ALX4 Expression in the Normal Breast and in Breast Cancer

by

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Laboratory Medicine and Pathobiology
University of Toronto

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2008

Abstract

Aristaless-like homeobox 4 is a homeodomain transcription factor that has important functions during mouse development. A recent report demonstrated that Alx4 expression is required in periductal stromal cells in the mouse mammary gland for normal mammary morphogenesis. To test the hypothesis that ALX4 is expressed in the normal human breast, and this expression is altered in breast cancer, immunohistochemistry was performed on normal and breast cancer tissue and breast tissue microarrays. In the normal breast, ALX4 was expressed in stromal fibroblasts and luminal epithelial cells, but not in myoepithelial cells. Expression was lost in breast cancer in both cell compartments. Upon global demethylation induced by 5-aza-2’-deoxycytidine, normal and breast cancer cell lines expressed ALX4, suggesting that hypermethylation may repress expression of ALX4 during malignant transformation of the breast. These results demonstrate that ALX4 may be used as a biomarker for breast cancer, and may act as a tumour suppressor.
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<th>Description</th>
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<tr>
<td>5-AZA-dC</td>
<td>5-aza-2’-deoxycytidine</td>
</tr>
<tr>
<td>ABCC6</td>
<td>ATP-binding cassette, subfamily C, member 6</td>
</tr>
<tr>
<td>ADH</td>
<td>atypical ductal hyperplasia</td>
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<tr>
<td>Alx</td>
<td>aristaless-like homeobox</td>
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<td>Alx4/ALX4</td>
<td>aristaless-like homeobox 4</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B cell</td>
<td>bone marrow cell</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1, early onset</td>
</tr>
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<td>BRCA2</td>
<td>breast cancer 2, early onset</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDH1</td>
<td>cadherin 1, type 1, E-cadherin (epithelial)</td>
</tr>
<tr>
<td>CDH13</td>
<td>cadherin family member 13</td>
</tr>
<tr>
<td>Chx10/Vsx2</td>
<td>visual system homeobox 2</td>
</tr>
<tr>
<td>CK</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine guanine dinucleotide, linked by a phosphate bond</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>Csfr1/CSF1</td>
<td>colony stimulating factor 1</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DME/DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>dpc</td>
<td>days post-coitum</td>
</tr>
<tr>
<td>DPP IV</td>
<td>dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
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<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GST</td>
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<td>hALX4 N term</td>
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<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<td>HMEC</td>
<td>human mammary epithelial cell</td>
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<td>Hox/HOX</td>
<td>homeobox</td>
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<tr>
<td>HPP1/TMEFF2</td>
<td>transmembrane protein with EGF-like and two follistatin-like domains 2</td>
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<tr>
<td>IDC</td>
<td>invasive ductal carcinoma</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MEC</td>
<td>mammary epithelial cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>MG</td>
<td>mammary gland</td>
</tr>
<tr>
<td>MINT1/APBA1</td>
<td>amyloid beta (A4) precursor protein-binding, family A, member 1</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumour virus</td>
</tr>
<tr>
<td>MS-APPCR</td>
<td>methylation-sensitive arbitrarily primed PCR</td>
</tr>
<tr>
<td>MSP</td>
<td>methylation-specific PCR</td>
</tr>
<tr>
<td>MSX2</td>
<td>msh (muscle segment homeobox) homeobox 2</td>
</tr>
<tr>
<td>MT1A</td>
<td>metallothionein 1A</td>
</tr>
<tr>
<td>p16/CDKN2A</td>
<td>cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFM</td>
<td>parietal foramina</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>Prrx</td>
<td>paired related homeobox</td>
</tr>
<tr>
<td>PSS</td>
<td>Potocki-Shaffer syndrome</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PyMT</td>
<td>polyoma virus middle T-antigen</td>
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<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
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<tr>
<td>RASSF1A</td>
<td>ras association domain family member 1</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPRM</td>
<td>reprimo, TP53 dependent G2 arrest mediator candidate</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>Rx1</td>
<td>retinal homeobox 1</td>
</tr>
<tr>
<td>SALL3</td>
<td>sal-like 3</td>
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<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
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<tr>
<td>STK11</td>
<td>serine/threonine kinase 11</td>
</tr>
<tr>
<td>T cell</td>
<td>thymus cell</td>
</tr>
<tr>
<td>TDLU</td>
<td>terminal ductal lobular unit</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end bud</td>
</tr>
<tr>
<td>TGFα</td>
<td>transforming growth factor alpha</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarray</td>
</tr>
<tr>
<td>TP53</td>
<td>tumour protein p53</td>
</tr>
<tr>
<td>ZPA</td>
<td>zone of polarizing activity</td>
</tr>
<tr>
<td>UDH</td>
<td>usual ductal hyperplasia</td>
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</table>
Introduction

Mammogenesis is a multi-stage process in which key changes coincide with stages of embryonic, sexual and reproductive development. Mammary gland development initiates during embryogenesis and resumes in the female with rapid changes at puberty. In adult life, constant remodeling during the estrous cycle and during pregnancy entails continuous cycles of proliferation and regression. Ultimately, a lactating gland is produced to fulfill the primary function of this organ: to synthesize milk for the nourishment of offspring. In the human breast, the dynamic process of morphogenesis engenders frequent opportunities for aberrances to arise in development which may increase susceptibility to carcinogenesis. Such changes, when they occur, must be checked by cell repair mechanisms, or may otherwise lead to breast cancer.

The mouse mammary gland is often used as a model for the human breast, and a basic understanding of both mouse and human mammogenesis throughout the various stages is necessary in order to study the development of breast cancer. After first considering mammary morphogenesis in the mouse and human, I will compare the architecture of the adult mouse and human mammary glands. Patterning of the mammary gland requires reciprocal interactions between stromal and epithelial cell compartments. To demonstrate the fundamental role of intercellular signaling networks in the mouse and human mammae, I will focus on the influence of the microenvironment on development of the glandular epithelium. Finally, I will discuss how aberrant expression and signaling play a role in breast cancer, and address mechanisms that may contribute to changes in normal expression.

The aim of this project is to illustrate the importance of ALX4 expression in the normal human breast, its aberrant expression in breast cancer and the modulation of this expression by
epigenetic mechanisms. My results will demonstrate that loss of ALX4 expression may be used as an early biomarker for breast cancer, and that ALX4 may function as a tumour suppressor whose expression is silenced by methylation.

1 Mouse mammary gland development

1.1 Embryonic development

During mouse embryogenesis, mouse mammary gland development initiates as a result of reciprocal signaling between the epithelial and mesenchymal cell compartments. At 10 to 11 days post-coitum (dpc), an elevated epidermal mammary line can be seen extending from the anterior to the posterior limb bud (Figure 1; Richert et al., 2000). Five pairs of epithelial placodes form at defined positions along the anterior-posterior axis via fragmentation of the mammary lines (Veltmaat et al., 2004). These localized epithelial thickenings appear in a precise order around 11 dpc: pair 3, pair 4, pairs 5 and 1, then pair 2 (Maireux et al., 2002; Veltmaat et al., 2004). By 12 dpc, the placodes transform into spherical buds which increase in size by migration of cells from the epidermis (Veltmaat et al., 2003). These buds invaginate the underlying mesenchyme and enter a resting phase for 24 hours. During this time, the adjacent fibroblasts within the mesenchyme condense around the buds (Kimata et al., 1985). By 13 dpc, a concentric layer of specialized fibroblasts two or three cells thick can be seen surrounding the epithelial buds (Kratochwil, 1969).

Sexual dimorphism in mouse mammary gland development first becomes apparent at this stage. At 13.5 dpc, in the male embryo, mesenchymal cells under the influence of testosterone condense around the neck of the epithelial bud, disconnecting it from the epidermis, and inducing apoptosis in these cells (Durnberger et al., 1978; Kratochwil and Schwartz, 1976). In the female embryo, the buds are no longer externally visible.
Figure 1. Embryonic development of the mouse mammary gland. An anterior-posterior elevated milk line first appears between 10 and 11 dpc. As a result of signalling from the mesenchymal compartment, the mammary line resolves into epithelial placodes. Migration of cells from the epidermis transforms the epithelium into buds. Cells from the adjacent primary mesenchyme condense around the epithelial structure to form a thin concentric layer, distinct from the more distal secondary mesenchyme. In the male, mammogenesis arrests at this stage as the mammary mesenchyme separates the bud from the epidermis, resulting in necrosis of the epithelial tissue. In the female, the secondary mesenchyme which will eventually develop into the mammary fat pad signals to the epithelium, which proliferates, forming a mammary sprout which grows toward the presumptive fat pad. By 18.5 dpc, the sprout has ramified to form a rudimentary ductal tree that is present at parturition.

At 14 dpc, a more distal secondary mesenchyme condenses to form the fat pad precursor, which consists of a pluripotent population of stem cells that may differentiate into fat, fibroblast, nerve and endothelial cells (Sakakura et al., 1987; Sakakura et al., 1982). Around 15 dpc, this fat pad precursor accumulates clusters of fat cells separated by connective tissue septa (Hovey et al., 1999). As the secondary mesenchyme of the presumptive fat pad signals to the epithelium, epithelial development resumes at 15.5 dpc with rapid cell proliferation at the tip of the epithelial bud (Veltmaat et al., 2003). A mammary sprout thus forms and grows towards the fat pad.
precursor. Within the presumptive fat pad, fat cells gradually accumulate lipid (Hovey et al., 1999).

The epithelial sprouts elongate and early branching gives rise to a rudimentary ductal tree with 10-20 branches, present at parturition (Veltmaat et al., 2003). Mesenchyme induces differentiation of the nipple skin from the epidermis a few days before birth (Foley et al., 2001). The proximal regions of the partially canalized branches fuse to form the primary duct, which opens at the skin surface via the nipple (Veltmaat et al., 2003). Thereafter, mammary ducts elongate slowly to match the growth of the animal. This period of relative quiescence precedes the rapid morphological changes that occur in response to hormonal influences at puberty.

1.2 Pubertal development

At puberty, estrogen released from the ovaries stimulates formation of terminal end buds (TEBs) at the tips of growing ducts (Figure 2; Silberstein et al., 1994). The TEB is a bilayer structure consisting of an inner mass of body cells surrounded by an outer layer of cap cells (Williams and Daniel, 1983). Body cells are believed to give rise to the inner layer of luminal epithelial cells of the subtending duct, while cap cells give rise to the outer layer of myoepithelial cells (Silberstein, 2001a). Cap cells are also thought to contribute to the luminal epithelial layer, and may include pluripotent stem cells which can differentiate into the multiple cell types which constitute the mammary gland (Williams and Daniel, 1983). Apoptosis of the innermost body cells occurs progressively in TEBs, forming lumens within the ducts (Humphreys, 1999; Humphreys et al., 1996).

Proliferation of epithelial cells within the TEBs and migration of these cells allow ductal extension into the fat pad (Williams and Daniel, 1983). Branching morphogenesis proceeds by cell proliferation, differentiation and migration, remodelling of the ducts and the extracellular
matrix, ductal elongation, and bifurcation of the TEBs until the ducts have reached the limits of the fat pad. Differentiated myoepithelial cells produce the basement membrane, and periductular fibroblasts synthesize components of the stroma (Richert et al., 2000). As growth proceeds, spacekeeping becomes apparent as TEBs which encounter other end buds or the limits of the fat pad regress (Williams and Daniel, 1983). These end buds become quiescent terminal ducts which are encased by basement membrane and stroma (Richert et al., 2000).

**Figure 2. Postnatal mouse mammary morphogenesis.** A From parturition until the onset of puberty, a rudimentary ductal tree (arrowhead) is present in the mammary gland. At puberty, under the influence of circulating estrogen released from the ovaries, terminal end buds (TEBs; asterisks) form at the tips of growing ducts. Growth proceeds by proliferation, differentiation, migration, elongation, remodeling and bifurcation of the TEBs until the ducts have reached the limits of the fat pad, by the end of puberty. B The terminal end bud is a bilayer structure that consists of an outer layer of cap cells and an inner layer of body cells. Body cells give rise to the luminal epithelial cells of the subtending duct while cap cells give rise to the myoepithelial layer, and may also contribute to the luminal cell layer. The TEB is surrounded by a loosely associated layer of stromal cells within the extracellular matrix.
1.3 Adult development

In response to the estrous cycle, the adult virgin mammary gland undergoes minor changes with the formation of lateral and alveolar buds (Richert et al., 2000). Continual cycles of proliferation and differentiation followed by apoptosis maintain tissue size homeostasis until pregnancy (Andres and Strange, 1999; Robinson et al., 1995).

During pregnancy, lobuloalveolar development proceeds under the influence of progesterone and prolactin (Brisken et al., 1999; Brisken et al., 1998). Following side-branching, alveolar buds form and differentiate into alveoli, which begin to secrete milk proteins and lipids one day prior to parturition (Richert et al., 2000). During lactation, fat from the stromal compartment is metabolized and the alveoli expand with milk (Elias et al., 1973). In response to the pituitary hormone oxytocin, the myoepithelial cells contract, forcing milk from the alveoli into the ducts (Emerman and Vogl, 1986; Leng et al., 2005).

Following approximately three weeks of lactation, weaning results in involution of the mammary gland (Elias et al., 1973). Epithelial cells within secretory alveoli undergo apoptosis, and the occupied area is replaced by increasing amounts of fat and stroma (Quarrie et al., 1995; Quarrie et al., 1996; Strange et al., 1992). This remodeling proceeds for a period of 21 days after weaning until the mammary gland of the parous animal resembles that of the adult virgin, with the persistence of some alveoli (Richert et al., 2000).

1.4 Cell populations in the adult virgin mouse mammary gland

The adult virgin mammary gland comprises a bilayer ductal structure consisting of an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells delimited by a closely applied basement membrane (Figure 3; Williams and Daniel, 1983). In addition, basal
cells located near the basement membrane are believed to act as stem cells of the mammary gland (Sonnenberg et al., 1986; Stingl et al., 2006). The parenchyma is situated in a stromal context which includes cellular and acellular components, namely adipocytes, fibroblasts, various migratory blood cells, endothelial and nerve cells, as well as collagens, proteoglycans and glycoproteins (Silberstein, 2001b).

Reciprocal signalling between the mammary epithelium and stromal constituents determine in large part the development of both the mouse and human mammae. While the mouse offers a useful and manipulable system to study these interactions, important interspecies differences exist in mammary development which will be addressed prior to further discussion of the stromal influence on mammary morphogenesis and breast cancer. The next section describes human breast development from the fetus to adult life, and is followed by a comparison of the mouse and human mammae.

Figure 3. Comparison of the mouse and human mammary glands. Haematoxylin and eosin staining illustrates differences between the microenvironments of the mouse and human mammary glands. In the mouse, bilayer ducts are delimited by a thin layer of connective tissue (arrowheads) and loosely associated stromal cells, surrounded by a large population of adipocytes (A). In contrast, the functional unit in the human breast, termed the terminal ductal lobular unit, consists of ductules embedded in a loose intralobular stroma (intra). Lobules are separated by a dense fibroconnective, largely acellular, interlobular stroma (inter). Differences in cell microenvironment between the human and mouse mammary glands may imply differences in expression patterns and cell-cell interactions, which may help to explain interspecies variation in morphogenesis and aberrations of normal development.
2 Human breast development

The mouse and human mammary glands are highly similar in structure and function but key differences exist during patterning as well as in the architecture of the adult tissue (Howard and Gusterson, 2000; Parmar and Cunha, 2004). As it is for the mouse, human mammary development is a dynamic process in which remodeling is mediated by hormones and growth factors. Moreover, broad variations in the size, shape and rate of breast morphogenesis suggest the interplay of multiple elements (Howard and Gusterson, 2000). This section describes the morphological changes that occur during normal breast development. As a multitude of genetic, epigenetic and environmental factors are known to play a role in breast tumourigenesis and cancer progression, these will be addressed hereafter in a discussion of breast cancer.

2.1 Prenatal development

Human breast development initiates in the embryo after the three major body axes are established. While morphogenesis typically begins during the fourth week of gestation (Parmar and Cunha, 2004), subsequent patterning events show high temporal variability as they occur primarily in relation to fetal size, not age (Howard and Gusterson, 2000); therefore, prenatal development is simply described here in a sequential manner.

In both males and females, a milk line or mammary crest first appears as a condensation of epithelial cells in the thoracic region of the embryo (Figure 4; Howard and Gusterson, 2000; Parmar and Cunha, 2004). The epithelium forms a nodule that evaginates into the underlying mesenchyme, which closely surrounds the growing breast bud (Howard and Gusterson, 2000). During the bud stage, deeper mesenchymal cells further condense around the enclosed epithelial bud. A characteristic pattern of vascularization is observed, and hair follicle formation in the vicinity of the breast anlagen is inhibited. In the center of the solid breast bud, a cleft forms then
deepens, and the bud begins to branch. Nipple formation initiates from the epidermis, and mesenchymal cells differentiate into fibroblasts, smooth muscle cells, endothelial cells and adipocytes (Parmar and Cunha, 2004). As the lumen enlarges, secondary buds appear, producing a cloverleaf shape (Howard and Gusterson, 2000). These buds elongate, canalize and branch, forming sprouts which invade the mesenchyme. Ducts are separated by islands of fat within the connective tissue by six months of gestation (Parmar and Cunha, 2004).

Figure 4. Fetal and infant development of the human breast. An elevated mammary milk line appears around 4 weeks of fetal life. Epithelial cells form a nodule, which penetrates the underlying mesenchyme. The nodule grows into a bud, surrounded by a closely applied mesenchyme around which deeper mesenchymal cells condense. A cleft forms in the bud and deepens as the bud branches. Canalization and ramification of the epithelial structure result in sprouts which invade the mesenchyme. Mesenchymal cells differentiate into fibroblasts, smooth muscle cells, endothelial cells and adipocytes. At birth, the mesenchyme proliferates to form the everted nipple. The ductal system may consist of rudimentary ducts or well-developed acini surrounded by a specialized intralobular stroma (intra) which includes cells different than those in the interlobular stroma (inter). Both rudimentary and more complex acinar structures may be secretory. During the first 2 years of life, the epithelium differentiates and involutes. Further development resumes in girls at puberty.
2.2 Infant development

At birth, in both boys and girls, the ductal system opens onto the skin surface. Proliferation of the underlying mesenchyme and the surrounding skin gives rise to the everted nipple and areola, respectively (Howard and Gusterson, 2000). Epithelial tissue is surrounded by a loose connective tissue separate from the dense collagenous tissue of the rest of the breast (Anbazhagan et al., 1991). Extensive variation in breast development is observed, with the presence of simple ducts or specialized acini distended with secretions that may be discharged from the nipple in both sexes (Figure 4; Anbazhagan et al., 1991; Howard and Gusterson, 2000). In breast tissue with developed acini, an encasement by the surrounding loose, cellular connective tissue of the specialized intralobular stroma is observed distinct from the largely acellular interlobular stroma.

During the first two years after birth, the lining epithelium differentiates then involutes (Anbazhagan et al., 1991; Howard and Gusterson, 2000). Thereafter, small ductal structures remain quiescent in a fibroblastic stroma until puberty, when female breast development proceeds and male breast development is arrested (Howard and Gusterson, 2000).

2.3 Pubertal development

At puberty, both the epithelium and stroma, which consists of fibrous and fatty tissue, resume growth in adolescent girls (Howard and Gusterson, 2000). Following the proliferation of connective tissue, ductal extension proceeds by elongation and both dichotomous and sympodial branching (Russo and Russo, 1987). Blunt-ended ducts branch and acquire bulbous and solid TEB-like tips, which show high proliferation (Howard and Gusterson, 2000; Monaghan et al., 1990). Primary ducts that emanate from the nipple give rise to subsidiary ducts, which lead to segmental ducts and smaller subsegmental ducts, which in turn lead to terminal ducts that give
rise to blind-ended ductules (Figure 5; Howard and Gusterson, 2000). The functional unit of the breast, known as a terminal duct lobular unit (TDLU), consists of the ductules arising from a terminal duct and their closely associated intralobular stroma. Growth continues at the periphery of the epithelium as it expands within the fat pad. Lobules remain separated from one another by interlobular stroma.

### 2.4 Adult development

Growth proceeds throughout adult life with increases in connective tissue preceding ductal extension (Parmar and Cunha, 2004). Ductules within the TDLU are surrounded by a layer of fibroblasts (Eyden et al., 1986). Continuous changes occur during each menstrual cycle, with sequential proliferation and apoptosis of the epithelial cells (Ferguson and Anderson, 1981). Stroma, which also responds to hormonal influences, may eventually occupy 80% of the adult breast (Figure 3; Hovey et al., 1999; Figure 3; Parmar and Cunha, 2004).

During pregnancy and lactation, lobules increase in number and fat is lost within the breast (Howard and Gusterson, 2000). Less differentiated lobular structures coexist alongside secretory acini (Russo and Russo, 1987). Throughout lactation, acini are dilated and contain milk, which is secreted through the nipple. At weaning, involution occurs and secretory epithelial cells undergo apoptosis and phagocytosis, resulting in a decrease in the number of acini.

### 2.5 Postmenopausal involution

In the postmenopausal breast, fewer lobules and ducts are present (Howard and Gusterson, 2000). Intralobular stroma is replaced by collagen, while the epithelium and interlobular stroma are replaced by adipose tissue. A few acini and ducts, as well as thin strands of collagen, remain within the fat.
**Figure 5. The adult human breast.** Ducts emanate from the nipple and branch into smaller and smaller ducts during puberty. The smallest true duct is the terminal duct, which has an intralobular and an extralobular portion. The intralobular portion of the terminal duct and its associated ductules are surrounded by a loose intralobular stroma, and this functional unit is termed the terminal ductal lobular unit (TDLU). The specialized intralobular stroma is distinct from the dense interlobular stroma. In contrast to interlobular fibroblasts, fibroblasts in the intralobular stroma do not express DPP IV or TGFα. In addition, intralobular fibroblasts express lower levels of type XIV collagen than do interlobular fibroblasts. **FRONT** and **SIDE** images from http://www.mayoclinic.com.

2.6 Cell populations in the adult human breast

In the human breast, ducts are a bilayer epithelial structure consisting of an inner layer of luminal cells, separated from the basement membrane by a surrounding layer of myoepithelial cells (Howard and Gusterson, 2000). In acini, the luminal cells may contact the basement membrane between the processes of the discontinuous layer of flattened myoepithelial cells (Hamperl, 1970). Basal clear cells are present near terminal ducts and elsewhere in the breast epithelium, and have been proposed to function as putative stem cells (see below; Smith et al., 1984). The stromal cell compartment includes fibroblasts, adipocytes, macrophages, mast cells, neutrophils, T and B lymphocytes and blood vessel cells (Cabezon et al., 2007; Howard and Gusterson, 2000).

A key difference between mouse and human mammary cell populations relates to the structure of the human breast. Fibroblasts form an important component of the specialized intralobular stroma that surrounds TDLUs, separating the glandular epithelium from the more distant interlobular stroma (Howard and Gusterson, 2000). Interlobular, but not intralobular,
fibroblasts express the cell-surface peptidase dipeptidyl peptidase IV (DPP IV; Atherton et al., 1992) and the polypeptide transforming growth factor alpha (TGFα; Howard and Gusterson, 2000). In addition, interlobular fibroblasts synthesize more type XIV collagen than do intralobular fibroblasts (Atherton et al., 1998). These distinct fibroblast cell populations have not been noted in the mouse mammary gland. The interspecies differences in architecture and gene expression may have additional implications with respect to intercellular interactions. In the next section, a detailed comparison of the mouse and human mammary glands is provided.

3 Architectural differences between the mouse and human mammae

Differences between the mouse and human mammae first become evident during early patterning events. In the mouse, the functional unit of the mammary gland is the duct, a bilayer epithelial structure which is formed during puberty (Williams and Daniel, 1983). In the human, this unit is known as the terminal ductal lobular unit (TDLU) and has a considerably more complex structure, which fulfills the same function as the mouse duct (Howard and Gusterson, 2000). The TDLU includes the intralobular portion of a terminal duct and its associated lobules, all of which are typically bilayer structures. In later stages of development, differences in growth and differentiation also become apparent. During pregnancy in the female mouse, alveolar buds develop from the duct and undergo functional differentiation, allowing the production of milk one day prior to parturition (Richert et al., 2000). In contrast to this situation, the human TDLU is influenced by maternal hormones, and both sexes may produce secretions (‘witch’s milk’) from differentiated lobuloalveoli in the first few months of early life (Figure 4; Russo and Russo, 1987). Involution of the lobuloalveoli occurs shortly thereafter, and no further functional differentiation is seen until puberty (Anbazhagan et al., 1991; Howard and Gusterson, 2000). In
adulthood, the female breast becomes lactational during pregnancy with the development of secretory lobuloalveoli (Russo and Russo, 1987).

Important differences exist not only in the glandular epithelium, but also in the stromal compartments of the mouse and human mammary glands. To explain these interspecies differences, it has been proposed that the abundance of connective tissue has evolved to physically support the mammary gland with respect to weight and position within the mammal (Hovey et al., 1999). Upon histological examination, architecture of the stromal component of the mouse mammary gland is quite simple compared to that of the human breast (Figure 3). In the mouse, stroma is rich in adipose tissue with a relatively minor contribution from fibroblasts and connective tissue, which surround the ducts in a thin sheath (Hovey et al., 1999). In the human breast, TDLUs are surrounded by an abundance of intralobular connective tissue interspersed with fibroblasts. Therefore, glandular epithelial cells are not proximal to adipocytes as they are in the mouse. As a consequence, expression patterns and paracrine interactions may show variation between the mouse and the human mammae.

Experiments in nude mice have, in fact, demonstrated the species specificity of stromal-epithelial interactions. In mammary gland transplantation studies in which human mammary tissue was transplanted into the mouse fat pad, the human epithelial component survived but failed to display outgrowth (Sheffield and Welsch, 1988). Later experiments in which collagen-embedded fibroblasts of mouse or human origin or human fibroblasts alone were used to alter the stromal context allowed the successful outgrowth of human tissue in the mouse mammary fat pad (Parmar et al., 2002; Proia and Kuperwasser, 2006), underlining the importance of signaling between the two cell compartments. These experiments unequivocally demonstrated that interspecies dissimilarities in architecture of the mammary gland can imply differences in
stromal-epithelial interactions, which are important during normal breast development as well as in tumourigenesis. Therefore, a careful analysis is necessary to extrapolate from the mouse model to the human system. Specific stromal-parenchymal interactions in the normal and transformed mammary glands of the mouse and human will be addressed shortly. Prior to this discussion, some background on breast cancer is provided.

4 Breast cancer

Breast cancer is a pleiotropic disease that results from uncontrolled cell growth. One in nine women in Canada will develop breast cancer over the course of a lifetime, and one third of these women will die from this disease (CCS/NCIC 2008). Yet, the precise etiology of breast cancer is still unknown and largely confounded by the fact that it is a heterogeneous disease. Breast cancer initiates as a result of transforming events in a single cell, and progresses via the accumulation of additional changes which allow clonal expansion and selection (Burstein et al., 2004). These events first manifest as ductal hyperproliferation, which precedes ductal carcinoma in situ (DCIS). The transition from DCIS to invasive ductal carcinoma (IDC) occurs with the loss of the myoepithelial layer and the basement membrane (Polyak, 2007). Locally invasive breast cancer may eventually metastasize to secondary sites as determined by additional alterations.

Research into pathways and mechanisms underlying tumourigenesis, cancer progression and metastasis has provided tremendous insight into treatment options. However, a new paradigm emerging in cancer theory may help to explain why there is currently no cure for breast cancer. Two competing ideas, the clonal evolution and cancer stem cell theories, are described below.
4.1 Cancer theory

A long-held notion in cancer biology is that cancer arises in differentiated cells that accumulate a series of changes that confer increased growth and proliferation properties, and decreased susceptibility to programmed cell death (Figure 6; Hanahan and Weinberg, 2000). Loss of tumour suppressor expression or activity, overexpression or hyperactivity of oncogenes and elusion from apoptosis have been documented for a variety of cancers. These acquired characteristics allow transformed cells to survive past DNA repair mechanisms, progress through cell cycle checkpoints, outgrow normal cells, clonally expand to produce tumours, and eventually colonize secondary sites.

Figure 6. Clonal evolution versus cancer stem cell theories. In clonal evolution theory, a normal cell undergoes a series of successive alterations that confer selective growth advantages to the evolving cancer cell. Enhanced proliferation allows cancer cells to clonally expand to produce a tumour. Additional changes are required to allow invasion and metastasis. In cancer stem cell theory, a normal stem cell undergoes an alteration, resulting in a cancer stem cell. The cancer stem cell and its affected pluripotential progeny may self-renew. Progenitor cells acquire additional alterations that are passed on to daughter cells. The inherent self-renewing capacity of cancer stem and progenitor cells allows the exponential expansion of differentiated and undifferentiated cancer cells.
This classical clonal evolution theory has proven useful for the temporary treatment of a variety of cancers, allowing extended patient survival. However, these ideas concerning the etiology of cancer ignore the fact that cells require long periods of time to accumulate these series of changes, and most cells are eliminated in much shorter intervals during normal tissue renewal processes. Furthermore, accumulating data concerning patient treatment, recovery and relapse have demonstrated that chemotherapy and radiotherapy are unsuccessful as permanent cure solutions.

A newer body of evidence has suggested an alternate mechanism for oncogenesis, involving cells that are few in number, have a long lifespan and proliferate infrequently, escaping obliteration by current therapeutic methods that target numerous rapidly dividing cells (for a recent review, see Wicha et al., 2006). Research has shown that these cancer stem cells resemble and likely derive from normal tissue stem cells or progenitor cells in which mechanisms of self-renewal have been dysregulated. Both cell types are self-renewing, pluripotential, and divide infrequently. By symmetric division, a stem cell may produce two daughter stem cells (Figure 6); this process is believed to occur relatively frequently in cancer, leading to the exponential clonal expansion of stem cells, their committed lineages and the resulting terminally differentiated cells. During asymmetric division, each stem cell gives rise to a duplicate stem cell as well as a progenitor stem cell. Progenitor cells have a more limited self-renewal capability and can produce two daughter progenitor cells or, by asymmetric division, may ultimately produce a committed or differentiated cell type in addition to a like progenitor cell. Dysregulation of self-renewal processes may occur at the level of progenitor cells as well. In the breast and in breast cancer studies, researchers have developed several techniques to address the intrinsic ‘stemness’ properties of cells. Serial passage, transplantability, and the detection of unique stem cell surface
markers allow the identification of stem cells or cancer stem cells. Using these techniques, stem cells in the breast have been identified, and these are discussed below in more detail.

4.2 Mammary stem cells

Mammary morphogenesis occurs throughout life and requires frequent expansions of the local cell population. Stem and progenitor cells in the mammary gland fulfill this function, allowing a rapid increase in the number of cells, which subsequently differentiate into luminal, myoepithelial and alveolar cell types (Charaf-Jauffret et al., 2008). Multi-parameter sorting in conjunction with serial transplantation experiments in the mouse mammary gland allowed the definitive identification of $CD45^{-} Ter119^{-} CD31^{-} Sca-1^{low} CD24^{med} CD49f^{high}$ adult mouse mammary stem cells capable of individually regenerating an entire mammary gland $in vivo$ within six weeks (Stingl et al., 2006). These cells expressed keratins 5 (CK5) and 14 (CK14), smooth muscle actin (SMA), vimentin and smooth muscle myosin, markers of basal cells which had long before been proposed to act as stem cells (Sonnenberg et al., 1986). Similar results were also obtained by these authors using $CD45^{-} Ter119^{-} CD31^{-} Lin^{-} CD24^{+} CD29^{high}$ mouse mammary cells (Shackleton et al., 2006).

In the adult human breast, coimmunofluorescence was used to detect progenitor cells expressing CK5, CK8/18 and SMA (Boecker and Buerger, 2003). Cells positive for CK5 alone were deemed to be progenitor cells, capable of differentiating into $CK8/18^{+}$ luminal epithelial cells or $SMA^{+}$ myoepithelial cells via the $CK5^{+} CK8/18^{+}$ and $CK5^{+}/SMA^{+}$ intermediaries, respectively. The $CK5^{+}$ progenitor cells were few in number, and were largely observed in the luminal epithelial location, with some cells in a more basal location. Most luminal cells in the resting breast were found to be intermediary ($CK5^{+} CK8/18^{+}$) in phenotype, with terminal differentiation to $CK8/18^{+}$ cells occurring during pregnancy, suggesting that the glandular
epithelium in the resting breast is immature. Myoepithelial cells, in large part, consisted of both intermediary (CK5+/SMA+) and differentiated (SMA+) cells.

Immunofluorescence experiments on breast tissue with usual ductal hyperplasia (UDH) and ductal carcinoma in situ (DCIS) suggested that these two conditions might be caused by different defects, and not proceed via the same pathway (Boecker and Buerger, 2003). Because staining of UDH tissue demonstrated the presence of CK5+ precursor cells, the authors proposed that alterations leading to this benign lesion occur at the level of the CK5+ glandular precursor, when the cells are already committed to a glandular phenotype. In contrast, staining of DCIS tissue showed the presence of only differentiated CK8/18+ luminal cells, and no CK5+ progenitor cells, suggesting a defect in the luminal cell type. Based on additional analysis of the literature, these researchers proposed the classification of breast carcinomas with respect to cytokeratin expression (Boecker and Buerger, 2003). Recent studies based on gene expression profiling support this classification of breast cancer into several subtypes in relation to clinical outcome, and have corroborated these findings (Sorlie et al., 2006).

These experiments defined a new method to study breast cancer taxonomy, which aims to define the cells of origin, alterations and pathways involved in carcinogenesis in order to provide better, tailored treatment options which consider the pathogenesis of these disease subtypes. Additionally, these studies allowed the integration of cancer stem cell theory, genetic and epigenetic analyses, and histology, propelling our understanding of breast cancer forward. The next sections describe recent advances in our knowledge, addressing the genetic, epigenetic and environmental influences in breast cancer etiology.
4.3 Etiology of breast cancer

Recent evidence from gene expression profiling studies of breast tumours suggests that five molecular subtypes of breast cancer exist: basal-like, luminal A, luminal B, HER2+/ER− and normal breast-like (Dalgin et al., 2007; Perou et al., 2000; Polvay, 2007; Sorlie et al., 2001; Sorlie et al., 2006). Basal-like breast cancers show high expression of the basal markers CK5/6 or CK17 as well as laminin and fatty acid binding protein 7 (Sorlie et al., 2001; Sorlie et al., 2006), are triple-negative for estrogen receptor (ER), progesterone receptor (PR) and HER2, and have the worst prognosis (Perou et al., 2000). Luminal breast cancers express luminal CK8/18, and are PR+, HER2− and typically ER+ (Perou et al., 2000). Luminal A cancers have the best outcome of the five subtypes, are low grade, and have been proposed to arise in ER+ stem or progenitor cells (Melchor and Benitez, 2008; Sorlie et al., 2001). Luminal B tumours are typically higher grade, may be ER−, and are believed to arise in ER− stem or progenitor cells that may differentiate into ER+ or ER− cancer cells (Melchor and Benitez, 2008). The HER2+/ER− breast cancer subtype is characterized by low levels of ER expression and overexpression of genes in the 17q22.24 amplicon, including the HER2 oncogene, which predicts for poor survival (Perou et al., 2000; Sorlie et al., 2001). Normal breast-like cancers show high expression of genes expressed in basal and adipose cells, with low expression of genes expressed in luminal epithelial cells (Perou et al., 2000). These cancer subtypes can be defined based on the differential expression of a limited number of genes. Molecular signatures of the subtypes are present at the earliest stages of disease (atypical ductal hyperplasia, ADH) and are typically consistent through progression to DCIS and IDC. As these subtypes are associated with different clinical outcomes and responses to therapy, they likely follow distinct tumour progression pathways. Cell of origin, genetic, epigenetic and microenvironmental changes may help to explain this tumour heterogeneity.
Two hypotheses have been proposed with regards to the cell of origin to address the distinct nature of the breast cancer subtypes (Polyak, 2007). First, it is possible that a single stem or progenitor cell of origin may give rise to different tumour subtypes, each defined by the acquisition of specific genetic or epigenetic changes. In the alternative hypothesis, each tumour subtype may initiate in a different stem or progenitor cell. Likely, a combination of these events may occur, as has been suggested in a recent breast cancer review. The integration of various approaches, namely histopathology, molecular pathology, genetics and gene-expression analysis, led to a comprehensive hypothesis in which a gradually differentiating stem cell can undergo an initiating alteration at any point during differentiation, effectively resulting in a different cancer stem cell of origin for each breast cancer subtype (Sims et al., 2007). Further changes in the cancer-associated microenvironment are believed to indicate a co-evolution of the epithelial and stromal cell compartments that is tumour subtype-specific.

Understanding the unique etiology of these cancer subtypes can provide valuable insight for treatment options. As changes in the genetics or epigenetics of a cancer cell or the supporting microenvironment may contribute to the pathogenesis of breast cancer, the following section addresses these events.

4.3.1 Genetics of breast cancer

A number of genes have been implicated in breast cancer in both hereditary and sporadic cases. Up to ten percent of breast cancers are caused by germline mutations of identified susceptibility genes (Palacios et al., 2008). High-risk susceptibility genes include \textit{BRCA1}, \textit{BRCA2}, \textit{PTEN}, \textit{TP53}, \textit{LKB1/STK11} and \textit{CDH1}, and multiple low risk genes are also known to play a role in breast carcinogenesis (Campeau et al., 2008). Both \textit{BRCA1} and \textit{BRCA2} gene products are involved in DNA repair. In almost all \textit{BRCA1} and \textit{BRCA2} breast cancers, loss of
heterozygosity is observed (Osorio et al., 2002). BRCA1 tumours are often basal-like, poorly differentiated, highly proliferating, and ER-, PR- and HER2-negative (Breast Cancer Linkage Consortium, 1997; Lakhani et al., 2002; Breast Cancer Linkage Consortium, 1997; Wuyts et al., 2000). These breast carcinomas tend to have p53 mutations (Palacios et al., 2003; Sorlie et al., 2003). The TP53 gene product is itself a tumour suppressor which can induce apoptosis or cell cycle arrest, depending on its levels within the cell (Aylon and Oren, 2007). BRCA2 tumours are usually moderately or poorly differentiated, ER+, PR+ and HER2-, and resemble most closely the luminal cancer subtype (Bane et al., 2007). PTEN is another tumour suppressor as well as a growth regulator (Goberdhan and Wilson, 2003). PTEN mutations are frequently found in BRCA1 carcinomas (Saal et al., 2008). STK11 is a serine/threonine kinase which acts as a tumour suppressor by inhibiting the mTOR proliferation pathway (Wei et al., 2008). Mutations in this gene confer an increased risk of breast cancer (Hearle et al., 2006). Finally, the CDH1 product, E-cadherin, is a cell adhesion molecule. A variety of point mutations, insertions and deletions have been found in the coding sequence, and female CDH1 mutation carriers have an increased risk of developing lobular breast cancer (Mastracci et al., 2005).

While genetic alterations are perhaps the best characterized component in a multi-step carcinogenesis model, they are by no means the only method of altering gene expression. Studies of epigenetic changes in some of the aforementioned high-risk genes, as well as a host of other genes, have demonstrated the value of an added level of regulation. The following section addresses these mechanisms of epigenetic regulation.

4.3.2 Epigenetics of breast cancer

Epigenetic states may be inherited or acquired through environmental exposure. Epigenetic alterations that are known to contribute to breast cancer include changes in promoter
methylation and post-translational modifications of histones (Stearns et al., 2007). Both mechanisms may lead to a change in chromatin state, effectively establishing the accessibility of a gene to transcription machinery. A recent hypothesis has been proposed in which epigenetic change occurs as the first step in a multi-step carcinogenesis model originating in cancer stem cells (Feinberg et al., 2006). These changes occur early in tumourigenesis, in preneoplastic lesions and even in normal tissue from a cancer patient. Thus, changes in gene methylation or histone acetylation may serve as biomarkers for cancer risk, detection and prognosis.

DNA methylation occurs when a DNA methyltransferase (DNMT) adds a methyl group to a cytosine that precedes a guanine (a CpG dinucleotide), forming methyl cytosine. CpG dinucleotide-rich sequences termed CpG islands are typically present at the promoter region of most genes and are unmethylated, allowing for gene transcription. As all tumours show changes in DNA methylation at the earliest stages of development, alterations are likely clonally inherited from an affected precursor population (Feinberg et al., 2006). These changes have been proposed to affect ‘tumour-progenitor genes’ which regulate stemness in progenitor cells, resulting in an alteration of the self-renewal/differentiation balance of these cells. Epigenetic modifications result in chromosomal instability; thus, a genetic mutation in a tumour suppressor or oncogene is believed to subsequently occur in an epigenetically altered progenitor cell. Further epigenetic alterations may substitute for genetic mutations by modulating the expression of tumour suppressors and oncogenes. The result is a cell population with both epigenetic and genetic alterations that has the inherent capacity for self-renewal. Additional genetic and epigenetic changes may occur as the lesion evolves.

A number of studies have demonstrated that virtually all cases of DCIS and IDC show abnormal methylation or hypermethylation, in contrast to normal breast tissue and benign lesion
controls (for example, Pu et al., 2003; Subramaniam et al., 2008; Zhao et al., 2008). Abnormally methylated genes involved in growth, apoptosis, tissue invasion and metastasis and increased proliferation have been observed. For example, ER, PR, HOXA5, p16, CDH1, RAR and BRCA1 have all been reported to be abnormally methylated in breast cancer (Widschwendter and Jones, 2002). The cytosine analog 5-aza-2’-deoxycytidine (5-AZA-dC) acts as a DNA methyltransferase (DNMT) inhibitor. When incorporated into DNA, 5-AZA-dC irreversibly binds DNMT, effectively sequestering the enzyme and allowing the passive demethylation of DNA (Christman, 2002). Recent approval of this drug for the treatment of myelodysplastic syndrome underlines the harmful effects of abnormal DNA methylation and the potential therapeutic value of DNMT inhibitors (Stearns et al., 2007).

Chromatin structure is also influenced by post-translational histone modification. DNA is organized into nucleosomes in which chromatin is wound around an octamer of histones, allowing for a relaxed, transcriptionally active or condensed, inactive chromatin state. The acetylation state of histones is determined by a balance in the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which maintain open and closed states of chromatin conformation, respectively (Mottet and Castronovo, 2008). A reduction in histone acetylation has been observed in cancer, suggesting increased deacetylase activity (Ono et al., 2002; Yasui et al., 2003). HDAC inhibitors may induce apoptosis and cell-cycle arrest and decrease the expression of oncogenes and angiogenic factors (Stearns et al., 2007). Furthermore, HDAC inhibitors have also shown promising results in anti-cancer therapeutics in the clinical setting, and are being used to treat diffuse large B-cell lymphoma, laryngeal cancer, thyroid cancer and mesothelioma (Kelly et al., 2005; Kelly et al., 2003). Taken together, these results show that epigenetic regulation has profound effects on gene expression, disease and therapy.
While mammary epithelial cells are subject to their own intrinsic genetic and epigenetic influences, it is well established that morphogenesis of the epithelial cell compartment which forms the mammary gland proper is highly dependent on systemic and local cues throughout all stages of development (Parmar and Cunha, 2004). The following sections address these stromal-parenchymal interactions in mammary morphogenesis and cancer.

4.3.3 Stromal-epithelial interactions and the cell microenvironment

Hormones and growth factors exert strong influences either directly or indirectly on the mammary gland (Silberstein, 2001a). In addition, cell microenvironment plays a fundamental role in mammogenesis as epithelial cells interact with stromal cells and components of the basal lamina and extracellular matrix (Fata et al., 2004). Interestingly, gene expression studies have demonstrated that a multitude of secreted proteins and receptors are abnormally expressed in tumour cells, suggesting a role for cell-cell signaling in breast cancer (Allinen et al., 2004). As in normal mammary gland development, epithelial-mesenchymal interactions also play a fundamental role in breast tumourigenesis. The cell microenvironment includes fibroblasts, myofibroblasts, adipocytes, leukocytes and endothelial cells as well as molecules of the extracellular matrix, all of which may affect growth, survival, polarity and invasiveness properties of breast cancer cells. While examples abound in the literature, to illustrate more specifically the requirement for the stromal compartment in mammary development and carcinogenesis, the following section will examine in detail a few examples of stromal-epithelial interactions that have relevance in both the mouse and human contexts.

4.3.3.1 Mammary gland transplantation and humanization of the fat pad

Mouse mammary gland transplantation studies exploring the interactions of the stromal and epithelial cell compartments have typically used the cleared fat pad method in which the
native mammary epithelium is removed and replaced with an engraftment of mammary epithelial cells (Deome et al., 1959). Early experiments using human mammary epithelial cells (MECs) transplanted into the mouse fat pad demonstrated that the human cells could survive but failed to colonize the fat pad and develop outgrowths (Sheffield and Welsch, 1988). Further research showed that embedding human MECs in collagen prior to transplantation allowed epithelial outgrowth (Yang et al., 1994), and this method was improved to yield robust ductal morphogenesis with the addition of mouse or human fibroblasts into the collagen matrix, transplanted under the renal capsule (Parmar et al., 2002). These experiments supported the idea that stromal context is important in normal mammary epithelial morphogenesis.

Additional studies seeking to recapitulate human breast epithelial morphogenesis in an *in vivo* mammary gland were pioneered in the lab of Robert Weinberg (Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology). In these experiments, a ‘humanized’ fat pad was created by first injecting activated, immortalized primary human breast fibroblasts into the cleared mouse fat pad (Kuperwasser et al., 2004). Thereafter, a suspension of human breast epithelial organoids and fibroblasts were introduced into the humanized fat pad. Results showed that human fibroblasts had integrated into the mouse mammary stroma. Human ductal-lobular units formed during pregnancy and neovascularization was observed. In contrast to the methods previously used, these experiments demonstrated the development of normal, functional human breast tissue in the mouse mammary gland. These results were entirely dependent upon the presence of human mammary fibroblasts, confirming that stromal-epithelial interactions are essential for proper mammary development.

Further experiments were conducted by this group using hepatocyte growth factor (HGF), a growth factor important in both mouse and human mammary development. HGF signals are
mediated by the proto-oncogene MET, a receptor tyrosine kinase (Weidner et al., 1993). In the normal mouse and human mammary glands, HGF is expressed by fibroblasts but not epithelial cells, and participates in paracrine signalling (Niranjan et al., 1995). *In vitro* and *in vivo* assays have demonstrated that HGF is mitogenic, motogenic and morphogenic to mouse and human MECs (Berdichevsky et al., 1994; Kamalati et al., 1999; Niranjan et al., 1995; Soriano et al., 1995). Overexpression of HGF *in vivo* results in hyperplastic ductal branching and mammary tumours in the mouse mammary gland (Takayama et al., 1997; Yant et al., 1998). In addition, in human breast cancer, HGF and the MET receptor are overexpressed (Jin et al., 1997).

In experiments in which human mammary fibroblasts overexpressing HGF were used to humanize the mouse mammary fat pad, the subsequent engraftment of human MECs from one individual in the absence of admixed fibroblasts resulted in the development of human breast cancer in the mouse fat pad (Kuperwasser et al., 2004). In contrast, when these same MECs were mixed with normal human primary fibroblasts, only normal ductal and lobular structures were seen. These results thus showed that an alteration in the stromal environment can promote breast tumourigenesis by abnormal human breast epithelial cells.

4.3.3.2 Macrophages

Seminal studies describing the role of macrophages in normal mammary gland development and cancer are largely the work of Jeffrey Pollard and his group. Macrophages are a migrant stromal cell population found in every tissue (Gouon-Evans et al., 2002). In the pubescent mammary gland, macrophages are located at the necks of TEBs. In addition, macrophages within TEBs play a role in phagocytosing epithelial cells undergoing apoptosis. Depletion of leukocytes by exposure of mice to sublethal doses of radiation resulted in impaired epithelial branching with delay in TEB formation. These results, together with
immunohistochemical data, determined that macrophages in the mammary gland are required for normal morphogenesis.

The osteopetrotic (Csf1<sup>op/op</sup>) mouse has a frameshift mutation in the colony-stimulating factor 1 gene which generates a premature stop codon (Yoshida et al., 1990), leading to an absence of the macrophage growth factor and an overall decrease in the number of macrophages, macrophage progenitors and stem cells (Wiktor-Jedrzejczak et al., 1990; Wiktor-Jedrzejczak et al., 1992). In Csf1<sup>op/op</sup> mice which are afflicted with a number of conditions, TEB formation in the mammary gland is also delayed (Gouon-Evans et al., 2002). Fewer branches, a shorter ductal length and disorganized migration were observed in the homozygous mutant mouse. Both treatment with human recombinant CSF1 and targeted Csf1 restoration using a transgenic approach reestablished macrophage density in the mammary gland, rescued TEB formation and branching number and resulted in a significant increase in ductal length (Gouon-Evans et al., 2002; Van Nguyen and Pollard, 2002). These experiments demonstrated the influence of macrophages in the stroma on mammary epithelial morphogenesis. Moreover, additional studies have shown that Csfl is expressed by epithelial cells at the terminal end bud and pregnancy stages (Ryan et al., 2001), illustrating the importance of crosstalk in stromal-epithelial interactions. Presumably, mammary epithelial cells express Csfl to recruit macrophages during relevant periods of growth, and macrophages are in turn necessary for proper epithelial morphogenesis to proceed. In the absence of Csfl expression by epithelial cells, a deficiency of macrophages in the mammary gland results in impaired epithelial morphogenesis.

The Csf1<sup>op/op</sup> mouse harbours a number of additional defects, including failure of the mammary gland to develop normally during lactation (Pollard and Hennighausen, 1994). Specifically, incomplete ductal arborization during pregnancy and precocious development of
lobulo-alveoli were observed. The failure of alveoli to secrete vesicular contents into the lumen resulted in the inability of mothers to nurture pups. Restoration of humoral CSF1 to normal physiological levels partially corrected the lactation defect. Therefore, the influence of macrophages on postnatal mammary gland development occurs at multiple stages, in the pubescent and adult mouse.

Mouse studies addressing the role of macrophages in breast cancer have also been performed. Transgenic mice expressing the polyoma middle T antigen under control of the mouse mammary tumour virus promoter (MMTV-PyMT) are susceptible to developing mammary tumours (Guy et al., 1992). Crossing these mice with Csf1<sup>op/op</sup> mice allowed researchers to determine the effects of macrophages on tumour development (Lin et al., 2001). The absence of Csf1 delayed the development of invasive, metastatic tumours, while transgenic expression of Csf1 in this mouse model accelerated progression and increased lung metastasis. Results demonstrated that Csf1 expression promotes progression to malignancy and that macrophages interact with mammary epithelial cells not only in normal development, but also in cancer progression. Interestingly, in the human condition, CSF1 expression is higher in invasive breast cancer than in intraductal cancer (Scholl et al., 1994). Additionally, infiltrating leukocytes, a high percentage of which are macrophages, are observed in invasive breast cancer (Tang et al., 1992). These results may have clinical significance with respect to human breast cancer, as macrophage infiltration may result in breast cancer associated with a worse prognosis. Thus, stromal cells may potentiate tumour progression via paracrine factors that are as important in transformation as are the properties of the glandular epithelial cells themselves.

Finally, in the mouse, immunohistochemical data have revealed the Csf1 receptor, Csf1r, to be uniquely localized to macrophages during postnatal development (Gouon-Evans et al.,
Human studies of breast tissue have shown CSF1R to be expressed in both macrophages in the stroma and in epithelium during pregnancy (Sapi et al., 1998). This observation suggests that different expression patterns may be observed between the mouse and human mammary glands. Altogether, studies on macrophages have demonstrated the requirement for a specific stromal cell type and cell-cell signaling pathway in both mouse and human mammary development. While the same conclusions may be drawn in both species as to the importance of paracrine signaling between the macrophages in the stroma and the glandular epithelium, CSF1 signaling in the human breast may, in addition, involve an autocrine component not observed in the mouse (Gouon-Evans et al., 2002).

### 4.3.3.3 Homeobox genes

Homeobox genes are fundamental in morphogenesis and are also known to play an important role in normal mammary gland and breast cancer development, in both stromal and epithelial cells (Chen and Sukumar, 2003; Lewis, 2000). This section provides an overview of the structure and function of homeobox genes, following which we examine the role of aristaless-like homeobox 4 (Alx4) as a stromal influence in mouse mammary gland development.

Homeodomain transcription factors are integral to patterning of the embryo during vertebrate development. First identified in Drosophila, the homeodomain is an evolutionarily conserved DNA-binding motif with a helix-turn-helix structure (Gehring et al., 1994). The homeobox which encodes the homeodomain is 180 bp in length, producing a 60-amino acid motif with DNA sequence specificity.

Paired domain proteins constitute a large class of homeodomain proteins which can be divided into two families. Paired domain proteins, like the paired protein of Drosophila, contain both a paired domain and a paired-type domain. Paired-type domain proteins, like the
*Drosophila* protein aristaless, harbour a paired-type homeodomain in the absence of a second DNA binding motif. Paired-type domain proteins can bind specific palindromic DNA sequences with high affinity as either homodimers or heterodimers together with other paired-type family members (Wilson et al., 1993; Wilson et al., 1995).

Paired-type homeodomain proteins are characterized by a glutamine at position 50 (Q50) of the homeodomain, unlike paired domain proteins which instead harbour a serine residue. In addition, hydrophobic residues at positions 28 and 43 in the homeodomain are critical for high affinity cooperative binding (Wilson et al., 1995). Crystallographic analyses have shown that the highly conserved arginine 5 of the homeodomain sits in the minor groove of DNA, where it makes physical contacts with both DNA strands. Paired-type domain proteins also often contain a conserved 22-amino acid paired-tail motif at the C-terminus (Mathers et al., 1997) which modulates the transactivation function of the protein (Brouwer et al., 2003; Simeone et al., 1994).

Most homeodomain proteins can bind the consensus binding sequence 5’TAAT 3’ (P1/2 site) as monomers (Gehring et al., 1994). However, the formation of a DNA-dimer complex results in increased binding affinity as well as biological specificity. Paired-type homeodomain proteins specifically recognize palindromic repeats of this consensus monomer sequence separated by a variable number of spacer nucleotides (Wilson et al., 1993). Optimal spacing is conferred by the identity of amino acid residue 50 within the homeodomain. The paired-type Q50 residue dictates preferential binding to P1/2 sites separated by three spacer nucleotides (P3 site, 5’ TAATnnnATTA 3’).

In the next section, I will describe a specific mouse homeodomain protein that was first identified and characterized in our lab ten years ago. Since then, numerous studies have
suggested that mouse Alx4 and human ALX4 may play a role in mammary development, breast cancer and other diseases.

5 Mouse aristaless-like homeobox 4

5.1 Identification and characterization

Aristaless-like homeobox 4 (Alx4) was first identified by two independent groups in separate two hybrid screens of mouse embryonic cDNA libraries. In one study, Alx4 was isolated and cloned in a screen of an embryonic day (E) 10.5 cDNA library for proteins that interact with FosB; this interaction was later deemed to be spurious (Qu et al., 1997a). In another study, Alx4 was independently identified in a yeast two-hybrid screen of an E11 mouse library based on the interaction of its paired-like homeodomain with the N-terminal portion of p130, a member of the pRB family of negative regulators of cell cycle progression (Hudson et al., 1998; Wiggan et al., 1998). In vitro binding assays using pRB and p107 confirmed that the pRB family of proteins can interact with Alx4 (Wiggan et al., 1998).

Characterization of Alx4 revealed 5’ and 3’ untranslated regions of 191 bp and over 4 kb, respectively (Qu et al., 1997a). Alx4 has two translation initiation sites corresponding to proteins of 383 and 399 amino acids, with predicted molecular weights of 41.3 and 42.9 kDa, respectively (Hudson et al., 1998; Qu et al., 1997a). The protein contains a homeodomain which shares 90% identity with the homeodomains of the vertebrate proteins Alx1 and Alx3 and 77% identity with that of the Drosophila protein aristaless, as well as a conserved paired-tail of 22 amino acids at the carboxyl terminus which is believed to modulate homeodomain function. The remaining sequence of the Alx4 protein shares only about 30% homology across the length of the other proteins. Alx4 is a transcription factor which is also related to the other paired-type domain proteins Prrx1/mHox, Prrx2/S8, Rx1 and Chx10 (Qu et al., 1997b; Wiggan et al., 1998). The
characteristic features of the paired-type homeodomain class of proteins are conserved in Alx4, namely a glutamine residue at position 50 of the homeodomain, hydrophobic residues at positions 28 (valine) and 43 (alanine), and the absence of a paired domain (Qu et al., 1997a). The Alx4 paired-type homeodomain may bind P1/2, P2, P3, P4 and P5 sequences (Hudson et al., 1998; Qu et al., 1998; Qu et al., 1999). In addition, the N-terminal region of Alx4 contains a proline-rich domain, which may function as an activation domain (ten Berge et al., 1998). Reporter assays using Alx4 deletion mutants have demonstrated that both this sequence and a second proline-rich region C-terminal to the paired-like homeodomain are necessary for optimal activity of a promoter construct consisting of three P3 sites in tandem with a CAT reporter (Hudson et al., 1998).

5.2 Expression

Experiments in transfected cells have demonstrated that Alx4 is a nuclear transcription factor (Hudson et al., 1998). Northern analysis of RNA samples from mouse embryos at different stages of development and in a panel of adult mouse tissues revealed a transcript of 6.5 kb in E9.5 and E11.5 embryos (Qu et al., 1997a). Western analysis confirmed the expression of two Alx4 products in E10.5 and E12.5 mouse embryos (Hudson et al., 1998; Qu et al., 1997a). No expression was seen in the brain, heart, liver, kidney, intestine, muscle, spleen or testes (Qu et al., 1997a).

In situ hybridization studies revealed that Alx4 is first expressed in mesenchymal cells of the craniofacial region at E8.25 and can be seen as late as E18.5 in the developing embryo (Hudson et al., 1998; Qu et al., 1997a). At various stages, Alx4 is expressed uniquely in mesenchymal cells in the first branchial arch, the limb buds, the ventral body wall, the genital tubercle and the umbilical vessels (Qu et al., 1997b). Notably, Alx4 expression is restricted to the
mesenchyme at sites of mesenchymal-epithelial interaction, during development of hair, whisker, teeth, bone and mammary tissues (Hudson et al., 1998). *Alx4* is rarely expressed in the epithelial cell compartment in the mouse embryo.

### 5.3 *Alx4* mouse mutants

Characterization of mouse mutants has revealed the importance of Alx4 during development. Mice homozygous for the targeted null mutation *Alx4*<sup>tm1qw</sup> (*Aristaless 4; targeted mutation 1; Ron Wisdom) produce no Alx4 protein, and display defects of the ventral body wall which lead to gastroschisis and death in over 98% of embryos, a delay in parietal bone formation, and preaxial polydactyly of all limbs (Qu et al., 1997b). The *Strong’s luxoid (lst<sup>D</sup>*) allele resulted from chemical mutagenesis experiments and has a point mutation in the homeodomain of Alx4 (R206Q) at conserved arginine residue 5, which sits in the minor groove of DNA (Qu et al., 1998). Though Alx4<sup>lstD</sup> is expressed at levels similar to that of wild-type Alx4, this mutation abrogates its ability to bind DNA. The *Strong’s luxoid Albany (lst<sup>Alb</sup>*) allele consists of a gene deletion which arose from independent chemical mutagenesis experiments (Flaherty et al., 1992; Qu et al., 1998). The *Strong’s luxoid Jackson (lst<sup>J</sup>*) allele developed spontaneously and has a small 16 bp deletion within the homeodomain (Qu et al., 1998; Takahashi et al., 1998). Homozygous mutants for any of these three alleles exhibit similar defects which, in addition to the aforementioned characteristics, include hemimelia of the tibia, dorsal alopecia, and male genital anomalies (Forsthoefel, 1962; Forsthoefel, 1963; Qu et al., 1997b; Qu et al., 1998). Observed differences in phenotype are likely due to strain-specific modifier genes (Forsthoefel, 1962; Qu et al., 1998). In contrast to the severe homozygous mutant condition, all heterozygotes display only polydactyly of one or more limbs, showing that mutations in *Alx4* exhibit semidominant inheritance.
5.4 Alx4 in development

Preaxial polydactyly in Alx4 mutants is caused by duplication of the zone of polarizing activity (ZPA), a signaling centre which specifies anterior-posterior vertebrate limb patterning, with ectopic expression of Sonic hedgehog (Shh), HoxD13 and fibroblast growth factor-4 in the anterior mesenchyme. These results demonstrate that Alx4 is a negative regulator of ZPA formation during limb bud development and suggest that Alx4 may be a target of retinoids or Hox genes involved in patterning (Qu et al., 1997b; Qu et al., 1998). The small size of the ectopic ZPA implies that other proteins in the same family may compensate for loss of wild-type Alx4 expression. Alx3, which shares a similar expression pattern in the limbs, craniofacial region and ventral body wall, has been proposed as a candidate for functional redundancy (ten Berge et al., 1998). Studies examining craniofacial defects in Alx3/Alx4 double mutant mice have corroborated the overlapping functions of these two genes, as double homozygous mutants display the worst phenotype of all genotype combinations (Beverdam et al., 2001).

A number of other studies have demonstrated a role for Alx4 during development. Bone Morphogenetic Proteins (BMPs) are soluble signaling factors that are important in embryonic patterning. In limb morphogenesis, a null mutation of Alx4 enhances the penetrance and expressivity of the heterozygous BMP4 null phenotype, resulting in preaxial polydactyly of both hindlimbs instead of one (Dunn et al., 1997). These results confirm the importance of Alx4 in limb development.

5.5 Alx4 in the mammary gland

Recently, our lab has shown that Alx4 is required in a subset of mesenchymal cells for normal ductal morphogenesis to occur in the pubescent mouse mammary gland (Joshi et al., 2006). Alx4 is expressed in a subset of stromal cells adjacent to terminal end buds during
puberty and alveoli during pregnancy. Mammary gland transplantation studies using recombinant wild-type epithelium and lstD mutant stroma, or vice versa, determined that Alx4 is required in stromal cells during puberty for normal mammogenesis to proceed. In the absence of functional Alx4 in these stromal cells, branching morphogenesis is impaired, with disorganized and overlapping ducts, atypical size variations, defective TEB formation and bifurcation, and a delay in fat pad invasion. These results suggest that ALX4 expression may play an important role in development of the human breast.

6 Human ALX4

6.1 Identification and characterization in patients with parietal foramina

Human aristaless-like homeobox 4 (ALX4) shares 90% sequence similarity at both the nucleotide and amino acid levels, and shares all of the aforementioned structural features with the mouse protein (Figure 7). Human ALX4 was initially identified by three independent groups studying the Potocki-Shaffer syndrome (PSS; Mavrogiannis et al., 2001; Wu et al., 2000; Wuyts et al., 2000), a contiguous gene deletion syndrome caused by monosomy of 11p11.2 whose symptoms include multiple exostoses and parietal foramina (PFM2) with possible associated cortical and vascular anomalies as well as epilepsy and, occasionally, dysmorphic features, mental retardation and genital abnormalities (Bartsch et al., 1996; Potocki and Shaffer, 1996; Valente and Valente, 2004; Valente et al., 2004).

In a contiguous gene deletion syndrome, each feature is purported to be caused by haploinsufficiency of a specific gene in the deleted region. Haploinsufficiency of muscle segment homeobox gene 2 (MSX2) caused by deletions or loss of function mutations in the gene at the 5p34-q35 locus had earlier been shown to result in the non-syndromic autosomal dominant
condition parietal foramina 1 (PFM1), a defect of the skull vault in which ossification of the parietal bones is delayed or incomplete. However, the absence of MSX2 mutations in some patients afflicted with PFM, as well as the presence of PFM within the context of other diseases such as the chromosome 11 deletion syndrome PSS, suggested genetic heterogeneity for this phenotype.

In one of the first studies to identify human ALX4, researchers hypothesized based on phenotypic similarity that a homologue of MSX2 located on the chromosome 11p proximal region could specifically cause biparietal foramina in PSS (Wu et al., 2000). BLASTN searches revealed a short homologous sequence which mapped to chromosome 11. FISH analysis determined its location on the proximal short arm at 11p11.2, the region deleted in PSS, and confirmed its heterozygous deletion in two patients with parietal foramina. Further analysis demonstrated that this human sequence had significant homology to the mouse Alx4 gene, which is located on chromosome 2, a region of conserved synteny with human 11p12q12. The human ALX4 coding sequence was determined to contain four predicted exons. Sequencing errors were later revised by another group who correctly sequenced the 1586 bp coding region (Mavrogiannis 2001).

Expression analysis by northern blot showed no human ALX4 transcript in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, uterus, small intestine, colon or peripheral blood leukocyte samples (Wu et al., 2000). However, RT-PCR using parietal bone from a deceased newborn as well as a lymphoblast cell line definitively demonstrated human ALX4 expression for the first time.
Using a different approach, an independent group of researchers performed linkage analysis with \textit{EXT2} to define the minimal candidate region on chromosome 11 associated with PSS (Wuyts et al., 2000). Sequencing of this region and the use of the combined gene, EST and exon prediction program NIX identified \textit{ALX4} as a candidate gene for PFM. Further sequence analysis determined the coding region of \textit{ALX4} encodes a 411-amino acid protein with high sequence similarity to its mouse and chicken counterparts, including 100\% sequence similarity over the homeobox region. In two of three families afflicted with PFM but with no mutations in the \textit{MSX2} coding region or splice junctions, mutation analyses demonstrated the presence of distinct family-specific point mutations in \textit{ALX4} in affected members but not in healthy family members or control subjects. No further analysis of the remaining family was undertaken. Interestingly, additional studies of a large Chinese pedigree affected by PFM which excluded mutations in \textit{ALX4} and \textit{MSX2} by linkage analysis suggested the possible involvement of a third gene located at 4q21-q23 (Chen 2003). To date, all potential gene candidates at this locus have been excluded by sequence analysis of the coding regions (Chen 2003).

A separate study which also used linkage mapping to identify candidates responsible for PFM noted variable degrees of penetrance and expressivity among the patients examined (Mavrogiannis et al., 2001). \textit{ALX4} was identified just outside of the critical region for PSS and was selected as a good candidate for PFM based on the phenotype of mice homozygous mutant
for Alx4, which display delayed ossification of the parietal bones. Independent characterization of *ALX4* demonstrated the presence of a dual translation initiation site, as seen in the mouse, which results in two proteins of 395 and 411 amino acids. Three distinct *ALX4* mutations, all point mutations in the homeodomain, were identified in affected members of four families. Thus, parietal foramina is likely the result of haploinsufficiency of functional ALX4. Correct dosage of ALX4 was therefore determined to be a requirement for proper human skull ossification.

Additional case studies using genetic linkage analysis and FISH have shown strong support for these early results, identifying further mutations including a putative dominant negative mutation in the homeodomain, and have suggested the possible role of position effects on undefined enhancer sequences where mutations have not been identified (Bremond-Gignac et al., 2005; Gentile et al., 2004; Hall et al., 2001; Mavrogiannis et al., 2006; Wakui et al., 2005). To date, no studies have examined the possibility of non-genetic alterations of *ALX4* or other candidates in PFM, though these should perhaps be considered. As we will see, evidence exists for the regulation of *ALX4* expression by epigenetic mechanisms in other diseases. Thus, it is important to consider both genetic and epigenetic alterations that may modulate ALX4 expression in order to assess ALX4 as a candidate for disease.

### 6.2 Epigenetic mechanisms of regulation of *ALX4* in cancer

Before its identification and characterization, the association between *ALX4* and breast cancer was first noted by researchers studying CpG island methylation (Yan et al., 2000). Differential methylation hybridization (DMH) was developed as a high-throughput tool for the genome-wide screening of CpG island hypermethylation in breast cancer. Its application to a panel of paired breast tumour and normal samples led to the identification of 30 CpG island loci which were hypermethylated in invasive ductal carcinoma, including many sequences which had
not yet been characterized. Of these, one sequence would later be established as a part of the gene ALX4 (GenBank accession number: Z58446; Ebert et al., 2006), a CpG island which resides within the first intron. Histological grading and dichotomization of the samples revealed that poorly differentiated high-grade breast tumours exhibit a higher frequency and level of hypermethylation than do moderately- or well-differentiated lower-grade tumours. While not all tumour samples demonstrated hypermethylation at all loci examined, this study suggested that the hypermethylation of genes including ALX4 in breast cancer could act as a clinically relevant parameter which reflects the presence and stage of this disease.

In a study examining methylation in colon cancer, researchers applied a novel immunological method to enrich for methylated sequences in the colon cancer cell line SW48. Microarray analysis of methylated DNA-enriched sequences versus non-enriched sequences, followed by PCR, demonstrated the methylation of ALX4 in SW48 cells (Weber et al., 2005). The location of methylated CpG islands was further refined using a CpG island microarray, which revealed methylation of the ALX4 promoter region. Similar analysis of methylation-enriched DNA from three adenocarcinoma samples and matched controls showed methylation of ALX4 in all normal and cancer samples tested, with some variation in methylation levels. These results suggest that ALX4 methylation may be prognostic in some, but not all, colon cases. Importantly, the authors did not examine expression levels of ALX4 to determine if changes in expression correspond to differences in methylation levels.

In a larger study examining methylation in colon cancer, several techniques were used to confirm the hypermethylation of ALX4 in cancer versus normal samples. Methylation-sensitive arbitrarily primed PCR (MS-APPCR) using genomic DNA from the colon tissue samples of patients with colon adenomas and adenocarcinomas with or without lymph node involvement or
metastasis, or from normal colon tissue, allowed the identification of sequences hypermethylated in lesional colon samples, including intron 1 of ALX4 (Ebert et al., 2006). Methylation-specific qPCR (MethyLight) analysis confirmed these results and demonstrated that ALX4 is methylated to a greater extent in colon cancer versus non-cancer samples. Where ALX4 was found to be methylated in the primary colon cancer, ALX4 was also methylated in the corresponding liver metastasis. However, a few samples showed no methylation of either the primary cancer or the metastatic site, and a single sample showed methylation of the metastasis but not of the primary colon cancer. Overall, the high concordance in ALX4 methylation at primary and metastatic sites suggests that ALX4 methylation is an early event in carcinogenesis which is persistent throughout progression, and therefore may act as an early diagnostic marker for the onset of colon cancer. Bisulfite sequencing confirmed the increased methylation of ALX4 in colon cancer versus matched normal colon samples.

Additional studies were undertaken using four colon cancer cell lines, LoVo, HT29, T84 and DLD-1, which were shown by the MethyLight assay to exhibit a high degree of methylation (Ebert et al., 2006). Treatment of these cell lines with the demethylating agent 5-aza-2’-deoxycytidine (5-AZA-dC) followed by RT-PCR analysis demonstrated that ALX4 can be derepressed and thereby expressed in LoVo cells. While experiments conducted on HT-29, T84 and DLD-1 did not show expression of ALX4 following treatment with 5-AZA-dC, levels of ALX4 expression might have been below the limits of detection of RT-PCR, as suggested by the authors. These results showed for the first time that the expression of ALX4 can be silenced by methylation, though it is not clear whether this regulation is direct or indirect.

In the other gastrointestinal cancer samples examined, ALX4 was more frequently methylated in cancer versus normal tissue (Ebert et al., 2006). Methylation of ALX4 at metastatic
sites was further analyzed in colon cancer and one case each of breast, gastric and renal cancer. In colon cancer, breast and gastric cancer metastases to the liver, ALX4 was methylated more frequently than in nonlesional liver. In the single case of renal cell metastasis to the liver, ALX4 was not methylated in either normal or disease tissue. These results suggest that ALX4 may be methylated in a host of cancers and that this methylation may be useful as a diagnostic tool. However, the level of methylation required to consider a gene to be methylated may be a tissue-specific parameter which should be defined in pilot studies.

Serum levels of ALX4 methylation were found to be significantly higher in patients with colon cancer versus noncancer controls (Ebert et al., 2006). Diagnosis parameters yielded a sensitivity of 83.3% and specificity of 70%. While performance of ALX4 as a biomarker is suboptimal, its use would nevertheless still be more beneficial than all commonly used biomarkers. Higher sensitivity and specificity could potentially be achieved with the use of a panel of multiple markers.

Similarly, assessment of plasma levels of ALX4 methylation revealed that, in the subset of patients with precancerous lesions that exhibited methylation of ALX4 in plasma DNA, ALX4 was more frequently methylated in the plasma of patients with advanced lesions than in that of patients with smaller adenomas and those with polyps without advanced intraepithelial neoplasia. These results suggest that the presence or absence of methylated ALX4 in the plasma could act as an indicator to warrant additional diagnostic tests (Ebert et al., 2006).

An independent group studying methylation in colon cancer found similar results. The DNA from normal colon tissue and microdissected colon adenoma and cancer lesions was extracted and modified by bisulfite treatment (Zou et al., 2007). A panel of candidate genes including ALX4 was selected based on previous methylation studies. ALX4 was found to be
methylated in 4/4 colorectal cancers, and in 0/4 normal colon tissue samples, and was selected for further investigation. DNA from colon tissue samples demonstrated that more frequent and higher levels of ALX4 methylation were detected in colon adenomas and cancers than in normal colon tissue. Furthermore, methylation levels of ALX4 were higher for larger tumour sizes. Overall, ALX4 methylation was detected in 11% of normal samples, 89% of adenomas and 68% of cancer cases examined. The use of ALX4 methylation alone as a biomarker could achieve a specificity of 93% (7% false positive rate), with a sensitivity of 93% (7% false negative rate) for colon adenomas and 58% (42% false negative rate) for carcinomas. The assessment of ALX4 methylation levels in conjunction with the use of additional biomarkers would likely improve these parameters (Zou et al., 2007).

ALX4 was also found to be methylated in the colon cancer cell lines SW480, SW620, HCT15 and WIDR (Zou et al., 2007). In the colon cancer cell line SNUC4, both an unmethylated and a methylated allele were found. Following treatment with the demethylating agent 5-AZA-dC, RT-PCR results demonstrated that ALX4 expression was upregulated in all tested cell lines, suggesting that methylation may either indirectly or directly silence the expression of ALX4. The role of ALX4 in cancer is still not well understood. However, its methylation in preneoplastic lesions such as adenomas could suggest a role for ALX4 as a tumour suppressor as such genes are typically inactivated early in tumourigenesis.

Another group examined methylation as a prognostic indicator in urinary bladder cancer. MSP conducted on three urinary bladder cancer cell lines, 5637, SCaBER and T24, revealed the methylation of several genes, including ALX4 (Yu et al., 2007). The methylation of a single ALX4 allele in each of these cell lines was confirmed by bisulfite sequencing of the amplification product, corresponding to a fragment of exon 1. Further MSP profiling revealed methylation of
in 45.5% of urine sediment samples and 40% of bladder tissue samples from bladder cancer patients in China, and no methylation in any of the 3 controls for each case. Comparison of ALX4 methylation in the urine sediment of pre- and post-surgery bladder cancer patient groups revealed a significant decrease in ALX4 methylation after removal of the tumour. Thus, the incidence of methylation in the urine sediment of some post-surgery patients could potentially serve as an indicator for the successful removal of the tumour.

The projected use of methylation profiling on a panel of 11 genes found to be hypermethylated in bladder cancer, namely ALX4, SALL3, CFTR, ABCC6, HPP1, RASSF1A, MT1A, CDH13, RPRM, MINT1 and BRCA1, yielded a sensitivity of 91.7% with a specificity of 87% for the detection of bladder cancer from urine sediment. These results suggest the possibility of a clinically valid non-invasive test for the early detection of bladder cancer in both new patients and in those who have undergone surgical treatment (Yu et al., 2007).

Taken together, these studies demonstrate that ALX4 is methylated in a multitude of cancers. The occurrence of ALX4 methylation and hypermethylation in preneoplastic lesions and its persistence throughout the transformation process support the use of ALX4 methylation as a biomarker for the early detection of cancer and suggest the possible role of ALX4 as a tumour suppressor. Furthermore, as ALX4 expression may be induced in cancer cell lines by treatment with a demethylating agent, demethylation may be a mechanism by which ALX4 expression is lost in colon and possibly in other cancers.
Rationale

To date, neither the expression pattern nor the role of ALX4 in the normal human breast has been systematically characterized. Several lines of evidence suggest that human ALX4 may be expressed in the normal adult breast and that expression of ALX4 may be altered in a dysplastic phenomenon such as breast cancer. First, the human and mouse counterparts share high sequence similarity at both nucleotide and amino acid levels. Alx4 expression is observed during pubescent mouse mammary morphogenesis and is required for normal morphogenesis to proceed (Joshi et al., 2006). Additionally, homeobox family genes, which are fundamental in development and typically have conserved domains and functions across species, are important in human breast cell lines for both development and transformation (Chen and Sukumar, 2003). Furthermore, the abnormal methylation or hypermethylation of \textit{ALX4} in colon, bladder and breast cancer supports the notion that loss of ALX4 expression may contribute to malignant transformation in the breast. Thus, I hypothesize that ALX4 is expressed in the normal breast and that this expression is altered in breast cancer. Finally, as has been shown in other cancers, ALX4 expression is likely to be modulated by methylation in the breast.
Hypotheses and Objectives

1. ALX4 is expressed in the normal human breast.
   a) Validate the antibodies used for immunostaining.
   b) Determine the normal expression pattern of ALX4 in the human breast.
   c) Compare the expression of mouse Alx4 and human ALX4 in the mammary gland.

2. ALX4 expression is altered in breast cancer.
   a) Compare ALX4 expression in the normal and transformed human breast.
   b) Assess ALX4 expression in the normal breast and in breast cancer in an independent data set using a tissue microarray.

3. $ALX4$ expression is regulated by methylation in the normal breast and/or breast cancer.
   a) Determine if demethylation of normal and transformed breast cell lines alters $ALX4$ expression.
Materials and Methods

Sequence analysis

Mouse Alx4 (NM_007442) and human ALX4 (NM_021926) sequences were compared using the alignment tools bl2seq (http://www.ncbi.nlm.nih.gov/BLAST/) and DIALIGN 2.2.1 (http://bibiserv.techfak.uni-bielefeld.de/dialign/).

Cell culture and demethylation

Human cell lines were grown in incubators under the following conditions: 37°C, 5% CO₂ and high humidity. 293 HEK, MDA-MB-231, MCF7 and MDA-MB-468 cells were cultured in DMEM with 10% fetal bovine serum (FBS). T47D and DLD-1 cells were grown in RPMI-1640 with 10% FBS. T84 cells were grown in DMEM/F12 with 5% FBS. LoVo cells were grown in Ham’s F12 with 10% FBS. MCF10A cells were cultured as described (Debnath et al., 2003) in DMEM/F12 medium supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 µg/ml insulin. Human mammary epithelial cells (HMEC; Stampfer et al., 1980) were cultured in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 1 ng/ml cholera toxin, 10 µg/ml insulin and 5 µg/ml transferrin.

For demethylation experiments, cells were seeded in 6-well plates and grown in complete medium with 5-aza-2’-deoxycytidine (250nM, 500nM, 1µM, 2µM, 3µM, 5µM and/or 10µM) or an equivalent amount of DMSO for three to five days. Medium was changed daily. Cells were lysed in TRIzol prior to attaining confluency, and RNA was isolated as per the manufacturer’s instructions.

RT-PCR and PCR

RT-PCR for ALX4 was performed as previously described using 1 or 2 µg total RNA (Ebert et al., 2006), producing a 491-bp product. As a loading control, a 541-bp product of β-actin was used (Hoppe et al., 1992). ERα primers amplifying a 470-bp product were as described (Ferguson et al., 1995; Sharma et al., 2005).
PCR for ALX4 was performed as previously described using 0.5 µg total DNA (Wuyts et al., 2000), with the following correction for the exon 4 F primer 5’-AGGTGCTCTGGGGGAAGGGCGGAG-3’. Product sizes for exon fragments were as following: exon 1, 595 bp; exon 2, 400 bp; exon 3, 234 bp; and exon 4, 738 bp.

**Cloning and expression of the 5’ end of human ALX4**

TRIzol reagent (Gibco) was used as per the manufacturer’s instructions to isolate RNA from 293 HEK cells. A 385-bp fragment corresponding to most of exon 1 of human Alx4 (AF294629) was amplified with the Superscript One-Step RT PCR kit (Invitrogen) using primers 5’-TGCCATGGACGCCTACTACA-3’ and 5’-GCTGCCTTCCTGGAGTTGA-3’ under the following conditions: first-strand synthesis: 50°C, 30 minutes; first 2 cycles: 94°C, 5 minutes; 50°C, 5 minutes; 72°C, 5 minutes; additional 38 cycles: 94°C, 1 minute; 50°C, 1 minute; 72°C, 2 minutes; final extension: 72°C, 5 minutes. After treatment with Klenow, the fragment was cloned into the SmaI site of pGEM7Zf(+) (Pharmacia) and sequenced. The fragment was then excised from pGEM7Zf(+) and subcloned into pGEX2T via BamHI and EcoRI sites. To clone the fragment in frame with the glutathione S-transferase (GST) tag of pGEX2T, the construct was digested with ClaI, treated with Klenow and religated. This strategy replaced the restriction site ClaI with the site NruI, with which subsequent diagnostic digests were performed.

Cultures of *E. coli* transformed with the expression construct pGEX2T containing the 5’ end of hALX4 were grown overnight in LB medium at 37°C. Log phase growth was stimulated by the addition of fresh LB, followed by induction with IPTG at 30°C for 2 hours. Cells were then resuspended in lysis buffer with the protease inhibitors PMSF, aprotin and leupeptin, and sonicated for protein isolation. Protein purification on glutathione sepharose beads (GE Healthcare) was followed by elution in glutathione buffer, as per the manufacturer’s instructions.

**Blocking goat anti-human ALX4**

The goat anti-human ALX4 antibody (Santa Cruz Biotechnology) was incubated overnight at 4°C with the human ALX4 N-terminal (GST-hALX4 N term) peptide in the following molar ratios: 1:1; 1:10; and 1:50. The unblocked and blocked antibodies were used to detect GST-hALX4 N term fixed to a nitrocellulose membrane by western analysis.
Immunostaining of mouse tissue

Wild-type or heterozygous pubescent (4-6 weeks) female mice were sacrificed following the University of Toronto guidelines for Animal Care. Mammary glands 2, 3 and 4 were dissected, fixed in 4% paraformaldehyde for at least one night, dehydrated through graded alcohols, defatted in several changes of xylene and embedded in paraffin. Slides were prepared with 7 µm tissue sections and dried overnight at 37°C. Prior to staining, slides were preheated to 55°C, cleared in xylene and rehydrated through graded alcohols and several changes of distilled water. Antigen retrieval was by boiling in sodium citrate pH 6.0 for 10 minutes, followed by cooling.

Chromogenic staining (IHC) was performed with the Mouse Staining Kit (R&D) as per the manufacturer’s instructions with each blocking step extended to 30 minutes to 1 hour, incubation with the primary antibody overnight at 4°C, washing steps after antibody removal extended collectively to 1 hour, and counterstaining with haematoxylin for 10 minutes, followed by washing and mounting with aqueous mounting medium. Primary antibody dilutions were as following: anti-mAlx4 supernatant from hybridoma culture 1:10, and mouse monoclonal anti-p63 (Santa Cruz Biotechnology) 1:100.

Immunostaining of human breast tissue

All slides were dewaxed in xylene and rehydrated in graded alcohols, then in distilled water. For IHC, performed by the Pathology Research Program (TGH), antigen retrieval was performed in 10mM sodium citrate buffer (pH 6.0) in a decloaking chamber (Biocare) at 120°C for 2 minutes. For IHC, endogenous peroxidase and biotin activities were blocked using 3% hydrogen peroxide and a commercial avidin/biotin blocking kit (Vector Labs), respectively. Sections were incubated with the anti-hALX4 antibody (1:400), the blocked antibody (1:400) or anti-p63 (1:200) in a humidity chamber overnight at room temperature. After washing, the sections were incubated with biotinylated rabbit anti-goat IgG (Vector Labs) and horseradish-conjugated streptavidin (ID Labs). Colour development was with NovaRed solution (Vector Labs) and counterstaining was with Mayer’s haematoxylin. Sections were then dehydrated in graded alcohols, cleared in xylene and mounted in Permount (Fisher).
For IF, slides were dewaxed and rehydrated as above. Antigen retrieval was performed by boiling for 10 minutes in 10mM sodium citrate buffer (pH 6.0). Thirty minutes later, sections were blocked with 3% BSA in PBS for 1 hour. Sections were incubated with primary antibody diluted in 1% BSA in PBS overnight at 4°C. Dilutions were as following: goat anti-human ALX4 1:100; mouse anti-p63 1:100; mouse anti-E-cadherin (Santa Cruz Biotechnology) 1:50; mouse anti-vimentin (BD Biosciences) 1:100. After washing in PBS, sections were incubated with secondary antibody diluted in 1% BSA in PBS for 1 hour. Dilutions were as following: TRITC donkey anti-goat 1:200 and FITC donkey anti-mouse 1:200 (Jackson ImmunoResearch Laboratories). After washing, slides were mounted with fluorescence mounting medium with DAPI (Vector Laboratories).

Scoring and analysis

Following approval from the Research Ethics Board at Toronto General Hospital (TGH), archival formalin-fixed paraffin embedded human breast tissue was obtained in conjunction with our pathologist, Dr. Susan Done. Nine normal and 19 breast cancer samples were obtained for our pilot study. In addition, three breast tissue microarray (TMA) replicates were obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource upon approval from the Research Evaluation Panel. Two TMAs were stained for ALX4 and the third TMA was stained with blocked anti-human ALX4 as a negative control.

In our pilot study, staining was assessed independently by three observers, including one pathologist, for epithelial and stromal cells separately. The approximate percentage of each cell type stained and intensity of staining, either weak, moderate or strong, was determined for each normal sample (n=9). Tumour samples were assessed for staining of both cancer tissue and adjacent normal tissue (n=18 paired samples; 1 sample did not have normal tissue). Examination of this data set determined the score cutoff of >25% epithelial cells weakly stained and >50% stromal cells moderately stained to provide the highest sensitivity and specificity to detect ALX4 expression.

This scoring criteria was subsequently applied in the TMA analysis of 252 samples, including 40 normal samples and 212 cancer cases. ALX4 staining was assessed on duplicate slides as positive or negative, or rejected from further analysis for one of the following reasons: no tissue present (X), tissue damage precludes analysis (NA), no epithelium (NE), no normal
epithelium (NNE; used in the case of ‘normal’ samples), high background precludes interpretation (HB), edge effects preclude interpretation (EE). Samples which could not be scored in duplicate and samples in which scoring differed between slides were eliminated from further analysis. Remaining samples (n=123) were subjected to statistical analysis.

Fisher’s exact test was used to compare ALX4 expression in unrelated normal and breast cancer samples in our pilot study (n=28) and in our TMA analysis (n=123). McNemar’s paired test of proportions was used to compare ALX4 expression in tumour tissue and adjacent normal tissue (n=18).
Results

Validation of the goat anti-human ALX4 antibody

The untested commercial goat polyclonal antibody directed against human ALX4 was raised against a synthetic peptide corresponding to the N-terminus of human ALX4 (Figure 8A, red box). To determine whether the antibody could recognize the human protein, the 5’ end of \textit{hALX4} was cloned (Figure 8A) and expressed as a glutathione S-transferase (GST)-tagged fusion protein (GST-hALX4 N term) in bacteria. The peptide fixed to a nitrocellulose membrane was used for western analysis, and revealed that anti-hALX4 indeed recognizes the N-terminus of hALX4 (Figure 8B, left panel). This antibody was subsequently used for immunohistochemistry.

To ensure that observed staining was specific, the goat anti-human ALX4 antibody was incubated with a blocking peptide in a 1X, 10X and 50X molar ratio. Western analysis shows that the antibody, when incubated with a 10-fold or 50-fold excess of the N-terminus of hALX4, fails to recognize this same peptide fixed to a nitrocellulose membrane (Figure 8B, left panel). The membrane was subsequently stripped and reprobed with unblocked anti-hALX4 (Figure 8B, right panel), demonstrating that similar amounts of protein are present in each lane, but fail to be recognized by the 10X and 50X blocked antibodies. This experiment confirmed that the ALX4 antibody is specific for human ALX4. Furthermore, a 10X molar ratio of blocking peptide is sufficient to block recognition of ALX4 by anti-hALX4. This blocked antibody was used as a negative control for staining human breast tissue.
Figure 8. Blocking of the human ALX4 antibody. A The 5’ region of ALX4 was cloned from 293 HEK cells and expressed as a bacterial glutathione-S transferase (GST)-tagged fusion protein (GST-hALX4 N term). We confirmed that the commercial polyclonal goat anti-human ALX4 antibody (αhALX4) used for immunostaining, which was raised against a synthetic fragment of the N-terminal portion of human ALX4 (A, red box), recognizes GST-hALX4 N term by western analysis (B, left panel). We then showed that incubation of the anti-ALX4 antibody with an increasing concentration of GST-hALX4 N term (molar ratios of 1x, 10x and 50x) blocked the ability of the antibody to recognize GST-hALX4 N term fixed on a nitrocellulose membrane (B, left panel). The blocked antibody also failed to recognize hemagglutinin-tagged mouse Alx4 (HA-mALX4) overexpressed in 293 HEK cells (B, left panel). This membrane was stripped and reprobed with unblocked anti-ALX4 to confirm that similar amounts of protein are present in all lanes, but fail to be recognized by the blocked antibody (B, right panel). The blocked antibody was used as a negative control for immunohistochemistry in our study of normal and breast cancer samples, demonstrating the specificity of the antibody for ALX4.

ALX4 is expressed in normal human breast stromal and epithelial cells

To determine the expression pattern of ALX4 in the human breast, nine normal breast tissue samples were stained for ALX4. Samples showed variable levels of expression in stromal cells as well as in the epithelial cells of ductules and ducts (Figure 9, left and middle columns, respectively). Moderate to strong staining of the majority of stromal cells (closed arrowheads) was observed. In addition, epithelial cells were stained weakly to moderately, not at all or, for a
minority of cells, strongly (staining denoted by double arrowheads). The blocked antibody used as a negative control showed no staining in any of the samples (right column).

We noted a difference between the expression pattern of ALX4 in the human breast and that of Alx4 in the pubescent mouse mammary gland, as described in a recent report (Joshi et al., 2006). We therefore stained mouse mammary tissue to confirm the pattern of Alx4 expression.
Comparison of mouse and human mammary tissues revealed differences in Alx4/ALX4 expression (Figure 10). In the mouse, Alx4 expression is restricted to a subset of periductal stromal cells (closed arrowheads); no expression is observed in luminal or myoepithelial cells (double arrowheads). In the human breast, ALX4 expression is observed in both stromal and epithelial cell compartments, with stronger expression in stromal cells, and a mosaic expression pattern in epithelial cells, as described above. Thus, while Alx4/ALX4 is expressed in stromal cells in both the mouse and human mammary glands, epithelial expression is unique to the human mammary gland, and is absent from the mouse mammary gland.

We next wanted to determine the identity of ALX4-expressing cells in the human breast. In the pubescent mouse mammary gland, Alx4 is expressed in fibroblasts and absent from epithelial cells (Figure 10 and data not shown). To examine these cell types in the human breast, co-immunofluorescence was performed using the following antibodies: E-cadherin, a marker of the luminal epithelial cell layer; p63, a marker of myoepithelial cells; and vimentin, a marker of fibroblasts in the stromal cell compartment. These experiments showed that ALX4 is expressed in cells positive for E-cadherin (Figure 11A and B, double arrowheads) and is absent from p63-expressing cells (Figure 11B, closed arrowheads). In the stroma, ALX4 is co-expressed in vimentin-expressing cells (Figure 12, arrows).

**Figure 10. Alx4/ALX4 expression in the mouse and human mammary glands.** In the pubescent mouse mammary gland, Alx4 is expressed in periductal stromal cells (closed arrowheads) and is absent from epithelial cells (double arrowheads). In contrast, in the human breast, ALX4 is expressed moderately to strongly in the majority of stromal cells and weakly to moderately in a subset of epithelial cells.
ALX4 expression in breast cancer

In an initial experiment to determine if the expression pattern of ALX4 is altered in breast cancer, nineteen breast cancer tissue samples were stained with anti-hALX4. Though adjacent normal breast tissue showed staining in stromal (Figures 13 and 14, left column, closed arrowheads) and epithelial (double arrowheads) cells as described above, we noted a complete loss or reduction of ALX4 staining to background levels in both cell compartments in DCIS and IDC (right column). We therefore made no further distinction between DCIS and IDC samples. To analyze the difference in ALX4 expression between normal and cancer samples, we used a semi-quantitative method that considered the intensity of staining and the area stained in both cell compartments (see Materials and Methods). Based on this preliminary analysis, maximum sensitivity and specificity were achieved when positive staining was defined as greater than 50% of stromal cells moderately stained together with greater than 25% of epithelial cells showing weak staining. In the breast cancer samples, 17/19 samples showed a reduction of ALX4 staining to background levels or a complete absence of staining (Figures 13 and 14; Table 1).
of nine normal and 19 cancer samples using Fisher’s exact test showed this difference in ALX4 expression to be highly significant (Table 1; \( n=28, p < 0.000008 \)). In addition, of the 19 cancer samples, 18 sections contained adjacent normal tissue. Therefore, a paired test was performed to compare ALX4 expression in cancer tissue with that of adjacent normal tissue, revealing a highly significant difference in ALX4 expression (Table 1; McNemar’s paired test of proportions, \( n=18, p < 0.0002 \)).

**Tissue microarray analysis of normal and breast cancer samples**

To determine if ALX4 could be used as a biomarker for the detection of breast cancer in an independent data set, the criteria generated from our pilot study were applied to breast tissue microarrays which included both normal and cancer samples. Tissue microarrays (TMAs) were stained in duplicate for ALX4, and a third TMA was stained with blocked anti-hALX4 as a negative control (Figure 15 and data not shown). Of 252 tissue samples, 119 samples could not be scored in duplicate for technical reasons (see Materials and Methods). In addition, 10 cancer samples showed inconsistent scoring between replicates and were rejected from further analysis. The remaining 123 samples were scored according to criteria established in our pilot study. ALX4 expression in stromal (Figure 15, closed arrowheads) and epithelial (double arrowheads) cells was as described in the pilot study, and expression was absent or greatly reduced in cancer. Three normal samples and a single case of invasive cancer scored positive, while the 119 other cancer samples scored negative. Fisher’s exact test deemed this difference in ALX4 expression to be highly significant (Table 2; \( n=123, p < 0.0001 \)).

**ALX4 expression in human breast cell lines**

RT-PCR analysis demonstrated *ALX4* expression in 293 HEK and in the breast cancer cell line MDA-MB-468, but not in the normal breast cell lines HMEC and MCF10A, the breast
cancer cell lines T47D, MCF7 and MDA-MB-231, and the colon cancer cell lines DLD-1, T84 and HT-29 (Figure 16A and data not shown). To determine if the absence of \textit{ALX4} expression in the breast cell lines was due to a deletion of both copies of the \textit{ALX4} gene, the exons were amplified by PCR. Results show that all cell lines examined have at least one copy of each of the four exons of \textit{ALX4} (Figure 16B).

Methylation of \textit{ALX4} has been demonstrated in colon, bladder and breast cancer, and demethylation experiments have shown that \textit{ALX4} expression can be induced in colon and bladder cancer cell lines (Ebert et al., 2006; Yan et al., 2000; Yu et al., 2007; Zou et al., 2007). To next determine if \textit{ALX4} expression could be silenced by methylation in the breast, HMEC, MCF10A, T47D, MCF7, MDA-MB-231, T84, DLD-1 and LoVo cells were treated with the demethylating agent 5-aza-2’-deoxycytidine (5-AZA-dC). Treatment of HMEC, T47D, MCF7 and LoVo, followed by RT-PCR analysis, showed expression of \textit{ALX4} upon demethylation (Figure 17). The negative control DLD-1, the normal breast cell line MCF10A and the breast cancer cell line MDA-MB-231 did not express \textit{ALX4} after treatment. Demethylation of MDA-MB-231 was verified by demonstrating that \textit{ERα} expression is induced after treatment with 5-AZA-dC.

\textbf{Figure 12.} \textit{ALX4} is co-expressed with vimentin in the stromal compartment of the human breast. Coimmunofluorescence on normal human breast tissue shows that \textit{ALX4} colocalizes with vimentin, a marker of fibroblasts (arrows) in the stroma. \textbf{D}, ductules.
Discussion

ALX4 is expressed in stromal and epithelial cells in the human breast

Immunohistochemistry of a panel of normal breast sections revealed that ALX4 is expressed in the normal human breast in both stromal and epithelial cells (Figure 9). In stromal cells, staining was generally stronger than in epithelial cells, with most (>50%) stromal cells showing moderate to strong staining. In contrast, epithelial staining was more mosaic, with cells staining a range of intensities. In general, epithelial cells stained weakly to moderately (>25%), but some cells appeared not to express ALX4 at all, while others stained strongly. The negative control, in which anti-ALX4 was blocked with the N-terminus of ALX4, showed minimal staining approximating background levels.

This pattern of expression differs from that of the mouse mammary gland, in which ALX4 expression is restricted to a subset of stromal cells in the pubertal female mouse, and ALX4 expression is absent from the epithelium (Figure 10; Joshi et al., 2006). Differences in mammary gland size and architecture, including the proximity between specific stromal and epithelial cell types, may help to explain this difference (Figure 3; Hovey et al., 1999). In contrast to the mouse mammary gland, the human breast contains a large amount of stroma, including the tightly packed intralobular stroma and the loose interlobular stroma. Paracrine interactions between the stromal and epithelial cell compartments which are required for normal mammary gland development may differ in this species-dependent context.

Other authors have shown that signaling molecules important in mammary development may be differently expressed in the two species. One example is the progesterone receptor (PR) which exists in two isoforms, PRA and the longer PRB. PRA is involved in ductal development
and sidebranching while PRB plays a role in alveogenesis. In the mouse, though both PRA and PRB are expressed in mammary epithelial cells and absent from stromal cells, expression is spatially and temporally separated during puberty and adult life. While PRA is expressed in ducts in the adult virgin mouse mammary gland and decreases during pregnancy, PRB expression is seen in alveoli during pregnancy. In fact, PRA and PRB are rarely expressed in the same cell. In contrast, in the adult premenopausal human breast, PRA and PRB are coexpressed at approximately the same levels within the same cells. Additionally, in breast cancer, a higher ratio of PRA to PRB is associated with less differentiated, more aggressive tumours. These results demonstrate that the mouse model may not faithfully recapitulate all aspects of human development. Thus, it is necessary to perform downstream studies in a context which replicates the human mammary gland microenvironment (see below).

**ALX4 is expressed in normal breast luminal and fibroblast cells**

Differences in ALX4 expression observed between the mouse and human mammary glands precipitated the need to identify the cell types express ALX4 in the human breast. To determine which epithelial cells in the human breast stain for ALX4, co-immunofluorescence was performed using E-cadherin, a marker of the luminal epithelial layer, and p63, a marker of the myoepithelial layer. ALX4 colocalized to the nucleus of E-cadherin expressing cells (Figure 11A) but was omitted from p63-expressing cells (Figure 11B), indicating that ALX4 is expressed in luminal epithelial cells but not in myoepithelial cells. To determine if fibroblasts express ALX4 as they do in the mouse (Joshi et al., 2006), vimentin was used as a marker for fibroblasts in the stroma. Colocalization of the ALX4 and vimentin signals in stromal cells showed that ALX4 is expressed in fibroblasts in the human breast (Figure 12). These experiments demonstrated that, in addition to being expressed in fibroblasts as seen in the mouse mammary gland, ALX4 is expressed in human breast luminal epithelial cells. To take into account the
architecture of the human breast, it would be interesting to determine if the ALX4-positive fibroblasts are intralobular and/or interlobular by costaining with antibodies against DPP IV or TGFα, which are expressed by interlobular, but not intralobular, fibroblasts (Atherton et al., 1992; Howard and Gusterson, 2000).

In the pubescent mouse mammary gland, ALX4 was previously shown to be expressed in a subset of ERα-expressing stromal cells (Joshi et al., 2006). Furthermore, injection of prepubescent mice with 17β-estradiol resulted in stromal and epithelial cell proliferation, followed by the induction of ALX4 expression in stromal cells. These results demonstrate that ALX4 expression in the mouse mammary gland may be regulated by estrogen, though it is unclear whether estrogen activity is mediated by ERα or ERβ. In the human breast, ERα and ERβ are expressed in epithelial and, to a lesser extent, stromal cells (Pelletier and El-Alfy, 2000). Colocalization studies using antibodies against ALX4, ERα and/or ERβ would allow further comparison with the mouse mammary gland, and could help to determine whether ALX4 expression is modulated by estrogen in the human breast. Injection of 17β-estradiol into a humanized mouse mammary fat pad, followed by detection of increased expression of ALX4, would definitively show that ALX4 is downstream of estrogen and the ER.

Further analysis could be undertaken to determine if ALX4 is expressed in other cell types in the stromal cell compartment, including macrophages, mast cells, neutrophils, and T and B lymphocytes (Cabezon et al., 2007). These cells could be uniquely identified by CD68, mast cell tryptase, neutrophil elastase, CD3 and CD20 cy, respectively. Macrophages and eosinophils are required for normal mouse mammary gland development (Gouon-Evans et al., 2002). Mice that fail to recruit macrophages to the mammary gland during puberty have a similar phenotype to lstD.lstD mice which express an allele of ALX4 that cannot bind DNA, resulting in a delay in
mammary gland development, fewer TEBs and fewer branches (Gouon-Evans et al., 2000; Joshi et al., 2006). Thus, to begin to assess the possibility of a common pathway involving ALX4 and other signaling molecules expressed in macrophages, it would be interesting to first determine by co-immunofluorescence if ALX4 is also expressed in macrophages in the human breast.

**ALX4 expression is lost in breast cancer**

A preliminary examination of all normal and breast cancer tissue sections was performed to determine the optimal score cut-off to define positive staining. Based on this exercise, the score cutoff was defined as greater than 50% moderate staining in stromal cells together with greater than 25% weak staining in epithelial cells. Samples that did not meet these criteria were deemed unstained. In almost all (17/19) breast cancer samples examined, ALX4 staining was reduced to background levels or completely absent (Figures 13 and 14). Statistical tests of both unpaired and paired samples revealed a highly significant difference in ALX4 expression between normal and breast cancer samples (Table 1), though the significance of these results was likely inflated as the score cutoff was determined based on this initial data set (see Materials and Methods). Nevertheless, these preliminary results suggested that loss of ALX4 expression might potentially be used as a biomarker for breast cancer.

In order to assess both the validity of the score cutoff and the usefulness of ALX4 as a prognostic indicator for breast cancer, the established criteria were applied to an independent data set. All normal samples (n = 3) were positive for ALX4 while almost all breast cancer samples (119/120) showed a reduction or complete absence of ALX4 staining (Figure 15 and Table 2). Thus, tissue microarray staining for ALX4 using the score cutoff generated from our pilot study confirmed that ALX4 expression is indeed lost in breast cancer and can be used as a biomarker for breast transformation.
Figure 13. ALX4 expression is lost in DCIS. In patient samples with both DCIS and adjacent normal tissue, ALX4 expression was observed in both stromal (closed arrowheads) and epithelial (double arrowheads) cells in normal areas (left panel), but was reduced or absent in cancer tissue (right panel). Shown here are representative samples from three patients with DCIS. Insets: p63 staining of serial tissue sections shows nuclear expression in the myoepithelial cell layer, which delimits normal epithelial structures and DCIS lesions.

In addition to the observation that ALX4 expression is reduced or absent in breast cancer, it was noted in both the pilot study and the TMA analysis that ALX4 expression is lost in cases of both DCIS and invasive cancer (Figures 13, 14 and 15). These results suggest that ALX4
expression is lost early in the transformation process. Assessment of ALX4 expression at earlier stages of breast disease could pinpoint the stage at which ALX4 expression is lost. As breast cancer is believed to initiate as benign hyperplasia (Allred et al., 1993), a tissue microarray platform with benign breast disease cores could be examined.

Tumour suppressors are lost in the early stages of breast disease and other cancers (Oesterreich and Fuqua, 1999). Thus, ALX4 may possibly function as a tumour suppressor, and the early loss of ALX4 expression may play a role in tumourigenesis. To assess the role of ALX4 as a potential tumour suppressor in vivo, several cancer cell culture assays could be performed on normal and transformed breast cell lines. Wound, invasion, apoptosis and proliferation assays on breast cells engineered to overexpress \textit{ALX4} might demonstrate a decrease in cancer cell properties. Specifically, overexpression of \textit{ALX4} might be expected to delay wound healing and invasion, increase apoptosis and/or decrease proliferation. A problem that arises when considering these studies is the unique expression pattern of \textit{ALX4} in the human breast. As \textit{ALX4} is expressed in both stromal and epithelial cells, a paracrine signaling component may be important. Therefore, it might be necessary to use the conditioned media from primary breast fibroblasts which have been engineered to overexpress \textit{ALX4} as the culture medium for breast epithelial cells when performing these assays. Following the overexpression of \textit{ALX4} in either epithelial cells, fibroblasts, or both cell types, the aforementioned cell culture assays could help to determine whether \textit{ALX4} can act as a tumour suppressor via autocrine or paracrine signaling.

While the data presented demonstrates that ALX4 expression is lost in breast cancer, it is unclear if ALX4 expression is required for normal human breast development. Furthermore, as ALX4 is expressed in both fibroblasts and luminal epithelial cells, it remains to be seen whether ALX4 expression would be required in the stromal compartment, the epithelial compartment or
both. In order to address these questions, we may invoke the NOD/SCID mouse model with the ‘humanized’ mammary fat pad developed in the laboratory of Robert Weinberg (Kuperwasser et al., 2004). In this model, a cleared mouse mammary fat pad is first injected with human fibroblasts and subsequently grafted with human epithelial cells. Injection of normal breast fibroblast and epithelial cells produces epithelial outgrowths that resemble human TDLUs, while using transformed breast cells results in tumours which are highly similar to human breast carcinomas. The application of this model to dissect the contributions of stromal versus epithelial ALX4 in the human breast would involve the recombination of normal primary fibroblasts which express ALX4 with transformed primary epithelial cells which have lost ALX4 expression, and vice versa. We would expect normal ALX4-expressing human epithelial cells injected with normal ALX4-expressing human fibroblasts to produce normal TDLUs with no tumours. We could also predict that transformed breast epithelial cells with no ALX4 expression injected with breast cancer-associated fibroblasts that have lost ALX4 expression produce tumours. If stromal ALX4 expression, but not epithelial ALX4 expression, is required for normal morphogenesis as it is in the mouse (Joshi et al., 2006), then normal epithelial cells injected with transformed fibroblasts would result in tumours with a worse phenotype than would transformed epithelial cells injected with normal fibroblasts. Conversely, if epithelial ALX4 expression, but not stromal ALX4 expression, is necessary for normal human breast development, we would see a worse phenotype in the transformed epithelium/normal fibroblast chimeras than in the normal epithelium/transformed fibroblast mice. If all of the chimeric mice had similar phenotypes with some tumour development, this result would suggest that ALX4 expression is required in both the stromal and epithelial cell compartments, and would imply that the effects of ALX4 are mediated by both paracrine and autocrine signaling components in the human breast.
Figure 14. ALX4 expression is lost in IDC. In patient samples with both IDC and adjacent normal tissue, ALX4 expression was observed in both stromal (closed arrowheads) and epithelial (double arrowheads) cells in normal areas (left panel), but was reduced or absent in cancer tissue (right panel). Shown here are representative samples from three patients with IDC. Insets: p63 staining of serial sections shows nuclear expression in the myoepithelial cells of normal epithelial structures but no staining in IDC lesions, which lack a delimiting myoepithelial cell layer.
ALX4 expression is regulated by methylation in normal and transformed breast lines

A number of studies have shown that ALX4 is mutated in parietal foramina (PFM) and associated syndromes. From these studies, cases of PFM have emerged in which neither ALX4 nor MSX2, the gene that was first found to be mutated in PFM, are mutated. While some studies have suggested the involvement of a third locus in this condition, the gene has yet to be identified. In several recent colon cancer studies, ALX4 was found to be methylated in vivo and in vitro, suggesting a possible mechanism for the loss of ALX4 expression. However, not all samples showed methylation of ALX4. Furthermore, induction of ALX4 expression after demethylation could only be demonstrated for a single colon cancer cell line. In studies examining ALX4 methylation in human tissue, levels of ALX4 expression were not determined. Thus, while parietal foramina studies have examined genetic mutations, no consideration has been given to alternate mechanisms of gene regulation such as methylation. In colon cancer studies, epigenetic mechanisms have been considered as a means to silence ALX4 expression, but mutations in the ALX4 gene have not been examined, and expression has, in large part, not been addressed. To address these shortcomings, we examined both genetic and epigenetic mechanisms that might lead to loss of ALX4 expression in breast cancer.

Table 1. ALX4 expression is lost in breast cancer. Immunohistochemistry was performed on normal and breast cancer tissue, and staining was analyzed in a semi-quantitative manner. Samples in which >50% of stromal cells were moderately stained and >25% of epithelial cells showed weak expression were deemed positive for ALX4 (+). Samples which did not meet this cutoff were deemed unstained (-). Fisher’s exact test was used to compare ALX4 expression in 9 normal and 19 unrelated breast tumour samples (left panel). McNemar’s paired test of proportions was used to compare 18 breast tumour samples with adjacent normal tissue (right panel). In both cases, a highly significant difference in ALX4 expression was observed between normal and cancer samples.
To address a possible mechanism for the loss of ALX4 expression, RT-PCR was performed on a panel of normal (HMEC and MCF10A) and transformed (T47D, MCF7, MDA-MB-468 and MDA-MB-231) breast epithelial cell lines (Figure 16). RT-PCR results indicated that ALX4 is expressed in 293 HEK cells, from which the 5’ end of the gene was cloned. This cell line was used as a positive control for all other RT-PCR experiments. In contrast to the immunohistochemistry results which showed ALX4 expression in the normal breast, the normal breast lines HMEC and MCF10A did not express ALX4 by RT-PCR. As has been suggested by authors examining ALX4 expression in colon cancer cell lines, levels of ALX4 expression may fall below the limits of detection of RT-PCR. In fact, HMECs were occasionally seen to express low levels of ALX4 by RT-PCR (Figure 17 and data not shown) but these results were inconsistent. Another possible explanation is that these ‘normal’ breast cell lines are actually not normal. HMECs derive from primary human breast epithelial cells that were subjected to benzo[a]pyrene treatment and induced to have an extended life span, and have been shown to harbour genetic aberrations (Walen and Stampfer, 1989). In one study, HMEC expression of the runt-related transcription factor RUNX1 resembled that of several breast cancer cell lines more closely than it resembled RUNX1 expression by primary human mammary epithelial cells, suggesting that this cell line may not accurately reflect the normal breast epithelium (Lau et al., 2006). MCF10A is a spontaneously immortalized cell line derived from a patient with fibrocystic disease. It is not clear if ALX4 expression is altered in these patients in vivo. Furthermore, for all cell lines, culture for extended periods of time may result in adjustment to culture conditions, and artefacts may ensue.

In the breast cancer lines T47D, MCF7 and MDA-MB-231, consistent with immunohistochemistry results on human breast cancer tissue (Figures 6 and 7), no ALX4 expression was observed. In addition, the colon cancer line DLD-1 which was previously shown
not to express *ALX4* showed no expression (Ebert et al., 2006). In contrast, *ALX4* expression was observed in the breast cancer cell line MDA-MB-468, suggesting that *ALX4* expression is not lost in all cases of breast cancer, and levels of ALX4 expression may differ between cell lines.

**Figure 15. Tissue microarray staining confirms the loss of ALX4 expression in breast cancer.** Two replicate tissue microarray slides were assayed for ALX4 expression and a third replicate slide served as a negative control. Positive staining, in which at least 25% of epithelial cells stained weakly (double-headed arrows) and at least 50% of stromal cells stained moderately (closed arrowheads), was noted for all normal breast samples (3/3), while virtually all breast cancer samples (120/121) were unstained.
To determine whether the lack of ALX4 expression observed in most of these cell lines was due to a deletion of ALX4, the four exons and intron-exon boundaries were amplified. Of the cell lines examined, none showed a large deletion that could be detected by RT-PCR. However, a small deletion or a point mutation that could introduce a premature stop codon would not be detected by this method, leaving open the possibility that genetic alterations of ALX4 might result in loss of ALX4 expression. This possibility could be explored by amplifying and sequencing the entire transcript. Additionally, promoter and enhancer sequences have not been defined for ALX4, and regulatory regions within the 5’ and 3’ UTRs as well as the intronic segments may play a role in ALX4 expression.

To explore the possibility that epigenetic mechanisms of regulation could modulate ALX4 expression in the human breast, breast epithelial cell lines were treated with the demethylating agent 5-AZA-dC. As has been described, treatment of LoVo colon cancer cells showed induction of ALX4 expression and was used as a positive control, while treatment of DLD-1 and T84 colon cancer cells showed no expression, and was used as a negative control (Figure 17 and data not shown; Ebert et al., 2006). Interestingly, demethylation of HMEC resulted in the expression of ALX4. Therefore, ALX4 may be under the control of methylation in some normal breast epithelial cells though it is not known whether this regulation is direct or indirect. While it is possible that ALX4 is methylated as has been shown for ALX4 in colon cancer cell lines, it is also possible that...
Figure 16. Absence of ALX4 expression in breast cell lines is not due to a gene deletion. A ALX4 is expressed in 293 HEK and in MDA-MB-468 breast cancer cells but is not expressed at levels detectable by RT-PCR analysis in the normal human breast cell lines HMEC and MCF10A, the breast cancer cell lines T47D, MCF7 and MDA-MB-231, or the colon cancer cell line DLD-1. B PCR analysis using primers to amplify the four exons and intron-exon boundaries of ALX4 demonstrates that none of the normal breast (HMEC and MCF10A) and breast cancer (T47D, MDA-MB-468, MCF7 and MDA-MB-231) cell lines has a deletion of both copies of ALX4.

an upstream regulator of ALX4 is demethylated upon treatment with 5-AZA-dC. To determine if ALX4 is itself methylated in breast cell lines, it would be necessary to perform bisulfite sequencing to examine the methylation status of ALX4 before and after demethylation. The loss of methylation of a CpG island-rich promoter region after 5-AZA-dC treatment would support the idea that ALX4 may be directly regulated by methylation in breast cell lines.

Demethylation of the other ‘normal’ cell line MCF10A did not result in the expression of ALX4. Aside from the genetic reasons considered above, MCF10A may not have expressed ALX4 for several reasons. First, ALX4 may not be regulated by methylation in this cell line. That is, ALX4 itself may not be methylated and other regulatory mechanisms may silence ALX4 expression. Or, this may be true of any upstream regulator(s) in the case of indirect regulation. If, however, ALX4 is regulated by methylation in MCF10A, the dosage or length of treatment with 5’-AZA-dC may have been insufficient to induce expression of ALX4 to levels detectable by RT-PCR in this cell line. Additionally, a number of studies have demonstrated that methylated genes may also be regulated by histone deacetylation. Thus, treatment of cell lines with a combination of 5-AZA-dC and/or a histone deacetylase inhibitor such as trichostatin A could potentially induce expression of ALX4.
MDA-MB-231 showed no expression of ALX4 after 5-AZA-dC treatment. Expression of ERα in this ERα-negative cell line after the treatment period confirmed that the dosage and period of treatment were sufficient to allow demethylation. As above, these results suggest several possibilities: that ALX4 levels are below the limits of detection by RT-PCR, that ALX4 is not regulated by methylation or methylation alone in MDA-MB-231, and/or that genetic alterations preclude the expression of ALX4 in this cell line.

**Figure 17.** ALX4 may be reexpressed by demethylation in normal and breast cancer cell lines. Treatment with the demethylating agent 5-aza-2’-deoxycytidine induces expression of ALX4 in HMECs but not in MCF10A. The breast cancer cell line MDA-MB-231 does not express ALX4 after treatment but is demethylated, as can be seen by induction of ERα expression. The colon cancer cell lines DLD-1 and LoVo are shown as negative and positive controls, respectively.
Treatment of T47D and MCF7 resulted in weak expression of *ALX4* (data not shown). These results indicate that *ALX4* expression is regulated by methylation in these breast cell lines though, as described above, bisulfite sequencing would be needed to determine if this expression is direct or indirect. Taken together, these experiments demonstrate that methylation is one mechanism by which *ALX4* expression may be regulated in the normal breast and in breast cancer. It would be interesting to determine if *ALX4* (or an upstream regulator of *ALX4*) is more highly methylated in breast cancer cell lines than in normal breast cell lines. As has been shown in colon cancer studies, while *ALX4* may be methylated in the normal colon, hypermethylation is observed in preneoplastic lesions as well as in colon cancer. If this also holds true for *ALX4* in breast cancer, then both *ALX4* expression and *ALX4* methylation levels may be used as biomarkers in the early stages of breast cancer.
Conclusion

ALX4 is a homeodomain transcription factor involved in key stages of development in the mouse. Its expression in mesenchymal cells at sites of epithelial-mesenchymal interaction in numerous skin appendages suggests a role in paracrine signaling. The cell microenvironment and cell-cell interactions are well known contributors during mouse mammogenesis. The same can be said for human breast development, though architectural differences suggest that these interactions may be species-dependent. These results demonstrate that ALX4 is a transcription factor that participates in a signaling network that is required for normal human breast development. In this set of experiments, we demonstrated that ALX4 is expressed differently in the mouse mammary gland and in the human breast. In the human breast, paracrine signaling may play a role as it does in the mouse mammary gland. In addition, localization of ALX4 expression to some luminal epithelial cells suggests the influence of an autocrine component in human breast development. Interestingly, ALX4 expression is lost in both cell compartments in breast cancer. This loss occurs at an early stage, suggesting that loss of ALX4 expression may be used as a biomarker for breast tumourigenesis. However, in order to assess the usefulness of ALX4 expression in clinical diagnostics, it is necessary to determine whether loss of ALX4 expression occurs prior to neoplastic transformation of the breast. Interestingly, researchers have shown that ALX4 is hypermethylated in benign colon adenomas, suggesting that silencing of ALX4 expression is an early event in the transformation process. Immunohistochemistry performed on a tissue microarray of early stage and benign breast lesions paired with clinical follow-up could help to determine if ALX4 expression is lost in preneoplastic lesions in the breast. Furthermore, we showed that methylation is one mechanism that regulates ALX4 expression in normal breast and breast cancer cell lines. Loss of ALX4 expression by methylation
or other mechanisms may possibly contribute to tumourigenesis in early breast lesions. Finally, cancer cell culture assays, such as wound, migration, invasion, apoptosis and proliferation assays, could help to establish the role of \textit{ALX4} as a tumour suppressor. It would be interesting to determine if demethylation could induce expression of \textit{ALX4} and thereby promote cancer cell properties in culture.

The question remains as to the precise role of \textit{ALX4} in the normal breast and in breast cancer. Studies in the mouse have shown that \textit{ALX4} is expressed during stages of rapid proliferation in development. \textit{ALX4} expression in the mouse mammary gland appears to be spatially and temporally regulated to allow paracrine signaling necessary for development of the gland. \textit{ALX4} expression is highest at the tips of growing end buds, where proliferation of cap cells and body cells allows expansion of the mammary gland into the fat pad. In contrast, cap cells and body cells differentiate into the myoepithelial and luminal cells of the subtending duct closer to the neck of the TEB. \textit{ALX4} expression is lower in periductal regions than near the TEB. These results suggest a possible role for \textit{ALX4} in maintaining the undifferentiated stage of progenitor cells in the developing mouse mammary gland, believed to be located in the cap cell layer.

In the human breast, breast cancer almost always arises in luminal epithelial cells. Most luminal epithelial cells exist in an immature state and only fully differentiate into CK8/18$^+$ cells during pregnancy (Boecker and Buerger, 2003). Interestingly, in the resting adult premenopausal breast, approximately 4\% of luminal epithelial cells are CK5$^+$ progenitor cells, while the majority of luminal cells are intermediary CK5$^+$CK8/18$^+$ cells. If \textit{ALX4} does indeed maintain the undifferentiated state of progenitor cells, then the mosaic pattern of \textit{ALX4} expression observed in our human breast immunohistochemistry experiments might be explained. \textit{ALX4}
expression in luminal epithelial cells may be highest in a small proportion of CK5⁺ progenitor cells, and ALX4 expression may gradually be lost in CK5⁺CK8/18⁺ intermediaries (Figure 18). Fully differentiated CK8/18⁺ cells may not express ALX4 at all. Coimmunofluorescence on human breast tissue using cytokeratin-specific antibodies and ALX4 could determine the nature of the epithelial cells in which ALX4 is expressed, and support the idea that ALX4 is expressed in progenitor cells. In cases of DCIS and IDC, but not usual ductal hyperplasia, mostly CK8/18⁺ cells are seen. These observations support the model in which ALX4 is expressed in CK5⁺ progenitor cells and, at lower levels, in CK5⁺CK8/18⁺ intermediary luminal cells, but not in differentiated CK8/18⁺ luminal epithelial cells. In our model, the loss of an anti-differentiation signal, possibly ALX4, would mark the transition from a normal CK5⁺ progenitor cell to a CD44⁺CK5⁺ breast cancer stem cell (Al-Hajj et al., 2003; Horwitz and Sartorius, 2008). Clonal expansion in the absence of a differentiation signal would allow differentiation into CD24⁺CK8/18⁺ luminal cells (Al-Hajj et al., 2003; Boecker and Buerger, 2003; Shipitsin and Polyak, 2008), which do not express ALX4. This model could explain the loss of ALX4 expression observed in DCIS and IDC. Loss of ALX4 expression, possibly by methylation, may be restricted to one or more subtypes of breast cancer or, as our data suggest, may be a universal mechanism which governs the transition of the breast from a normal to a transformed state.

In conclusion, my results have demonstrated the importance of analyzing and understanding the applications and shortcomings of a model system. Experiments on human breast tissue and breast cell lines have demonstrated the usefulness of ALX4 expression and methylation as a novel biomarker for breast cancer. Combining our knowledge of ALX4 in other systems together with breast histopathology, stem cell theory and molecular taxonomy may help to define the precise role of ALX4 and other genes implicated in breast cancer. This integrative
approach may lead to a better understanding of pathways and mechanisms involved in breast cancer etiology, and potentially provide a host of new therapeutic targets.

**Figure 18. Model for the role of ALX4 in the normal breast and in breast cancer.** In the normal human breast, ALX4 is expressed in the majority of fibroblasts (1) and in a mosaic pattern in luminal epithelial cells (2 and 3). A small population of CK5+ stem cells (2, dark brown nuclei) express the highest levels of ALX4, which functions as an anti-differentiation factor and maintains the immature, largely undifferentiated state of ducts and ductules within the breast. Intermediary CK5+CK8+ cells (3, brown and beige nuclei) express variable levels of ALX4 as they gradually differentiate into CD24+CK8+ luminal cells (4, uncoloured nuclei), which no longer express ALX4. In a CK5+ breast stem cell, an initiating event (5) results in the loss of ALX4 expression by hypermethylation, producing a CD44+CK5+ breast cancer stem cell (6, red nucleus). The loss of expression of the anti-differentiation factor ALX4 results in a shift in the stemness versus differentiation balance, leading to the abnormal hyperproliferation of ALX4-deficient terminally differentiated CD24−CK8− luminal cells (7, black nuclei), as observed in DCIS.
References


