7* mRNA was detected in the epithelial compartment of the glands of virgin animals. Some stromal cells in the vicinity of the ducts in glands from virgin animals also appeared to express this gene, but it was at considerably lower levels than the epithelium (Fig. 2A). In the glands of pregnant animals, reduced expression of *Hoxb-7* was detected by Northern blot, but some of the ductal structures were shown to be strongly positive for *Hoxb-7* by the in situ technique (Fig. 2C). *Hoxb-7* expression was absent in the epithelial cells of the gland from lactating animals (not shown).

Expression of *Hoxa-1* and *Hoxb-7* in Mammary Cells in Culture Is Downregulated by an Exogenous BM

We had shown previously that cultivation of primary mammary epithelial cells on an exogenous BM matrix (EHS) (see under Materials and Methods) leads to induction of a near normal lactating phenotype [Aggeler et al., 1991]. We had also shown that two mammary cell lines CID-9 and SCp2 (see under Materials and Methods) turn on milk protein genes and undergo similar morphogenesis on such substrata. We therefore asked whether cultivation on EHS would also recapitulate the loss of these *Hox* genes observed in the lactating gland in vivo.

The two cell lines were cultured in the presence of lactogenic hormones either on EHS, where they acquire a three-dimensional organization, or directly on tissue culture plastic, where they form a flat, two-dimensional monolayer (Fig. 3A). Cells were harvested at different time points after plating, and total RNA was extracted and analyzed by Northern blot.

Hoxb-7 and *Hoxa-1* transcripts were downregulated in mammary cell lines plated on EHS (the same result was found in growth "factor-free" EHS). While this effect could be observed as early as 12 h after plating (not shown), it became more pronounced after 24 h and was sustained for approximately 72 h, the latest time point studied (Fig. 3B, B′, C, C′). Downregulation of these *Hox* genes preceded expression of milk protein genes in the cell lines (data not shown). This finding is interesting in a number of respects: it indicates that BM can downregulate both *Hoxb-7* and *Hoxa-1,* but it also indicates that either the BMs surrounding the epithelial cells of mammary glands from virgin and pregnant animals are different from the BM surrounding the lactating glands or that the epithelial cells have a different spacial relationship to the underlying BM (see under Discussion). The EHS matrix clearly mimics the latter. Furthermore, *Hoxa-1* appears to be subject to a less stringent regulation than *Hoxb-7* in vivo because it is suppressed in all normal epithelia at all stages of development.

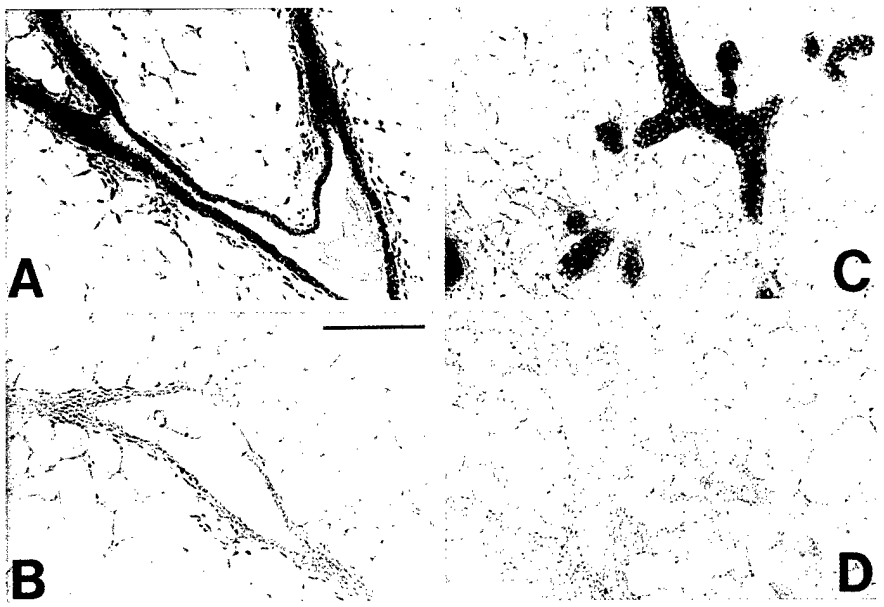


Fig. 2. Localization of *Hoxb-7* mRNA in the mammary gland by in situ hybridization. Sections of mammary glands from mature virgin (**A,B**) and early pregnant mice (**C,D**) were hybridized with *Hoxb-7* sense (B,D) or antisense (A,C) riboprobes. Please note that while some ducts were expressing *Hoxb-7* (C), the total expression was much lower in pregnant; lactating gland was negative (not shown). Scale bar = 100 μm.

Nevertheless, when EHS matrix was dried or cross-linked to confer a rigid structure [Chen and Bissell, 1989], neither *Hoxb-7* nor *Hoxa-1* was downregulated (not shown). This means that malleability of the substratum, and of the matrix molecules themselves, may be important in suppressing these *Hox* genes.

Signal Transduction by "Cell Shape" Versus Specific ECM Ligands

When mammary epithelial cells are placed on EHS, ECM-dependent changes in morphology precede ECM signaling through cell surface receptors, and the latter is dependent on the former [Roskelley et al., 1994]. Whereas EHS can provide both morphological and functional cues, culturing cells onto the nonadhesive substratum polyHEMA [Folkman and Moscona, 1978] induces only the "cell shape" response; that is, cells round up and cluster, but do not deposit, a functional endogenous BM, judged by the lack of β-casein expression and full differentiation [Roskelley et al., 1994] (Fig. 4A). Using the epithelial cell line SCp2, which is unable to deposit its own BM, we were able to separate the consequences of a change in cell shape and cytostructure (which would lead to altered signaling) from ECM biochemical signaling on *Hoxa-1* and *Hoxb-7* expression.

SCp2 cells were plated either on tissue culture plastic or on polyHEMA-coated dishes in the presence of lactogenic hormones. RNA was extracted at different times after plating and was analyzed by Northern blot.

Hoxa-1 transcript levels were markedly reduced in SCp2 cells plated on polyHEMA-coated dishes, compared to the level on tissue culture plastic. By contrast, *Hoxb-7* expression was not altered when the cells were clustered on polyHEMA (Fig. 4B). The pattern of expression of both genes persisted regardless of the length of culturing (from 24 to 72 h). This finding shows that inhibition of *Hoxa-1* expression depends primarily on epithelial cell shape modulated, in vivo, by the stroma and BM at any stage of the gland's development, whereas *Hoxb-7* requires additional signaling from the ECM. It is tempting to suggest that the regulation of expression of *Hoxa-1* depends on a putative "shape response element," whereas *Hoxb-7* gene expression relies on an ECM-response element [Schmidhauser et al., 1992; Roskelley et al., 1995].

Not surprisingly, this response to the microenvironment was abrogated in mammary tumor cells. A mammary tumor generated in transgenic animals [Sympson et al., 1995] expressed high levels of *Hoxa-1* (Fig. 5A), as re-

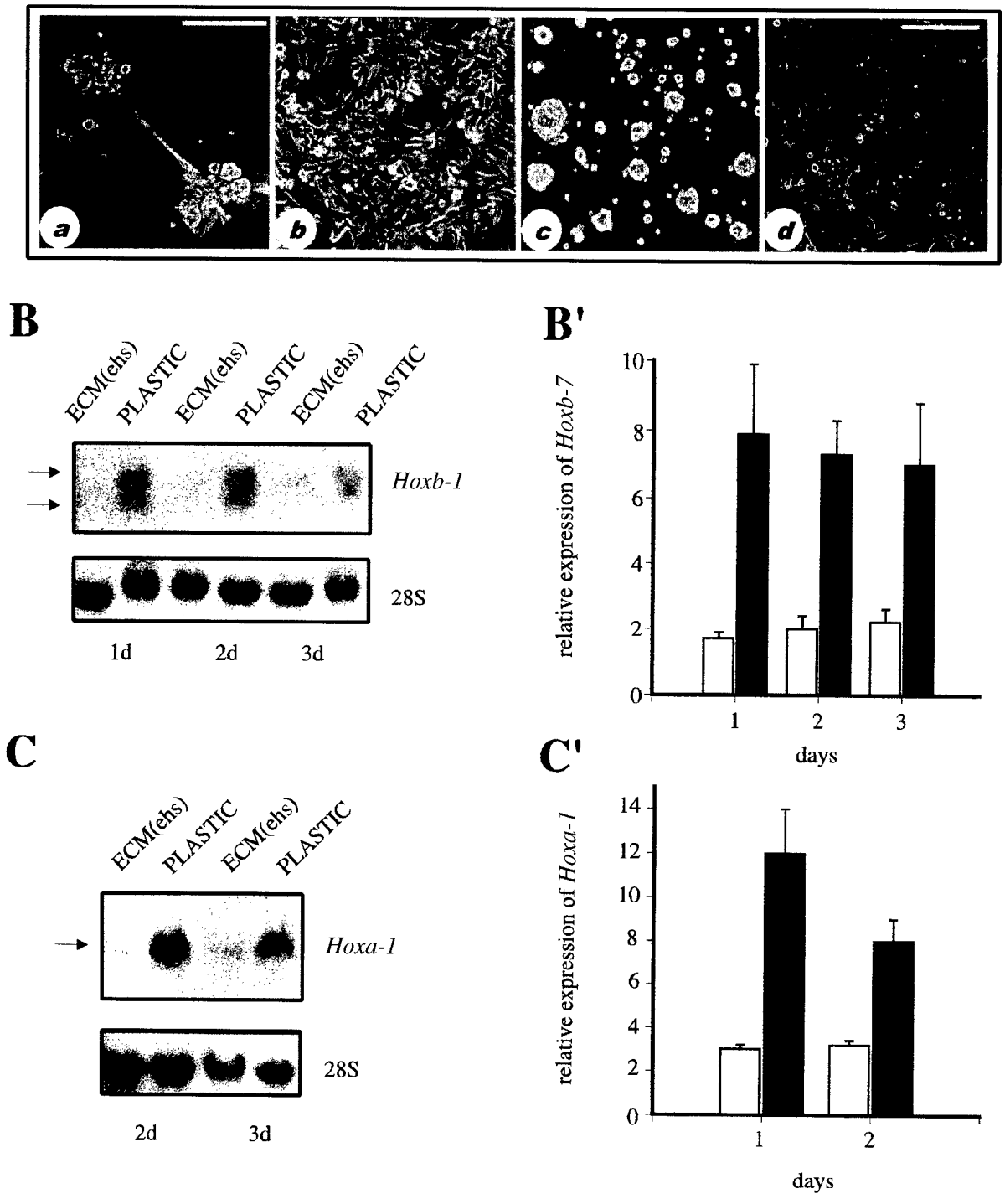


Fig. 3. Influence of an exogenous BM-like ECM on the expression of *Hoxa-1* and *Hoxb-7* in cultured mouse mammary cells. **A:** Morphology of CID-9 cells (a,b) and SCp2 cells (c,d) cultured for 2 days on a reconstituted BM (EHS) (a,c) or on tissue culture plastic (b,d). Scale bar = 200 μm. **B:** Northern blot of total RNA from cells cultured as described above for 3 days. Two *Hoxb-7* transcripts (B) and one *Hoxa-1* transcript **(C)** were identified (arrows). The same blots were stripped and rehybridized with a probe for 28S rRNA. Autoradiographs were scanned and analyzed densitometrically. *Hoxb-7* (**B'**) and *Hoxa-1* (**C'**) expression values were standardized against the corresponding value for 28S rRNA. Graphs show average values and standard deviations (s.d.) from at least three independent experiments. White bars, ECM/EHS; black bars, PLASTIC.

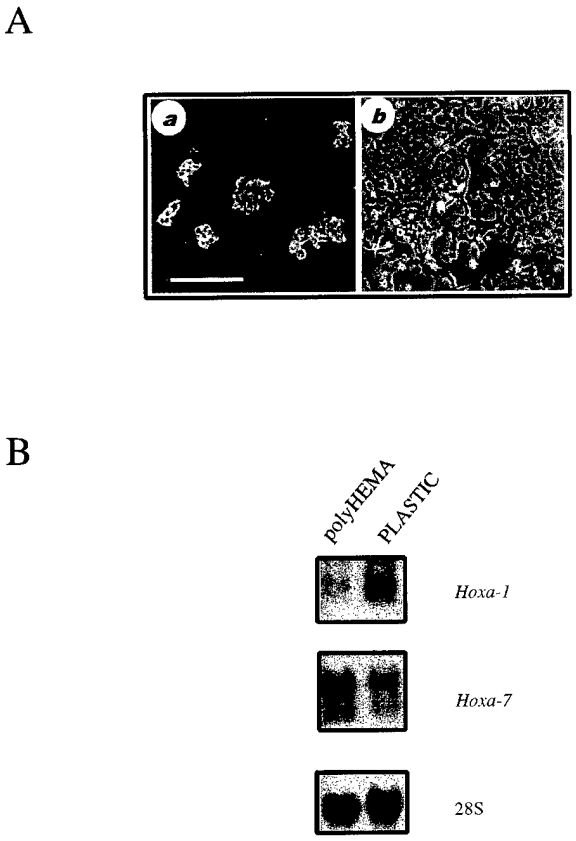


Fig. 4. Influence of cell shape on the expression of *Hoxa-1* and *Hoxb-7* in SCp2 cells. **A:** Morphology of mouse mammary epithelial cells (SCp2) cultured as nonadherent clusters on polyHEMA-coated dishes (a) or as a monolayer on tissue culture plastic (b). Scale bar = 200 μm. **B:** Northern blot of total RNA from cells cultured under conditions mentioned above. The same blot was probed consecutively for *Hoxa-1, Hoxb-7* and 28S rRNA.

ported for other mammary tumors by Friedmann et al. [1994]. A highly tumorigenic cell line (TCL-1) derived from this tumor [Lochter et al., 1997] was cultured on tissue culture plastic, on a reconstituted BM or on polyHEMA-coated dishes (Fig. 5B). RNA was extracted and analyzed by Northern blot. The expression level of *Hoxa-1* was slightly higher in TCL-1 cells than in the other two functional cell lines, whereas *Hoxb-7* was expressed at considerably lower levels in the tumor cell line (data not shown). *Hoxa-1* was downmodulated in CID-9 and SCp2 cells under these conditions (as we described above). Furthermore, its expression not only was not suppressed in TCL-1 cells cultured on an exogenous BM or on polyHEMA, but it was slightly increased compared to the level of expression on tissue culture plastic (Fig. 5C; cf. a and b with c).

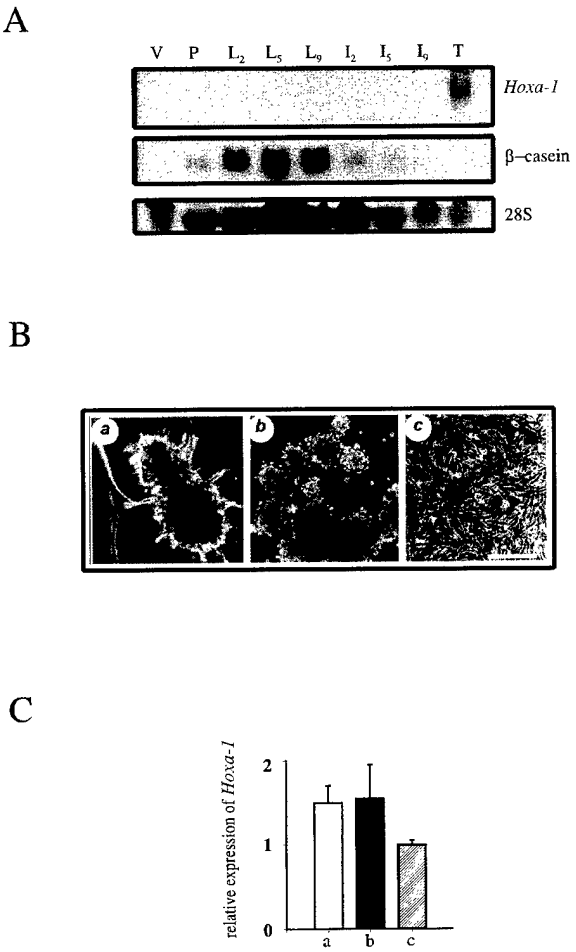


Fig. 5. Expression of *Hoxa-1* mRNA in normal mammary gland, in a mammary adenocarcinoma and in a derived mammary tumor cell line (TCL-1). **A:** Total RNA from glands of 70 days virgin (V); 13 days pregnant (P); 2, 5, and 9 days lactating (from left to right, L) and 2, 5, and 9 days involuting (from left to right, I) mice, as well as from a mammary tumor from stromelysin-1 transgenic mice (T) was analyzed by Northern blot. The same blot was sequentially hybridized with *Hoxa-1,* β-casein, and 28S rRNA probes. **B:** TCL-1 cells cultured on a reconstituted BM (a), on polyHEMA-coated dishes (b), or on tissue culture plastic (c). Scale bar = 200 μm. Total RNA from TCL-1 cells cultured on the above mentioned substrata was analyzed by Northern blot. Graph **C** shows relative expression values for *Hoxa-1* transcript standardized against the corresponding 28S rRNA band. The plotted values represent the average of three independent experiments, and s.d. are shown: a, EHS; b, polyHEMA; c, plastic.

Hoxa-1 Regulation and Cell Cycle Progression

Many nonmalignant cells, including primary mammary epithelial cells, CID-9 and SCp2 cell lines, when cultured on a BM gel or on polyHEMA, where cells are prevented from attachment and spreading, undergo growth arrest [Folkman and Moscona, 1978; Petersen et al.,

1992; Desprez et al., 1995]. By contrast, when these cells are allowed to spread on tissue culture plastic or on other substrata, they continue to proliferate for several days, even in the absence of serum [Boudreau et al., 1996]. If the observed downregulation of *Hoxa-1* in SCp2 cells on polyHEMA-coated dishes was related to an inhibition of cell-cycle progression, the absence of regulation in malignant cells could be due to an inability to suppress growth in these cells, even when they are grown on a reconstituted BM.

SCp2 cells were allowed to spread onto tissue culture plastic and then maintained for 24 h in the absence or presence of increasing concentrations of insulin (0–5 μg/ml), which was previously shown to be required for the growth of these cells. DNA synthesis was determined by BrdU incorporation (Fig. 6A). Despite a dramatic decrease (from 60% to <10%) in cell cycle progression observed in the absence of insulin (Fig. 6B), the expression of *Hoxa-1* was, in fact, slightly increased (Fig. 6C). This finding indicates that suppression of *Hoxa-1* expression in nonmalignant mammary epithelial cells cultured on polyHEMA is modulated by the change in cell shape rather than by a change in growth rate. The fact that mammary epithelial cells do not express *Hoxa-1* in either virgin or pregnancy states supports this conclusion. This finding also indicates that the inability of tumor cells to downregulate *Hoxa-1* as a result of clustering is not related to their loss of growth control, but to their inability to appropriately respond to the microenvironment [Petersen et al., 1992].

DISCUSSION

The mammary gland retains the plasticity to undergo repeated cycles of growth, morphogenesis and functional differentiation at the onset of each pregnancy. Furthermore, mammary epithelial cells are capable of recapitulating these morphogenic events when cultured under proper conditions [Barcellos-Hoff et al., 1989; Aggeler et al., 1991]. These observations lead to the idea that specific regulatory molecules must exist that are able to sense and integrate the variety of signals emanating from the cell's microenvironment (i.e., neighboring cells, ECM, growth factors, hormones) and process them in a coordinated manner so as to ensure the maintenance of cell fate and tissue identity. Homeobox-containing genes appear to be good candidates for the orchestration of this complex developmental phenomenon, in which form and function are dynamically intertwined.

Several homeobox-containing genes have been observed to display an altered pattern of expression in some malignancies when compared with the corresponding normal tissues [Cillo et al., 1992; De Vita et al., 1993]. Moreover, misregulation of certain homeotic genes can lead to cellular transformation in culture, as well as tumor formation in vivo [Aberdam et al., 1991; Song et al., 1992; Maulbecker and Gruss, 1993]. These observations suggest that in addition to their role in embryogenesis, homeobox-containing genes may play an important role both in controlling cell differentiation and in the multistep process of tumorigenesis.

Experimental evidence suggests that many of homeotic genes are involved in the regulation of morphoregulators, such as adhesion molecules. For example, *Hoxc-6, Hoxb-8,* and *Hoxb-9* can regulate N-CAM promoter activity in cotransfection experiments [F.S. Jones et al., 1992, 1993]. Overexpression of *Hoxd-3* alters the adhesive properties of a human erythroleukemia cell line (HEL), along with an increase in integrin αIIbβ3 [Taniguchi et al., 1995] and *Evx-1,* a homeobox-containing gene outside the *Hox* cluster, activates the tenascin-C (cytotactin) promoter [Jones et al., 1992].

What regulates the homeotic genes themselves is less understood. In the mouse mammary gland, *Hoxc-6, Msx-1,* and *Msx-2* expression are regulated by mammogenic hormones [Friedmann et al., 1994, 1996]. The latter further appear to play a role in stromal–epithelial interactions. However, it is difficult to decipher molecular mechanisms and interconnections between the regulatory pathways in vivo. We used a number of culture conditions developed in our laboratory to investigate broad signals that impinge on regulation of *Hoxa-1* and *Hoxb-7,* and which could not be studied easily in vivo. While a change in cell shape alone was sufficient to suppress *Hoxa-1* expression, it was not enough for *Hoxb-7* where the BM itself was also required for downregulation. This effect may be attributed to either a direct signal generated by a particular ECM ligand present in EHS preparations, ECM-dependent modulation of growth factors produced by the cells themselves [Streuli et al., 1993; Lin et al., 1995], formation of an endogenous BM as a result of the cultivation in

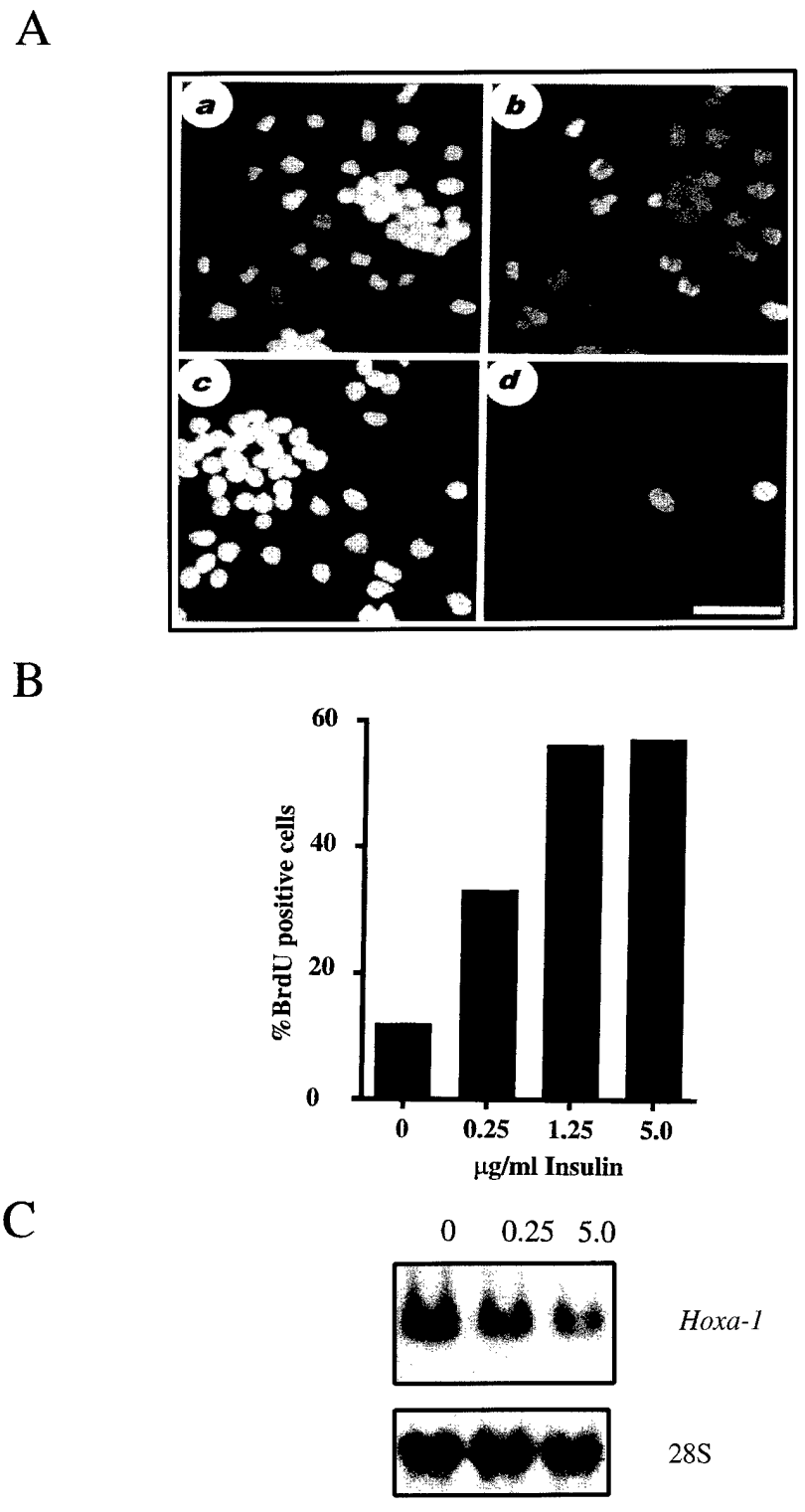


Fig. 6. Downregulation of *Hoxa-1* in SCp2 cells is not due to a decrease in the rate of cell-cycle progression. **A:** Cell proliferation assay. SCp2 cells cultured with 5 µg/ml of insulin (a,b) or in the absence of insulin (c,d). Nuclear DAPI staining (a,c) and detection of incorporated BrdU by indirect immunofluorescence (b,d). Scale bar = 50 µm. **B:** Percentage of BrdU-positive SCp2 cells cultured with increasing concentrations (0, 0.25, 1.25, 5.0 µg/ml) of insulin over 24 h. **C:** Northern blot analysis of *Hoxa-1* expression in SCp2 cells cultured as indicated above. Only those insulin concentrations that led to different cell proliferation indices are shown. The same blot was sequentially hybridized with *Hoxa-1* and 28S rRNA probes. Note that levels of *Hoxa-1* may be even lower in growing cells and not as the result of the absence of growth.

exogenous EHS [Petersen et al., 1992] or the acquisition of secretory epithelial polarity observed only when cells are plated onto this malleable substratum. As mentioned previously, dried EHS, which becomes rigid and promotes cell attachment and spreading [Chen and Bissell, 1989], did not suppress the expression of either *Hox* genes studied, supporting the conclusion that changes in cytostructure may be necessary.

It is interesting to note that the suppression of *Hoxa-1* and *Hoxb-7* in CID-9 and SCp2 mammary cells, mirrors the expression patterns of several milk proteins studied in our laboratory. The functional lactational phenotype is acquired by a hierarchy of ECM-dependent signals [Lin and Bissell, 1993; Roskelley et al., 1995]. A change in cell shape ("rounding"), in the absence of specific ECM signaling, leads to cessation of growth, reorganization of the cytoskeleton, changes in nuclear morphology and concomitant expression of the iron-binding protein lactoferrin [Roskelley et al., 1994, 1995]. This step is also sufficient to suppress *Hoxa-1* expression. A second level of complexity is required for the expression of β-casein. In this case, not only a cell shape change, but also biochemical signaling through β-integrin is necessary to induce β-casein expression and promoter activity [Streuli et al., 1991; Roskelley et al., 1994]. Additional morphogenic signals associated with cell–cell junctions, polarity, and three-dimensional organization are needed to turn on the expression of whey acidic protein (WAP) [Chen and Bissell, 1989; Lin et al., 1995]. The regulation of *Hoxb-7* appears to have characteristics in common with both β-casein and WAP. Although a BM overlay that induces β-casein expression [Roskelley et al., 1994] also modulates *Hoxb-7* on tissue culture plastic, the same overlay does not suppress *Hoxb-7* expression when applied to cells that were already preclustered on polyHEMA-coated dishes (data not shown). This finding suggests that a change in polarity may also be required for downregulation of this gene. Clustered cells on polyhema, although rounded and growth arrested, do not form hemidesmosomes and are not polar. Which mechanism is, in fact, involved is currently under investigation.

The pattern of expression of *Hoxb-7* in the mammary gland in vivo is intriguing. It is expressed in the epithelial cells of virgin animals, but transcript levels decrease appreciably through pregnancy and become virtually absent during lactation. As the gland involutes, expression increases until the gland is remodelled by day 9 of involution. It is therefore reasonable to suspect that *Hoxb-7* may indeed play a role in remodeling and in reestablishing ductal branching. The expression of this homeogene in the virgin gland at a time when ductal branching is occurring would be consistent with this hypothesis. The expression of *Hoxb-7* in the glands of both virgin and pregnant animals in vivo, despite the presence of a visible BM, may be attributable to differences in composition and structure of the ECM, the balance and the composition of integrins and/or other microenvironmental regulators. Also, it is important to note that the mammary gland is a double-layered tube in which myoepithelial cells are localized between the epithelium and the BM. It is known that the relation of the epithelial and myoepithelial cells changes as a function of developmental stage. Thus, one other possibility may be that in the gland of virgin and pregnant animals, the epithelium may be only in occasional contact with the BM, while in lactation the shape of the myoepithelial cell changes allowing a more direct contact between the epithelium and the BM [J.L. Jones et al. 1997; J.C. Jones et al. 1991; Emerman and Vogl 1986].

Our finding that *Hoxb-7* is suppressed in culture when a BM-like ECM is present further supports the "lactational" phenotype of these cells in culture [Aggeler et al., 1991]. Furthermore, the expression of *Hoxb-7* in cells plated on tissue culture plastic is consistent with the "involuting" phenotype of cells on this substratum. CID-9 and SCp2 cells on plastic apoptose when they reach confluence [Boudreau et al., 1995], have little or no milk protein expression but express tenascin [Jones et al., 1995] and *Hoxb-7* (this study).

Two different transcripts corresponding to *Hoxb-7* can be detected in mammary epithelial cells cultured on plastic, whereas only one is observed in normal mammary gland. Although different alternative-spliced variants have been reported for several *Hox* genes [Lopez, 1995], the absence of one isoform in the tissue and its presence in culture is intriguing and warrants further investigation in order to understand its biological significance as well as the molecular mechanisms that regulate its expression.

The fact that epithelial cells on tissue culture plastic express *Hoxa-1,* while the normal breast epithelium in vivo never does indicates that plastic substratum is an abnormal microenvironment for these cells. We know that cells on a flat or rigid substrata express and secrete many growth factors [Streuli et al., 1993; Lin et al., 1995] and inappropriate ECM components such as tenascin [P.L. Jones et al., 1995]. This finding also indicates that expression of *Hoxa-1* need not be limited to the malignant phenotype

only. SCp2 cells do not form tumors when injected into nude mice [S. Galosy, P.Y. Desprez, and M.J. Bissell, unpublished results) and downregulate *Hoxa-1* on polyHEMA-coated dishes and on a reconstituted BM. This regulated expression contrasts with the lack of regulation observed in tumor cells. *Hoxa-1* may thus be expressed in those tumors that have lost their ECM ("shape") responsiveness. This would be consistent with the observation that most human mammary carcinoma cell lines and primary cultures established from tumor biopsies were unresponsive to their microenvironment when grown within a BM [Petersen et al., 1992; Howlett et al., 1995; Weaver et al., 1995].

The availability of functional mammary epithelial cell lines and the existence of differences in the modulation of *Hoxa-1* and *Hoxb-7* could be used to further explore the nature of the regulatory pathways upstream and downstream of *Hox* genes in mammals as an alternative to genetic manipulations in the intact organism. These experiments are in progress.

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Regulated Expression of Homeobox Genes *Msx-1* and *Msx-2* in Mouse Mammary Gland Development Suggests a Role in Hormone Action and Epithelial–Stromal Interactions

Yael Friedmann[1] and Charles W. Daniel

Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, Santa Cruz, California 95064

The murine homeobox genes *Msx-1* and *Msx-2* are related to the *Drosophila msh* gene and are expressed in a variety of tissues during mouse embryogenesis. We now report the developmentally regulated expression of *Msx-1* and *Msx-2* in the mouse mammary gland and show that their expression patterns point toward significant functional roles. *Msx-1* and *Msx-2* transcripts were present in glands of virgin mice and in glands of mice in early pregnancy, but transcripts decreased dramatically during late pregnancy. Low levels of *Msx-1* transcripts were detected in glands from lactating animals and during the first days of involution, whereas *Msx-2* expression was not detected during lactation or early involution. Expression of both genes increased gradually as involution progressed. *Msx-2* but not *Msx-1* expression was decreased following ovariectomy or following exposure to anti-estrogen implanted directly into the gland. Hormonal regulation of *Msx-2* expression was confirmed when transcripts returned to normal levels after estrogen was administered to ovariectomized animals. *In situ* molecular hybridization for *Msx-1* showed transcripts localized to the mammary epithelium, whereas *Msx-2* expression was confined to the periductal stroma. Mammary stroma from which mammary epithelium had been removed did not transcribe detectable amounts of *Msx-2*, showing that expression is regulated by contiguous mammary epithelium, and indicating a role for these homeobox genes in mesenchymal–epithelial interactions during mammary development. © 1996 Academic Press, Inc.

INTRODUCTION

Mammary development and function are driven by a complex network of hormones acting systemically, which in turn influence peptide growth factors that regulate developmental events at the tissue level (Topper and Freeman, 1980; Dembinski and Shiu, 1987). Estrogen and progesterone are particularly crucial signals in growth and morphogenesis of the breast. It is likely that these hormones control regulatory genes that serve to coordinate developmental interactions and to specify pathway decisions in the developing gland. Because homeobox genes function as master regulators of embryonic events in a variety of organisms including the mouse (Morgan *et al.*, 1992; Balling *et al.*, 1989; Wolgemuth *et al.*, 1989; Ramirez-Solis *et al.*, 1993; Le Mouellic *et al.*, 1992), and because the expression of several *Hox* genes was recently reported in the mammary gland (Friedmann *et al.*, 1994), the mammary gland is a candidate target for hormone-regulated homeobox gene action.

In the mammary gland, genes regulating morphogenesis and growth are likely to be associated with epithelial–stromal interactions. With the onset of ovarian function at 3–4 weeks of age, the mouse mammary ductal system enters a phase of rapid growth and morphogenesis, in which continuing inductive interactions between mammary epithelium and contiguous stroma result in growth and patterning of a ductal tree that fills the adipose-rich stroma (Sakakura *et al.*, 1979). The ductal epithelial cells may follow one of several differentiative pathways depending on their position within the growth buds at the tips of the ductal branches, becoming cells for ductal walls, milk synthesis and secretion, or contraction (Williams and Daniel, 1983). In concert with this epithelial differentiation, the stroma adjacent to the growing duct becomes rich in fibrocytes, producers of

[1] Present address: Department of Oncology, P.O. Box 12000, Hadassah University Hospital, Jerusalem 91120, Israel.

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materials of the fibrous extracellular matrix which encases mammary ducts. (Williams and Daniel, 1983).

The homeobox is a relatively conserved 183-nucleotide sequence encoding a DNA-binding domain found in many genes playing key roles in *Drosophila* embryogenesis. These genes are classified according to their homeobox sequence and chromosomal location. Thirty-eight mammalian homeobox-containing genes, the *Hox* genes, are found in clusters on four chromosomes. Gene disruption and gain-of-function mutations generated in mice have shown that improper expression of *Hox* genes leads to developmental defects (Chisaka and Capecchi, 1991; Kessel and Gruss, 1991; Lufkin *et al.*, 1991), many of which represent homeotic transformations (Morgan *et al.*, 1992; Ramirez-Solis *et al.*, 1993; Small and Potter, 1993; Le Mouellic *et al.*, 1992).

Other mammalian homeobox genes are not located within these clusters and form smaller classes based on sequences that relate them to other conserved motifs such as *Pax* and *Oct* (Herr *et al.*, 1988; Epstein *et al.*, 1991; Hill *et al.*, 1991; Palmieri *et al.*, 1994; Corcoran *et al.*, 1993). The *Drosophila melanogaster* muscle segment homeobox (*msh*) gene contains a homeobox which is markedly divergent from that of any other characterized *Drosophila* genes. In *Drosophila, msh* is mainly expressed in the central nervous system and in segmented striated muscles of the body wall. In the mouse there appear to be three distinct msh-like genes, named *Msx-1, Msx-2,* and *Msx-3,* which are found at separate loci and are not clustered (Hill *et al.*, 1989; Robert *et al.*, 1989; Monaghan *et al.*, 1991; Holland, 1991). Closely related versions of *Msx-1* and *Msx-2* have been identified in a variety of vertebrate species including zebrafish (Ekker *et al.*, 1992), *Xenopus* (Su *et al.*, 1991), and the chick (Coelho *et al.*, 1991).

Msx-1 and *Msx-2* show a closely associated, interactive pattern of expression throughout early embryonic development (MacKenzie *et al.*, 1991a, 1991b; Monaghan *et al.*, 1991). The earliest expression of both genes is detectable in primitive streak mesoderm, followed by expression in neural crest cells and their derivatives. Later expression patterns have been examined by *in situ* hybridization methods in the development of several organs, including the mouse and chick limb bud (Nohno *et al.*, 1992; Davidson *et al.*, 1991; Robert *et al.*, 1991), mouse tooth bud (Mackenzie *et al.*, 1991a, 1992; Jowett *et al.*, 1993), chick heart (Chan-Thomas *et al.*, 1993), and chick craniofacial development (Nishikawa *et al.*, 1994). The results suggested that the two genes play a role in epithelial–mesenchymal interactions in these developing organs.

In a previous paper we reported expression in the mammary gland of several genes from the four *Hox* clusters and the altered expression of some during tumorigenesis (Friedmann *et al.*, 1994). In this paper we describe the expression of *Msx-1* and *Msx-2* RNA in different stages of mammary development. To move closer to a functional analysis, we have examined the effects of experimentally altering tissue interactions and manipulating mammogenic steroids.

METHODS

Animals. C57BL/crl mice were used for collection of the inguinal mammary glands in all RNA preparations. Virgin mice were chosen randomly from multiple cages to minimize the chances of selecting animals in a particular stage of estrus. For involuting glands, pups were weaned 10 days after birth and the following day was counted as Day 1 of involution. Thoracic glands of virgin and pregnant C57BL/crl mice were used for *in situ* hybridization.

Surgery. Ovariectomy was carried out at about 5 weeks of age (animal weight 16–17 g) and glands were collected 4 weeks later to allow complete mammary regression. When estrogen implants were used, they were implanted subcutaneously 6 weeks after ovariectomy and glands were collected 4 days later. This staging was necessary because after estrogen replacement therapy, the mammary glands from ovariectomized animals more closely resemble developing glands in younger 6-week animals.

The inguinal fat pads were cleared of epithelium at 3 weeks of age by removing the portion of the gland containing epithelium and cauterizing the nipple area and associated blood vessels (DeOme *et al.*, 1959).

Implants. EVAc (Elvax 40P) was a gift from DuPont Chemical Co. (Universal City, CA). Anti-estrogen ICI 164,384 was a gift from ICI Pharmaceutical (Cheshire, England). 17 β-Estradiol is from Sigma (E-8875). Implant preparation is described in detail elsewhere (Silberstein and Daniel, 1982). Briefly, anti-estrogen was dispersed in 0.125 ml of EVAc that had been dissolved in dichloromethane (20% w/v). This mixture was quick-frozen and evaporated under vacuum, and the polymer matrix with entrapped chemical was then cut to form pellets containing 250 μg anti-estrogen and surgically implanted (typical implant weight, 0.5 mg). 17 β-Estradiol was mixed with dichloromethane and serially diluted to a final dose of 50 ng implants per animal. Recipient mice were anesthetized with an interperitonial injection of Nembutal (60 $\mu g\ g^{-1}$ body wt) and the number 3 mammary glands (in the case of anti-estrogen) were exposed by reflecting the skin from a midline ventral incision. A small pocket was made in the mammary fat pad using Dumont forceps, which were then used to insert the implant. Estrogen implants were inserted through a small incision subdermally at the back of the neck. The skin was then closed with wound clips and the animals were allowed to recover in an atmosphere of 95% O_2/5% CO_2.

RNA preparation and Northern hybridization. Inguinal mammary glands were frozen in liquid nitrogen immediately after removal, and total RNA was prepared by the guanidine isothiocynate (4 *M*), cesium chloride (5.7 *M*) method (Ausubel *et al.*, 1989). Total RNA from the glands (the number and age of animals used for each experiment are given in the figure legends) was isolated. In several cases poly(A)$^+$ RNA was purified by oligo(dt)–cellulose chromatography as described (Sambrook *et al.*, 1989). Five micrograms of poly(A)$^+$ enriched RNA, or 25 μg total RNA was electrophoresed in 1.0% agarose containing 2.3 *M* formaldehyde in Mops buffer (0.2 *M* morpholinopropane sulfonic acid, 50 m*M* sodium acetate, 5.0 m*M* EDTA, pH 7.0). RNA was transferred to a nylon transfer membrane (Magna NT, Micron Separation Inc., 0.45 μm) by the established procedure of Maniatis *et al.* (1982). Northern hybridizations were carried out under high stringency conditions, using ^{32}P-random-primed labeled (1×10^9 to 10×10^9 counts $minute^{-1}\ \mu g^{-1}$) murine *Msx-2* and *Msx-1* and human L7 cDNAs. The *Msx-2* fragment is ~400 base pairs (bp), derived from the 3′ end of the gene. It does not include the homeobox but does include ~200 bp of the 3′ UTR. The *Msx-1* fragment is ~850 bp, derived from the 3′ end

of the gene. It begins at the C-terminal half of the homeobox and includes ~550 bp of the 3′ UTR. Washes after hybridizations were in 0.1× SSPE/0.1% sodium dodecyl sulfate at 65°C. All Northern hybridizations were repeated at least twice using different blots.

In situ hybridization. Mammary glands from virgin and pregnant animals were fixed for 3 hr in 4% paraformaldehyde/PBS, dehydrated through a graded series of ethanols to xylene, and embedded in paraffin wax. Seven-micrometer sections were cut, floated on slides coated with 3-aminopropyltriethoxysilane (Sigma), and baked onto slides overnight on a slide warmer at 45°C. Sections were dewaxed through two changes of xylene and rehydrated through graded series of ethanols. Sections were then digested with proteinase K (1 μg ml^{-1} in 10 m*M* Tris–HCl, 5 m*M* EDTA, pH 7.5) at 37°C for 30 min and the reaction was stopped with two changes of H_2O and one wash in PBS for 2 min.

To avoid nonspecific binding of RNA probes, slides were prehybridized for 1 hr at 45°C with hybridization buffer (see below) that did not include the RNA probe. Slides were hybridized at 45°C for 16 hr under siliconized coverslips in a solution containing 50% formamide, 3 m*M* NaCl, 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 150 μg ml^{-1} tRNA, 1 mg ml^{-1} yeast total RNA, 10% dextran sulfate, 1% blocking solution (blocking reagent for nucleic acid hybridization, Boehringer-Mannheim Genius system kit), and 800 ng ml^{-1} digoxigenin labeled RNA probe. After hybridization, coverslips were removed in 2× SSPE and then slides were rinsed twice, for 1 hr each time in 0.2× SSPE at 50°C.

To avoid nonspecific binding of anti-digoxigenin antibody, slides were treated for 45 min at room temperature with 2% blocking solution in 100 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl and then for 45 minutes in BSA wash solution [1% BSA (Sigma A-7030), 0.3% Triton X-100, 100 m*M* Tris–HCl, pH 7.5, and 150 m*M* NaCl] at room temperature. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody in 2% blocking solution under siliconzed coverslips for 16 hr at room temperature. Coverslips were removed in BSA wash solution, followed by two more BSA washes, and then incubated in 2% blocking solution for 30 min followed by 2 min in a solution containing 100 m*M* Tris–HCl, pH 9.5, 100 m*M* EDTA, and 50 m*M* $MgCl_2$. To visualize probes, slides were incubated with a pair of colorimetric substances, NBT and x-phosphate, in the above solution (as described in Boehringer-Mannheim The Genius System User's Guide for Filter Hybridization, Version 2.0) for various times ranging from 5.5 to 24 hr. When the desired intensity was reached, the reaction was stopped in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5. Slides were dehydrated through graded alcohols into xylene and coverslipped.

Riboprobes were labeled with digoxigenin labeling mix (NTP labeling mixture 10×, Boehringer-Mannheim Catalog No. 1277 073), using the appropriate SP6 or T7 transcription system. All *in situ* hybridizations were repeated at least four times.

RESULTS

Expression of Msx-1 and Msx-2 Transcripts Levels during Mammary Gland Development

The expression level of *Msx-1* and *Msx-2* transcripts in various stages of mammary development was evaluated by Northern blot hybridization to poly(A)$^+$ enriched RNA isolated from mouse mammary glands at several stages of development. The Northern blot was hybridized consecutively with probes for *Msx-1*, *Msx-2*, and L7 (as a control for the amount of RNA and its integrity). *Msx-1* (~2050 bp) and *Msx-2* transcripts (~1300 and ~2300 bp) were present in glands from virgin animals and glands from animals during early pregnancy (5–8 days post coitus) (Fig. 1). Transcript levels of both genes decreased substantially in glands from animals in late stages of pregnancy (15–18 days post coitus). In lactating glands *Msx-2* expression was not detected, while *Msx-1* transcripts were seen at low levels. The transcript size of *Msx-1* in glands from lactating animals was somewhat smaller (~1.9 kb) than the transcript size from other stages of the mammary cycle.

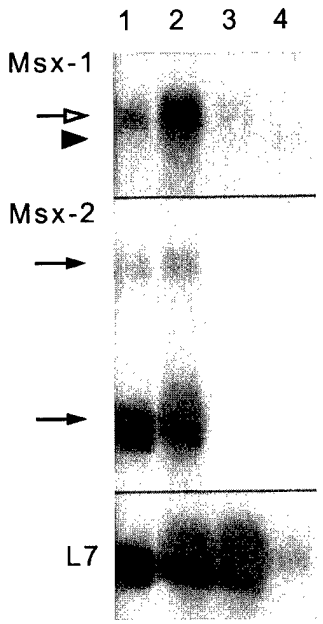

FIG. 1. RNA expression of *Msx-1* and *Msx-2* during stages of mouse mammary gland development. Lane 1: 25 immature virgin mice were taken; Lane 2: 7 mice were taken 5–8 days into timed pregnancies; Lane 3: five 15- to 18-days pregnant mice were taken; Lane 4: 3 animals were taken 3–4 days into lactation. Each lane contains 5 μg of poly(A)$^+$ RNA. L7 mRNA was used as a loading control. Open arrow points to *Msx-1* transcripts, solid arrows point to *Msx-2* transcripts. Arrowhead points to smaller *Msx-1* transcript observed only in lactating glands.

The final stage of the mammary cycle is involution, in which, following weaning, secretory tissue is destroyed by apoptotsis as the gland reorganizes to a form resembling its prepregnancy state. Total RNA was extracted from glands of mice that were lactating for 10 days before pups were weaned, and their glands were removed at several time points during involution. Figure 2 shows the expression of *Msx-1* and *Msx-2* during involution. *Msx-1* was expressed at low levels in the first 3 days of involution (lanes 2 and 3). In the fourth day of involution expression increased and remained at similar levels thereafter. *Msx-1* transcript size in glands that were involuting for 2 days was the same as in lactation. In the third day after weaning both transcripts

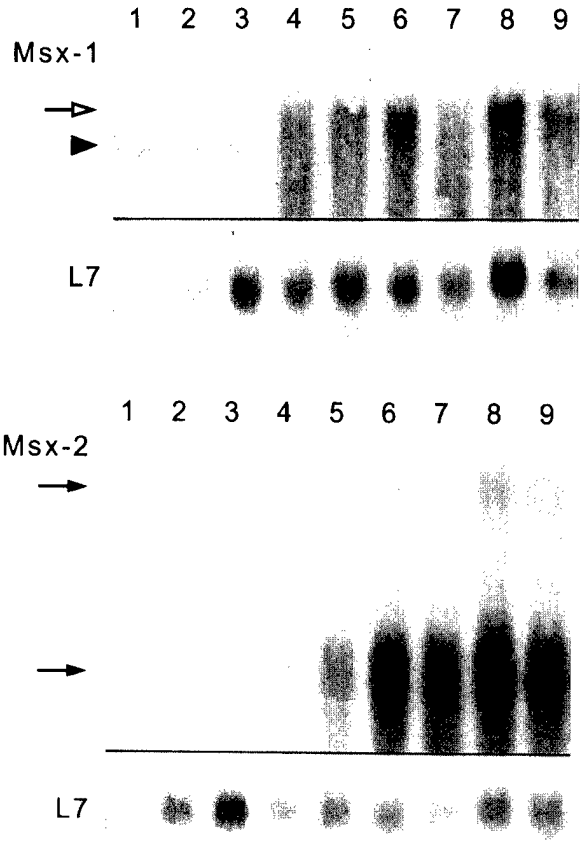

FIG. 2. Northern analysis of *Msx-1* and *Msx-2* in involuting mammary glands. Mice were lactating for 10 days before pups were removed. The next day is considered the first day of involution. RNA was extracted from glands involuting for different numbers of days. The Northern analysis was performed as described under Materials and Methods. Lane 1: lactating glands. Lane 2: 2 days involuting. Lane 3: 3 days involuting. Lane 4: 4 days involuting. Lane 5: 6 days involuting. Lane 6: 8 days involuting. Lane 7: 10 days involuting. Lane 8: 12 days involuting. Lane 9: 14 days involuting. Each lane contains 20 μg total RNA. Lanes 1–3: three animals were taken. Lanes 4–5: five animals were taken. Lanes 6–9: six animals were taken. Solid arrows point to *Msx-2* transcripts. Open arrow points to *Msx-1* transcript. Arrowhead points to *Msx-1* smaller transcript in lactating and early involuting glands.

were visible, after which the larger transcript size, that which was detected in other stages of gland development, was the predominant one. *Msx-2* transcripts could not be detected in the first 3 days of involution. Expression increased gradually in Days 4–8 and then reached a plateau.

Msx-2 Expression in Glands from Ovariectomized Animals and after Estrogen Replacement

To determine if *Msx-1* and *Msx-2* transcript levels are regulated by ovarian secretions, we isolated RNA and made a Northern blot from glands of animals that had been ovariectomized at two time points during the gland development. Ovariectomy was performed either at the age of 5 weeks, when the mouse initiates estrus cycles (puberty), or at the age of 12 weeks when the mouse is already mature and cycling (adulthood). After ovariectomy, mice were allowed to recuperate for 4 weeks, a time period that was determined adequate for ovarian steroids to be depleted from the tissues, when glands were taken.

The expression level of *Msx-1* in glands from ovariectomized mice was similar to that in glands from intact controls (Fig. 3). On the other hand, the expression of *Msx-2* was lower in glands from ovariectomized animals at both time points compared to glands from intact controls (Fig. 3), suggesting that *Msx-2* RNA level is up-regulated by ovarian secretions.

To further test this hypothesis, *Msx-2* was hybridized to mammary gland RNA from animals that were ovariectomized at 5 weeks of age, and in which estrogen was restored by subcutaneous implants at 11 weeks of age. Glands were collected 4 days after estrogen was implanted. When estrogen was replaced in animals that were ovariectomized, *Msx-2* levels (Fig. 4, lane 2) returned to levels found in glands from intact animals (Fig. 4, lane 3).

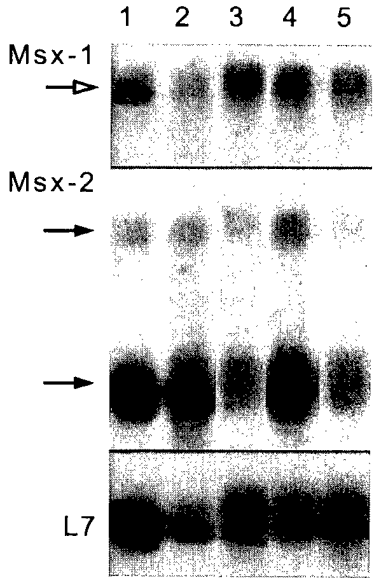

FIG. 3. RNA expression of *Msx-1* and *Msx-2* in the mammary gland in response to ovariectomy. Poly(A)$^+$ RNAs were extracted from glands of twenty 5-week-old endocrine intact mice (lane 1); fifteen 8- to 9-week-old endocrine-intact mice (lane 2); thirty 9-week-old mice that were ovariectomized at 5 weeks of age (lane 3); fifteen 16-week-old endocrine-intact mice (lane 4); twenty-five 16-week-old mice that were ovariectomized at 12 weeks of age (lane 5). Northern analysis was performed as described under Materials and Methods. Each lane contains 5 μg of poly(A)$^+$ RNA. Solid arrows point to *Msx-2* transcripts. Open arrow points to *Msx-1* transcript.

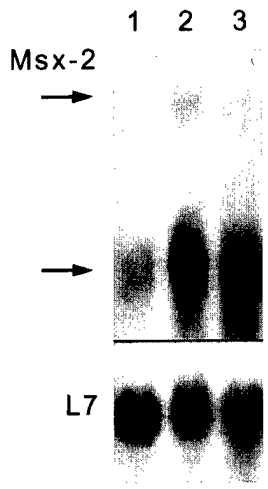

FIG. 4. Expression of *Msx-2* RNA in the mammary gland in response to estrogen replacement in ovariectomized mice. RNA was extracted from mammary glands of mice that were ovariectomized at the age of 5 weeks, let mature for 6 more weeks, when estrogen was added subcutaneously. Glands were collected 4 days later (lane 2). RNA levels are compared to RNA from glands of 6 weeks endocrine-intact animals (lane 3) and to glands from mice that were ovariectomized at 5 weeks of age and let mature for an additional 6 weeks prior to glands collection (lane 1). For each lane 10 animals were taken. Northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

Expression of *Msx-2* RNA was also examined in glands that were treated with pure anti-estrogen in endocrine-intact animals. The pure anti-estrogens are estrogen antagonists without the estrogenic properties that are, paradoxically, associated with many conventional anti-estrogens. Pure anti-estrogens have been shown to have a highly localized inhibitory effect on mammary epithelial growth and morphogenesis when tested in a natural endocrinological and physiological milieu (Silberstein *et al.*, 1994). EVAc implants, containing the pure antiestrogen ICI 164,384, were implanted directly into the mammary gland in a concentration that was shown to influence only the implanted gland, leaving other glands in the same animal unaffected (Silberstein *et al.*, 1994). RNA was extracted from glands treated with anti-estrogen and from untreated contralateral glands. Glands that were treated with anti-estrogen show reduced levels of *Msx-2* RNA compared to the untreated glands (Fig. 5). These results support the hypothesis that *Msx-2* RNA is up-regulated by ovarian secretions, of which estrogen is a component.

Spatial Localization of Msx-1 and Msx-2 RNA in the Mammary Gland

In situ hybridization was performed on sections of mammary gland tissue using gene-specific probes. Figure 6A shows *Msx-2* RNA localized to the periductal stromal cells, where these cells are forming extracellular matrix (ECM) in coordination with ductal growth. ECM is maintained around the ducts in the quiescent gland of the nonpregnant mouse. In glands from pregnant mice *Msx-2* is localized to stromal cells adjacent to mammary ducts (Fig. 6B) and in most cases not around the developing secretory alveoli or away from gland.

To further test the involvement of *Msx-2* in epithelium–stroma interactions, we examined mammary gland-free fat pads from which the epithelial component had been surgically ablated in prepubertal mice (DeOme *et al.*, 1959). As adults these mice carry inguinal glands consisting solely of mammary adipose stroma which is devoid of any mammary epithelial component. RNA extracted from gland-free fat pads was probed with *Msx-2* and no transcripts were detected, even after a long exposure time (Fig. 7). *In situ* hybridization did not show any detectable *Msx-2* messages either (not shown). This indicates the essential role of epithelium in inducing mesenchymal expression of *Msx-2*.

Figure 6C shows *Msx-1* transcripts localized to the epithelium in glands from pregnant mice. Epithelial localization was found in glands from virgin mice as well (not shown).

DISCUSSION

In a previous paper (Friedmann *et al.*, 1994), we described the expression of *Hox* genes in mouse mammary gland development, in precancerous lesions, and in malignancy. Here we extend these experiments to include developmental studies on expression of two homeobox-containing

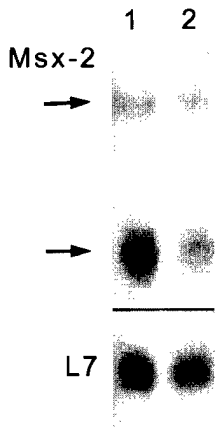

FIG. 5. RNA expression of *Msx-2* in the mammary gland in response to implanted anti-estrogen. RNA was extracted from glands of five pubescent mice that were implanted with 100 μg/gland of anti-estrogen ICI 164,384 for 4 days (lane 2) or from untreated contralateral glands (lane 1). Northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

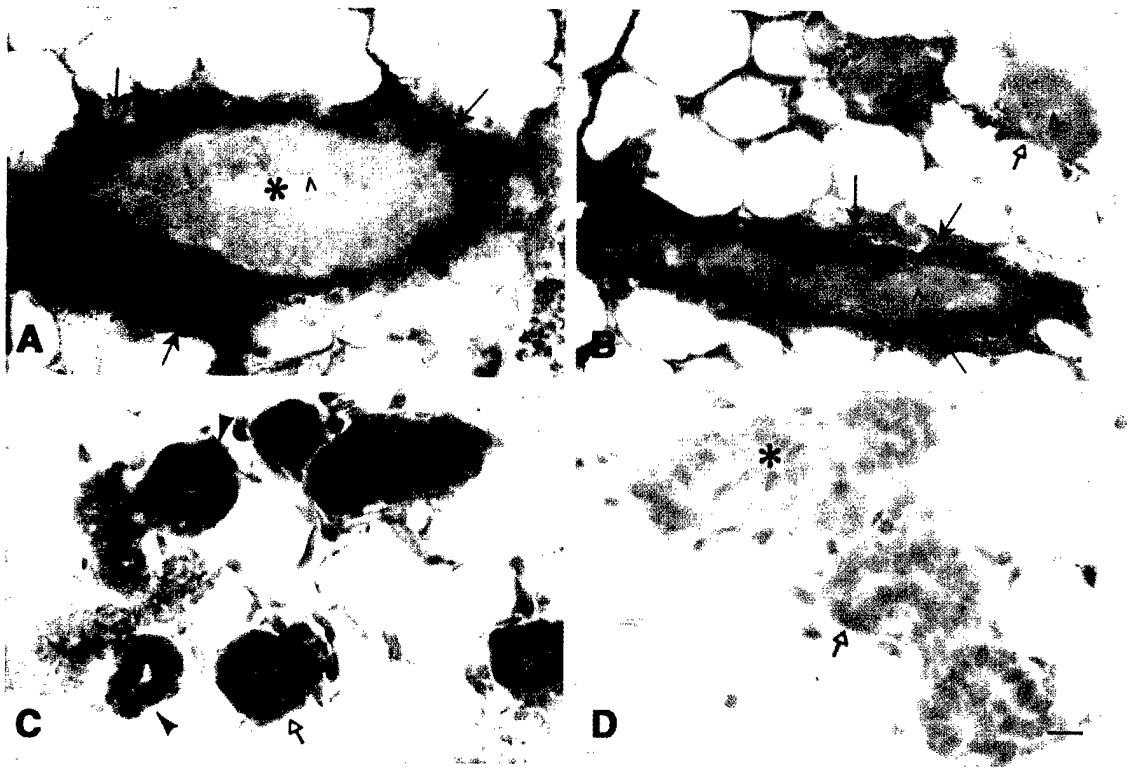

FIG. 6. Spatial expression of *Msx-1* and *Msx-2* mRNA in mammary gland. Riboprobes were labeled with DIG-11-UTP. Fragments labeled were the same as described for Northern blot hybridization (see Materials and Methods). (A) *Msx-2* expression in a gland of mature, virgin mouse. (B) *Msx-2* expression in a gland of a pregnant mouse. (C) *Msx-1* expression in mammary gland of a pregnant mouse. (D) *Msx-1* sense control probe in a gland of a pregnant mouse. It is representative of the controls for other stages of gland development for *Msx-1* and *Msx-2* sense probes. Solid arrowheads point to *Msx-1*-positive epithelial cells. ⟩ points to *Msx-2*-negative epithelial cells. Solid arrows point to *Msx-2*-positive periductal stromal cells. Open arrows point to lobule-alveolar structures in pregnant glands. Asterisks indicate lumens. Bar, 15 μm.

genes, *Msx-1* and *Msx-2*, which are located on chromosomes 5 (Hill *et al.*, 1989) and 13 (Bell *et al.*, 1993), respectively, and are not linked to other known homeogenes. *Msx-1* and *Msx-2* RNAs were expressed during mammary gland development in a stage-dependent manner, appearing in the virgin animal, declining during pregnancy and lactation, and increasing again at the later stages of involution. The expression patterns and the different expression levels of the two genes during the various stages of the gland development indicate differential regulation of *Msx-1* and *Msx-2*, as well as developmental regulation of their expression in the mammary cycle. Absence of detectable expression of *Msx-2* and low levels of expression of *Msx-1* during lactation may be due in part to high levels of milk protein transcripts that may dilute other mRNAs, as seen by the L7 loading control. The smaller size of *Msx-1* transcripts from lactating glands may indicate that an alternative protein with a possible different role is produced.

Increasing levels of expression of both *Msx-1* and *Msx-2* as involution progresses may indicate that both genes participate in the later stages of glandular reorganization, rather than being required for earlier apoptotic events. Though *Msx-1* is expressed from the beginning of involution, the expression level is low and the transcript size in the second day after weaning is the same as in the lactating gland. This indicates that the detected RNAs were probably leftover from lactation, and they may not participate significantly in the early stages of involution. *Msx-1* synthesis *de novo* appears to start only after 4 days postweaning, supporting a role for this gene in the later stages of involution. Both *Msx-1* and *Msx-2* transcripts showed a relatively high degree of degradation during involution on Northern blots. Because there is extensive tissue rearrangement during involution, this degradation is probably part of this process and may be specific to certain classes of transcripts, as the L7 control does not show the same degree of degradation.

The mammary gland is an endocrine target organ of considerable complexity. The ovarian steroids, estrogen and progesterone, are critically involved in the stimulation of mammary growth at puberty and during pregnancy (Lyons, 1958; Nandi, 1958), and genes that are involved in regulating tissue-specific responses to these hormones are likely to

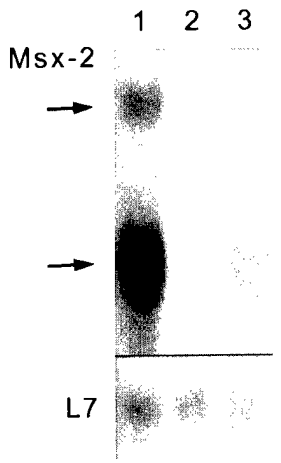

FIG. 7. RNA expression of *Msx-2* in gland-free fat pad. RNA was extracted from glands whose epithelial component had been removed in the 3-week-old mice. Glands were collected 2 months after surgery (lane 2). Expression is compared to levels in glands of epithelium-intact mature virgin mice (lane 1) and to glands of ovariectomized mice (lane 3). For lanes 1 and 3, 5 animals were taken; for lane 2, 15 animals were taken. The northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

be directly or indirectly influenced by levels of circulating steroids. If *Msx-1* or *Msx-2* are involved in the development of the mammary gland, their activity is expected to be linked to mammogenic endocrine secretions, however indirectly. To test this, glands from ovariectomized animals were examined for the expression of *Msx-1* and *Msx-2* RNA. *Msx-1* transcript levels did not change conspicuously in response to ovariectomy. On the other hand, *Msx-2* transcripts levels decreased in glands from animals that were ovariectomized either at puberty or at adulthood relative to glands taken from same age intact animals, suggesting that ovarian secretions up-regulate *Msx-2* expression. When estrogen was replaced in ovariectomized mice, *Msx-2* levels returned to levels similar to those seen in glands in endocrine-intact mice.

This indicates that estrogen has the potential to regulate *Msx-2* expression, but the question of whether physiological, circulating levels of endogenous estrogen could do so remained unanswered. *Msx-2* expression was then studied in glands that were treated *in situ* with anti-estrogen. As with ovariectomized animals, transcripts levels declined, but in this case only the treated glands were affected, while untreated contralateral glands displayed normal levels of *Msx-2* expression. Because the anti-estrogen used belongs to a class of agents that lacks estrogenic activity (Wakeling and Bowler, 1988; Wakeling *et al.*, 1991), this experiment demonstrates that estrogen is required for and normally functions as a regulator of *Msx-2* expression. More work should be done to determine if the effect is direct or through downstream mediators.

Msx-1 and *Msx-2* belong to a family of genes which are related to the *Drosophila Msh* genes. Although a precise function has yet to be established for any of the *msh*-related genes, their spatial domains of expression, as well as features of their regulation, suggest that they are key participants in basic developmental processes. Msx-1 and Msx-2 are involved in epithelial–mesenchymal interactions in developing organs, including the mouse and chick limb buds (Davidson *et al.*, 1991; Robert *et al.*, 1991), mouse tooth development (Jowett *et al.*, 1993; Satokata and Maas, 1994), and mouse and human craniofacial bone development (Satokata and Maas, 1994; Liu *et al.*, 1994, 1995; Jabs *et al.*, 1993). Epithelium–mesenchyme interactions are crucial to the development of the mammary gland (Sakakura *et al.*, 1976; Sakakura, 1987), and localization of *Msx-1* and *Msx-2* transcripts by *in situ* hybridization was expected to provide insights into whether these genes play a morphogenetic role in mammary development.

In glands from virgin and pregnant animals, *Msx-1* was localized to the mammary epithelium. *Msx-2* RNA was found in stroma closely associated with epithelial elements, not in epithelial cells and not in stroma distant from epithelial elements. In pregnancy, *Msx-1* expression was detected in epithelium of the ducts as well as the developing alveoli. *Msx-2* expression was associated mainly with stromal cells surrounding ducts and not with cells in the less abundant stroma associated with lobule-alveolar structures. The close physical association of cells displaying *Msx-1* and *Msx-2* transcripts with mammary epithelium strongly suggests that these homeogenes play a role in the inductive interactions occurring between mammary epithelium and stromal cells of the mammary fat pad.

Msx-2 expression was not detected in epithelium-free fat pad. This indicates that mammary epithelium is required for the expression of *Msx-2* in contiguous periductal stroma. The absence of *Msx-2* expression in stromal cells associated with blood vessels indicates a degree of specificity in this tissue interaction. Interestingly, *Msx-1* expression and *Msx-2* expression, which are normally found in the mesoderm underlying the apical ectodermal ridge in developing chick limbs, are not maintained in a limbless mutant that is unable to form an apical ridge (Robert *et al.*, 1991). The mutant can be rescued by grafting normal ectoderm to the limb field, leading to expression of these homeobox genes. Our results indicate that mesodermal expression of *Msx-2* in the mammary gland also requires contiguous mammary epithelium. In the case of the breast, an additional regulatory element is indicated by the influence of endogenous estrogen on *Msx-2* expression.

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Expression and Functional Role of E- and P-Cadherins in Mouse Mammary Ductal Morphogenesis and Growth

CHARLES W. DANIEL, PHYLLIS STRICKLAND, AND YAEL FRIEDMANN

Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

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Mammary ducts, and the highly mitotic terminal end buds from which they are derived, consist of two layers of ectodermally derived epithelium, forming a tube-within-a-tube structure. We investigated the role of Ca^{2+}-dependent cell–cell adhesion molecules in maintaining the integrity of these layers. Immunostaining showed abundant E-cadherin on the lateral membranes of end bud body cells and ductal lumenal cells, but no P-cadherin. The basally located cap cells and their differentiated descendants, the ductal myoepithelial cells, displayed only P-cadherin. We investigated the functional significance of this pattern of cadherin expression *in situ* by surgically implanting small, slow-release plastic implants releasing function-blocking antibodies. End buds exposed to a monoclonal antibody to E-cadherin showed disruption of the body epithelium, with epithelial cells floating freely in the lumen. Epithelial DNA synthesis, which is normally very high in these growth buds, abruptly declined. That this reduction in growth was not due to cell damage was shown by spontaneous reaggregation of the cells into a normal epithelium, with resumption of DNA synthesis, when the blocking antibody was depleted. A monoclonal antibody to P-cadherin had no effect on the lumenal layer but partially disrupted the basally located cap cell layer. These data indicate that spatially selective expression of E- and P-cadherins is required for mammary tissue integrity, which is in turn a prerequisite for normal rates of DNA synthesis.

INTRODUCTION

The mammary gland differs from most other organs in that growth and patterning occur in the juvenile, and in rodents the gland is readily accessible for observation and experiment. Even though development of the mammary ductal tree takes place in the sub-adult, it is embryonic-like with respect to active branching morphogenesis and the continuing inductive interactions between the epithelium and its contiguous stroma (Sakakura *et al.*, 1979; Cunha *et al.*, 1992). Mammary end buds, which represent the growth points for ductal morphogenesis, are of special interest because by their turning, branching, and alterations in growth rate, patterning of the ductal tree is achieved. The end bud is the structure that interprets morphogenetic signals from surrounding stroma. Within the end buds, epithelial cells are channeled into spatially segregated subpopulations of the ducts, where they differentiate and become mitotically inactive. Because of this apical growth habit, a longitudinal section through a terminal end bud and its duct provides both a spatial and a temporal picture of histogenesis, in which the rapidly dividing and unspecialized cap cells of the end bud differentiate into myoepithelium of the subtending ducts. Body cells of the end bud are a distinct, multilayered epithelium that becomes the single-layered lumenal tissue of the mammary duct.

The cadherin multigene family of Ca^{2+}-dependent cell–cell adhesion molecules is generally regarded as having a fundamental morphoregulatory role in the embryo and as participating in stabilization of more differentiated tissues (Geiger and Ayalon, 1992). Cadherins undergo spatiotemporal changes in expression during embryonic development, and E- and P-cadherins, in association with cell–substratum adhesion factors, are particularly implicated in the patterning of epithelial sheets and tubes (Takeichi, 1988). We studied the expression and functional activity of cadherins in mammary end buds and their subtending ducts to determine if these calcium-dependent adhesion systems are responsible, at least in part, for the sharp separation between basal and lumenal cell populations, which is a conspicuous feature of mammary ductal morphogenesis.

MATERIALS AND METHODS

Animals and Tissues

The thoracic No. 2 and No. 3 mammary glands of $5\frac{1}{2}$-week-old female C57/B1 mice were used in all experiments.

Immunolocalization

Antibodies ECCD-1 for blocking mouse E-cadherin, ECCD-2 for immunostaining mouse E-cadherin, and

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PCD-1 for both blocking and immunostaining mouse P-cadherin were a gift from M. Takeichi. Mammary glands were removed from anesthetized mice at the indicated times. Flattened glands were quick frozen on dry ice and stored at −80°C. Cryosections (5 μm) were thaw-mounted on silanized slides, postfixed in 1:1 acetone/methanol at −20°C for 10 min, air dried, and stored at −20°C. Slides were defrosted, washed in PBS, and successively treated with 0.2% glycine in PBS 2×5 min, H_2O_2/methanol for 30 min, and 5% milk in PBS for 30 min, with PBS washes between each step. Antibody ECCD-2 diluted 1:500 and PCD-1 diluted 1:20 in milk/PBS were applied to slides that were then coverslipped, sealed with rubber cement, and incubated at room temperature overnight. The slides were then washed in PBS/1% goat serum and the biotinylated goat anti-rat secondary antibody (Amersham) diluted 1:200 was applied for 1 hr. Slides were washed in PBS, and Vectastain ABC reagent (Vector Labs), regular or elite, respectively, was applied for the time specified, followed by washing in PBS. The peroxidase substrate was applied for 10 min and sections were lightly counterstained with hematoxylin, dehydrated, and coverslipped.

For whole-gland immunostaining we employed a modified version of the procedures used on embryos (Dent *et al.*, 1989; Le Motte *et al.*, 1989). Glands were removed, flattened onto strips of stiff paper, and fixed overnight in 4:1 methanol:DMSO at 4°C. Tissue was bleached for 5 hr in 4:1:1 methanol:DMSO:30% H_2O_2 at room temperature and stored at −20°C in 100% methanol from overnight to several weeks. Tissue was hydrated to PBS through a graded series of methanols, washed in two 1-hr changes of PBSMT (PBS, 5% milk, 0.1% Triton X-100), and incubated at 4°C overnight in primary antibody diluted in PBSMT; 1/100 ECCD2 and 1/20 PCD. Tissue was washed in PBSMT 2× for 1 hr at 4°C, and 3× for 1 hr at room temperature followed by secondary antibody HRP (horseradish peroxidase) goat anti-rat (Chemicon) diluted 1/300 in PBSMT and incubated overnight at 4°C. Tissue was incubated in primary antibody for 20 min in PBT (0.01% Triton X-100 in PBS) followed by 10 min in substrate without H_2O_2 and 10–15 min in substrate with H_2O_2. The reaction was stopped with a 5-min wash in PBT and tissue was dehydrated through a graded series of ethanols, cleared in xylene, and stored in methyl salicylate for examination. Some samples were subsequently embedded in paraffin and sectioned at 6 μm to determine antibody localization more precisely.

Antibody Neutralization of Cadherins in Vivo

Blocking antibodies were lyophilized and incorporated into EVAc (DuPont) plastic pellets as described, using bovine serum albumen as carrier (Silberstein and Daniel, 1982). Implants containing 200–300 μg of lypholate were inserted into fat pads in front of the advancing end buds and left for 12, 24, 30, 48, and 72 hr. To monitor the cells engaged in DNA synthesis, animals were injected with 100 μci [^{3}H]thymidine (78 Curies/m*M*) or Brdu (5′-bromo-2′-deoxyuridine) (0.01 cc/g body wt of 25 μg/ml PBS) 1 hr before glands were taken and fixed in Tellysesniczkys fixative (10% formalin, 5% acetic acid, 70% EtOH). Glands were processed for whole-mount examination followed by paraffin embedding and sectioning at 5 μm. Effects on histoarchitecture and DNA synthesis were evaluated relative to contralateral control glands that had been implanted with equivalent amounts of rat IgG or nonblocking antibody to E-cadherin, ECCD-2.

RESULTS

Spatial Expression of E- and P-Cadherins in Ductal Morphogenesis

Mammary glands were immunostained and examined for E- and P-cadherins both in whole-mount preparations and in histological sections. Figure 1a shows a mammary ductal tree terminating in highly mitotic end buds that are actively penetrating the surrounding adipose stroma. When whole glands were immunostained with ECCD-2 to E-cadherin, the multilayered mass of epithelial body cells was deeply stained, whereas the basal layer of cap cells was not (Fig. 1b). In histological sections E-cadherin was conspicuously displayed on cell membranes of body cells (Fig. 2a and 2c). These multilayered body cells displayed E-cadherin on all cell surfaces except the membranes directly exposed to the lumen (Figs. 2c and 2e). Cap cells and their descendants, the myoepithelial layer of the subtending mammary ducts, did not display detectable E-cadherin.

P-cadherin, in contrast, was localized in the basally located cap cells of end buds (Figs. 1c, 2b, and 2d) and in small clusters of cells within the lumenal compartment (Fig. 2d). These may represent groups of cap cells that have detached from the basal lamina and migrated into the interior, as reported using microcinematography (Williams and Daniel, 1983). It is postulated that these detached patches of cap cells are visualized because they temporarily retain membrane-associated P-cadherin.

In differentiated mammary ducts the same sharp distinction between the distribution of E- and P-cadherins was observed. In lumenal tissue, which in these ducts is usually one or two cells in thickness, staining for membrane E-cadherin was more intense than in the end buds (Fig. 2e). Membrane surfaces facing the lumen were again unstained, as were the myoepithelial cells. The basally located, longitudinally aligned myoepithelial

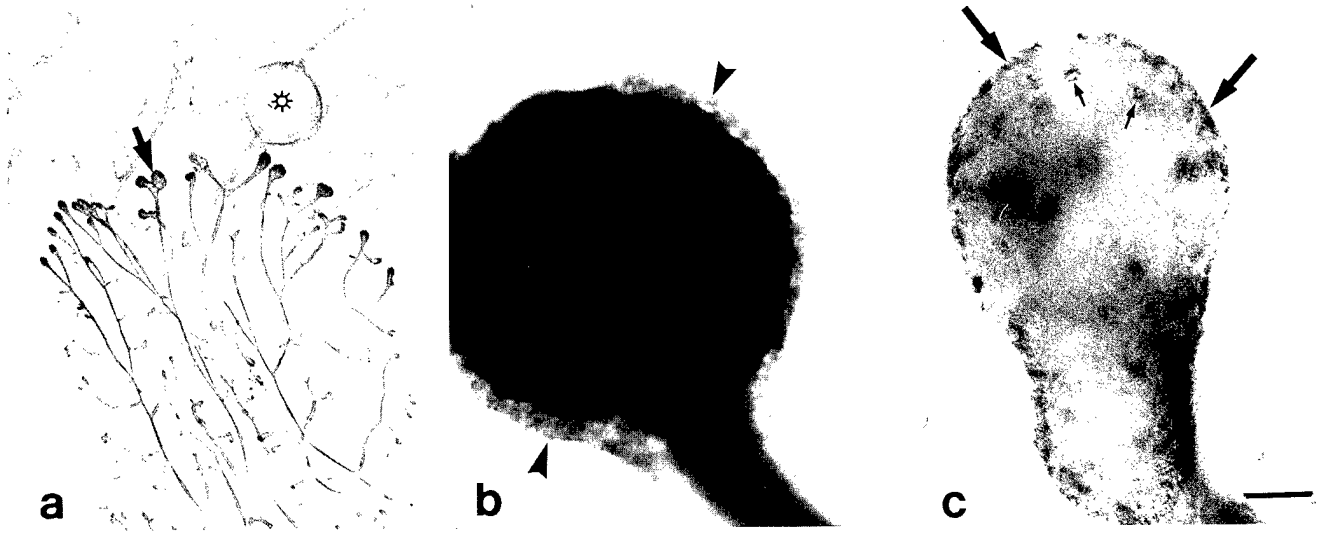

FIG. 1. Whole-mount preparations of a No. 3 thoracic mammary gland taken from 5-week-old virgin mice. (a) Hematoxylin-stained gland showing a branching system of mammary ducts terminating in enlarged end buds (arrow). An EVAc pellet has been implanted into the fatty stroma ahead of the advancing end buds (*). (b) An end bud and a portion of its subtending duct immunostained for E-cadherin with antibody ECCD-2. The multilayered body epithelium is darkly stained, obscuring the end bud lumen. The basal layer of cap cells (arrowheads) is unstained. (c) An end bud and a portion of its subtending duct immunostained for P-cadherin with antibody PCD-1. The cap cells are stained (large arrows), whereas the interior epithelium shows only occasional patches of P-cadherin immunostaining (small arrows). Bar, 1.6 mm in a, 28 mm in b, and 25 mm in c.

cells, like their progenitor cap cells of the end bud, were decorated with P-cadherin in a punctate distribution, presumably reflecting the distribution of adherans junctions (Fig. 2f).

Blocking Antibodies Applied in Situ

Slow-release plastic implant technology presents an opportunity to test the effects of blocking antibody directly in the mammary gland. Miniature pellets made from DuPont's ELVAX (EVAc) are nondenaturing to proteins, are capable of sustained release, and do not initiate an inflammatory reaction in the host (Silberstein and Daniel, 1987). EVAc implants have found use in investigations on the effects of exogenous materials acting locally at precisely determined locations within the mammary gland (Silberstein and Daniel, 1982; Daniel *et al.*, 1989).

Slow-release pellets were implanted directly ahead of the advancing end buds in 5-week female mice, in which the diffusion pattern could influence both the buds and the subtending ducts (Fig. 1a). Antibody ECCD-1 to E-cadherin produced dramatic effects in 12 hr in the end bud (Fig. 3b). Multilayered cells of the lumenal compartment, which had previously shown specific staining with antibody to E-cadherin, loosened and were found freely floating in the lumen. The cap cells of the end buds and the myoepithelial cells of the subtending ducts remained associated with each other and attached to the basal lamina, where they formed a sack enclosing the freely floating lumenal cells. In order to determine whether these effects of blocking antibodies were reversible, we examined the treated glands at later time points. At 72 hr, when the antibody had been largely depleted (Silberstein and Daniel, 1982), the end buds had regained their characteristic structure, apparently due to reassembly of the disaggregated lumenal cells (Fig. 3c).

The blocking antibody PCD-1 to P-cadherin had comparable but less dramatic effects on the cap cell population (Fig. 4). At 30 hr of treatment the majority of cap cells in end buds remained in place, but clusters of loosened cells were found between the basal layer and the lumenal population (Figs. 4b and 4c). These clusters appeared to represent regions in which the cap cell layer of the end buds had been more than one cell in thickness and were therefore susceptible to disaggregation by antibody. In areas where the cap cells were monolayered and each cell was attached to the basal lamina, antibody to P-cadherin was unable to dissociate the tissue. At 78 hr pockets of dissociated cells had disappeared and histoarchitecture was normal (Fig. 4d).

In subtending ducts, treatment with the E-cadherin blocking antibody ECCD-1 caused partial disassembly of the lumenal tissue by 12 hr, with epithelial cells floating freely in the lumen (Fig. 3e) while other cells remained attached to the basal lamina (Fig. 3e, arrow), presumably through integrin-mediated adhe-

FIG. 2. Immunostaining patterns of E-cadherin (a,c, and e) and P-cadherin (b,d, and f) in histological sections of mammary end buds and ducts. E-cadherin is found on membranes of interior epithelium except for those cells directly facing the lumen of end buds and ducts (c and e, arrowheads). Basally located cap cells do not display E-cadherin (a and c, arrows) nor do myoepithelial cells along the mammary ducts (e, arrow). P-cadherin is located in the cap cells (b and d, arrowheads) and their differentiated descendants, the myoepithelial cells along the duct (f, arrow). Small clusters of epithelial cells staining for P-cadherin are seen in the multilayered interior epithelium (d, arrows). Bars, 72 μm in a and b, 36 μm in c through f.

sions to the basal lamina or perhaps adhesions of unknown type to myoepithelial cells. Like the end bud, normal tissue morphology was restored at 72 hr (Fig. 3f). Treatment with the blocking antibody PCD-1 to P-cadherin had no obvious effect (not shown), probably because the myoepithelium is entirely monolayered and each cell is anchored to the basal lamina (Williams and Daniel, 1983).

Effects of Blocking Antibodies on DNA Synthesis in Situ

Prior to the removal of glands for the blocking experiments described above, mice were injected with either [^{3}H]thymidine for autoradiography or bromodeoxyuridine for immunolocalization of cells engaged in DNA synthesis. Both methods yielded equivalent results. In 12 hr, the DNA synthetic index (percentage of cells in S-phase)

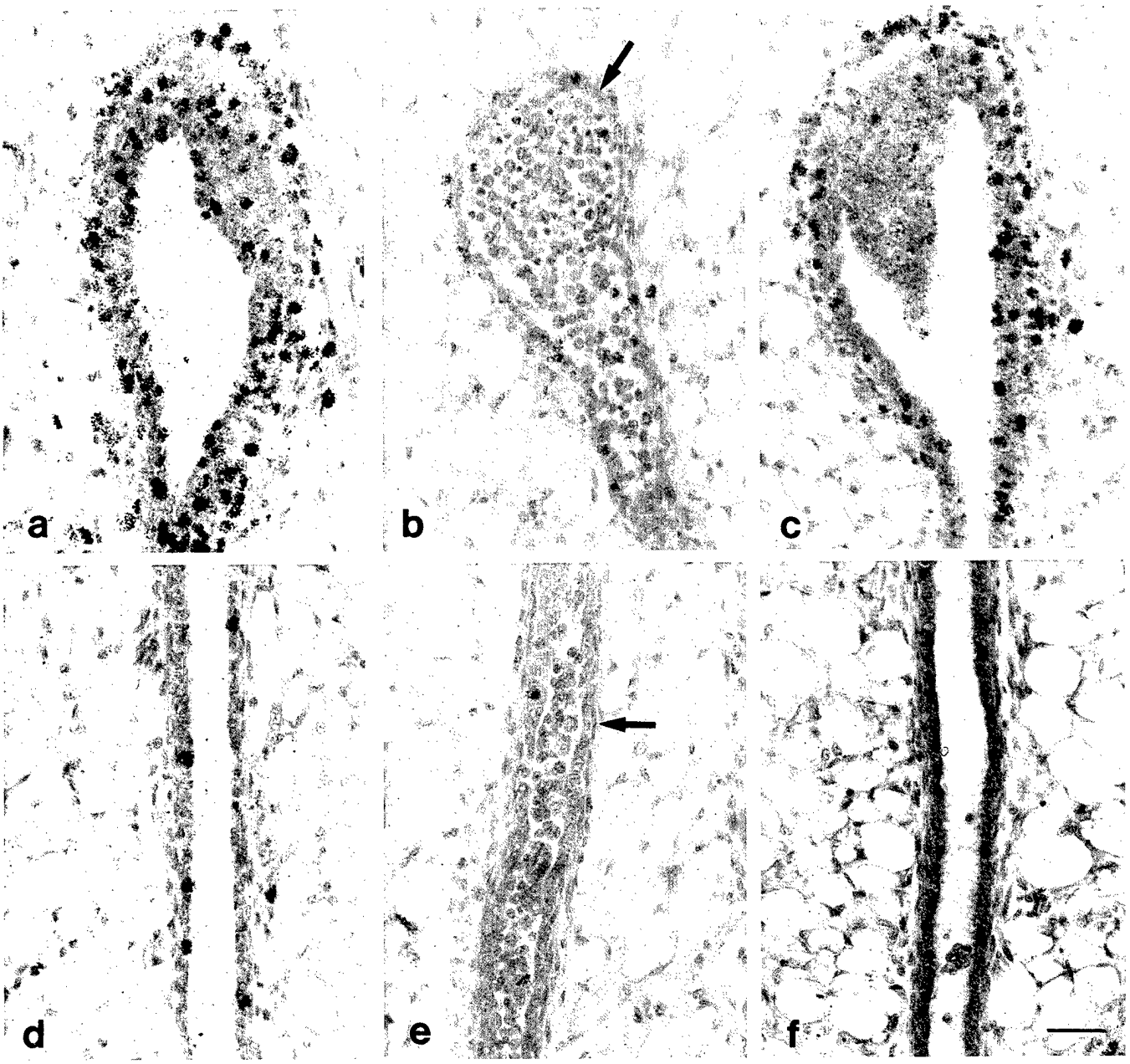

FIG. 3. Blocking antibody ECCD-1 applied to mammary end buds *in situ*. In these autoradiographs cells engaged in DNA synthesis display black silver grains over their nuclei. (a and d) Normal tissue architecture at Time 0. At 12 hr the lumenal epithelium of end buds was dissociated, with loosened cells filling the lumen (b). DNA synthetic cells were reduced in number at 12 hr compared to Time 0 (a) and 72 hr (c). In ducts, the lumenal epithelium was also dissociated at 12 hr (e). At 72 hr normal tissue architecture was restored (f). Cap and myoepithelial cells were relatively unaffected (b and e, arrows). Bar, 70 μm.

in end buds treated with ECCD-1, antibody to E-cadherin fell to 7% compared to 18% in control end buds in the same animal treated with nonblocking antibody to E-cadherin (Figs. 3a, 3b, and 5a). DNA synthesis remained reduced until 72 hr after treatment, when normal growth levels of DNA synthesis resumed, coincident with the reassembly of normal tissue structure from disaggregated cells (Fig. 3c). DNA synthesis was largely unaffected in cap cells (Fig. 5b). Blocking antibody PCD-1 to P-cadherin caused only small changes in DNA synthesis of lumenal cells (Fig. 5c), but cap cell DNA synthesis was reduced and remained so through 78 hr (Fig. 5d).

DISCUSSION

The essential morphoregulatory role of specific adhesion factors in tissue reconstruction and histogenesis

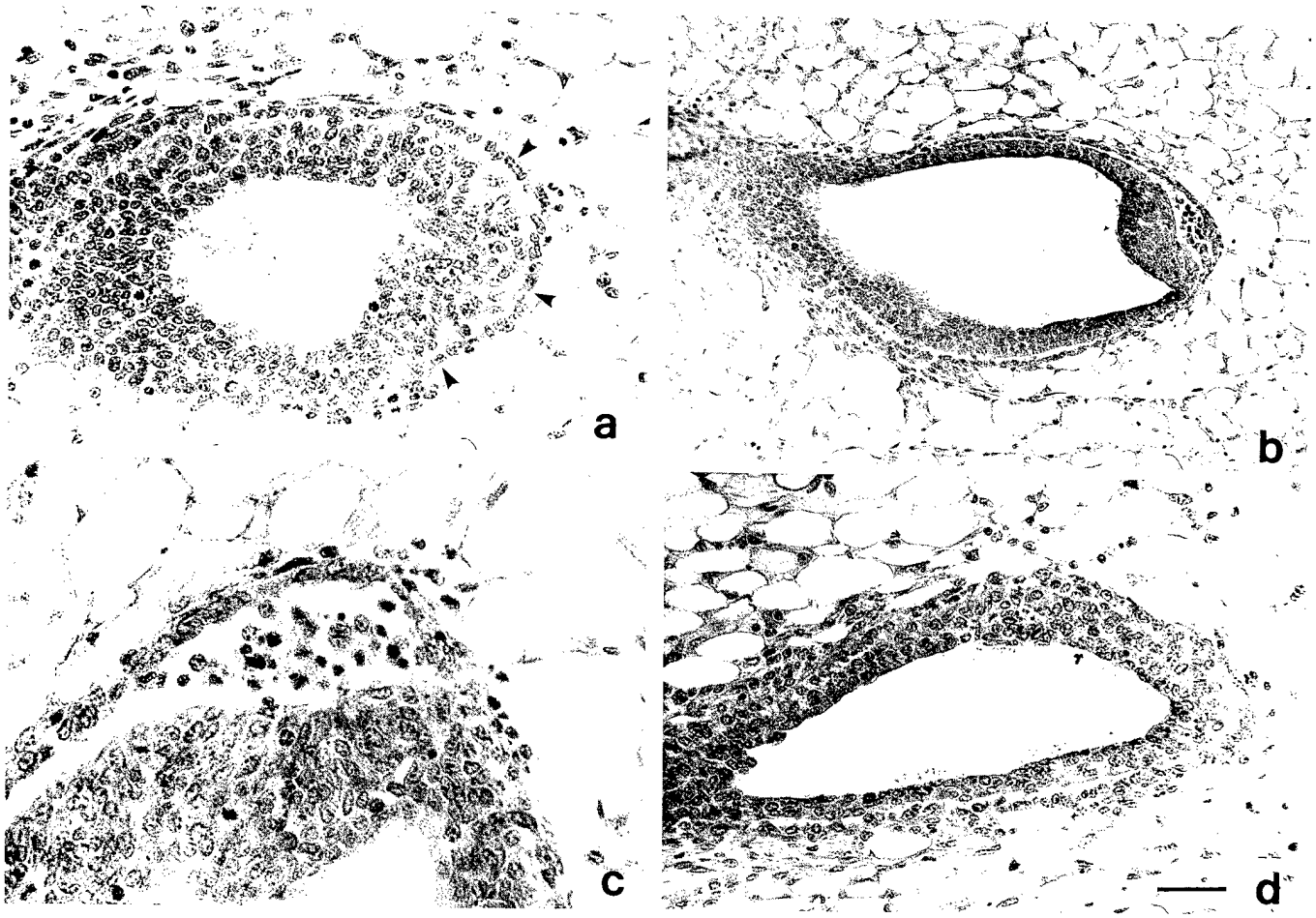

FIG. 4. Blocking antibody PCD-1 to P-cadherin applied to mammary end buds *in situ*. The well-organized cap cell layer at Time 0 of exposure is shown in a (arrowheads). At 30 hr (b and c) patches of cap cells are loosened and appear to be floating in the space between cap and lumenal cell layers. At 78 hr normal architecture was restored (d). Ducts treated with PCD-1 appeared to be unaffected by treatment (not shown). Bar, 25 μm in a and d, 50 μm in b, and 17 μm in c.

has become a central paradigm of development (Edelman *et al.*, 1990). The cadherins represent a multigene family of calcium-dependent adhesion molecules that mediate many homophilic cell–cell adhesive interactions in the initial formation of epithelial aggregates and in their subsequent growth, migration, folding, and bending during morphogenesis (Takeichi, 1991; Geiger and Ayalon, 1992). Together with cell–extracellular matrix adhesive interactions, cadherins trigger morphogenetic processes leading eventually to the assembly of organs, in which these adhesive systems may continue to play an essential role in the stabilization and maintenance of differentiated tissues through specialized adherens-type cell adhesions.

The mouse mammary gland represents a well-developed model for the study of organogenesis and is unique in that its development occurs mainly in the subadult animal where large size and ready accessibility confer experimental advantages over organs that develop mainly *in utero*. During puberty the rapidly growing terminal end buds penetrate the fatty stroma, creating an arborizing system of mitotically inactive, differentiated epithelial tubes from which secretory alveoli arise during pregnancy. The role of adhesive interactions during ductal branching morphogenesis has not been reported, although E-cadherin expression has been observed in alveolar tissues in the mouse (Streuli *et al.*, 1991) and human (Oka *et al.*, 1993) and in cell lines derived from human breast tissues (Bracke *et al.*, 1993; D'souza and Taylor-Papadimitriou, 1994). Attachment of mammary epithelial cells to the basal lamina through integrin receptors is also known to play an essential role in alveolar morphogenesis and secretory differentiation (Streuli, 1993).

The aim of the present experiments was to investigate the expression of E- and P-cadherins and, using function-blocking antibodies, their functional role in segregating subpopulations of epithelial cells in the end buds

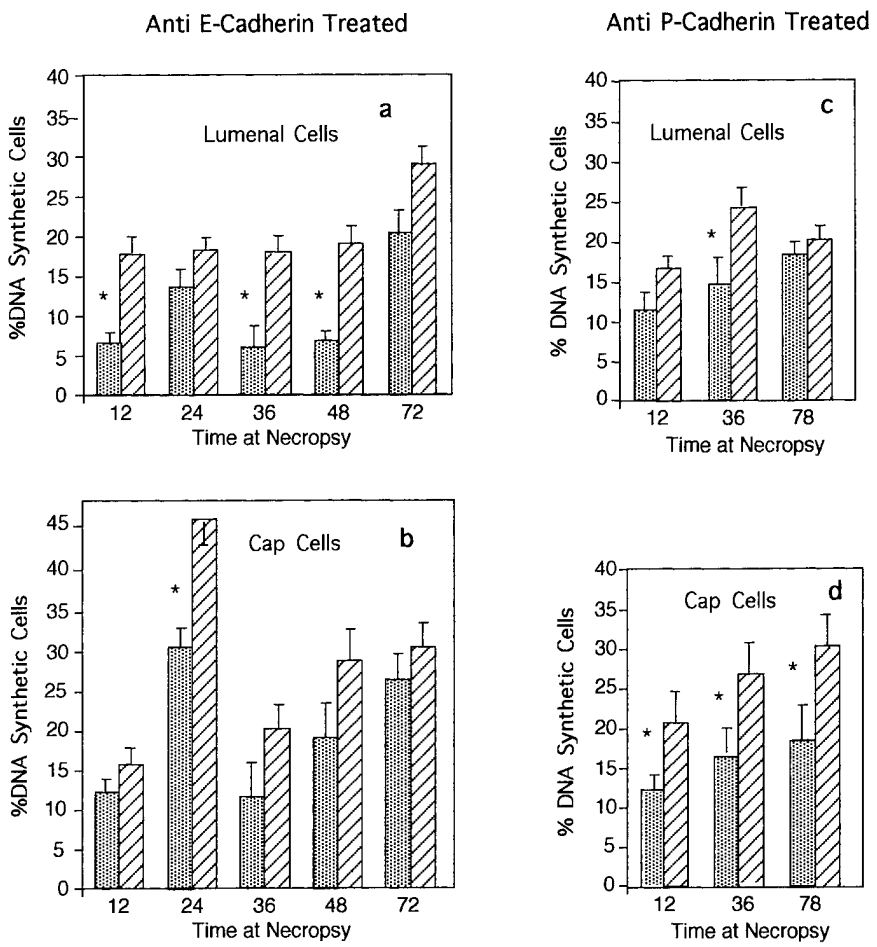

FIG. 5. Effects of blocking antibodies on DNA synthesis *in situ*. Shaded bars, glands implanted with function-blocking antibody; hatched bars, glands with control implants consisting of nonblocking antibody ECCD-2 to E-cadherin (a and b) or nonimmune rat IgG (c and d). Asterisks indicate that experimental tissues differed from the contralateral control tissues at a 0.95 confidence level or better. Vertical bars indicate standard errors. (a) End bud lumenal cells exposed to implant releasing antibody ECCD-1 to E-cadherin. (b) End bud cap cells exposed to implant releasing antibody ECCD-1 to E-cadherin. (c) End bud lumenal cells exposed to implant releasing antibody PCD-1 to P-cadherin. (d) End bud cap cells exposed to implant releasing antibody PCD-1 to P-cadherin.

as they proliferate and are channeled into the lumenal and myoepithelial compartments of their subtending ducts. A second aim was to examine the possible effects of altered adhesion on epithelial DNA synthesis. Longitudinal sections of elongating ducts showed bulbous, multilayered end buds consisting of two readily identifiable and clearly separated cell populations, the multilayered body (lumenal) cell compartment and the basally located monolayer of cap cells. Immunostaining revealed a discrete, nonoverlapping pattern in which cap cells displayed moderate levels of membrane-associated P-cadherin, whereas E-cadherin was present on all body cell membranes with the exception of those directly bordering the lumen. In the more differentiated tissues of the subtending duct this discrete pattern was maintained; myoepithelium, derived from cap cells, displayed P-cadherin, whereas lumenal cells showed strong immunostaining for E-cadherin, suggesting that the segregation of these tissue layers may arise from selective expression of these and perhaps other adhesion factors.

Cap cells and their myoepithelial derivatives appear to be topologically continuous with the basal layer of the epidermis (Daniel and Silberstein, 1987), although a direct developmental relationship has not been described. During development the mouse epidermis also expresses cadherins, though in a less discrete spatial distribution, with E-cadherin expressed in both the basal and the intermediate layers, and P-cadherin only in the basal layer (Nose and Takeichi, 1986; Hirai *et al.*, 1989b). This diffuse pattern of E-cadherin distribution may function in permitting cells derived from the basal layer to move into intermediate layers for differentiation into keratinocytes. In the mammary gland, where cadherin distribution is apparently nonoverlapping, cadherins may be

associated with the spatially discrete segregation of basal and lumenal cell layers.

We observed occasional clusters of cells in the lumenal compartment of the end bud staining anomolously for P-cadherin (Figs. 1c and 2d). These are probably identical to cell clusters shown by histology and by time-lapse microcinematography to dislodge from the cap layer and sink into the lumenal compartment (Williams and Daniel, 1983). It is postulated that these migratory cap cells acquire E-cadherin, enabling them to migrate between tissue layers while continuing for a time to display P-cadherin. It is not possible to verify the acquisition of E-cadherin by these cells because their membranes are closely contiguous with those of surrounding body cells. The nature of these migratory cells is unknown, but it is interesting to speculate that, because the cap cells are the least differentiated of identifiable mammary cell types, they may represent the source of mammary stem cells that are dispersed throughout the mammary tree (Faulkin and DeOme, 1960) and which have been tentatively identified as clear-staining cells (Smith and Medina, 1988).

Using slow-release plastic implants to expose selected regions of the gland *in situ* to blocking antibodies, we found that antibody to E-cadherin induced disruption of the multilayered body epithelium of the end bud, resulting in cells freely floating in the space contained within the basal lamina and its adherent basal cells (Fig. 3b). This result indicates that E-cadherin is required and could be sufficient for integrity of the lumenal tissue. Interestingly, reassociation of these loosened cells occurred after depletion of the antibody (Silberstein and Daniel, 1982), restoring normal tissue architecture (Fig. 3c). This reaggregation indicates both the reversible nature of the antibody treatment and the ability of these disaggregated cells to reconstruct histotypic structures, an ability usually associated with embryonic tissues (Townes and Holtfretter, 1955; Moscona, 1961). Exposure of mammary ducts to blocking antibody to E-cadherin also resulted in disaggregation of lumenal epithelium and the appearance of freely floating cells, but in this case many of the basally located cells appeared to retain associations with either the basally located myoepithelium or the basal lamina itself (Fig. 3e).

Treatment with antibody to P-cadherin had no effect on lumenal cells but produced partial disruption of the basally located cap cell layer (Figs. 4b and 4c). Here pockets of disaggregated cells were found next to cap cells that appeared to be normally arranged, presumably adhering to the basal lamina by integrins (Streuli, 1993). Occasional patches of disaggregated cells may represent areas in which the cap cells had become multilayered, losing their attachment to the basal lamina and becoming susceptible to loosening by antibody.

Experiments with cells transfected with cDNAs encoding different cadherins have demonstrated that forced expression of specific adhesive factors can give rise to aggregation and highly selective segregation (Nose *et al.*, 1988). In our experiments, the histotypical sorting of mammary cells *in situ* is conceptually consistent with this, but adds the dimension of tissue reconstruction in an adult rather than an embryonic organ.

An interesting consequence of exposure to blocking antibodies was the approximately $2\frac{1}{2}$-fold decline in DNA synthesis observed in end bud lumenal cells following tissue disaggregation (Figs. 3b and 5a). Declines as much as 5-fold were observed in other experiments (not shown). This decline was sustained through a gradual and progressive reaggregation, but was reversed following tissue reconstruction at 72 hr when DNA synthesis returned to normal levels (Fig. 3c). This decline in DNA synthesis was specific and was not observed in glands implanted with control pellets containing either rat IgG or nonblocking ECCD-2 (Fig. 5a). The observed association of a decline in DNA labeling index with disaggregation of epithelial cells suggests that these nontransformed mammary epithelial cells require normal cell–cell associations to permit passage through the cell cycle. The opposite is true with transformed cells, and there exist numerous reports of increased growth and malignant potential associated with decreased cadherin expression (Shiozaki *et al.*, 1991; Oka *et al.*, 1992; Gamallo *et al.*, 1993; Moll *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993).

We conclude that in the normal gland, regulation of DNA synthesis and cell division requires at least some degree of tissue-level structure, for which cadherin-mediated adhesions are required. Because loss of tissue structure is associated with inhibition of DNA synthesis, a lack of cadherin-mediated cell adhesions cannot, in itself, account for loss of growth regulation in malignancies.

In lung epithelial morphogenesis studied in explant cultures, partial disruption of tissue structure was observed in response to antibodies to E- and P-cadherins, which appeared to be reversible when antibodies were removed (Hirai *et al.*, 1989a). Although DNA synthesis was not studied, growth of the explanted epithelial tissues appeared to occur normally in the presence of antibodies. The same blocking antibodies were shown to partially disrupt morphogenesis in organ cultures of embryonic mouse skin, in which, interestingly, secondary effects on dermal condensation were observed (Hirai *et al.*, 1989a).

In the present study, precise spatial distribution of cadherins is associated with the maintenance of the "tube-within-a-tube" architecture that is required both for the proper channeling of presumptive tissues into

their proper location within the duct and for the normally high rates of DNA synthesis and cell division required for growth. Together with cell–substrate adhesions, the cadherin-based adhesion systems play a central role in mammary growth and patterning of epithelial ducts in the mammary gland.

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Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands

G. SHYAMALA*†, X. YANG*, G. SILBERSTEIN‡, M. H. BARCELLOS-HOFF*, AND E. DALE*

*Division of Life Sciences, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720; and ‡Department of Biology, University of California, Santa Cruz, CA 95064

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ABSTRACT **In this report we document the creation of transgenic mice in which the native ratio of A and B forms of progesterone receptor (PR) has been altered by the introduction of additional A form as transgene. We also show that in these mice there is an aberration in mammary development. In ovariectomized prepubertal PR-A transgenic mice, end buds with unusual morphology persist after ovariectomy, and in young adult nonovariectomized mice, mammary glands have extensive lateral branching. The glands of adult mice also exhibit ductal hyperplasia with a disorganized basement membrane and decreased cell–cell adhesion, features commonly associated with neoplasia. Because progesterone is a mitogenic hormone in mammary glands and PR is required for mammary development, these data provide direct evidence that *in vivo* a regulated expression of the two isoforms of PR is critical for appropriate cellular response to progesterone and that for mammary glands this may have major implications to carcinogenesis.**

Progesterone receptor (PR) belongs to the superfamily of steroid receptors and mediates the action of progesterone in its target tissues (1, 2). In normal mammary glands of both rodents and humans, progesterone promotes the proliferation of epithelial cells (3–6). High levels of PR gene expression are associated with the end bud cells of the growing duct (7), the putative progenitors of the ductal cells (8), and in the mature female, the epithelial cells of the duct that give rise to lobulo-alveolar outgrowths also express high levels of PR (7). Direct evidence for the importance of PR in mammary development is revealed in PR-null mutant mice that exhibit a marked impairment in lobulo-alveolar development (9).

PR exists in two molecular forms, the A and B forms whose expression is regulated by two promoters (10, 11). The ratio of the two forms varies among target tissues (2), suggesting that their differential expression may be critical for appropriate cellular responsiveness to progesterone (12). In the same cell, the A and B forms can have different functions and the activity of the individual form of the receptor varies among different types of cells (13, 14). Also, depending on the cell and promoter context, the A form can either inhibit or enhance the activity of the B form (14). The A and B forms of PR also modulate estrogen receptor (ER) and estrogen-dependent gene expression (15–17). All these observations strongly suggest that an imbalance in the expression and/or activities of the two forms of PR can have important consequences to normal mammary development, which requires a coordinated action of estrogen and progesterone among its various cell types (18). Also, to the extent that an aberration in normal developmental processes can serve as a trigger for carcinogenesis, an imbalance in the expression and/or activities of the two forms of PR can also have implications to mammary carcinogenesis.

Almost all studies to date have employed *in vitro* models to investigate the relative actions of the A and B forms of PR, by using either immortalized or tumorigenic cell lines; such studies, although informative, however, cannot be extrapolated to normal developmental processes. Therefore, we have created transgenic mice in which the native ratio of A/B forms of PR has been altered by introduction of additional A form as transgene. In these mice, there is an aberration in mammary development characterized by extensive lateral branching. These glands also exhibit ductal hyperplasia and a disorganized basement membrane, features commonly associated with neoplasia.

MATERIALS AND METHODS

Construction of Transgenic Mice. There was a possibility that in transgenic mice, carrying an imbalance in the normal ratio of the two forms of PR, pregnancy might be jeopardized. Therefore, we used a binary transgenic system in which the GAL-4 gene, driven by the murine cytomegalovirus (CMV) promoter (CMV-GAL-4 mice), served as the transactivator of the PR-A gene, carrying four GAL-4 binding sites (UAS; UAS-PR-A mice). Crossing the CMV-GAL-4 mice with UAS-PR-A mice resulted in bigenic mice carrying additional PR-A gene.

For construction of CMV-GAL-4 plasmids, the GAL-4 gene was excised from the plasmid of pGATB (19) as a *Hin*dIII fragment and the *Hin*dIII site was modified to *Eco*RI prior to insertion into the unique *Eco*RI site of plasmid pSV2NeoCMV; this vector, derived from pSV2 Neo (20), carries the murine CMV promoter (21) upstream from the inserted sequence. For construction of UAS-TATA-PR-A plasmid, the first intron of mouse PR (cloned by this laboratory) was inserted into its proper position in the PR cDNA (22), previously digested with *Bam*HI to remove the first ATG; this fragment was then fused to the UAS-TATA fragment excised from pUAST (19) and was inserted in place of the CMV-PR cDNA in pCNmPR3, previously constructed by this laboratory (22). A schematic representation of the two constructs is shown in Fig. 1. Both DNA constructs were tested in cultured cells to confirm the GAL-4/UAS transactivation (data not shown). The parent plasmids containing the respective transgenes were digested with appropriate restriction enzymes to release the transgene(s) and purified prior to microinjection into the pronuclei of mouse zygotes. Transgenic

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Abbreviations: PR, progesterone receptor; ER, estrogen receptor; CMV, cytomegalovirus.

†To whom reprint requests should be addressed at: Lawrence Berkeley National Laboratory, Division of Life Sciences, Building 74, University of California, Berkeley, CA 94720. e-mail: Shyamala_Harris@lbl.gov.

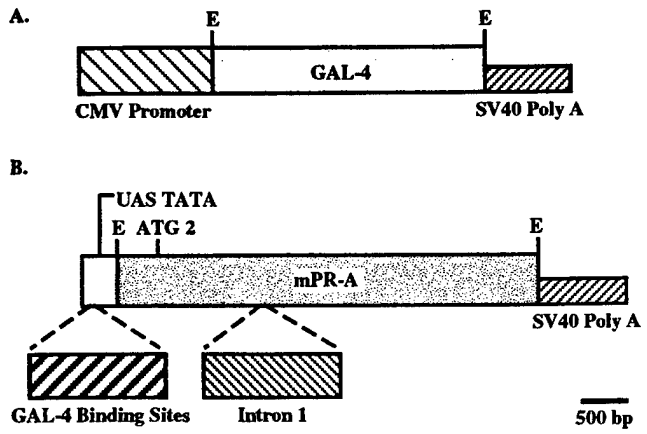

FIG. 1. Schematic representation of plasmid construction for the binary system. (*A*) Insertion of the GAL-4 gene into the CMV promoter expression plasmid containing simian virus 40 splice and polyadenylylation sequences. (*B*) mPR cDNA (A form with only ATG 2) containing intron 1 and simian virus 40 splice and polyadenylylation sequences fused to UAS-TATA fragment containing four GAL-4 binding sites. E, *Eco*RI.

mice were identified initially by Southern blot analysis, and once the founder lines had been established, they were routinely screened by PCR using tail DNA.

Analysis for Transgene and Endogenous Genes' Expression. Transgene and endogenous PR expression was examined by reverse transcription-coupled PCR. The oligonucleotide primers for detecting various transgene expression were as follows: PR-A transgene (forward from PR cDNA, PR-2527, 5′-CGAATTGATCAAGGCAATTGGT-3′; reverse from the simian virus 40 termination sequence, 5′-AGACACTCTATGCCTGTGTGGAG-3′), GAL-4 transgene (forward from GAL-4 untranslated leader (UTL), 5′-GAAGCAAGCCTCCTGAAAGA-3′; reverse GAL-4 784, 5′-CACTGAAGCCAATCTATCTG-3′), and endogenous PR gene (forward from mPR UTL, 5′-AAAAGGGGAGCTTGGGTCGT-3′; reverse, PR-440, 5′-CAAAGAGACACCAGGAAGTG-3′).

Indirect Immunofluorescence Assay. For examining the immunolocalization of PR, E-cadherin, and laminin in individual mammary glands, an indirect immunofluorescence assay was performed with a secondary antibody conjugated to fluorescein isothiocyanate, as described (22). The antibody used for analysis of PR was prepared against synthetic peptide corresponding to amino acid residues 376–394, selected from the amino-terminal half of the mouse PR sequence (23); this antibody reacts with both the A and B forms of murine PR (22). Antibody to E-cadherin, originally obtained from M. Takeichi (Kyoto University, Japan) was provided by C. Daniel (University of California, Santa Cruz). Antibody to laminin was purchased from Telios Pharmaceuticals (San Diego).

Whole-Mount Preparation and Histological Analysis. The entire number 4 inguinal mammary gland was removed and fixed in Carnoy's solution (acidic ethanol) at room temperature. The tissues were washed in 70% ethanol, rinsed in distilled water, and stained overnight in carmine solution [0.2% carmine/0.5% aluminum potassium sulfate (both from Sigma)] at room temperature. The stained tissue was dehydrated through graded series of ethanol, cleared in toluene, and stored in methyl salicylate. For histological examination, structures of interest in whole mounts were excised and embedded in paraffin, sectioned at 4 mm, and stained with hematoxylin/eosin by standard procedures.

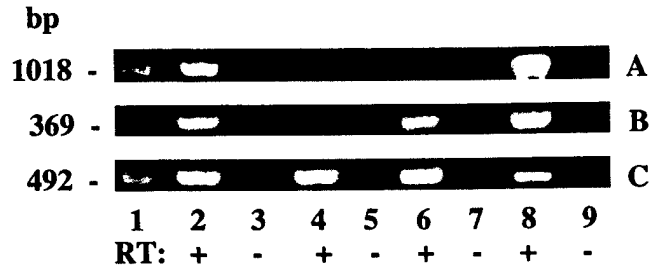

FIG. 2. Reverse transcription-coupled PCR analysis of gene expression. RNA from mammary glands of nonovariectomized bigenic (lanes 2 and 3), bigenic ovariectomized for 2 weeks (lanes 8 and 9), nonovariectomized monogenic GAL-4 (lanes 6 and 7), and transgene-negative (lanes 4 and 5) mice was subjected to PCR analysis either without (−RT) or (+RT) after reverse transcription. (*A*) PR-A transgene expression corresponding to the expected fragment of 1031 bp. (*B*) GAL-4 gene expression corresponding to the expected fragment of 360 bp. (*C*) Endogenous PR expression corresponding to the expected fragment of 460 bp. Lanes 1 represent standard DNA with molecular weights indicated on the left.

RESULTS

Analysis for PR Transgene Expression. At present, we have examined two sets of bigenic mice (TG 32/91 and TG 32/42) obtained by crossing the same GAL-4 founder with two different lines of UAS/PR-A trangenic mice that, so far, have

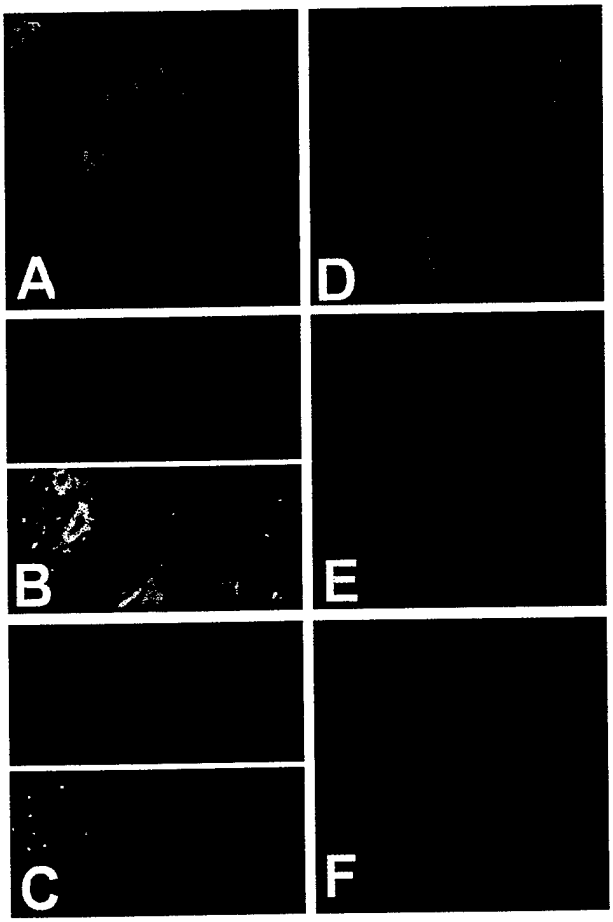

FIG. 3. Immunolocalization of PR. Mammary glands from control PR-A transgene-negative (*A* and *B*) and PR-A transgenic (*C–F*) were analyzed for PR (green color) by indirect immunofluorescence. (*B–D*) Glands from mice ovariectomized at 5 weeks of age and analyzed 2 weeks later. (*A*) Glands from intact 7 week old. (*E* and *F*) Glands from intact 14 week old mice. (*B Lower* and *C Lower*) Nuclei (in the same sections as *Upper*) stained with 4,6-diamidino-2-phenylindole (blue color) to illustrate that the lack of immunoreactivity in *B* was not due to the lack of ductal epithelium. In all cases, without the primary antibody, there was no immunoreactivity (*F*). (Original magnification: ×100.)

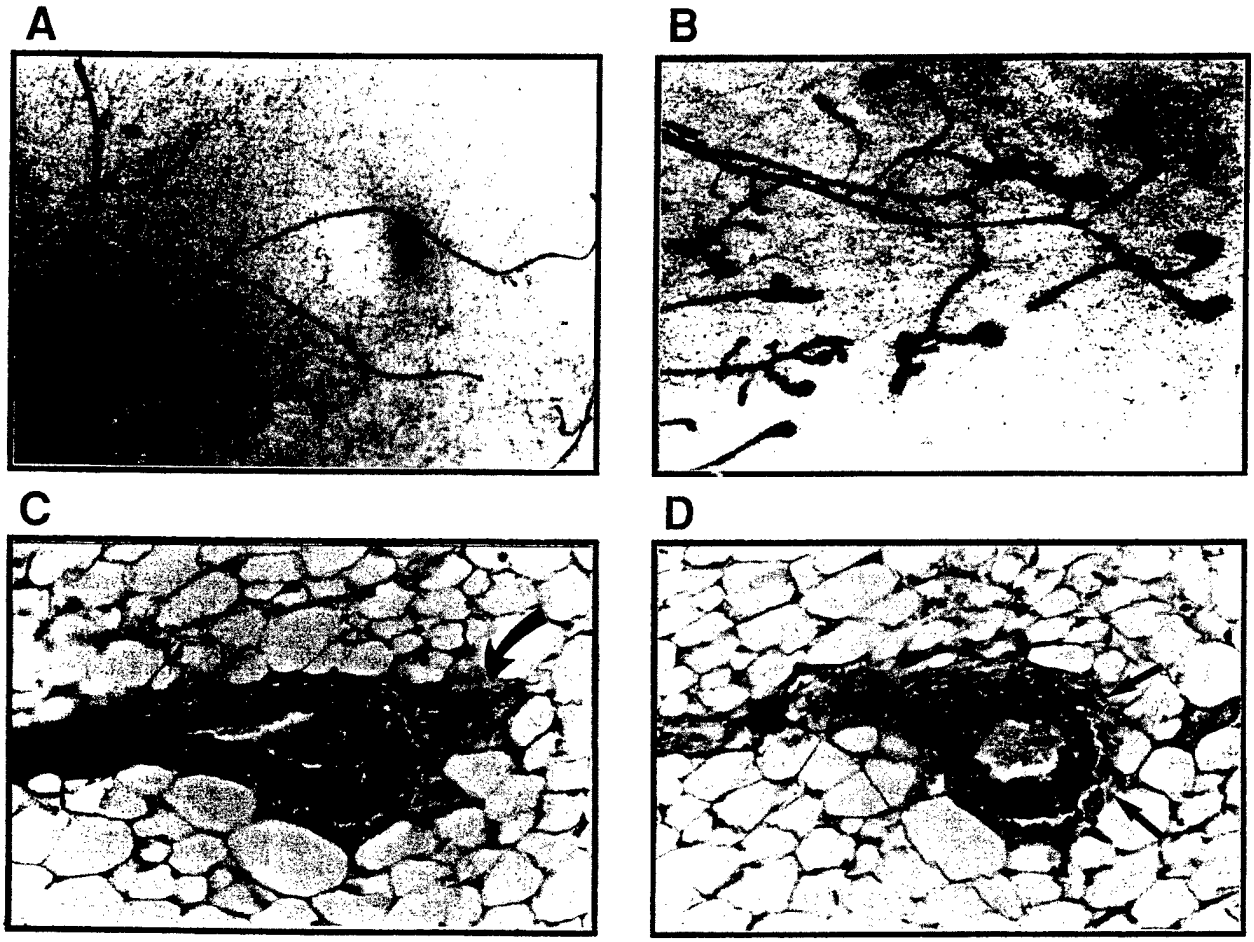

FIG. 4. Morphological and histological characteristics of mammary glands in prepubertal PR-A transgenic mice. Micrographs (*A* and *B*) of and histology (*C* and *D*) of mammary glands from mice ovariectomized at 5 weeks of age and examined 2 weeks later are shown. In controls glands from PR-A transgene-negative mice (*A*), there are no end buds in contrast to glands of PR-A transgenic mice (*B*). (*C*) An end bud with unusual fibrous cap (curved arrow). Arrows in *D* show disruption in the continuity of cap cells.

not shown any appreciable differences with respect to their mammary morphology and histology. TG 32/91, the best characterized bigenic mice, will be described herein. In all experiments, littermates negative for PR-A transgene were used as controls and on occasion, when these were not available, transgene-negative mice of same age were used.

PR-A transgene expression was found in mammary glands of bigenic TG 32/91 mice (Fig. 2*A*, lanes 2 and 8) and not in glands of monogenic TG 32 mice carrying only the GAL-4 gene (Fig. 2*A*, lane 6) or in glands of mice negative for both GAL-4 and PR-A transgenes (Fig. 2*A*, lane 4). GAL-4 gene expression was found in the mammary glands of both TG 32/91 and TG 32 mice (Fig. 2*B*, lanes 2, 6, and 8) and not in the glands of transgene-negative mice (Fig. 2*B*, lane 4). In contrast, as expected, endogenous PR expression was found in the mammary glands of all mice (Fig. 2*C*). The identity of all PCR products were confirmed by Southern blot analysis (data not shown). The transgene expression was unaffected by ovariectomy (Fig. 2*A*, compare lane 2 with lane 8), although, as expected, endogenous PR expression was lower in the glands of ovariectomized mice (Fig. 2*C*, compare lane 2 with lane 8).

Analysis for PR by Indirect Immunofluorescence. Mammary glands of nonpregnant females are composed primarily of nonepithelial cells and PR is present only in a subpopulation (23) of the epithelial cells; this results in a low overall concentration of PR so that analyses of PR in individual mammary glands by biochemical assays is not feasible. Therefore, to verify that the introduction of additional A form of PR as transgene had indeed increased the steady-state levels of PR, an indirect immunofluorescence assay was performed in individual mammary glands of ovariectomized prepubertal and adult mice. Ovariectomy was performed to reduce the contribution of the endogenous PR gene expression and at the same time allow the detection of PR arising from the transgene. As expected, the levels of PR were greatly diminished and virtually undetectable in the glands of transgene negative mice ovariectomized at 5 weeks of age (Fig. 3*B*) as compared with the glands of control intact transgene negative mice (Fig. 3*A*). Analysis of mammary glands of TG 32/91 (hereafter referred to as PR-A transgenic) mice, also ovariectomized at 5 weeks of age, readily revealed that they contained much higher levels of PR as compared with their transgene-negative counterparts (Fig. 3, compare *B* and *C*). Similarly, the mammary glands of adult ovariectomized PR-A transgenic mice also contained more immunoreactive PR than glands of ovariectomized adult transgene negative mice (data not shown). Thus, overall, the immunolocalization of PR confirmed a higher level of PR expression in PR-A transgenic mice.

In the glands of ovariectomized prepubertal PR-A transgenic mice, immunostaining was also observed in structures resembling end buds (Fig. 3*D*). This was surprising because end buds usually regress when there is a cessation in growth after ovariectomy or after antiestrogen treatment (24). Also, in the glands of nonovariectomized adult PR-A transgenic mice, immunoreactive PR was present in several ducts composed of more than one layer of cells (Fig. 3*E*). This was unusual because normal mammary ducts in young nulliparous mice are composed of a single layer of epithelial cells (see Fig. 3*A*),

suggesting that in PR-A transgenic mice there may be an aberration in mammary development.

Morphological and Histological Characterization. An overall comparison of mammary glands of 5- to 6-week-old prepubertal PR-A transgenic females with their transgene negative counterparts did not reveal any major differences; in both cases, 50–60% of the fat pads had been filled with ducts and several end buds, characteristic of a growing tissue were present (data not shown). However, after ovariectomy, the end buds in the mammary glands of control mice regressed (indicating a cessation in growth), whereas the end buds in transgenic mice persisted (Fig. 4, compare *A* with *B*). The degree of end-bud persistence was variable among individual mice but after prolonged ovariectomy, end buds disappeared in all mice. Histological analyses revealed that some of these end buds had unusual fibrous caps (Fig. 4*C*) and disruption in the continuity of cap cells (Fig. 4*D*).

From whole-mount analyses of mammary glands of young adult mice (10–14 weeks old), the degree of ductal branching appeared to be similar for both PR-A transgenic and control mice; i.e., the ducts at each level of branching appeared to be present in roughly the same numbers. However, the glands of adult PR-A transgenic mice had extensive lateral branching and also contained some very thick ducts (Fig. 5, compare *B* and *C* with *A*). The extensive lateral branching from mature secondary ducts sometimes resulted in a gland resembling that of an early pregnant female (Fig. 5*B*); however, often the lateral branches terminated in bulbous structures, and in contrast to normal ducts, the ducts in transgenic mice exhibited extraordinary numbers of buds growing from what are normally growth-quiescent zones (Fig. 5*C*). Peculiar morphology was also apparent at the tips of the ducts of transgenic mice that exhibited clustered buds compared with the smooth structure characteristic of normal terminal ducts (as shown in Fig. 5*A*).

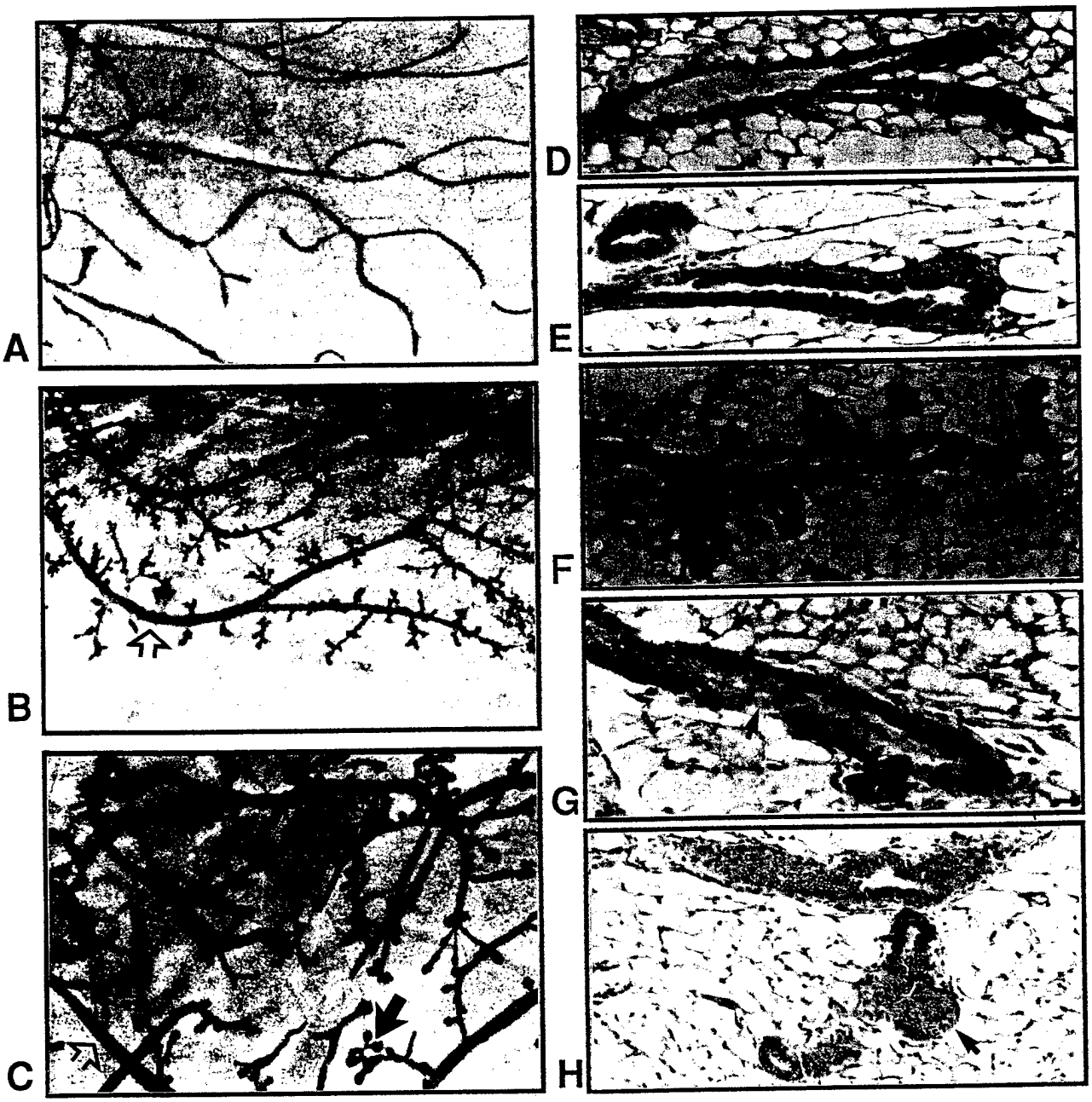

FIG. 5. Morphological and histological characteristics of mammary glands in adult PR-A transgenic mice. Whole-mounts (*A–C*) and histology (*D–H*) of mammary glands from young (10–14 weeks old) control PR-A transgene-negative (*A* and *D*) and PR-A transgenic mice (*B, C,* and *E–H*) are shown. In *B* and *C*, open arrows show thick ducts and solid arrow shows clustered buds at the tip of ducts. In *G*, arrow shows an indistinct epithelial–stromal boundary, and in *H*, arrow shows disorganized masses of cells at the tip of a duct.

Histological analyses revealed that the thickening of the duct wall in the glands of transgenic mice was the result of ducts composed of multilayered cells, in contrast to the monolayer associated with the normal duct (Fig. 5, compare *E* with *D*). Increased budding in the mammary glands of transgenic mice was also evident when ducts of similar lengths from control and transgenic mice were compared (Fig. 5, compare *F* with *D*). Some of the ducts in transgenic mice also exhibited regions with indistinct epithelial–stromal boundary (Fig. 5*G*) and multiple branched outgrowths consisting of disorganized masses of epithelial cells were seen at the tip of some ducts (Fig. 5*H*).

Disruption of Basement Membrane Integrity and Cell–Cell Interaction in Mammary Epithelium of PR-A Transgenic Mice. In normal mouse mammary glands during puberty, the end bud body cells (which give rise to ductal cells) express E-cadherin and exposure to anti-E-cadherin antibodies causes a disorganization of ductal epithelial cells and their detachment (25). A decreased expression of E-cadherin has also been shown to be associated with a reduced ability by human mammary epithelial cells to undergo morphogenesis *in vitro* (26). Cadherins are cell adhesion molecules (27) and play an important role in cell–cell interaction and also in cell–matrix interactions; this is because cells interact with basement membrane by means of adhesion receptors that allow the cells to migrate on extracellular matrix components, such as laminin. Therefore, to determine whether the disruption in the architecture of mammary glands in PR-A transgenic mice was the result of a derangement in the mechanisms regulating normal cell–cell adhesion and cell–matrix interactions, we examined the pattern of immunostaining for E-cadherin and laminin. As shown in Fig. 6, in the mammary glands of PR-A transgenic mice, laminin staining was discontinuous at the base of epithelial cells, indicative of a disruption in the basement membrane (Fig. 6, compare *A* with *B*). Similarly, in the mammary glands of PR-A transgenic mice, E-cadherin staining exhibited a disorganized pattern (Fig. 6*D*), whereas in the glands of control mice, E-cadherin was present in an organized manner outlining the epithelial cells (Fig. 6*C*).

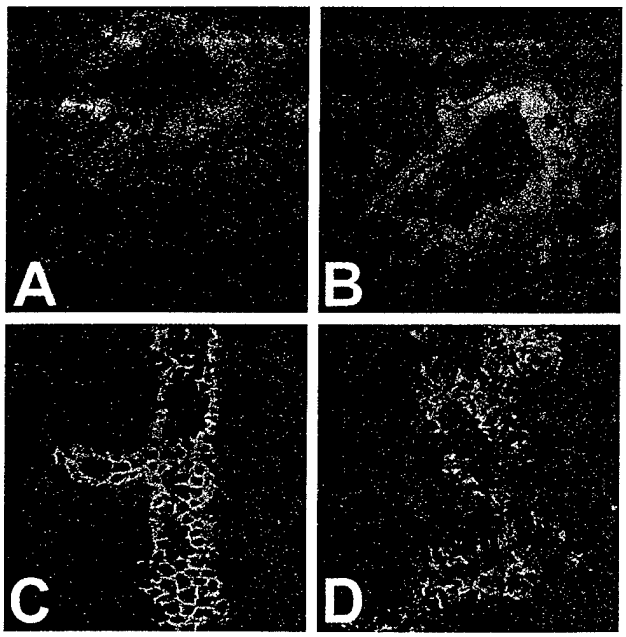

FIG. 6. Immunolocalization of E-cadherin and laminin. Laminin immunoreactivity (*A* and *B*), green color (nuclei are blue), in mammary-duct cross-sections circumscribes the mammary epithelium of PR-A transgene-negative mice (*A*) but is discontinuous and decreased in the gland of PR-A transgenic mice (*B*). Cadherin immunoreactivity (green color) delineates the epithelial cells in this branching duct of transgene negative mice (*C*), which is greatly diminished and disorganized in the epithelium of PR-A transgenic mice (*D*). In all cases, without primary antibody, there was no immunoreactivity (data not shown).

DISCUSSION

In this report, we document the creation of transgenic mice in which the native ratio of A to B forms of PR has been altered by introduction of additional A form of PR as transgene. We also show that the mammary glands of these PR-A transgenic mice have an aberrant morphology. Normal mammary development requires a coordinated action of estrogen, progesterone, and glucocorticoids. Also, the action of each of these steroids has a relative dominance depending on the developmental state (28). Studies with human PR have established that, *in vitro*, PR-A can modulate the action of PR-B, ER, and also other steroid receptors (12). Furthermore, it also appears that in the mammary epithelium, there may be distinct lineage limited progenitor cells capable of giving rise to either ductal or lobulo-alveolar growth (29) and PR is present only in a subpopulation of mammary epithelial cells (23). Therefore, when the complexity of steroid hormonal regulation of normal mammary development and the complexity of PR-A action is considered, at present, we can only speculate on the mechanisms whereby an overexpression of PR-A can result in an abnormal mammary phenotype.

It is well established that for ductal growth accompanying puberty, ER and estrogen are essential (24, 30). End buds are the indicators of ductal growth representing the site of both intense mitotic activity (24) and apoptosis (31). Indeed, an inhibition of apoptosis in the end buds can have an effect on the structural organization of end buds (31). As such, the persistence of end buds upon ovariectomy in prepubertal PR-A transgenic mice and their atypical organization suggest that during this developmental state, overexpression of PR-A may have interfered with ER action.

The mammary glands in adult PR-A transgenic mice have extensive adventitious lateral branching and also contain ducts composed of multilayered cells. Progesterone augments DNA synthesis in the epithelial cells of mouse mammary ducts (4) and extensive lateral branching usually accompanies pregnancy (3, 8) and requires PR (9). Therefore, it is likely that in PR-A transgenic mice, due to the increase in steady-state levels of PR, there is an increased responsiveness to progesterone. Indeed upon ovariectomy, there is a loss in the thickening of ductal walls, which, however, reappear upon administration of progesterone (data not shown). Regardless, it is clear that in PR-A transgenic mice, there is a derangement in the epithelial-cell replicative homeostasis. Therefore, although PR-A (directly or indirectly) can trigger the epithelial growth and lateral branching, a regulated growth may require the coordinated action of PR-A, PR-B, and ER that is disrupted with the overexpression of PR-A.

An important feature of the mammary glands of PR-A transgenic mice is the disruption in the organization of the basement membrane and a decrease in cell–cell adhesion. This is clearly abnormal because during pregnancy, when there is an extensive epithelial cell proliferation and lateral branching, the basement membrane remains intact (32). In human breast, basement membrane proteins undergo differential distribution during the menstrual cycle (33), indicating their potential regulation by ovarian hormones. In rodents, normal mammary development can be disrupted when there is an inhibition in the deposition of extracellular matrix (34, 35). Therefore, if the integrity of the basement membrane indeed requires a coordinated action of ovarian steroids, it is conceivable that this can be disrupted in PR-A transgenic mice, Mammary hyperplasia with a disorganized basement membrane and decreased cell–cell adhesion are characteristics generally associated with

mammary epithelial cells that have acquired invasive properties. As such, the characteristics of mammary glands of PR-A transgenic mice strongly suggest that they may have a high predisposition to become tumors.

In summary, our present studies provide *in vivo* evidence that a regulated expression of the two PR isoforms is critical for appropriate responsiveness to progesterone. Furthermore, they also provide direct evidence that an aberration in the mechanisms regulating the differential expression of the two isoforms of PR can have major implications to mammary carcinogenesis.

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Altered expression of the WT1 Wilms tumor suppressor gene in human breast cancer

GARY B. SILBERSTEIN*†, KATHARINE VAN HORN*, PHYLLIS STRICKLAND*, CHARLES T. ROBERTS, JR.‡, AND CHARLES W. DANIEL*

*Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064; and ‡Department of Pediatrics, Oregon Health Sciences University, Portland, OR 97201-3042

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ABSTRACT **The product of the WT1 Wilms tumor suppressor gene controls the expression of genes encoding components of the insulin-like growth factor and transforming growth factor β signaling systems. The role of these growth factors in breast tumor growth led us to investigate possible WT1 gene expression in normal and cancerous breast tissue. WT1 was detected by immunohistochemistry in the normal mammary duct and lobule, and the patterns of expression were consistent with developmental regulation. In a survey of 21 infiltrating tumors, 40% lacked immunodetectable WT1 altogether and an additional 28% were primarily WT1-negative. Cytoplasmic, but not nuclear, localization of WT1 was noted in some tumor cells and WT1 was detected, sometimes at high levels, in more-advanced estrogen-receptor-negative tumors. In this highly malignant subset, the tumor suppressor protein p53, which can physically interact with WT1, was also sometimes detected. WT1 mRNA was detected in normal and tumor tissue by reverse transcription-coupled PCR. Alternative splicing of the WT1 mRNA may regulate gene targeting of the WT1 protein through changes either in its regulatory or zinc-finger domains. The relative proportions of WT1 mRNA splice variants were altered in a random sample of breast tumors, providing evidence that different tumors may share a common WT1-related defect resulting in altered regulation of target genes.**

Normal growth and differentiation of the mammary gland depend on endocrine hormones that act in concert with locally produced growth factors such as the insulin-like growth factors (IGFs) and members of the transforming growth factor β (TGF-β) family. Multiple lines of evidence support the role of IGFs, acting through the IGF-I receptor (IGF-IR), in normal mammary growth and morphogenesis and in mammary tumorigenesis (1–6). *In vivo*, IGF-I, supplemented with estrogen, orchestrated normal ductal growth and morphogenesis when administered adjacent to regressed mammary epithelium in the rat (7). IGFs are potent mitogens in numerous breast cancer cell lines and expression of the IGF-IR, which is found in high concentrations in primary breast cancers, is crucial for tumor cell proliferation; blockade with an IGF-IR-specific antibody inhibited IGF-stimulated cell division *in vitro* and tumor formation *in vivo* (2). The TGF-β system appears responsible for the normal inhibition of mammary growth (8–10). Paradoxically, expression of TGF-β in breast tumors is correlated with metastasis and poor prognosis, and TGF-β can stimulate the tumorigenicity of breast cancer cell lines in nude mice (11–14). The genes encoding the IGF-IR, IGF-II, and TGF-β, as well as WT1 itself, are among the targets of the product of the Wilms tumor suppressor gene WT1 (15–18), which encodes a transcription factor consisting of an amino-terminal regulatory domain and a carboxyl-terminal domain composed of four Cys_2His_2 zinc-finger motifs responsible for DNA and RNA binding (19, 20). An alternative splice site in each of these domains results in four isoforms of WT1 mRNA (21). Mutations in the WT1 gene are associated with a subset of Wilms tumors, the most common pediatric renal cancer (22–24). It has been previously proposed that, during normal renal development, WT1 functions to suppress an IGF-II/IGF-IR autocrine loop to effect differentiation of the renal epithelium and that loss of WT1 function contributes to Wilms tumorigenesis through constituitive activation of this loop (25).

The causative role of the loss of the WT1 transcription factor in the etiology of a human tumor and its regulation of genes encoding at least two growth factors and a tyrosine kinase known to be important in mammary duct growth regulation and breast cancer cell proliferation led us to investigate possible WT1 expression in the normal and cancerous breast. We now report, to our knowledge, the first evidence that WT1 protein is present in normal breast tissue and appears to be developmentally regulated and that a high percentage of breast tumor cells express little or no WT1 protein. WT1 mRNA was also detected, and differences in the proportions of alternatively spliced WT1 mRNAs correlated with normal versus cancerous status.

EXPERIMENTAL PROCEDURES

Tissue. Specimens used for immunohistochemical analysis were obtained directly after surgical excision, transferred immediately to chilled (4°C) 4% paraformaldehyde in phosphate-buffered saline (PBS), and fixed for 3 h. Additional specimens of fixed sectioned breast tumors were provided by the University of Michigan Breast Cell/Tissue Bank, where histological grading, steroid hormone receptor status, and p53 and cERB2 expression were determined. Specimens used for RNA extraction were quick-frozen in liquid nitrogen immediately after excision. Histological typing of these specimens was determined by Kelly R. O'Keefe, Dominican Hospital, Santa Cruz, CA. Steroid hormone receptor status was available only for a subset of these samples.

Immunohistochemistry. Fixed tissue was dehydrated through a graded series of ethanols to xylene and embedded in paraffin wax. Tissue was then sectioned at 7 μm and mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma). The anti-WT1 antibody used in this study was WT(C-19) (sc-192; Santa Cruz Biotechnology) directed against an epitope corresponding to the 9 amino acids at the carboxyl terminus of the human WT1 protein. It was used at 1:200 dilution in PBS. A second anti-WT1 antibody, WT(180) (sc-846; Santa Cruz Biotechnology) specific for the amino terminus was used at a 1:10 dilution. Sections were incubated with antibody overnight at room temperature and antibody binding was detected with the avidin-biotin-peroxidase system protocol for the Vectastain standard kit with the following additional blocking steps: aldehyde groups were blocked using 0.2% glycine in PBS for two 5-min periods; endogenous

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Abbreviations: IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; TGF-β, transforming growth factor β.

†To whom reprint requests should be addressed.

peroxidases with 0.3% hydrogen peroxide were blocked in methanol for 30 min; nonspecific proteins were blocked with 2% dried milk in PBS for 30 min and 5% goat serum in PBS for 3 h instead of 30 min (Vector Laboratories).

Evidence for Specificity of Anti-WT1 Antibody C-19. In COS7 cells, nuclear staining by C-19 was observed only in transfectants containing WT1-expressing constructs; transfectants expressing a mutant WT1 lacking the carboxyl-terminal domain did not stain nor did untransfected cells (26). In this same study, C-19 colocalized with each of four independently raised anti-WT1 monoclonal antibodies in COS cells as well as in mouse testis and kidney tissue (26). WT1 expressing and nonexpressing cell lines (identified by Northern blot hybridization) were tested for staining with C-19 antibodies or monoclonal antibodies, and expressing cell lines all showed characteristic nuclear staining pattern with C-19 or monoclonal antibodies, whereas nonexpressing cells were not stained (26). In separate studies, buffalo rat BRL-3A cells and human glomerular epithelial cells were shown to express WT1 and demonstrated nuclear staining with C-19 (27, 28). Additionally, Western immunoblotting of antibody C-19 gave a single band of correct size in extracts from CHO cells containing a WT1 expression vector (29). Finally, transfection of epitope-tagged WT1 into a variety of WT1-negative cell lines, followed by immunostaining and/or Western immunoblotting with C-19 and an anti-FLAG (M2) monoclonal antibody verified the specificity of C-19 in our own hands.

The specificity of WT1 immunostaining in our system is supported by the determination that a second independently raised polyclonal antibody, WT(180) (sc-846; Santa Cruz Biotechnology), directed against the amino-terminal domain of WT1 gave the same staining pattern as C-19 (data not shown), and preincubation of C-19 with its cognate peptide greatly reduced nuclear staining (Fig. 1*A Inset*).

RNA Preparation and Reverse Transcription Reactions. Frozen samples were pulverized under liquid nitrogen, and a primary extraction of total RNA was performed with Purescript reagents (Gentra Systems) and was followed by secondary purification with a Qiagen total RNA midi kit (Qiagen, Chatsworth, CA). Reverse transcription reactions used an $NN(T)_{33}$ primer (CLONTECH) with Moloney murine leukemia virus reverse transcriptase and a standard buffer (Promega) in a 20-μl reaction volume containing 10 μg of human breast tissue total RNA or 3 μg of human kidney total RNA. High molecular weight reaction products were purified from the reaction mixture by centrifugal filtration using a Millipore filter with a 30,000 molecular weight cut-off. Purified DNA/RNA was resuspended in 25 μl of RNase-free water for PCR. Samples of human kidney and uterus RNA were from (CLONTECH).

PCR. One-tenth of the purified DNA/RNA volume was subjected to a first-round PCR amplification (94°C for 1 min, 58°C for 2 min, and 72°C for 3 min for 30 cycles) using *Taq* polymerase (Fisher Scientific) in a 50-μl reaction volume in a Perkin–Elmer model 9600 thermal cycler. For the second round, a 1:25 dilution of first-round reaction mixtures containing mammary samples or a 1:50 dilution of the kidney or uterus RNA was made in fresh reaction mixtures containing a second set of primers and the same program was repeated for an additional 25 cycles. WT1-specific PCR primer sequences were selected using MACVECTOR primer selection software (IBI-Kodak) and are as follows: F3 (20-mer), 5′-TTGTGATGGCGGACAAATTC-3′; F1 (21-mer), 5′-GGAATCAGATGAACCTAGGAG-3′; B5 (25-mer), 5′-CGTTTCTCACTGGTCTCAGATGCCG-3′; F11 (24-mer), 5′-AGGTTTTCTCGCTCAGACCAGCTC-3′; B1 (20-mer) 5′-GCCACCGACAGCTGAAGGGC-3′; B3 (20-mer), 5′-TTGTGATGGCGGACMAATTC-3′.

Gel electrophoresis in 2% agarose was used to resolve both splice variants. Low EEO agarose (Fisher Scientific) in 1× TAE (47) was used to resolve the 51-bp difference in the exon 5 variants. "Metaphor" high-resolution agarose (FMC) in 1× TBE (47) was used to resolve the 9-bp difference in the KTS variants.

WT1 Southern Blot Hybridization. Probe was a digoxigenin-labeled 645-bp human WT1 cDNA fragment spanning exons 7 through 10 and into the 3′ untranslated region. Hybridization was at 45°C overnight followed by two 30-min washes in 0.1× standard saline citrate/0.1% SDS at 65°C. Signal was detected by chemiluminescent substrate diluted 1:1 in basic buffer (Lumi-Phos 530, Genius buffer 3; Boehringer Mannheim, Indianapolis, IN).

Determination of the Relative Proportions of Alternative Splice Variants. Each sample of total RNA from a reduction mammoplasty or breast cancer patient was subjected to at least two reverse transcription reactions and from two to six primary (round 1) PCR amplifications prior to amplification with splice-specific primers. For each sample therefore, up to six replicate PCR amplification reactions were carried out with multiple samplings of the reverse-transcribed RNA. Within the normal or tumor groups, the data from all patients gave essentially the same results and, therefore, were pooled, and the occurrence of signals for unspliced and spliced forms, appearing either independently or together, were expressed as a percentage of the total number of occurrences observed for each form.

RESULTS

WT1 expression in normal breast tissue was investigated by immunohistochemistry with a polyclonal antibody directed against the WT1 carboxyl terminus. Normal mammary ducts are constructed of cells from two developmental lineages. The first, which always stained heavily with antibodies to WT1, is the myoepithelium, a sheath of contractile cells (Fig. 1*A*, open arrow) that overlays cells of the second lineage that line the duct lumen. The latter population can be further subdivided on the basis of nuclear morphology and chromatin density. Cells with rounded nuclei and diffuse chromatin were mostly WT1-positive (Fig. 1*A*; solid triangles). Cells with this appearance have been described in the mouse mammary gland and are considered to be less-differentiated probable stem cells that to give rise to lobular cells or new ducts (30–33). The second ductal cell type has a polygonal nucleus with compact chromatin, features that are considered characteristic of differentiated cells. Some of these cells were also positive for WT1 (Fig. 1*A*).

The complex expression pattern for WT1 observed in ductal cells was not seen in the normal lobule, where staining was more uniform, presumably reflecting the fact that these more-differentiated presecretory cells constitute a relatively homogeneous population (Fig. 1*E*). As with the duct, the myoepithelial cells investing lobules stained heavily for WT1.

In contrast to normal mammary epithelium, tumor cells often lacked detectable WT1 protein. All the cells of one ductal tumor, for example, uniformly lacked immunodetectable WT1; the nuclei of these tumor cells were monomorphic and similar to putative less-differentiated cells described for the normal duct (compare the nuclear morphology in Fig. 1*C* with Fig. 1*A*, triangle in box). The WT1-deficient phenotype can be established early in tumorigenesis, as seen in an example of carcinoma *in situ*, a situation where tumor elements are still contained within an otherwise normal duct (Fig. 1*B*). As pictured, a multilayered tumor that is negative for nuclear WT1 protein lies directly to the left of a normal-appearing duct wall containing numerous WT1-positive cells. Lobular tumors were also WT1-negative (Fig. 1*F*). In the pictured example, infiltrating tumor cells formed WT1-negative acinar-like structures or small islands, the larger of which had myoepithelial-like cells at their periphery that stained for WT1.

Changes in the intracellular localization of WT1 protein accompanied tumor progression. WT1 was detected in the cytoplasm of tumor cells in the carcinoma *in situ* but not in the infiltrating tumor from the same patient (Fig. 1 *B* and *C*, respectively). Cells of a second infiltrating ductal carcinoma had either cytoplasmic or nuclear WT1, suggesting that further differentiation of WT1 expression may have occurred after

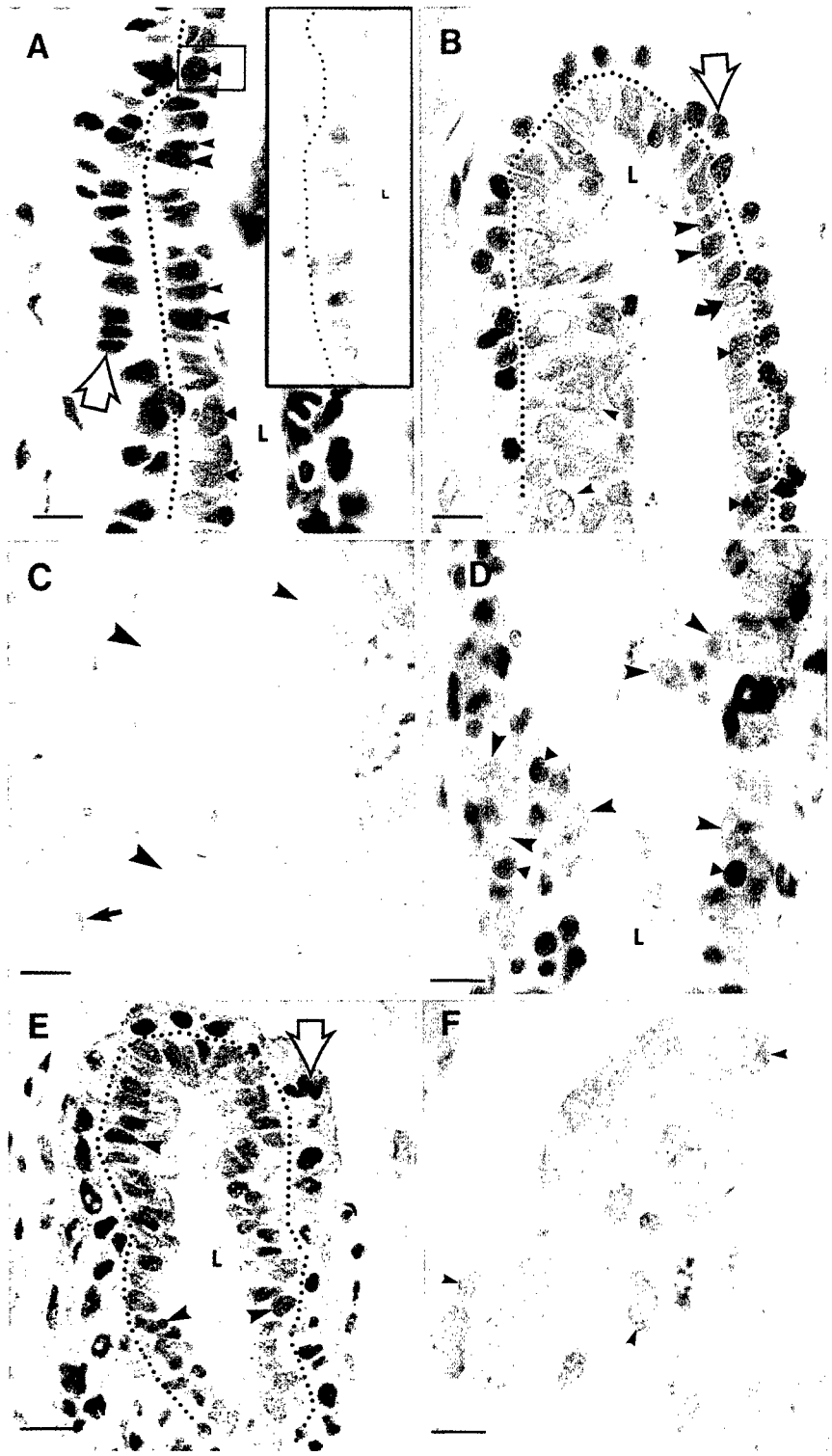

FIG. 1. Photomicrographs illustrating immunolocalization of WT1 protein in the epithelial cells of normal and cancerous human breast tissue. Cells that are positive for WT1 protein stained brown; the blue nuclear stain is hematoxylin. (*A*) Nuclear morphology and WT1 staining characteristics of normal mammary ductal cells. Cells with oval nuclei and diffuse chromatin were WT1-positive (triangles and box in the center). Cells with polygonal nuclei and compact chromatin were positive (large arrowheads) or negative (small arrowhead) for WT1. The latter stained deeply with hematoxylin. Myoepithelial cells were WT1-positive (open arrow; dotted line delineates the boundary between lumenal and myoepithelial cells). L, ductal lumen. (*Inset*) Antibody preincubated with cognate peptide shows reduced staining. Myoepithelial cells are on the left of dotted line. (Bar = 15 μm.) (*B*) Ductal tip partially involved with carcinoma *in situ*. Normal cellular arrangement occurred on the right side of the duct; a multilayered tumor appears on left side. L, lumen. The top of the L points to transitional zone between the normal and tumorous sides of the duct. Immunostaining of monolayer of normal-appearing ductal cells on the right side of the duct: some ductal cells with oval nuclei and diffuse chromatin were WT1-positive (triangles); fewer were WT1-negative (curved arrow). Numerous cells with polygonal nuclei were WT1-positive (large arrowheads). The tumor element consists primarily of cells with large oval WT1-negative nuclei containing diffuse chromatin. Some cells in this mass had WT1-positive cytoplasm (small arrowheads). Myoepithelial layer lies outside dotted line. (Bar = 15 μm.) (*C*) Infiltrating ductal carcinoma. Tumor cells have proliferated to form masses (large arrowheads) within which all cells were WT1-negative. A band of infiltrating tumor cells is visible (small arrowhead). This tumor and the previously described tumor *in situ* are from the same patient. WT1-positive stromal cell is positive control for immunostaining. (Bar = 20 μm.) (*D*) Nuclear and cytoplasmic immunostaining for WT1 in infiltrating ductal carcinoma. WT1 immunostaining subdivides this population roughly in half on the basis of cytoplasmic versus nuclear localization of the protein. Cells with only cytoplasmic WT1 immunoreactivity (arrowheads) are intermixed with cells having nuclear stain. In addition, there are some cells that have both cytoplasmic and nuclear WT1 (triangles). (Bar = 20 μm.) (*E*) Normal mammary lobule. Normal-appearing lumenal epithelium stained with moderate intensity for WT1 (arrowheads). The nuclei of the myoepithelial cells stained deeply for WT1 (open arrow; dotted line delineates boundary between myoepithelial and lumenal cells). (Bar = 20 μm.) (*F*) Infiltrating lobular carcinoma. Section from vicinity of normal lobule (*E*). The nuclei of tumor cells are uniformly negative for WT1; however, some cells showed cytoplasmic staining (arrowheads). (Bar = 15 μm.)

clonal transformation (Fig. 1*D*). Cytoplasmic staining for WT1 was also detected in a lobular carcinoma (Fig. 1*F*).

To determine the frequency of the WT1-negative breast tumor phenotype, levels of expression were investigated in tissues from 21 patients with infiltrating mammary carcinoma (Table 1). All histopathological grades of ductal tumor were represented and patients ranged in age from 29 to 88 years. In 40% of all tumors studied, WT1 protein was undetectable. Where tumor cytology was heterogeneous, a majority cell type (estimated by inspection to be 50% or more of the tumor cells) was usually identifiable and in 28% of these tumors, the majority of the cells were WT1-negative. A higher percentage of lobular tumors compared with ductal carcinomas (83% versus 67%) had a majority of WT1-negative cells (data not shown). A correlation was noted between estrogen receptor status and WT1 expression; 78% of the lower-grade receptor-positive tumors lacked WT1 compared with only 40% for receptor-negative counterparts (data not shown).

The detection of the p53 tumor suppressor protein and estrogen-receptor-negative status are associated with higher-grade more-aggressive cancers that have the poorest clinical outcomes (34). Although the sample numbers were too small to assess statistical significance, the observation that two of three WT1-positive high- grade tumors also expressed p53 should be noted, because WT1 has been shown to physically associate with and modify p53 action (35). Unlike p53, the expression of the c-ERB2

Table 1. WT1 protein expression: Survey of immunostaining in normal and cancerous breast tissue

Tissue	WT1 Neg	WT1 Pos
Tumor	15	6
Normal	1	14

In 60% (9 of 15) of the tumors surveyed, WT1 immunostaining was absent in greater than 90% of the tumor cells (estimated by inspection). In the remaining 6 tumors, 50% or more of the tumor cells were estimated to be WT1-negative. Source of normal tissue: ducts and lobules with normal-appearing cellular architecture and nuclear morphology were often found in the vicinity of tumors and were used as controls (Normal).

protooncogene was not correlated with either tumor grade or WT1 expression (data for clinical parameters is not shown).

The plus- and minus-KTS variants of WT1 have different DNA and RNA binding specificities and hence must transcriptionally control different constellations of genes (36, 37). In addition, a WT1 plus-KTS splice variant was recently shown to bind in a sequence-specific fashion to mRNA, implicating plus-KTS variants in post-transcriptional regulation of gene expression (28). Should WT1 be involved in gene-regulatory perturbations associated with breast cancer initiation or maintenance, we reasoned that tumor-related WT1 action on different sets of genes would be likely and could be deduced through detection of altered expression of KTS variants. For this reason we undertook a detailed analysis of the expression of the two classes of WT1 KTS variants (Figs. 2 and 3*A*) in tissue from the normal and cancerous breast.

By using nested pairs of PCR primers and Southern blot hybridization to detect low levels of target, a WT1-specific probe detected only the plus-KTS form(s) in samples from normal tissue from reduction mammoplasties, whereas, in addition to plus-KTS variants, minus-KTS signals of various intensities appeared for each tumor (Fig. 3*A*). As expected, both the plus- and minus-KTS forms were detected in the kidney and in the plasmid controls. Plus- and minus-exon-5 variants appeared as expected in all tumors and in the kidney control (Fig. 3*B*). In normal tissue, plus-exon-5 form(s) were the only ones expressed except for patient 16, where the minus form also appeared. Our splice-variant detection strategy was tested by coamplifying plasmids containing cDNA for either the WT1 message variant with both splices or neither splice. The appearance of amplification products of the predicted size validated the nested PCR system (Fig. 3*A*). Kidney tissue also expressed both splicing forms of KTS and exon 5 as predicted (Fig. 3) (21, 38).

A replicate PCR for the sample from patient 16 resulted in amplification of only the plus form, indicating that in some of our PCR trials, variants may not have been detected. In fact, when there exists a low abundance of two or more *bone fide* targets for a primer pair, as with the WT1 splice variants, a stochastic sampling error in which only a single target is amplified is likely if not inevitable. This pitfall has been studied in detail by Taberlet *et al.* (39), who demonstrated its avoidance by multiple PCR trials to detect all possible targets. The same study indicated that the frequency with which a target is detected will depend on its relative abundance; in multiple PCR trials, the more abundant targets will appear most frequently. This suggested to us that replicate amplifications

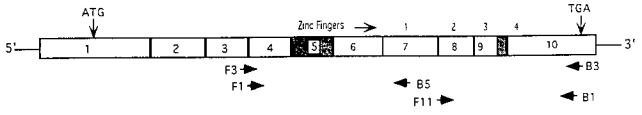

FIG. 2. WT1 gene structure, splice variants, and detection scheme. The 10 exons of WT1 are shown as boxes and the alternatively spliced 51-base and 9-base sequences encoded by exon 5 and the 3′ end of exon 9 (KTS) are shaded. The location of the four zinc-finger motifs are noted above the boxes and the positions of the forward (F) and reverse (B) oligonucleotide primers relative to the transcript are shown.

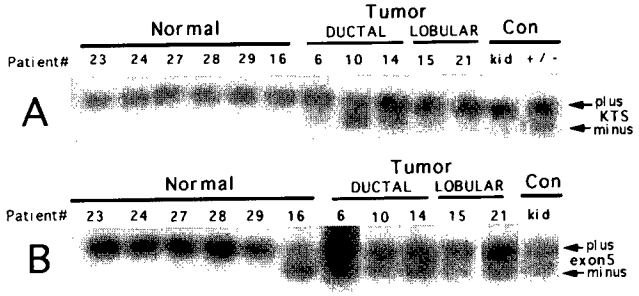

FIG. 3. Detection and analysis of WT1 mRNA splice variants in normal (reduction mammoplasty) and cancerous breast tissue. (*A*) Reverse transcription-coupled PCR and Southern blot hybridization analysis of KTS splice variants for presence or absence of the 9-base KTS sequence. Upper row of signals, plus KTS; lower row, minus KTS. Normal (control) is kidney and a mixture of plus- or minus-KTS WT1-containing plasmids. Round 1 primers, F3/B3; round 2 primers, F11/B1. Two rounds of 30 and 25 amplification cycles for mammary gland WT1 mRNA; kidney WT1 mRNA could be detected with a single round of 30 cycles. (*B*) Reverse transcription-coupled PCR and Southern blot hybridization analysis of exon 5 splice variants. Upper row of signals, plus exon 5; lower row, minus exon 5. Normal (control) is kidney. Round 1 primers, F3/B3; round 2 primers, F1/B5.

could be used to quantify the relative proportions of the WT1 splice variants in tumor versus normal samples.

A multiple sampling scheme was, therefore, used to further investigate the proportions of KTS and exon 5 splice variants, the results of which were consistent with the initial PCR trial and add important details. Data on previously undetected splice variants showed that in normal breast tissue, the minus forms of each variant were present at significant levels, in the 20–30% abundance range (Fig. 4). In tumor tissue, this ratio was less pronounced due to a relative increase in the minus-KTS and minus-exon-5 variants to 40–50% of the splice variant mixture. Finally, breast tumors are often composed of a heterogeneous mixture of tumor and stromal cells. Although the latter must contribute to the WT1 mRNA pool, the relatively low levels of immunodetectable WT1 seen in the stroma and the random patient population, which should reflect a variety of epithelial/stromal ratios, suggest that systematic variation in stromal contribution between normal and tumor tissue is not likely to account for the observed differences in splice abundance.

DISCUSSION

The known tumor suppressor action of the WT1 gene in the developing kidney and its regulation of the genes encoding the mammotrophic IGF-IR/IGF-II system, as well as the mammary growth inhibitor TGF-β1, make it a candidate gene for action in the mammary gland. The goal of the current study was to determine whether WT1 was expressed in normal human breast tissue, and if so, whether this expression was altered in mammary tumors. Our key findings are that WT1 protein and mRNA are expressed in the normal breast and that levels and subcellular localization of WT1 protein and the alternative splicing of WT1 mRNA were significantly altered in a random sample of breast tumors (Figs. 1 and 3).

In the normal breast, the level and patterning of WT1 immunostaining strongly indicated that WT1 gene expression is differentially regulated between developmentally divergent ducts and lobules as well as within the mature duct. Duct lumenal cell staining contrasted with the lobular pattern, the former with a patchy distribution versus the latter with the more uniform distribution, suggesting that WT1 may play different developmental roles within these structures (Fig. 1 *A* and *E*). Developmental regulation of WT1 has been described in the mouse urogenital system and in rat ovarian development (29, 40).

Interestingly, the higher frequency of WT1-negative lobular tumors compared with ductal tumors suggests that developmen-

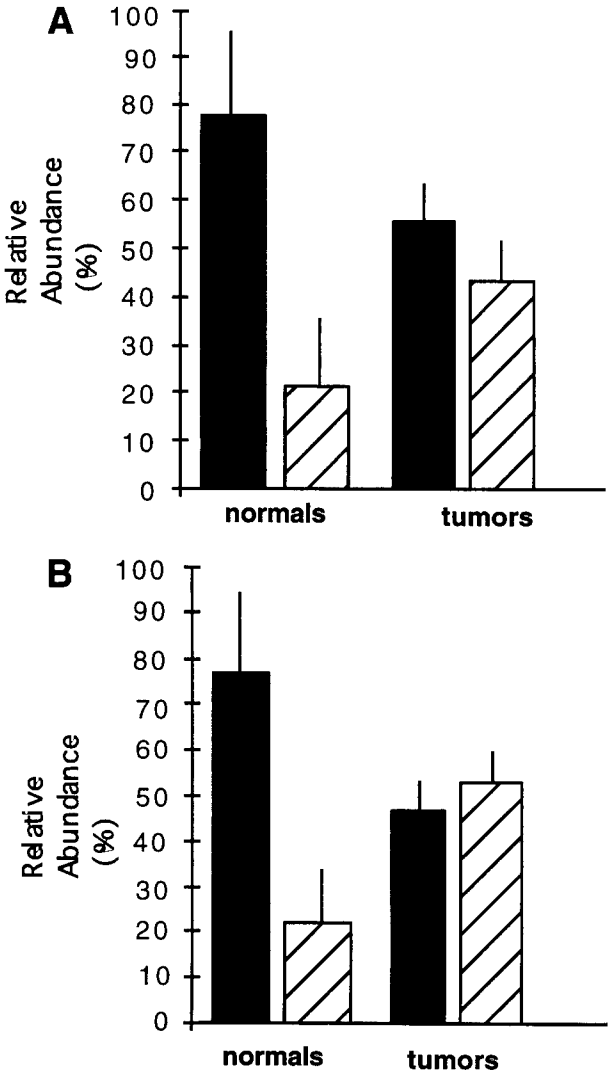

FIG. 4. Reverse transcription-coupled PCR analysis of the relative proportion of KTS and exon 5 splice variants in normal versus cancerous breast tissue. (*A*) KTS variants. Bars: solid, plus forms; hatched, minus forms. (Total number of PCRs: normal, $n = 31$; tumor, $n = 17$). Normal tissue samples ($n = 10$) consisted of tissue from eight reduction mammoplasty patients and two samples of normal breast tissue in the vicinity of tumors. As a group and based on age (ranging from 23 to 45 years), these noncancer patients were considered premenopausal with the possible exception of one individual (52 years). Six tumor samples were analyzed. Data are the mean ± SD. (*B*) Exon 5 variants. Bars: solid, plus forms; hatched, minus forms. (Total number of PCRs: normal, $n = 26$; tumor, $n = 15$.)

tal differences in expression may have sequelae in neoplasia. Within the duct, the distinctive differences in WT1 staining intensity between the myoepithelial and lumenal cell lineages and the subdivision of lumenal cells into WT1-positive and -negative populations encompassing putative stem- and differentiated-cell types are indicative of developmental regulation (Fig. 1*A*). The presence of WT1 protein in undifferentiated cells could be relevant both to normal development and tumorigenesis, as these cells are considered to be premitotic, poised to divide under the hormonal influences of pregnancy, for example. If we borrow from the Wilms tumor model, where ablation of WT1 action results in derepression of the IGF-IR and IGF-II (25), then in the mammary gland, suppression of this mammotrophic autocrine–paracrine loop by WT1 would normally maintain these cells in mitotic arrest and loss or inactivation of WT1 would lead to unregulated cell division and tumors.

The most striking features of WT1 immunostaining in breast tumors were (*i*) the absence of immunodetectable WT1 in a majority of tumor cells (Fig. 1 *C* and *F* and Table 1), (*ii*) cytoplasmic localization in a subset of tumor cells (Fig. 1 *B* and *D*), and (*iii*) high levels in some advanced tumors, suggesting possible overexpression (Fig. 1*D* and Table 1). All are indicative of breast-tumor-related perturbations of WT1 expression. Since significant reduction of WT1 mRNA was not detected in breast tumors (Fig. 3), the tumor-related changes in expression patterns of the alternatively spliced WT1 mRNA species must in part underlie the changes in protein expression noted above (Figs. 3 and 4). Consistent with this idea, in all tumors studied, the normal proportions of the splice variants were replaced with increased proportions of both minus-exon-5 and minus-KTS variants (Fig. 4). The appearance of this pattern in a random sample of breast tumors suggests that altered splicing of WT1 mRNA is characteristic of breast tumor tissue, potentially resulting in a change in the set of target genes subject to WT1 regulation (Fig. 3). The fact that the observed pattern was shared by ductal and lobular tumors derived from developmentally divergent tissues may mean that a cell type common to both tissues (e.g., an early progenitor cell) was affected prior to differentiation (Fig. 3).

General loss of WT1 protein was evident in a cancerous versus normal lobular tissue sample from the same patient (Fig. 1 *E* and *F*, respectively). The origin of WT1-negative ductal tumor cells is a more complex issue, given that normal ductal epithelium contains many WT1-negative cells. If the latter were selectively vulnerable to neoplastic transformation, however, this would still be consistent with a tumor suppressor function for WT1 in the normal breast.

In either case, whether nuclear WT1 expression is actively lost or WT1-negative cells are selected, the establishment of the WT1-negative tumor phenotype can occur early in tumorigenesis. Thus, in a carcinoma *in situ*, the incipient tumor was negative for nuclear WT1 while still contained in apparently normal duct (Fig. 1*B*), indicating that the WT1-negative status of the derivative frank carcinoma (Fig. 1*C*) was established at its inception. The persistence of the WT1-negative phenotype in this case would be consistent with continued tumor growth requiring the reduction or absence of WT1 expression.

The discovery of cytoplasmic, but not nuclear, WT1 in the cells of three tumors (Fig. 1 *B*, *D*, and *F*) suggests that inactivation of WT1 may occur by restricting its access to nuclear targets. Recent experiments have shown that cytoplasmic retention inhibits the normal regulatory functions of WT1 (27). Phosphorylation is a major post-translational mechanism regulating transcription factor action, and phosphorylation of WT1 by protein kinase A resulted in the cytoplasmic retention of WT1 protein, inhibiting the transcriptional suppressor activity of a WT1 reporter construct in 3T3 cells. Interestingly, in mammary epithelial cells *in vitro*, protein kinase A activation by cholera toxin stimulated proliferation (41) and, *in vivo*, locally elevated cAMP levels powerfully stimulated the growth of regressed ducts in the mouse mammary gland (42), effects that are consistent with the inhibition of a growth suppressor. Cytoplasmic retention has recently been suggested as a mechanism for the regulation of other transcription factors. The breast tumor suppressor gene product BRCA1, a nuclear phosphoprotein and putative transcription factor, is aberrantly localized to the cytoplasm in most breast cancer cells (27, 43), and in oligodendrocytes *in vitro*, the p53 tumor suppressor protein moves to the cytoplasm during proliferation and shifts to the nucleus during differentiation where it mediates differentiation or apoptosis (44). If we return to our observations on the subcellular localization of WT1, we conclude that mammary cells with cytoplasmic WT1 are likely to be under a different program of gene regulation than their counterparts with nuclear protein localization.

Finally, the higher-grade most-dangerous tumors showed interesting correlations among WT1 expression, estrogen receptor status, and p53 expression. In estrogen-receptor-positive tumors, the majority of cells lacked WT1 in 78% of the cases; however, in the more-advanced estrogen-receptor-negative counterparts,

the majority of cells lacked WT1 in only 40% of the cases (data not shown). In the latter, higher average staining levels suggested possible negative regulation of tumor WT1 by estrogen. In addition, there was a correlation in higher-grade tumors between WT1 and p53 expression. Recent cotransfection studies have shown that WT1 can stabilize p53 protein and inhibit p53-mediated apoptosis (35). In breast tumors, overexpression of p53, usually associated with its functional inactivation, is correlated with high levels of IGF-IR, whereas normal p53 protein repressed IGF-IR expression (45, 46). Overexpression of WT1 or, possibly, perturbation of WT1 splice variant expression in breast tumors might, therefore, interfere with the normal surveillance activities of p53. Conversely, p53 might under certain circumstances, interfere with normal WT1 action(s). Expression of c-ERB2, an epidermal growth factor receptor-related oncogene, was not obviously correlated with either tumor status or WT1.

In conclusion, a role for WT1 in mammary development is inferred from its differential localization in lobules versus ducts, as well as within ducts, where it was present in putative progenitor cells. With regard to WT1 and breast tumorigenesis, if WT1 regulates the IGF and TGF-β systems in mammary epithelium, then our observations indicate that it would be a candidate for a breast cancer tumor suppressor gene. This conclusion is based on the following points: (*i*) a high percentage of breast cancer cells lacked immunodetectable WT1, indicating that WT1 could be responsible for the overexpression of IGF-IR common to breast cancers; (*ii*) cytoplasmic localization of WT1 suggests that functional inactivation of WT1 occurs in a subset of tumors; (*iii*) nuclear WT1 protein was absent in tumors *in situ*, suggesting that altered WT-1 expression may coincide with the first appearance of tumor cells possibly reflecting an event related to cause; (*iv*) the WT1-negative phenotype was maintained in tumor masses, consistent with a requirement for on-going lack of expression in growing tumors; (*v*) common tumor-related perturbations of mRNA splice usage were demonstrated.

Given our data, it should now be of interest to elucidate those mechanisms that determine WT1 function in the normal and cancerous human breast and the possible role of WT1-regulated IGF and TGF-β action in mammary growth and tumorigenesis.

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REGULAR ARTICLE

Michael T. Lewis · Sarajane Ross
Phyllis A. Strickland · C. John Snyder
Charles W. Daniel

Regulated expression patterns of *IRX-2*, an Iroquois-class homeobox gene, in the human breast

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Abstract In the mouse mammary gland, homeobox gene expression patterns suggest roles in development and neoplasia. In the human breast, we now identify a family of Iroquois-class (IRX) homeobox genes. One gene, *IRX-2*, is expressed in discrete epithelial cell lineages being found in ductal and lobular epithelium, but not in myoepithelium. Expression is absent from associated mesenchymal adipose stroma. During gland development, expression is concentrated in terminal end buds and terminal lobules and is reduced in a subset of epithelial cells during lactation. In contrast to observations for many homeobox genes in the mouse mammary gland in which homeobox gene expression is lost on neoplastic progression, *IRX-2* expression is maintained in human mammary neoplasias. Data suggest *IRX-2* functions in epithelial cell differentiation and demonstrate regulated expression during ductal and lobular proliferation as well as lactation.

Key words Mammary gland · Differentiation · Neoplasia · Homeodomain · Cancer · Human

Introduction

Mammary gland development in rodents and humans appears similar (Daniel and Silberstein 1987; Russo and Russo 1987) and may be divided into a proliferative and a cyclical phase of development. During the proliferative phase, ductal outgrowth begins at the nipple with subsequent invasion of the mammary adipose stroma occurring during puberty. As ductal proliferation proceeds, lumenal epithelial and myoepithelial cell differentiation occurs primarily in the growing terminal end bud located at the end of elongating ducts. In humans, terminal lobules are also formed as precursors to the mature pre-secretory lobules. The cyclical phase of development is initiated by pregnancy with terminal differentiation of lobule-alveolar secretory cells and lactation. After weaning, the cycle is completed by apoptosis (death) of secretory cells in involution. After involution, the remodeled gland resembles the morphology of the mature state.

Genetic control of mammary epithelial cell type differentiation (Smith 1996; Chepko and Smith 1997) and functional differentiation are poorly understood. Candidate regulators include homeobox genes which specify eukaryotic cell fate during development (Manak and Scott 1994; Thesleff et al. 1995). In the mouse, homeobox genes may function throughout mammary proliferative development and lactation and may contribute to development of mammary cancers (Friedmann et al. 1994; Stuart et al. 1995; Friedmann and Daniel 1996; Phippard et al. 1996). Loss-of-function of at least one homeobox gene, *Hoxd-10*, results in defects during lactation (Carpenter et al. 1997; Lewis, unpublished).

In searching nucleotide sequence databases for novel homeobox genes expressed in the human breast, we identified a partial cDNA represented as an Expressed Sequence Tag (EST) and initiated an expression study of this gene, ultimately designated *IRX-2*, in the human breast and associated cancers. In this paper, we present evidence that *IRX-2* is differentially expressed in major mammary epithelial cell lineages and that its expression is further regulated during both the proliferative and cyclical phases of human breast development.

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M.T. Lewis · S. Ross · P.A. Strickland · C.W. Daniel (✉)
Department of Biology, Sinsheimer Laboratories,
University of California, Santa Cruz, CA 95064, USA
e-mail: daniel@darwin.ucsc.edu;
Tel.: +1–831 459–4171; Fax: +1–831 459–3139

C.J. Snyder
Dominican Hospital, 1555 Soquel Drive,
Santa Cruz, CA 95060, USA

A

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                10         20         30         40         50         60         70         80         90
IRX1    TCCCGTCCCA AGAACGCCAC CAGGGAGAGC ACCAGCACGC TGAAGGCCTG GCTCAACGAG CACCGCAAGA ACCCCTACCC CACCAAGGGC
IRX2    GCGTACCGGA AGAACGCCAC AAGGGACGCC ACGGCTACCC TCAAGGCCTG GCTCAACGAG CACCGCAAGA ACCCCTACCC CACCAAGGGC
IRX3    GGTCGCCGAA AGAACGCGAC CCGGGAGACC ACCAGTACAC TCAAGGCCTG GCTCAACGAG CACCGCAAAA ACCCCTACCC CACTAAGGGT
IRX4    ---CGGCGCA AGAACGCCAC GCGCGAGACC ACCAGCACGC TCAAGGCCTG GCTGCAGGAG CACCGCAAGA ACCCCTACCC CACCAAGGGC
IRX5    GGGCGGCCCA AGAACGCCAC CCGCGAGAAC ACCAGCACGC TCAAGGCCTG GCTCAACGAG CACCGCAAGA ATCCCTACCC CACCAAGGGC

              100        110        120        130        140        150        160        170        180
IRX1    GAGAAGATCA TGCTGGCCAT CATCACCAAG ATGACCCTCA CCCAGGTGTC CACC (R1 primer)
IRX2    GAGAAGATCA TGCTGGCCAT CATCACCAAG ATGACCCTCA CCCAGGTGTC CACCTGGTTC GCCAACGCGC GCCGGCGCCT CAAGAAAGAG
IRX3    GAGAAGATCA TGCTGGCCAT CATCACCAAG ATGACCCTCA CCCAGGTGTC CACC (R1 primer)
IRX4    GAGAAGATCA TGCTGGCCAT CACCACCAAG ATGACCCTCA CACAGGTCTC CACC (R1 primer)
IRX5    GAGAAGATCA TGCTGGCCAT CATCACCAAG ATGACCCTCA CGCAGGTCTC CACC (R1 primer)
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B

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                        Hexapeptide-like                                    Homeodomain
Antp                    LYPWMR                              RKRGRQTYTRYQTLELEKEFHF---NRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN
mrr                     YHPYD---AAFAGYPF--NSYGMDLN-GARRKNATRETTSTLKAWLNEHKKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKEN
caup                    YYSYDP-MSAYGGLLVSNSSYGASYDLAARRKNATRESTATLKAWLSEHKKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKEN
ara                     YYSYDPTLAAYG--------YGPNYDLAARRKNATRESTATLKAWLNEHKKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKEN
IRX-2a                  SYPY----------------GDP----AYRKNATRDATATLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKEN
IRX-3                   YYPYERTLGQYQYERYGAVELSGA----GRRKNATRETTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVST (R1 primer)
IRX-4                   YYPYEPALGQYPYDRYGTMD-SGT-----RRKNATRETTSTLKAWLQEHRKNPYPTKGEKIMLAITTKMTLTQVST (R1 primer)
IRX-5                   YYPY----GQFQY--------GDP----GRPKNATRENTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVST (R1 primer)
IRX-1                   FYPY----GQYQF--------GDP----SRPKNATRESTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVST (R1 primer)

CONSENSUS               YYPY    GQYQY       GDP     RRKNATRESTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKEN
100%                     +++    ++                    +++++  ++++++++ ++++++++++++++++++ ++++++++++
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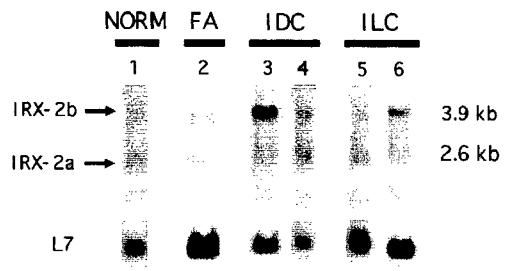
C



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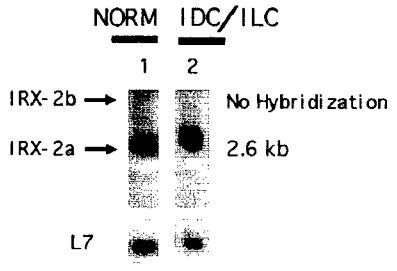
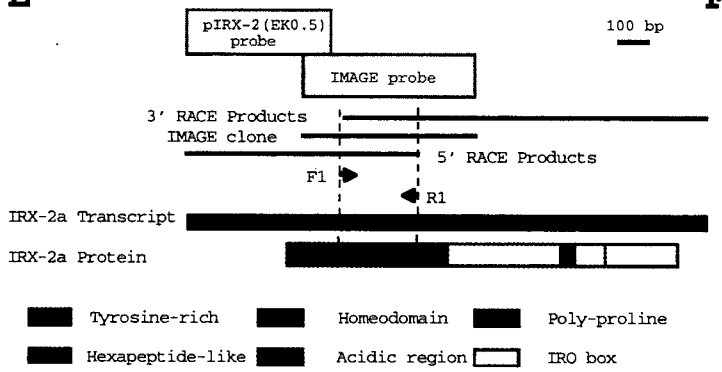


E



F

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LTQVSTWFANARRRLKKENKMTWTPRNRSEDEEEEENIDLEKNDE
DEPQKPEDKGDPEGPEAGGAEQKAASGCERLQGPPTPAGKETEGS
LSDSDFKEPPSEGRLDALQGPPRTGGPSPAGPAAARLAEDPAPHY
PAGAPAPGPHPAAGEVPPGPGGPSVIHSPPPPPPPAVLAKPKLWS
LAEIATLSDKVKDGGGGNEGSPCPPCPGPIAGQALGGSRASPAPA
PSRSPSAQCPFPGGTVLSRPLYYTAPFYPGYTNYGSFGHLHGHPG
PGPGPTTGPGSHFNGLNQTVLNRADALAKDPKMLRSQSQLDLCKD
SPYELKKGMSDI (417 a.a.)
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G

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ara       CCENGRPIMTDPVSGQT--VCSCQ
caup      RCENGRPIITDPVSGQT--VCSCQ
mrr       CCDTGRTIYTDPVSGQT--ICSCQ
IRX-3     CCESTQRSVSDVASGSTPAPALCC

CON.      CCE GR I TDPVSGQT   CSCQ
           +       +  ++ +    +
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H

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ara       KMTWEPKNRTDDDDDALVSDDEKDKEDLE
caup      KMTWEPKNKTEDDDDGMMSDDEKEKDAAD
mrr       KMTWEPRNRVDDDDANIDDDDDKNTEDND
IRX-2a    KMTWTPRNRSEDEEEEENIDLEKDNEDEP

CON.      KMTWEP NR  DDD     DDEK  ED
          ++++ + +   +       +  +
```

I

```
ara       KPKIWSLADTV
caup      KPKIWSVADTA
mrr       KPRIWSLADMA
IRX2a     KPKLWSLAEIA

CON.      KPKIWSLAD A
          ++  ++ + +
```

Materials and methods

Human tissues

Breast tissue was obtained from mastectomy or reduction mammaplasty patients in accordance with protocols and ethical standards reviewed by our Institutional Review Board. All participating patients were informed of the nature of the study and gave written consent for use of their tissues prior to surgery. For each patient and tissue type (i.e., normal or tumor), independent tissue samples were immediately frozen in liquid nitrogen (for RNA extraction) or fixed in 4% paraformaldehyde:phosphate-buffered saline (PBS) (for in situ hybridization). Tumors were either non-metastatic or metastatic; tumor grades ranged from I to III. Detailed pathology data are available from the authors by request.

Identification of the IRX family and cloning via RACE (rapid amplification of cDNA ends) PCR

To expand our studies of homeobox genes into the human breast, we searched the dbEST database (Boguski et al. 1993) and identified a breast-derived partial cDNA containing a novel homeobox [Expressed Sequence Tag (EST); identification: 152453; GenBank accession: R46202, R46296]. This partial cDNA clone was obtained through the IMAGE consortium and found to be lacking both 5' and 3' ends upon sequencing.

To clone the full-length cDNA corresponding to the IMAGE clone 152453, oligonucleotide primers were designed to the IMAGE clone homeobox and used in RACE polymerase chain reaction (PCR). Primer sequences were: sense "F1": 5'-GCCACGGCTACCCTCAAGGCCTGGCT-3'; antisense "R1": -5'-AGGCCGGCGCGCGTTGNCGAACCA-3'. Template was an adaptor-ligated breast cDNA library (Marathon-ready, Clontech). The library-specific adaptor primer "AP1" was used with "F1" and "R1" in RACE PCR as recommended to generate 3' and 5' ends, respectively.

Fig. 1 **A** *IRX* homeobox alignment. *Asterisks* denote pairwise differences; *periods* denote identities. The position of the "R1" PCR primer is noted. **B** IRX hexapeptide-like and homeodomain amino acid sequences aligned with relevant portions of araucan (*ara*), caupolican (*caup*) and mirror (*mrr*). *Dashes* indicate gaps. *Vertical lines* show pairwise identity between Antennapedia (*Antp*) and mirror and between araucan and IRX-2a. *Plus signs* below the Iroquois-class consensus designate amino acids 100% conserved. The position of the "R1" PCR primer is noted. **C** Northern hybridization: gene-specific pIRX-2 (EK0.5) probe. *Lane 1* represents all normal (*NORM*) samples (n=11). *Lane 2* represents a fibroadenoma (*FA*). *Lanes 3* (representing three tumors) *and 4* (representing nine tumors) depict transcript patterns observed in infiltrating ductal carcinomas (*IDC*). *Lanes 5* (representing one tumor) *and 6* (representing two tumors) depict transcript patterns observed in three infiltrating lobular carcinomas (*ILC*). Control hybridization using a probe for the L7 ribosomal protein mRNA was used to assess loading and is shown below. **D** Northern hybridization: homeobox-containing IMAGE probe. *Lane 1* represents all normal samples tested (n=7). *Lane 2* represents all tumors tested (*IDC:* n=4/*ILC:* n=1). The small transcript (~1.7 kb) detected by this probe is probably derived from a related *IRX* gene but the possibility of additional transcripts derived from *IRX-2* cannot be ruled out. Control hybridization using a probe for the L7 ribosomal protein mRNA is shown below. **E** Schematic diagram of the *IRX-2a* cDNA and protein. Extent and locations of probes used are shown above the RACE products and IMAGE clone. Locations of the F1 and R1 primers are shown by *arrows*. Amino acid motifs are noted by *color*. **F** *IRX-2a*-translated protein. Motifs noted according to Fig. 1E. **G** Cysteine-rich motif found in some Iroquois-class proteins. **H** Acidic region (partial). **I** IRO box (extended two amino acids to include a conserved alanine). Annotations for **G**, **H** and **I** are as for **B**

While screening 5' and 3' RACE PCR products for cDNAs corresponding to the IMAGE clone, we isolated fragments of transcripts derived from four additional IRX genes. Nucleotide sequence data were immediately deposited in the GenBank sequence database under the following accession numbers: IMAGE clone 152453 (U90309); *IRX-2a* (U90304); *IRX-1* (one isolate) (U90308); *IRX-3* (two identical isolates) (U90305); *IRX-4* (one isolate) (U90306); and *IRX-5* (one isolate) (U90307).

RNA isolation

Mammary tissue was ground in liquid nitrogen to a fine powder. Total RNA was isolated using the Purescript system followed by an additional purification by column chromatography (Qiagen). Total RNA from human uterus, kidney, salivary gland and lung was purchased from Clontech.

Hybridizations

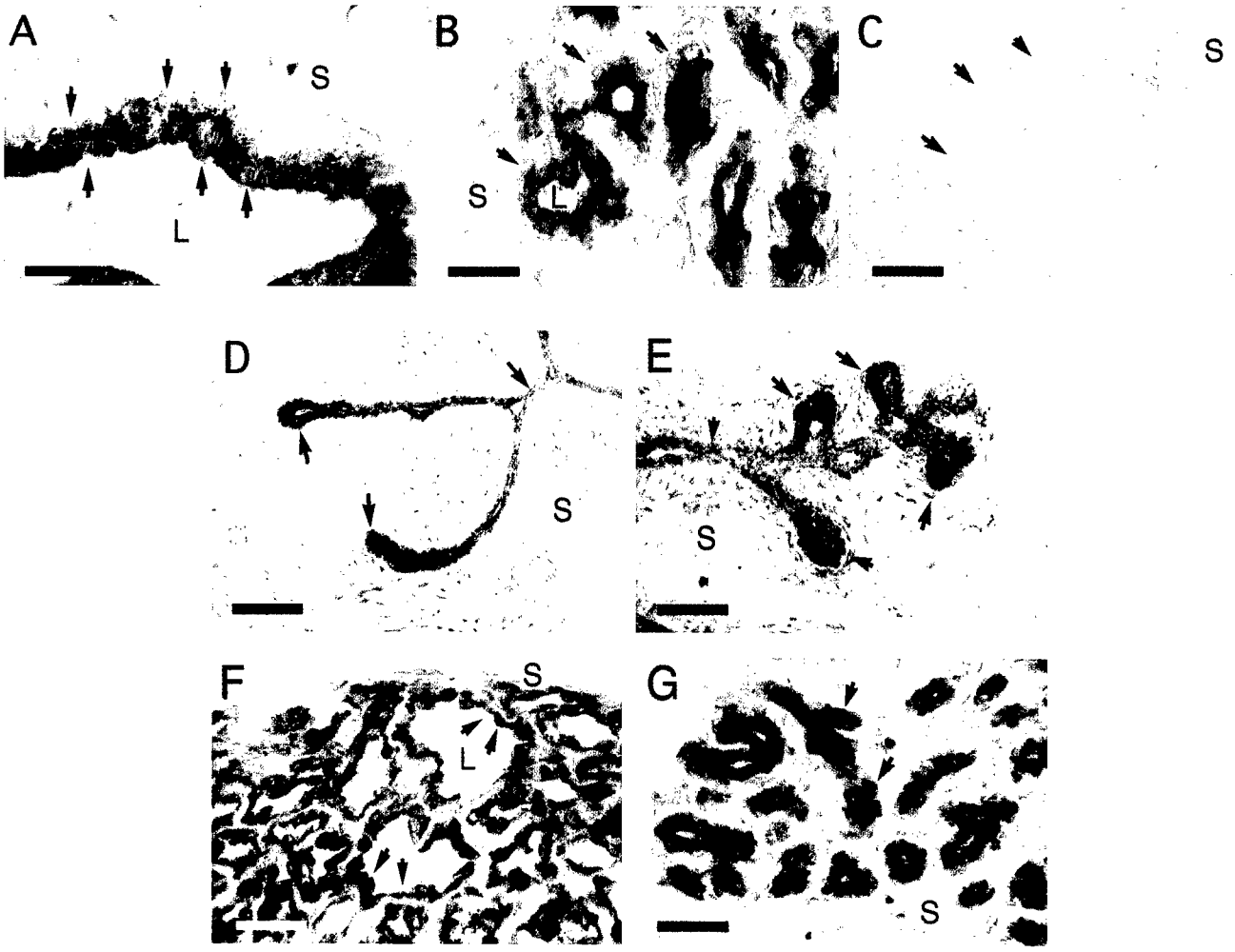
Probe preparation and Northern hybridizations were performed as described (Friedmann and Daniel 1996). The location and extent of probes are depicted in Fig. 1E. In situ hybridization was performed as described (Friedmann and Daniel 1996) using 500 ng/ml antisense or sense pIRX-2 (EK0.5) riboprobes with an additional RNAse A treatment [5 µg/ml RNAse A in 10 mM TRIS, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl, 37°C, 15 min] between the first and second stringency washes to unequivocally ensure gene-specific hybridization. In control hybridizations, the pIRX-2 (EK0.5) probe does not cross-hybridize with any of the other cloned IRX genes at high stringency (final washes 2×30 min, 0.1×SSC; 0.1%SDS at 65°C); the homeobox-containing IMAGE probe cross-hybridizes with all of the cloned IRX genes at this stringency. Northern hybridizations using the IMAGE probe were done at "unusually high stringency" to obtain gene-specific results (final washes 2×30 min, 0.1×SSPE; 0.1% SDS; 15% formamide at 70°C).

Results and discussion

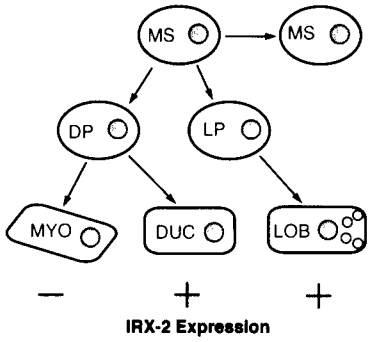
To expand our studies of mammary-associated homeobox genes into the human breast, we searched the dbEST database (Boguski et al. 1993) and identified a breast-derived partial cDNA containing a novel homeobox (obtained through the IMAGE consortium; identification: 152453; GenBank accession: R46202, R46296).

While screening 5' and 3' RACE PCR products for cDNAs corresponding to the IMAGE clone, we isolated fragments of transcripts derived from four additional IRX genes (Fig. 1A,B) giving presumptive evidence for expression of at least five different IRX genes in the adult human breast. To our knowledge, this is the first report of homeobox gene expression of any kind in the human breast. Homologs of this gene family were subsequently identified in *Drosophila* (the Iroquois complex genes) (Gomez-Skarmeta et al. 1996; McNeill et al. 1997) and shown to be required for proper neural patterning. In addition, homologs were recently identified in both mouse and *Xenopus* (Bosse et al. 1997; Bellefroid et al. 1998; Gomez-Skarmeta et al. 1998) and shown to function in the development of the vertebrate nervous system.

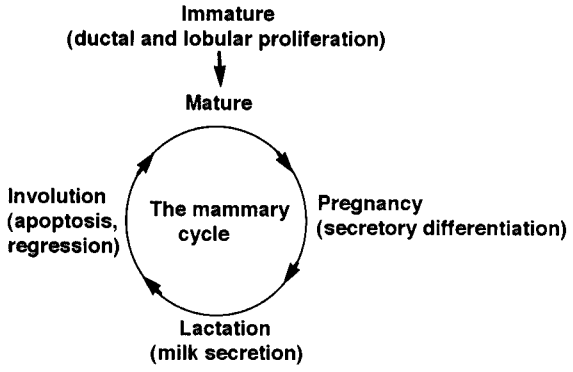
BLAST program database searches (Altschul et al. 1990) showed the IRX homeodomains to be ~90% iden-

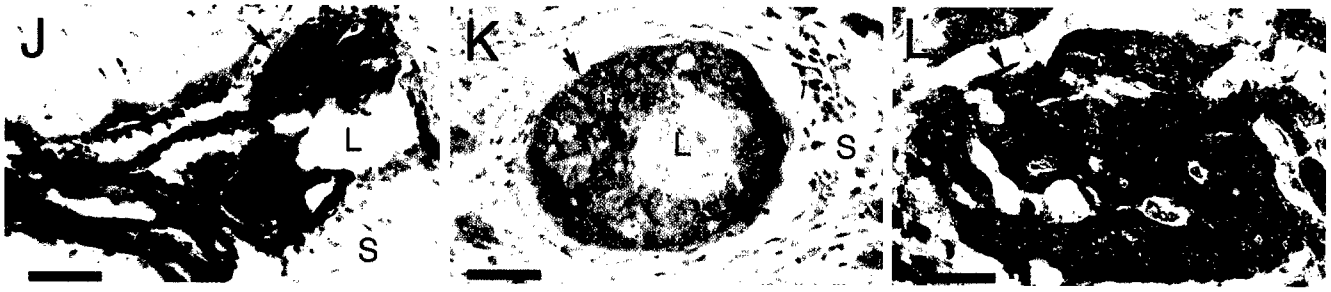


H Mammary Epithelial Cell Differentiation



I Phases of Mammary Gland Development





tical to the Iroquois-class homeodomains encoded by the *Drosophila* genes *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mrr*) and between 92% and 93% identical to one another (Fig. 1B) (Gomez-Skarmeta et al. 1996; McNeill et al. 1997). The designation *IRX-2* was ultimately assigned to the gene corresponding to the IMAGE cDNA. Each IRX protein contains a hexapeptide-like motif (Fig. 1B) (Chang et al. 1995; Chan et al. 1996). Thus far, only IRX-3 shows a cysteine-rich motif (Fig. 1G).

Northern hybridization using the gene-specific pIRX-2 (EK0.5) probe (which lacks the homeobox) against normal (n=11), benign fibroadenoma (FA) (n=1), infiltrating ductal carcinoma (IDCs) (n=12) and infiltrating lobular carcinoma (ILCs) (n=3) RNAs detected two main transcripts at 2.6 kb and 3.9 kb, designated *IRX-2a* and *IRX-2b*, respectively (representative samples are shown in Fig. 1C). Interestingly, unusually high stringency hybridizations using the IMAGE probe (which contains the homeobox) on normal and tumor samples identified what appeared to be only the single 2.6-kb *IRX-2a* transcript. Since the homeobox-containing IMAGE probe does not hybridize with the 3.9-kb *IRX-2*b transcript, these data suggest that this transcript does not contain a closely related homeobox (Fig. 1D). A similar set of alternatively spliced trancripts both with and without a homeobox has been demonstrated for the mouse *HoxA1* gene (LaRosa and Gudas 1988). Reliable quantitation is not possible using primary tissue samples due to gross variation in the epithelial content of each tissue (evident upon histological analysis) and the unavoidable contamination of tumor tissue with various amounts of normal tissue at the surgical margins. Therefore, we decline to speculate at this time whether *IRX-2* might be misexpressed in some tumors. The question of misregulation of IRX genes will be better approached using microdissection and RNA analysis techniques recently developed for this purpose (Bonner et al. 1997).

To determine whether *IRX-2* expression was mammary specific in the adult, we also conducted Northern analysis on RNA from adult human lung, uterus, salivary gland and kidney using the gene-specific pIRX-2 (EK0.5) probe (data not shown). *IRX-2* messages were weak but detectable in all four non-mammary samples, indicating that expression in the adult is not breast specific.

Cloned, overlapping 5' (n=4) and 3' (n=3) RACE products comprise a 1.8-kb *IRX-2a* cDNA (exclusive of the polyA tail) that contains a complete open reading frame for the 417-amino acid IRX-2a protein (Fig. 1E,F). In addition to the homeodomain and the hexapeptide-like sequences, the translated cDNA shows acidic and poly-proline regions, and an IRO box (Bürglin 1997) (Fig. 1F–I). Attempts to isolate an *IRX-2b* cDNA via RACE PCR or standard phage-based cDNA library screens were unsuccessful, perhaps due to poor representation resulting from a high G+C content as is observed in the *IRX-2a* cDNA (60–90%, window size=50 bp).

IRX-2 expression was investigated by in situ hybridization using the pIRX-2 (EK0.5) probe against normal breast tissue (n=8). In mature tissue, lumenal epithelium of ducts (Fig. 2A) and alveoli (Fig. 2B) express *IRX-2* as evidenced by accumulation of blue-black stain in the cytoplasm of these cells; myoepithelium, adipose stroma, and a small subpopulation of ductal epithelial cells (Fig. 2A) do not express. Sense-strand probe shows no hybridization (Fig. 2C). During the proliferative phase of development, immature tissue shows concentrated expression in terminal end buds and terminal lobules (Fig. 2D,E) becoming reduced in differentiated subtending ducts. During the cyclical phase of mammary development, in lactating tissue (Fig. 2F), ~18% of alveolar epithelial cells show undetectable or reduced expression (cf. Fig. 2B). Uniform expression is re-established in late-stage involuting tissue (Fig. 2G). Tissue from pregnant and early involuting patients was unavailable for study.

Evidence for regulated expression of *IRX-2* is threefold. First, as summarized in Fig. 2H, *IRX-2* is differentially expressed in discreet epithelial cell lineages, being undetectable in differentiated myoepithelial cells but

Fig. 2 Expression of *IRX-2* transcripts in normal development and neoplasia. In situ hybridization using the gene-specific pIRX-2 (EK0.5) probe (detecting both *IRX-2a* and *IRX-2b* transcripts). Expression is evident by accumulation of a blue-black precipitate stain. Ductal and alveolar lumens (*L*) and adipose stroma (*S*) are identified when possible. **A** Major duct showing staining in lumenal epithelial cells. *Red arrows* indicate examples of unstained myoepithelial cells within the myoepithelial cell layer; *black arrows* indicate occasional unstained lumenal epithelial cells. Identification of myoepithelial cells is based on well-established morphological and positional criteria as well as comparison with published immunohistochemical studies using these tissues (Daniel and Silberstein 1987; Russo and Russo 1987; Taylor-Papadimitriou and Lane 1987; Silberstein et al. 1997). Bar 27 μm. **B** Alveoli. Epithelial cells are uniformly stained. *Red arrows* indicate unstained myoepithelial cells. **C** Sense control probe showing a completely unstained lobule. *Black arrows* indicate alveoli. **D** Terminal end buds (*black arrows*). Staining decreases in the subtending duct (*red arrow*). **E** Terminal lobule. *Black arrows* indicate developing alveolar buds which stain heavily for *IRX-2* transcripts. Staining is reduced in the subtending duct (*red arrow*). **F** Lactating tissue in which an increased proportion of epithelial cells (~18%) stain poorly for *IRX-2* transcripts. *Red arrows* indicate unstained epithelium; *black arrows* indicate strongly staining epithelium. **G** Late-stage involuting tissue (not yet completely remodeled). *Black arrows* indicate alveoli in which uniform *IRX-2* expression is re-established in all epithelial cells. **H** A current model for mammary epithelial cell differentiation (Smith 1996) and summary of *IRX-2* expression in differentiated cell types (*MS* mammary stem cell, *DP* ductal progenitor cell, *LP* lobule-alveolar progenitor cell, *MYO* myoepithelial cell, *DUC* differentiated ductal cell, *LOB* differentiated lobule-alveolar cell). The pattern of *IRX-2* expression in each differentiated cell type is shown below. **I** Summary of major phases of mammary gland development. *IRX-2* expression patterns in four of the five developmental phases are represented in panels **A–G** demonstrating changes in *IRX-2* expression through the mammary cycle. **J** Fibroadenoma. *Red arrow* identifies the characteristic network of epithelial cells within the duct. **K** Infiltrating ductal carcinoma. *Red arrow* indicates neoplastic epithelial cells filling the ductal lumen. **L** Infiltrating lobular carcinoma (*ILC*) (*red arrow*). An entire lobule is shown in which histotypic structure is lost (cf. **G**). Hybridizations using the IMAGE probe (which likely detects all five known IRX genes at this stringency) were qualitatively similar to those shown above (data not shown). *Bars* 27 μm (**A, B**), 220 μm (**C–E**), 70 μm (**F, G, J**), 80 μm (**K**), 220 μm (**L**)

readily detected in lumenal ductal and more highly expressed in alveolar epithelial cells. Second, a small subpopulation of lumenal ductal epithelial cells do not express *IRX-2* (Fig. 2A). Finally, with reference to Fig. 2I, there are distinct changes in *IRX-2* expression during different phases of mammary gland development, with concentrated *IRX-2* expression in terminal structures in immature tissue, uniform expression in mature and late-stage involuting tissue, and reduced expression in ~18% of alveolar epithelial cells during lactation.

In tumors (FA, n=1; IDC, n=7; ILC, n=3), *IRX-2* expression is maintained (Fig. 2J–L) regardless of tumor type, grade, receptor status, or metastatic state. In contrast, expression of 9 of 11 normally expressed mouse homeobox genes is lost in neoplastic progression (Hox complex genes B6, B7, C6, C8, D4, D8, D9, D10 and the non-complex gene Msx-2) (Friedmann 1995). Therefore, *IRX-2* may serve as an excellent marker for mammary epithelial cell identity during tumor progression since it is observed in the epithelial component of even high-grade metastatic tumors.

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

Michael T. Lewis[1,‡], Sarajane Ross[1], Phyllis A. Strickland[1], Charles W. Sugnet[1], Elsa Jimenez[1], Matthew P. Scott[2] and Charles W. Daniel[1,*]

[1]Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA
[2]Departments of Developmental Biology and Genetics, Howard Hughes Medical Institute, 279 Campus Drive, Stanford University School of Medicine, Stanford, CA 94305, USA
‡Present address: Department of Physiology and Biophysics, University of Colorado School of Medicine, Box C240, Room 3802, Denver, CO 80262, USA
*Author for correspondence (e-mail: daniel@darwin.ucsc.edu)

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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

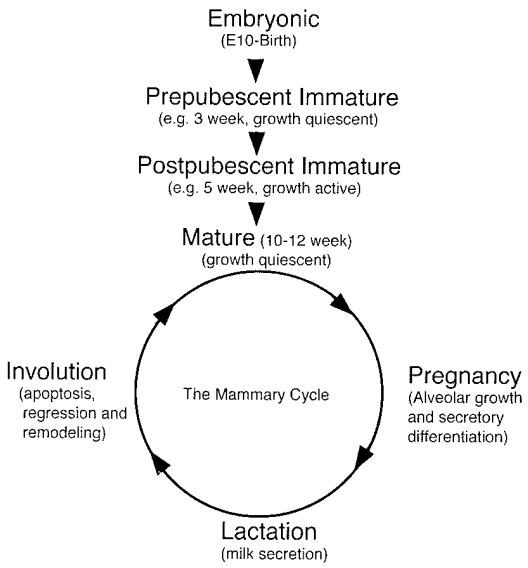

Fig. 1. Phases of mammary gland development. Proliferative development in virgin animals is represented by the linear portion of the diagram from embryonic day 10 (E10) through maturity. Cyclical development initiated by pregnancy is represented by the circular portion of the diagram.

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.

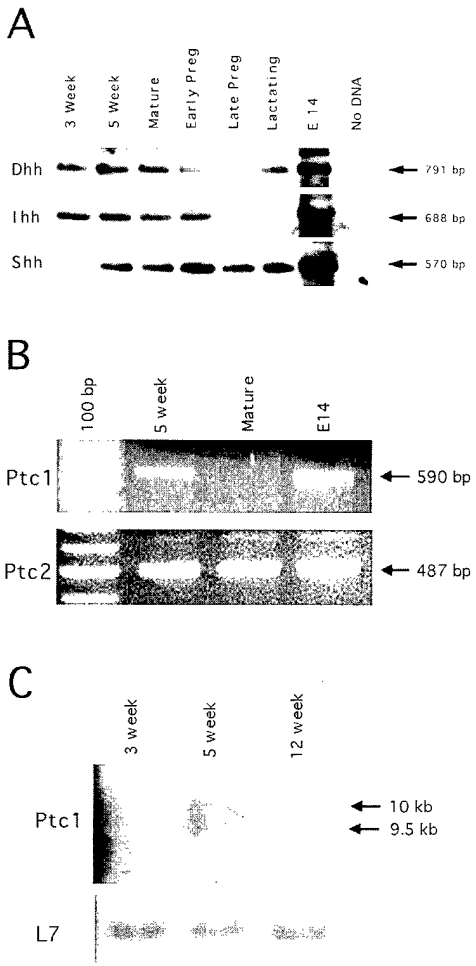

Fig. 2. Hedgehog network component expression – non-quantitative RT-PCR and northern blot hybridization. (A) Hedgehog gene expression detected by Southern blot hybridization of products from RT-PCR at different stages of mammary development. Each hybridizing band was of the expected size for each of 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

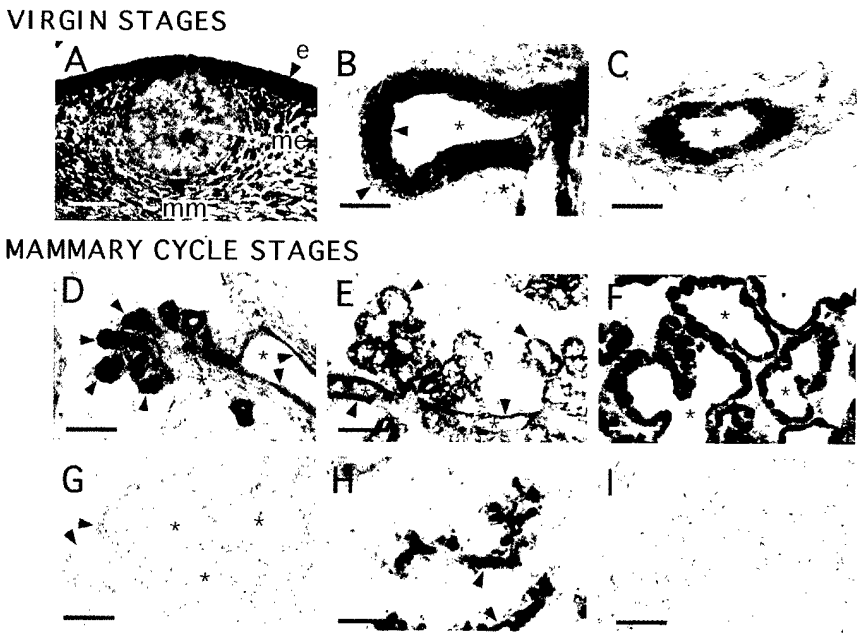

Fig. 3. In situ hybridization of *Ptc1* during embryonic and postnatal mammary development. Expression is detected by the accumulation of a blue-black precipitate. Selected lumenal spaces are denoted by red asterisks. (A) Embryonic day-14 mammary bud. Me, mammary epithelium; mm, mammary mesenchyme; e, 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

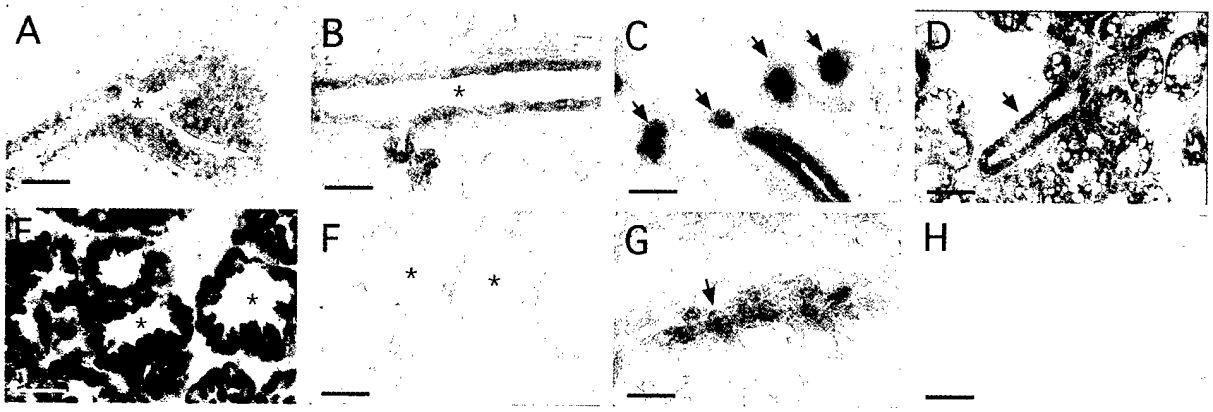

Fig. 4. In situ hybridization of *Ihh* during postnatal mammary development. Expression is detected by the accumulation of a blue-black precipitate. Red asterisks denote selected lumenal spaces. (A) 5-week terminal end bud showing body cell expression. (B) Duct of a 12-week mature gland with a sidebranch. 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).

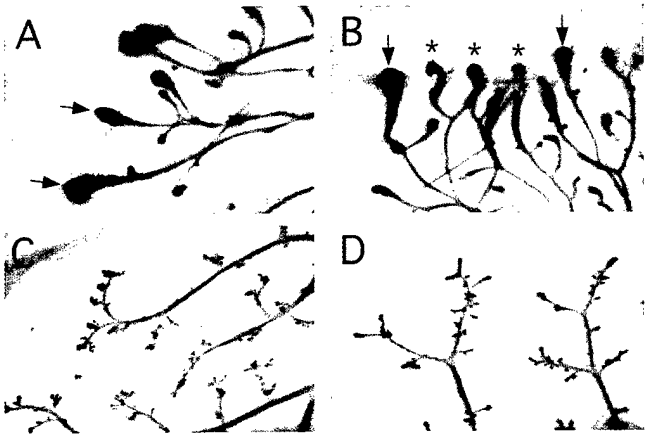

Fig. 5. Whole gland morphological analysis during proliferative development in virgin animals. (A) Wild-type, 5-weeks old. Terminal end buds (black arrows) and subtending ducts appear well formed. (B) *Ptc1* heterozygote, 5-weeks old. TEB are generally normal (black arrows) but a subset of TEBs are clearly 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.

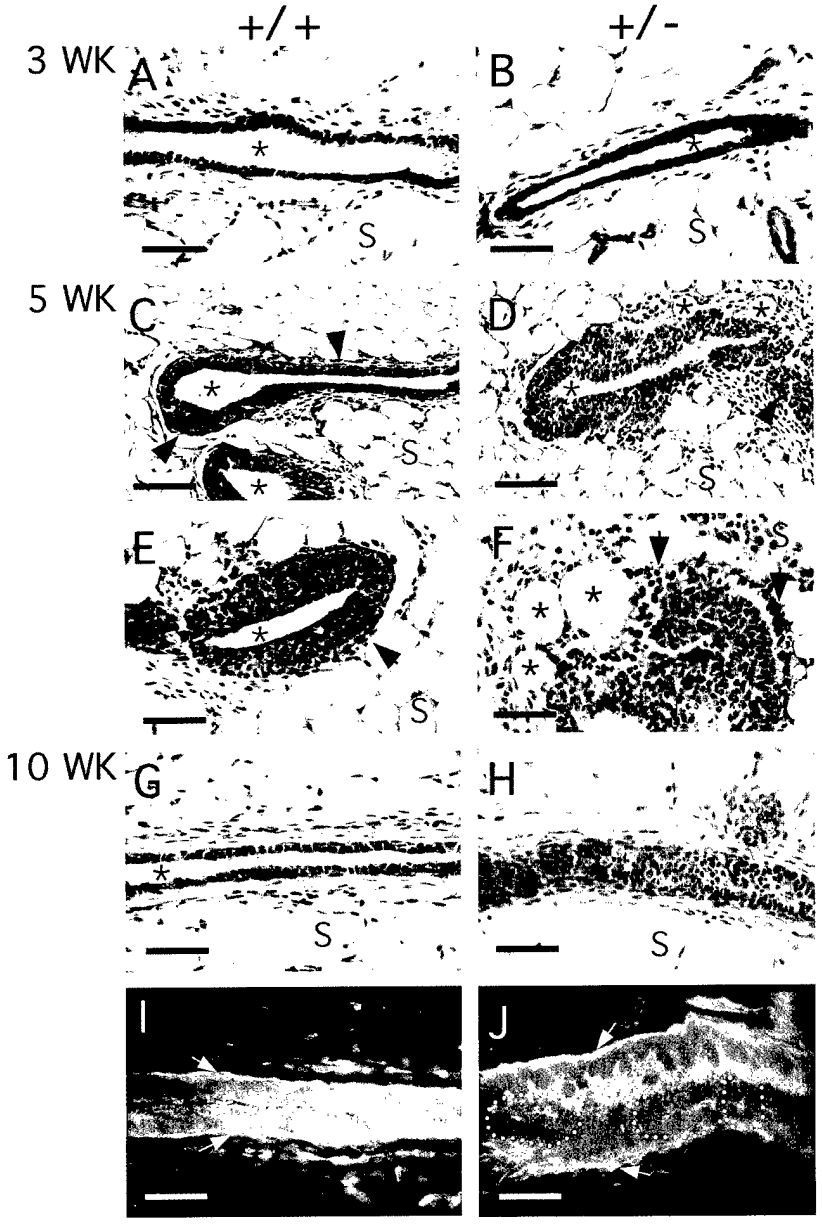

Fig. 6. Histological comparison of glands during development in virgin animals. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column. A-H are stained with hematoxylin and eosin; I-J are stained 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,

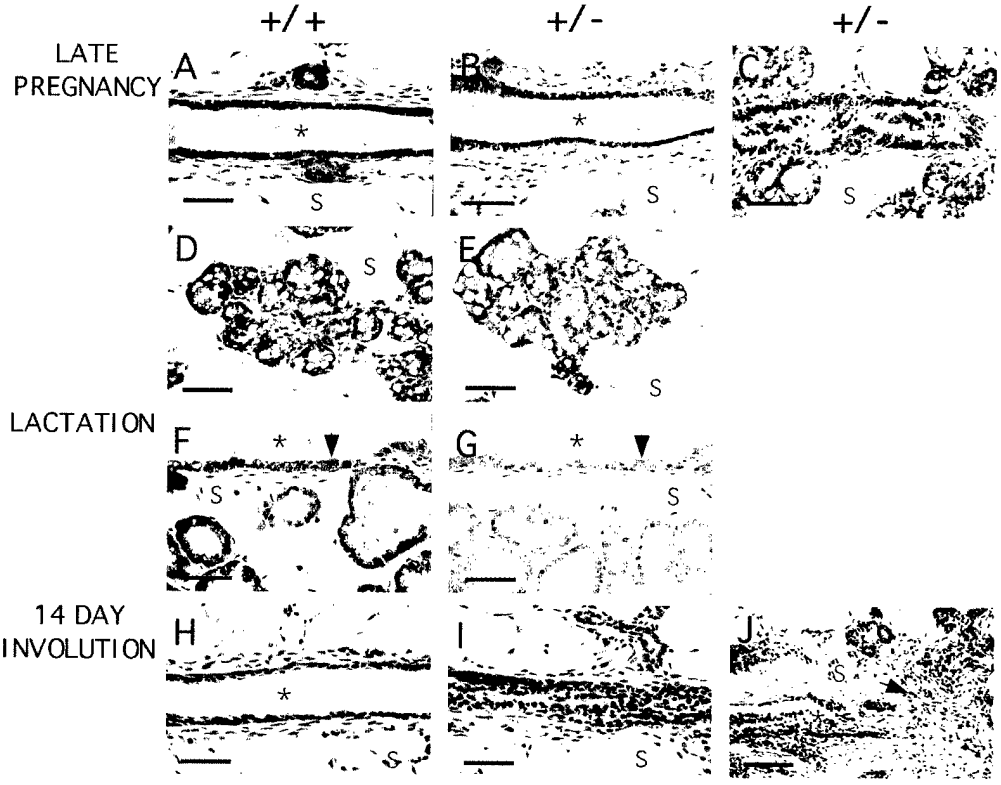

Fig. 7. Histological analysis of glands during the mammary cycle. Developmental stages are shown on the left. Genotype of the animal from which the glands are derived is shown at the top of each column of panels. Red asterisks denote ductal lumens; a red letter 's', adipose stroma. (A) Mammary duct. 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

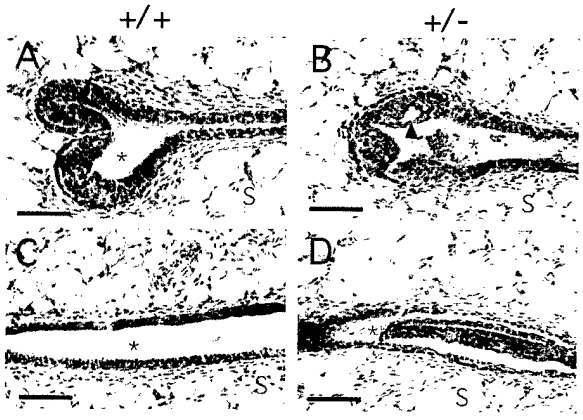

Fig. 8. Whole mammary gland transplant into nude mice. Genotype of the transplanted mammary gland is noted above the panel columns to which they refer. Red asterisks denote lumenal spaces; a red letter 's', adipose stroma. (A) Terminal end bud showing well-ordered cap and body cell layers. (B) Terminal 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.

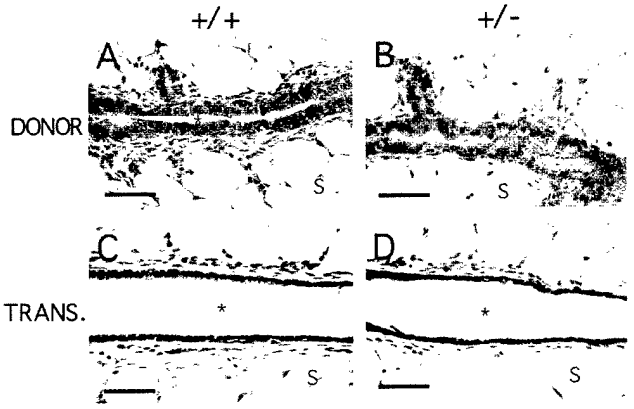

Fig. 9. Epithelial transplantation into cleared fat pads. Tissue source is noted on the left; genotype of the epithelium is noted above the panel columns to which they refer. Red asterisks denote lumenal spaces; a red letter 's', adipose stroma. (A) Mammary duct showing an open lumen and monolayer of 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.

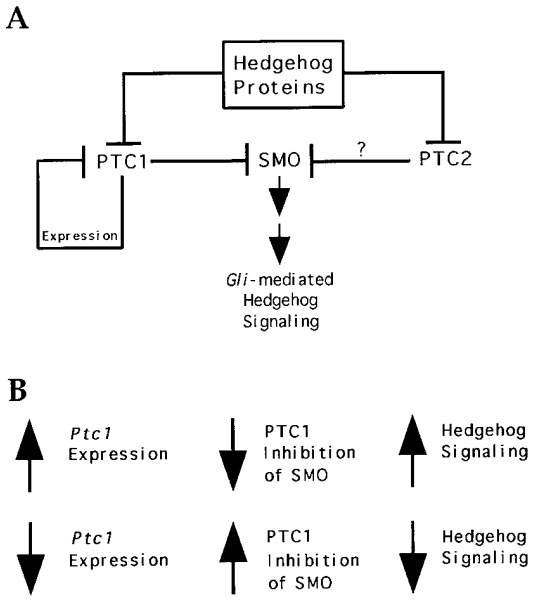

Fig. 10. Summary of a portion of the model for hedgehog signal transduction in mammals. (A) Schematic of functional interactions among the hedgehog proteins, PTC1, PTC2 and SMO leading to GLI-mediated control of target gene expression. (B) A generalized summary of *Ptc1* gene expression relative to protein 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.

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