

## Review

# Homeobox genes in mammary gland development and neoplasia

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## Abstract

Both normal development and neoplastic progression involve cellular transitions from one physiological state to another. Whereas much is being discovered about signal transduction networks involved in regulating these transitions, little progress has been made in identifying the higher order genetic determinants that establish and maintain mammary cell identity and dictate cell type-specific responses to mammotropic signals. Homeobox genes are a large superfamily of genes whose members function in establishing and maintaining cell fate and cell identity throughout embryonic development. Recent genetic and expression analyses strongly suggest that homeobox genes may perform similar functions at specific developmental transition points in the mammary gland. These analyses also suggest that homeobox genes may play a contributory or causal role in breast cancer.

**Keywords:** breast cancer, embryogenesis, homeodomain, *HOX* genes, organogenesis

## Introduction

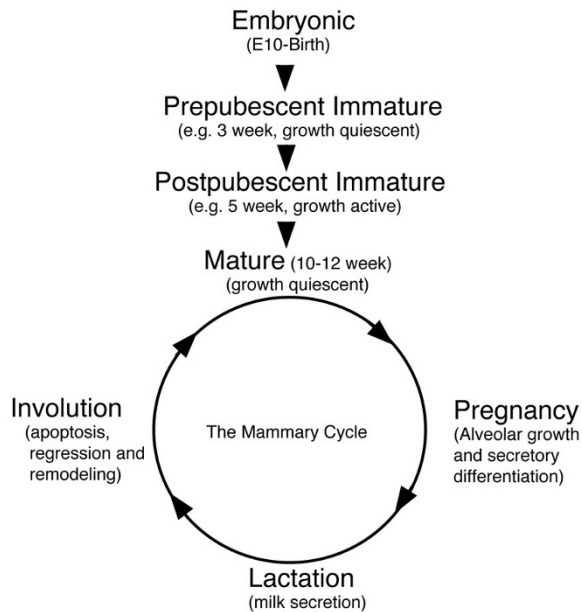
The mammary gland is a remarkable organ with respect to its development and functional differentiation. It is also remarkable with respect to the consequences on mammalian life should development become abnormal, leading either to lactational failure or, most importantly, mammary cancer.

Unlike most mammalian organs, which develop primarily embryonically with a more or less linear progression toward functional maturity, development of the mammary gland is primarily postpubertal and may be divided into both a linear and a cyclical phase (Fig. 1) (see [1–3] for detailed reviews). These phases can be characterized further as a series of highly orchestrated transitions, or switches, in which critical developmental decisions are made concerning cell differentiation, pattern formation and cell function. While mutations have been identified that block or delay most of these transitions (primarily in signal transduction networks), the

higher order genetic determinants of cell identity that dictate cell type specific responses are largely unknown.

Among other candidates, one superfamily of genes presents itself as capable of regulating developmental decisions during these transitions: the homeobox genes. As a general principle, homeobox genes encode transcription factors that play key roles in the determination and maintenance of cell fate and cell identity [4–7]. Homeobox genes share a common nucleotide sequence motif (the homeobox) encoding the roughly 61 amino acid homeodomain. The homeodomain, in turn, is a helix-turn-helix DNA-binding domain of the functional transcription factor. Evolutionary relationships and family classifications are determined based upon the degree of identity and similarity among homeodomains followed by comparative analyses of amino acid sequences both amino-terminal and carboxyl-terminal to the homeodomain [8–10]. These terminal sequences vary considerably from protein to

**Figure 1**



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

protein and, indeed, may demonstrate no evidence of evolutionary or functional relationship whatsoever.

Homeobox genes are found in animals ranging from hydra to humans (as well as fungi and plants). Over evolutionary time, the number of homeobox genes has increased and their functions have been reengineered to meet the demands of increasingly diverse developmental processes. To date, there are well over 100 homeobox genes identified in the human, with a comparable number of homologs identified in the mouse [10].

In mammals, homeobox genes reign over the specification of the overall body plan and are known to play key roles in a variety of developmental processes including central nervous system and skeletal development, limb and digit specification, and organogenesis. Mutations in homeobox genes can cause dramatic developmental defects including loss of specific structures as well as ‘homeotic transformations’, in which one body part or segment is converted to the likeness (identity) of another. Some homeobox genes appear to serve cell autonomous functions in differentiation and cell cycle control; others serve noncell autonomous functions such as pattern formation and mediation of reciprocal tissue interactions.

At least five difficulties present themselves when trying to understand the functions of individual members of such a

large superfamily of genes. First and foremost, exceptionally little is known about the number and identity of target genes controlled directly or indirectly by any mammalian homeobox gene, although it appears that much of the *Drosophila* genome is under homeobox gene control [11]. Second, only slightly more is known about upstream genes that control homeobox gene expression and function. Recent efforts in both *Drosophila* and mice demonstrate that establishment and maintenance of restricted homeobox gene expression is controlled, in part, by combinatorial activity of trithorax and polycomb group genes (which themselves are highly regulated) [12–15]. Third, homeobox genes do not generally act alone to determine cell identity. Rather, in many cases, it appears to be the combinatorial spatially and temporally regulated pattern of homeobox genes functioning in a given cell (a ‘homeobox code’) that determines the cell’s identity [6,16,17]. In fact, in some cases, important functions of a given homeobox gene can be masked by compensatory function of a related homeobox gene. Fourth, to further complicate the issue, some homeodomain proteins are known to interact physically with other homeodomain proteins (eg PBX and MEIS or PBX and HOX) or other cofactors to control downstream target gene specificity [18–20]. Finally, many homeobox gene mutations are (or will be) embryonic or perinatal lethal, making analysis of the development of adult organs particularly challenging. This current state of affairs makes it difficult to develop mechanistic models for the function of a given homeobox gene, especially in the mammary gland where none of these issues have been investigated adequately.

In addition to roles in normal development, altered homeobox gene function is implicated in the development of cancers, particularly leukemias and rhabdomyosarcomas, as well as those of the breast, prostate, kidney, colon, skin and brain. With the exception of a role in breast cancer, this subject has been reviewed extensively [21–24].

Homeobox genes clearly occupy a prominent position in the developmental regulatory hierarchy, yet homeobox genes have received little attention with respect to mammary gland organogenesis, functional differentiation and cancer. It is the purpose of this review to highlight a few known and suspected roles of homeobox genes in controlling developmental decisions and neoplasia in the mammary gland, and to point out some gaping holes in our understanding that study of homeobox genes may help to fill.

## Transitions and switches in mammary gland development

### The linear phase

#### *Differentiation of the embryonic mammary gland*

The mammary epithelium is an ectodermal derivative. As such, among the first distinctions that must be made is the differentiation of presumptive mammary epithelium from tissue that would otherwise form skin, hair follicles or other

ectodermally derived structures. This differentiation occurs in at least two major stages. The first stage begins about day 10 of gestation (E10) with the establishment of the mammary streaks, two lines of epidermally derived thickened epithelium that run anterior→posterior, symmetrically displaced off the ventral midline. These streaks represent the first morphological evidence of mammary pattern formation and differentiation.

The second stage occurs around E11 with the definition of the nipple region. Presumptive mammary epithelium forms a lens-shaped disk that becomes associated with underlying condensed mammary mesenchyme. The mammary epithelium continues to grow to form a bulb-shaped mammary bud that elongates and invades the condensed mesenchyme. Mammary epithelial cell identity is firmly established as early as E12.5 (the bud stage), as evidenced by the ability to transplant the presumptive mammary gland into a cleared fat pad and regenerate a ductal tree (CW Daniel, G Robinson, personal communication).

#### *Invasion of the mammary fat pad precursor mesenchyme*

As the mammary bud elongates into a mammary sprout, it reaches a second mesenchyme, the fat pad precursor mesenchyme, and undergoes a small amount of branching morphogenesis to form the rudimentary gland of the neonate. Several genes are known to act either at or before this critical transition to allow further growth or to control establishment of sexual dimorphism of the mouse mammary gland (eg *Lef1*, *PThRP*, *PPR1*) [25–27]. Tissue recombination experiments demonstrate that mammary mesenchyme and fat pad mesenchyme affect the growth of mammary epithelium in dramatically different ways, suggesting fundamental differences in their identities and biological properties with respect to tissue interactions (see [2]). How these differences are established is unknown.

#### *Growth initiation, cellular differentiation and growth arrest*

From birth to puberty, the gland remains rudimentary and relatively growth quiescent. At puberty, ovarian hormones stimulate rapid and invasive ductal elongation driven by growth of a structure called the terminal end bud, which consists of four to six layers of relatively undifferentiated 'body cells' and a surrounding single layer of 'cap cells'. These two populations differentiate into luminal epithelial cells (also consisting of multiple cell types) and myoepithelial cells, respectively, as the subtending duct is formed [1,28]. Whereas a variety of individual cell types are known to exist and their developmental capacities have begun to be explored, virtually nothing is known about how these cell lineages and fates are established in the first place.

Upon reaching the limits of the fat pad at ductal maturity, ductal elongation ceases and terminal end buds regress to leave a branched system of differentiated ducts. These ducts will remain relatively quiescent as long as the animal

remains virgin. How this growth control is achieved and maintained is not known, although it probably involves members of the transforming growth factor- $\beta$  superfamily.

#### **The cyclical phase: lobuloalveolar differentiation, lactation, involution and gland remodeling**

Hormonal changes during pregnancy initiate a cyclical phase of development in which there is a dramatic transition from a predominantly ductal to a predominantly lobuloalveolar gland morphology. Lobuloalveolar progenitor cells located within the ducts proliferate to form alveolar buds, which further differentiate to form the alveoli. Near midpregnancy, the alveolar epithelium acquires the capacity to produce milk proteins (the stage I transition of lactogenesis) but secretory function is inhibited. At parturition, inhibition of secretory function is released and these cells begin to secrete large quantities of milk (the stage II transition of lactogenesis). Upon weaning, milk secretion ceases and the gland involutes. During involution, most alveolar cells undergo apoptosis (programed cell death), while a residual epithelial population remodels itself back into a ductal tree to await the next pregnancy.

Of course, there are known hormonal and growth factor signals that control some of these transitions, but little is known about how epithelial and stromal cells are poised to respond to such signals or how the resultant differentiated state is maintained.

#### **Homeobox genes in embryonic and postnatal mammary gland development**

Judging from their properties in the development of other organs, it is likely that homeobox genes may function at many of the transition points already described. The approach that has been most useful thus far in investigating mammary homeobox gene function is to determine which homeobox genes are expressed in the gland, characterize when and where they are expressed throughout development, and assay their function *in vitro* or, preferably, *in vivo* (Table 1). Unfortunately, many of the current studies are in their early stages and have been limited to a single detection technique (eg reverse transcription polymerase chain reaction) or to a particular developmental stage with no further analysis. Selected analyses, conveniently divided by gene family, are now highlighted with historical context whenever possible.

#### **Hox/HOX**

In the mouse (and human), 39 known *Hox* (*HOX*) complex genes are arranged in four paralogous gene clusters, one on each of four different chromosomes. These genes are primarily responsible for anterior–posterior patterning of the body and limbs but organ-specific functions are also known. Targeted disruption (knockout) strains exist for most *Hox* genes, as do a limited number of overexpressing transgenic strains. Both types of mutants have rarely been examined for

mammary defects and tumors, and overall phenotypic analysis of single gene mutations has been somewhat hampered by functional compensation from paralogous genes within the complex itself. Nevertheless, it is already clear that some *Hox* genes are critically important for proper mammary gland development and function (Table 1).

Early immunohistochemical studies and screens based on polymerase chain reaction first detected homeobox gene expression in mammary epithelial cell lines and tumors [29,30]. Subsequently, Friedmann and coworkers identified several *Hox* genes that were expressed in the normal mouse mammary gland and associated neoplasias (Table 1) [31\*,32]. *In situ* hybridization demonstrated that many of these genes are expressed either in the mammary epithelium or in the periductal stroma, or both (Fig. 2). Several genes, including *Hoxc-6*, *Hoxc-8*, *Hoxd-8*, *Hoxd-9* and *Hoxd-10*, appeared to be regulated developmentally. Most intriguing was the demonstration that expression of some homeobox genes was sensitive to manipulation of the estrogen level. Together, these data provided the first substantive clues that homeobox genes are important for normal mammary development.

Soon after, Srebrow *et al* [33\*] identified five *Hox* genes expressed in CID-9 mouse mammary epithelial cells. When these cells are cultured on reconstituted basement membrane in the presence of lactogenic hormones, they form alveolar-like hollow spheres and differentiate to express milk proteins. Expression of two genes, *Hoxa-1* and *Hoxb-7*, was shown to be downregulated by exogenous basement membrane, suggesting that homeobox gene expression may be modulated *in vivo* by interactions between epithelial cells and components of the basement membrane. Such interactions have been shown to be critical for mammary differentiation and function, and tend to be altered on neoplastic progression [34–37].

Taking cue from these studies, Chen and Cappechi [38\*] demonstrated compromised mammary function in mutant mice carrying various deletions of paralogous genes *Hoxa-9*, *Hoxb-9* and *Hoxd-9*. Single mutant lines disrupted for either *Hoxa-9* or *Hoxb-9* (heterozygous at the remaining relevant *Hox* loci) showed only a small decrease in newborn survival, while the *Hoxd-9* disruption alone reduced survival to below 50%. Double mutant combinations showed synergistic interactions that reduced newborn survival well below the additive expected values for each single mutation, suggesting functional cooperativity. Dams with the most severely affected genotypes (*Aabbdd*, *aaBbdd* and *aabbdd*) demonstrated marked hypoplasia of the mammary gland after parturition with gland morphology resembling that of a midpregnant animal.

During embryogenesis, *Hoxb-9* and *Hoxd-9* are each expressed in condensed mammary mesenchyme sur-

rounding the epithelial bulb, suggesting roles in mesenchyme condensation or in modulation of epithelial–mesenchymal interactions during early mammary gland development [38\*]. Unfortunately, *in situ* hybridization against *Hoxa-9* and *Hoxb-9* using adult tissues has not been performed. However, Friedmann has examined expression of *Hoxd-9* throughout postnatal development and showed it to be expressed highly in periductal fibroblasts and ductal epithelium in the virgin, but only expressed weakly in these cell types during pregnancy. Interestingly, *Hoxd-9* showed enhanced expression in developing alveolar epithelium relative to subtending ducts, consistent with its apparent role in alveolar differentiation during pregnancy.

*In situ* hybridization has also demonstrated expression of *Hoxd-10* in both epithelium and stroma with elevated levels in the epithelium of developing and secreting alveoli. These observations suggested a role in lobuloalveolar differentiation or the transition from pregnancy to lactation. Consistent with this hypothesis, targeted disruption of *Hoxd-10* led to lactation failure in a significant percentage of homozygous mutant animals ([39]; Lewis MT, Daniel CW, unpublished results). In glands of affected animals, alveolar development progressed through late pregnancy but failed, in whole or in part, to make the transition to lactation. In severely affected animals, alveoli failed to expand and increased pup mortality was observed. Together with the data for the group 9 paralogs, these observations firmly establish a functional role for *Hox* genes in alveolar differentiation and function.

### ***Msx***

*Msx* is a small family of three related genes. In the mouse, at least two members (*Msx-1* and *Msx-2*) appear to play roles in mediating inductive tissue interactions during organogenesis. Only *Msx-1* and *Msx-2* have been examined in the mouse mammary gland [40\*,41\*].

Expression of *Msx-1* and *Msx-2* has been demonstrated in mammary buds during embryogenesis, with both genes expressed in the epithelium [41\*]. In contrast, in postnatal mice, *Msx-1* and *Msx-2* are expressed in reciprocal tissue compartments, with *Msx-1* expressed in the epithelium and *Msx-2* expressed in the periductal stroma (Fig. 2). The expression of *Msx-2* in the fat pad was dependent on the presence of mammary epithelium. Results are suggestive of a fundamental transition in epithelial–stromal signaling that leads to tissue compartment switching of *Msx-2* expression during organogenesis.

Expression studies also suggested a potential role in mediation of hormone responses. The *Msx-2* mRNA was downregulated in glands of ovariectomized and antiestrogen treated animals, and upregulated by estrogen replacement in ovariectomized animals. So far, *Msx-1*, *Msx-2* and *Msx-1/Msx-2* targeted disruption strains have been exam-

Table 1

## Summary of homeobox gene expression and function in mammary gland development and neoplasia

Gene family	Gene name <sup>a</sup>	Expression detected in <sup>b</sup> :	Method <sup>c</sup>	Expression altered in neoplasias?	Possible regulation?	Mammary or <i>in vitro</i> phenotype?	Reference
<i>Hox/HOX</i>	<i>Hoxa-1</i>	MG (UD) Scp2, CID-9	RT-PCR; Northern	OE (freq.)	Extracellular matrix components Retinoic acid		[31*,33*]
	<i>HOXA-1</i>	MCF7	RT-PCR				[63,30]
	<i>Hoxa-5</i>	CID-9	RT-PCR				[33*]
	<i>Hoxa-7</i>	MG	RT-PCR				[31*]
	<i>HOXA-7</i>	MG	RT-PCR				1
	<i>Hoxa-9</i>	MG	RT-PCR				[38*]
	<i>HOXA-10</i>	MCF7	RT-PCR				Vitamin D
	<i>Hoxb-6</i>	MG	RT-PCR; Northern	Lost			[31*]
	<i>HOXB-6</i>	MCF7	RT-PCR				[30]
	<i>Hoxb-7</i>	MG (E+S)	Northern; <i>in situ</i>	UE	Extracellular matrix components; developmental	OE in SkBr3 cells: bFGF induction; increased proliferation; decreased growth factor dependency	[33*,57]
	<i>Hoxb-8</i>	CID-9	RT-PCR				[33*]
	<i>Hoxb-9</i>	CID-9 E12.5 Embryo (MM)	RT-PCR; <i>in situ</i>			KO: lactational defects	[33*,38]
	<i>Hoxc-6</i>	MG	Northern	Lost	Estrogen (-); developmental		[31*]
	<i>HOXC-6</i>	MCF7; MCF7D; Hs578Bst; MCF10F; T47D	cDNA screen; RT-PCR		Variable		[57,65]
<i>Hoxc-8</i>	MG	Northern	Lost	Estrogen (-); developmental		[32]	
<i>HOXC-10</i>	MG	PCR of cDNA library				2	
<i>Hoxd-3</i>	MG (UD)	Northern	OE (rarely)			[32]	
<i>Hoxd-4</i>	MG (E)	In situ	Lost			[32]	
<i>Hoxd-8</i>	MG	Northern	Lost	Developmental		[32]	
<i>Hoxd-9</i>	MG (E+S); E12.5 Embryo (MM)	RT-PCR; Northern; <i>in situ</i>	Lost	Developmental	KO: lactational defects	[32,38*]	
<i>Hoxd-10</i>	MG (E+S)	Northern; <i>in situ</i>	Lost	Developmental	KO: lactational defects	[32,39]; 2	
<i>Hoxd-11</i>	MG (UD)	Northern	No			[32]	
<i>Hoxd-12</i>	MG	RT-PCR; Northern (UD)	OE (myc tumor)			[31*]	
<i>Msx</i>	<i>Msx-1</i>	E13.5 Embryo (E); MG (E)	Northern; <i>in situ</i>	Maintained but variable	Developmental	KO: none detected in embryos	[40*,41*]
	<i>Msx-2</i>	E13.5 Embryo (E); MG (S)	Northern; <i>in situ</i>	Lost	Estrogen; developmental	KO: MG fail to form	[40*]; 3
Iroquois related	<i>IRX-1</i>	MG	RT-PCR				[48*]
	<i>IRX-2</i>	MG; primary tumors	RT-PCR; Northern; <i>in situ</i>	Maintained. expression levels or splice form usage may be altered in some tumors	Developmental		[48*]

**Table 1 continued**

Gene family	Gene name <sup>a</sup>	Expression detected in <sup>b</sup> :	Method <sup>c</sup>	Expression altered in neoplasias?	Possible regulation?	Mammary or <i>in vitro</i> phenotype?	Reference
	<i>IRX-3</i>	MG	RT-PCR				[48*]
	<i>IRX-4</i>	MG	RT-PCR				[48*]
	<i>IRX-5</i>	MG	RT-PCR				[48*]
Paired domain	<i>Alx-4</i>	MG (S)	<i>In situ</i>		Expression associated with actively condensing periductal stroma	KO: none detected	[45]
<i>HSIX</i>	<i>HSIX-1</i>	MCF7		OE	Expressed in S phase of cell cycle	OE in MCF7 abolishes X-ray induced cell cycle arrest in G2 phase	[58*]
POU domain	<i>OCT-1</i>	MCF7;MCF10; SkBr3; MDA-MB453	RT-PCR; Western EMSA				[54]
	<i>Oct-1</i>	MG (lact.)	RT-PCR				[53]
	<i>OCT-2</i>	MCF7; MCF10; SkBr3	RT-PCR; Western EMSA				[54]
	<i>OCT-3</i>	MG (UD); MCF7; MCF10; SkBr3; primary tumors	RT-PCR	OE (freq.)		Form with a 5 amino acid deletion detected in MCF7 cells	[54]
	<i>Oct-3</i>	MG (lact.)	RT-PCR				[53]
	<i>OCT-11</i>	MG (UD) MCF7; SkBr3; MDA-MB453; primary tumors	RT-PCR; EMSA	OE (freq.)			[54]
	<i>Pit-rs1</i>	MG (lact.)	RT-PCR				[53]
	<i>Pou4f1 = Brn-3</i>	MG (lact.)	RT-PCR				[53]
	<i>Pou4f-rs1 = Brn-3R</i>	MG (lact.)	RT-PCR				[53]
	<i>Pou3f4 = Brn-4</i>	MG (lact.)	RT-PCR				[53]
	<i>Pou3f-rs1 = POU-III A</i>	MG (lact.)	RT-PCR				[53]
Engrailed related	<i>En-1</i>	MG	Northern	UE (freq.)	Developmental		[32]
	<i>En-2</i>	MG (UD)	Northern	UD		KO: none detected	[32]
<i>MOX</i>	<i>MOX1</i>	MG					[52]
<i>MEIS</i>	<i>MEIS2a</i>	MG	RT-PCR of cDNA library				4
	<i>MEIS2d</i>	MG	RT-PCR of cDNA library				4
	<i>MRG1a</i>	MG	RT-PCR of cDNA library				4

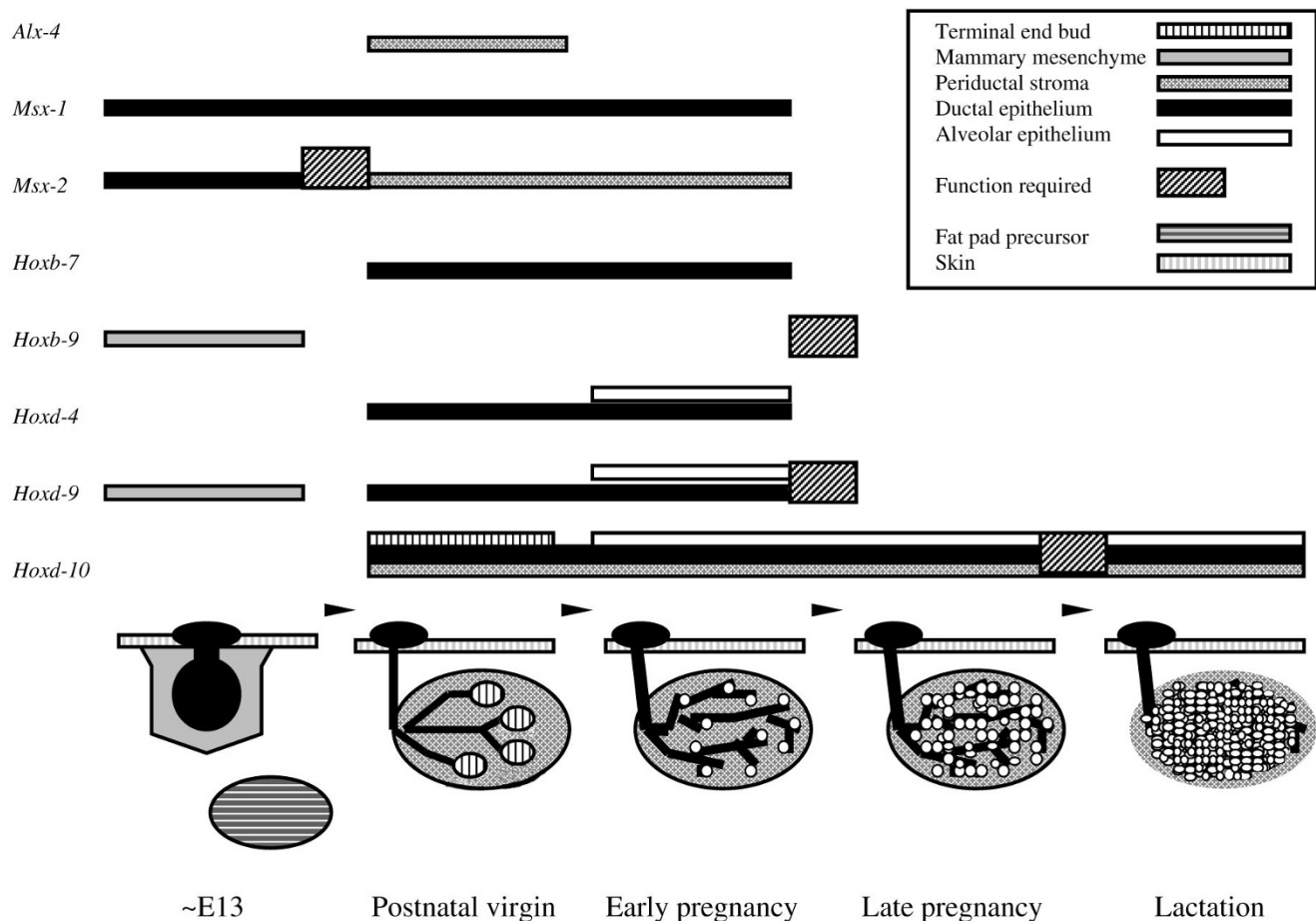
bFGF, basic fibroblast growth factor; E, epithelium; EMSA, electrophoretic mobility shift assay; freq., frequently; KO, knockout mutation; lact., lactating; M, mammary mesenchyme; MG, mammary gland; Northern, Northern blot assay; OE, overexpressed; RT-PCR, reverse transcriptase polymerase chain reaction; S, periductal stroma; UD, undetectable; UE, underexpressed.

<sup>a</sup>Human genes are uppercase; mouse genes are lowercase. <sup>b</sup>Cell lines are listed by their common designation. If expression was detected in the intact mammary gland, the tissue compartment is shown. <sup>c</sup>Not all detection techniques have been used on all tissue types/cell lines shown. <sup>1</sup>Friedmann Y, Daniel CW, unpublished results; <sup>2</sup>Lewis MT, Daniel CW, unpublished results;

<sup>3</sup>Maas R, unpublished results, cited in [41\*]; <sup>4</sup>Lewis MT, unpublished results.



Figure 2



Tissue distribution of homeobox gene expression through mouse mammary gland development. Selected stages of mammary gland development are depicted with reference to the expression patterns of several homeobox genes as demonstrated by *in situ* hybridization. Expression at a given stage is shown by a bar above the stage. Bars are pattern coded to represent a unique tissue compartment or epithelial structure. Transition points affected by a given homeobox gene mutation are denoted by a hatched box above the arrow representing the transition.

ined for mammary defects only during embryogenesis. No defects in embryonic mammary gland development were observed in *Msx-1* mutants but *Msx-2* and *Msx-1/Msx-2* animals reportedly showed developmental defects as early as E13.5 ([41\*]; Maas R, unpublished results cited in [41\*]).

As with other organs, data from the mammary gland are consistent with a role in mediation of tissue interactions. Because homozygous *Msx-1* and *Msx-2* mutations are embryonic lethal, analyses of *Msx* gene function in the adult mammary gland are largely absent. Transplantation rescue experiments and gland reconstitution assays using embryonic tissue should be extremely useful in further examining *Msx* gene function in the adult mammary gland [42–44].

#### Paired domain class

##### *Aristaless-like*

The *Aristaless-like* gene *Alx-4* is expressed in mesenchymal condensations of tissues whose development is

dependent on epithelial–mesenchymal interactions [45]. In the mammary gland, *Alx-4* expression was associated primarily with actively condensing stroma at the neck of terminal end buds (Fig. 2). *Alx-4* mutants failed to complement Strong's luxoid (*lst*) mutants and the two strains show similar defects [46,47]. Interestingly, mammary glands of *lst* mice appear normal, as do other ventral (but not dorsal) structures in which *Alx-4* is expressed [45–47], although it is not clear to what extent the mammary glands from these animals were analyzed.

#### Iroquois-related

At least five members of the Iroquois-related homeobox (*IRX*) gene family are expressed in the human mammary gland [48\*]. One member, *IRX-2*, was shown to be expressed in discrete epithelial cell lineages being found in luminal epithelium of both ducts and alveoli, but not myoepithelium. *IRX-2* also showed developmental regulation: expression was enhanced in terminal buds and

terminal lobules of the immature gland but uniformly distributed in mature glands. During lactation, some alveolar epithelial cells (~18%) showed reduced levels of mRNA relative to adjacent strongly expressing cells. Uniform epithelial expression was reestablished upon involution. Mutational analyses of these genes are underway in several laboratories.

#### **Engrailed-related**

Both Engrailed-related (*En*) *En-1* and *En-2* have been examined in the mouse mammary gland by Northern hybridization [32]. *En-2* was not detected in any tissue examined; in contrast, *En-1* was expressed at virtually constant levels through early pregnancy but was not detectable during late pregnancy or lactation. Homozygous *En-1* mutations are embryonic lethal. Because neither embryonic glands nor epithelial transplants have been examined, it remains possible that *En-1* serves a function in the mammary gland near the early→late pregnancy transition [49–51].

#### **Other homeobox genes**

Several other homeobox genes have been identified either in intact mammary glands or from cDNA library screens. These include members of the POU domain containing, *MOX*, and *MEIS* gene families ([52–54]; Lewis MT, Daniel CW, unpublished results). No functional or detailed expression data are yet available. Given the known importance of the POU and *MEIS* gene families in development and cancer, these should be among the first to be evaluated for mammary function.

#### **Intrinsic versus extrinsic function: the Forkhead transcription factor *Fkh-5/Mf3***

*Forkhead*-related genes encode transcription factors containing a divergent 'winged helix' homeodomain. One of these genes, *Fkh-5/Mf3*, was shown to be required for brain development and postnatal growth with most homozygous null animals dead at birth. Surviving adult homozygous females were unable to feed their pups, yet did not show overt defects in mammary gland morphology or secretory function. Further analysis showed a defect in the milk letdown response. This defect was alleviated by injection with oxytocin and was probably caused by improper hypothalamic development [55,56].

This example illustrates the important point that the mammary gland cannot be considered an isolated organ. Rather, it must be considered an organ profoundly influenced by, and dependent upon, proper neuroendocrine function. Given that many homeobox genes affect development of the central nervous system and endocrine organs, it becomes imperative to demonstrate experimentally (eg via transplantation) that observed mammary defects are intrinsic to the gland and are not a downstream consequence of an extrinsic physiologic dysfunction.

#### **Homeobox genes in mammary neoplasia**

Embryogenesis and oncogenesis share several common features. Among them, both processes require cell proliferation, modulation of cell death (apoptosis), cell motility, invasion of surrounding tissue and neovascularization. In embryogenesis, the cells that accomplish these tasks are generally relatively undifferentiated. Similarly, in cancer progression, cells that contribute to neoplasias tend to appear relatively undifferentiated, or dedifferentiated, as the case may be.

This consideration leads to the following hypotheses. If cells require accurate spatial and temporal regulation of homeobox gene expression during embryogenesis to acquire the proper differentiated state (identity), misexpression of homeobox genes could lead to failure of differentiation, loss of the differentiated state or adoption of an alternative cell identity (a homeotic transformation). The expression profile of homeobox genes (and their targets) may then be taken as a crude measure of the identity or functional state of a given cell. If so, misregulation of homeobox genes during neoplastic progression may be indicative of progressive alteration of epithelial cell identity. If such cells consequently possessed inappropriate characteristics (eg loss of cell cycle control, decreased apoptosis, altered cell–cell adhesions, altered hormone and growth factor responses or increased protease expression, to name a few), misexpression of homeobox genes could easily contribute to cancer initiation or progression.

While evidence has accumulated that altered homeobox gene function plays a causal role in development of other types of cancers, particularly leukemias, there is only circumstantial evidence for their involvement in mammary neoplasia. However, when taken in aggregate and in the context of cancer in other organs, a number of observations suggest a contributory role of homeobox genes in the initiation or progression of mammary cancer. Again, the more suggestive existing data can be easily recounted by gene family and are now summarized.

#### ***Hox/HOX***

Expression of all eight normally expressed homeobox genes examined (*Hoxb-6*, *Hoxb-7*, *Hoxc-6*, *Hoxc-8*, *Hoxd-4*, *Hoxd-8*, *Hoxd-9* and *Hoxd-10*) was lost on neoplastic progression in a selected population of mouse hyperplasias and tumors [32]. Conversely, three genes not normally expressed were activated in subsets of tumors (*Hoxa-1*, *Hoxd-3* and *Hoxd-12*).

Thus far, it appears that loss of function mutations simply affect developmental progression but that gain of function mutations impact neoplastic progression in unpredictable ways. For example, loss of function mutations of *Hoxa-9/Hoxb-9/Hoxd-9* or of *Hoxd-10* resulted in developmental failure, rather than tumor formation. As for gain



of function mutations, whereas overexpression of human *HOXA-10* in MCF7 cells promoted cell cycle arrest in G1, overexpression of murine *Hoxb-7* in SkBr3 mammary cancer cells caused increased proliferation and decreased growth factor dependency [57]. These observations suggest that the consequences of a given *Hox* gene mutation cannot be predicted *a priori*, but will depend entirely on the nature of the mutation and on the battery of downstream genes misregulated as a result.

### **HSIX1**

*HSIX1* is expressed toward the end of S phase of the cell cycle in MCF7 cells [22,58\*]. Overexpression of *HSIX1* in these cells abolished the G2 cell cycle checkpoint in response to irradiation, leading to inappropriate entry into mitosis. Interestingly, very low expression of *HSIX1* was observed in the normal mammary gland, but elevated expression was observed in 44% of primary breast cancers and in an astonishing 90% of metastatic lesions. These data represent the only known involvement of homeobox genes in mammary cell cycle control at the G2 checkpoint. Given that loss of this and other checkpoints is associated with mammary cancer [59–62], these data represent the strongest indication to date that homeobox genes may contribute to neoplastic progression in the mammary gland.

### **Teasers**

#### ***Msx***

*Msx-1* expression was maintained in all tumors examined [40\*]. In contrast, *Msx-2* became undetectable in neoplasias. Given its reportedly critical role in embryonic mammary gland development, loss or alteration of stromal *Msx-2* function in the adult mammary gland might be expected to have profound effects on interactions between the neoplastic epithelium and its associated stroma.

#### ***IRX***

Similar to the expression pattern observed for *Msx-1*, *IRX-2* expression was also maintained in all tumor types examined [48\*]. However, in some tumors, there was evidence of either increased expression or altered ratios of the two known transcripts. Since the *IRX-2b* transcript lacks the homeobox found in the *IRX-2a* transcript, target gene regulation may be expected to be altered.

#### **POU domain**

At least four POU class homeobox genes have been shown to be expressed in various human breast cancer cell lines, including OCT1, OCT2, OCT3 and OCT11 [54]. OCT3 and OCT11 were not detected in the normal human breast samples examined. Since OCT3 is generally considered to be an embryonic transcription factor, it is conceivable that these tumor cells adopted a more embryonic cell identity. Interestingly, Oct3 has also been detected in the normal mouse mammary gland during lactation [53], but the significance of this observation is not known.

### **Conclusion**

Important questions remain with respect to homeobox gene function in the normal and neoplastic mammary gland. The studies summarized in this review represent only a small fraction of the homeobox genes likely to be involved. What has become evident from the more extensive studies is that, whereas a given homeobox gene may be expressed in regulated patterns throughout development (eg *Msx-2*, *Hoxd-9*, *Hoxd-10*), mutation of that gene may overtly affect only a single transition point or developmental stage (Fig. 2). If this general observation holds for other homeobox genes, functional analysis will require *in vivo* phenotypic evaluation of mutant animals throughout mammary organogenesis, including embryogenesis.

Despite the temptation to try, a unified model for homeobox gene function in the mammary gland is unlikely to be developed given the diverse functions and complex interactions known for many of these genes. As a consequence, the mechanistic details of how the various homeobox genes dictate mammary gland identity, form and function will require years of effort and careful attention to information derived from other model systems, particularly *Drosophila*. However, a general framework can be postulated based on data gathered thus far and by analogy with known roles in other organs. In all likelihood, homeobox genes control mammary gland development and function by sequential activation or inactivation of specific sets of homeobox genes at specific developmental transition points. For example, *Hoxa9*, *Hoxb9* and *Hoxd9* appear to be important for alveolar development near midpregnancy, while *Hoxd10* appears to be required at the next major transition point, the onset of lactation. As already suggested by currently available expression data, layered on top of this temporal regulation will no doubt be tissue specific and cell type specific regulation of gene function (Fig. 2).

With respect to normal development, it is interesting to recount what homeobox gene mutations have not been reported to cause in the mammary gland (yet?). So far, no alterations in nipple placement (patterning and cell identity), no alterations in mammary gland number (patterning and cell identity), no homeotic transformation of mammary tissues to other ectodermally derived tissues (eg those of the sweat or sebaceous glands, skin or hair follicle) and no demonstrated function during postnatal virgin development or early pregnancy have been observed.

As for a possible role in the development of mammary cancer, the initial case is far weaker than one might hope. However, lack of solid evidence at this early stage should not be taken as an indication that the hypothesis is fundamentally flawed. There are still a huge number of targeted disruption strains as well as appropriately engineered strains that overexpress candidate oncogenic homeobox

genes to be made and examined. For example, notably absent from current studies, or poorly understood, are those gene families that have been implicated causally in development of other types of cancer, including *PBX*, *PAX* and *MEIS*.

Homeobox genes clearly represent attractive candidates for the control of mammary development and neoplastic progression. Unfortunately, given the limitations recounted in the introduction of this review, analysis of homeobox gene expression and function in mammary gland development and neoplasia will be an exceptional undertaking. Fortunately, the mammary gland has several advantages over many other developmental models that bypass many of these potential problems. Identification of target genes, upstream regulators and cofactors will be enhanced significantly by the coupling of mouse developmental genetics with powerful gene expression analysis techniques such as DNA microarray and real-time reverse transcription polymerase chain reaction, for which the mammary gland provides ample material. The problem of embryonic or perinatal lethality, particularly with animals carrying combinatorial mutations, can be addressed using two powerful approaches. First, engineered mouse strains carrying tissue-restricted gene disruptions or overexpressing transgenes can be generated, which should allow genetic analyses of otherwise lethal or detrimental mutant combinations. Second, transplantation techniques have been developed that allow transplantation of mammary epithelium or intact mammary glands from embryonic and postnatal sources into wild type host animals. Such manipulations allow development of otherwise moribund mammary tissue in a physiologically relevant *in vivo* setting.

The study of homeobox gene function in the mammary gland is truly a daunting task but, given what we already know, it should be well worth the effort.

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