**Multi-author Review**

# Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer

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Cell. Mol. Life Sci. 64 (2007) 3161 – 3184
1420-682X/07/243161-24
DOI 10.1007/s00018-007-7386-2


Cellular and Molecular Life Sciences

# Molecular links between mammary gland development and breast cancer

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Online First 22 October 2007

**Abstract.** During its lifetime, the mammary gland undergoes many phases of development and differentiation. Much of this occurs during puberty, when the ductal epithelium expands by branching morphogenesis, invading the surrounding fat pad to form an organised mammary tree. Throughout its existence, the epithelium will go through several cycles of proliferation and cell death during pregnancy, lactation and involution. Many of the signalling mechanisms which control the initial invasion of the fat pad by the epithelium, and regulate its continuing plasticity, can be harnessed or corrupted by tumour cells in order to support their aberrant growth and progression towards invasion. This is true not just for the epithelial cells themselves but also for cells in the surrounding microenvironment, including fibroblasts, macrophages and adipocytes. This review examines the complex web of signalling and adhesion interactions controlling branching morphogenesis, and how their alteration can promote malignancy. Current *in vivo* and *in vitro* mammary gland models are also discussed. (Part of a Multi-author Review)

**Keywords.** Pubertal development, breast cancer, hormones, TEB (terminal end bud), TDLU (terminal ductal lobulo-alveolar unit), signalling, adhesion, microenvironment.

## Introduction

The principal function of the mammary gland is to provide the newborn with milk, a unique bodily fluid that has a dual role in nutrition and immunological protection. This gland was considered such a significant feature that it became the basis for defining the class Mammalia [1]. The mammary gland is a unique organ in that it undergoes the majority of its development after birth. This post-natal development occurs in discrete phases linked to sexual development and reproduction [2]. Much of our knowledge about this process is drawn from experiments on mouse models, which must be extrapolated to humans, while accounting for both the genetic and physical differences between these species. Development of the mammary gland begins during foetal development, and a rudimentary gland is present at birth. Further epithelial growth is hormone-dependent and begins at puberty, when the virgin gland develops highly proliferating structures called terminal end buds (TEBs), known as terminal ductal lobulo-alveolar units (TDLUs) in humans, which grow and branch to form the mature epithelial ductal tree (Fig. 1).

TEBs are bilayered structures with a hollow central lumen, an inner layer of luminal cells and an outer layer of myoepithelial cells, outside of which lies a highly crosslinked basement membrane (BM) layer. At the tip of the TEB are the cap cells, loosely adhering epithelial cells which lack cytoplasmic polarity, steroid receptors and a well-organised cytoskeleton [3]. The TEBs are highly motile structures which bifurcate repeatedly and invade through surrounding fatty stroma, responding to local promotional and inhibitory signals to form the final 'open' architecture of the gland. Behind TEBs, mature ducts sprout laterally to form side branches. Unlike the process of ductal elongation, formation of these lateral branches requires invasion of the ductal epithelium through a

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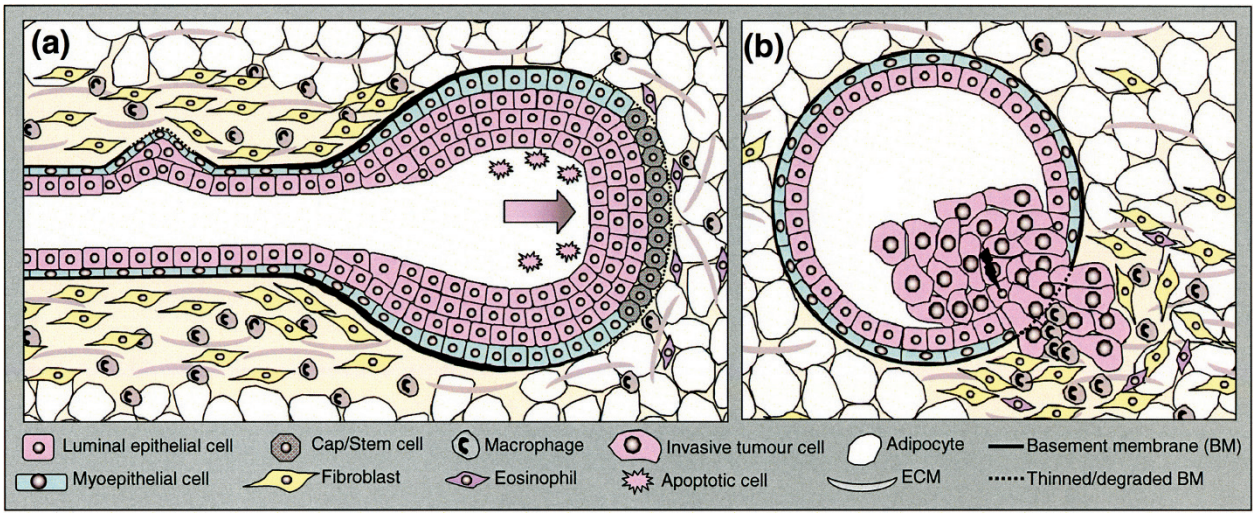


**Figure 1.** Representation of some common microenvironmental influences on (*a*) pubertal terminal end buds (TEBs) and (*b*) invasive tumours. TEBs are thought to move through the stroma by a combination of 'pushing' force due to high rates of proliferation and a constricting periductal BM, and 'pulling' force due to migratory signals from surrounding extracellular matrix (ECM) and stromal cells. The BM at the TEB tip is thinner than ductal BM, possibly due to partial degradation by proteases or reduced synthesis of components by cap cells. The cap cells at the tip of the TEB are putative stem cells for both the luminal epithelial and myoepithelial lineages. Stromal cells (fibroblasts, macrophages, eosinophils, adipocytes) aid TEB invasion by production of growth factors, promigratory elements and ECM proteases. Similar mechanisms support tumour cell invasion, although aberrant signalling by both epithelial and stromal cells leads to increased/dysregulated propagation of signals. The tumour cells are thought to escape from the duct via a combination of analogous 'pushing' and 'pulling' forces, while infiltrates of stromal cells secrete proteases to degrade the BM and create a path for tumour cells to invade.

mature BM and periductal stroma, and is thus regulated by distinct cues. Branching morphogenesis is controlled through systemically released steroid and peptide hormones, which induce local paracrine signals between the epithelial and stromal cells of the mammary gland, to ultimately control the precise development of the mature gland. This structure allows for further development of the gland during cycles of pregnancy and lactation. During early pregnancy, lateral buds extend from the main ducts, and at a later stage these differentiate, filling the interductal stroma with lobuloalveolar structures containing secretory epithelial cells, and stretching the myoepithelial layer into an open conformation. The secretory cells are stimulated after birth to produce milk, and the contractile myoepithelial cells constrict in response to oxytocin to aid milk release. Upon weaning, the lobuloalveolar compartment undergoes massive apoptotic cell death and remodelling in a process called involution, to restore the simple ductal architecture [2, 4]. Therefore, although the mammary gland may be in a dormant state throughout much of its lifetime, it must be ordered in such a way that it can receive, assimilate and respond to diverse signals at the onset of pregnancy.

During this complex developmental cycling, the epithelial compartment undergoes many rounds of proliferation, remodelling and cell death. The notion that the pathways controlling this process can be harnessed to promote tumorigenic processes is not a new one [2, 5–7]. Pubertal mammary development involves invasion of ductal epithelial structures into surrounding stroma in a highly directed manner, with recruitment of fibroblasts, immune cells and ECM components around the TEBs, and thinning of BM at the leading end [3]. During the transition from *in situ* to invasive breast cancer, proliferating tumour cells within intact ducts recruit numerous stromal cells and ECM proteins to the outside of the ducts and induce secretion of proteases such as MMPs, leading to BM degradation and subsequent invasion of tumour cells into the stroma [6]. In spite of the obvious disparity between the highly regulated process of development and the less organised environment of invasive cancer, many identical mechanisms and signalling pathways may in fact regulate both activities. This applies not only to the pubertal gland – the mammary gland remodelling which occurs during involution is also developmentally programmed and highly ordered, involving massive cell death of secretory epithelia, and requiring recruitment and activation of fibroblasts, immune cells and other stromal components. This activated stromal environment is strikingly similar to that present in invasive breast cancer, and several studies have shown that the stromal microenvironment during involution is tumour promoting [8, 9]. This suggests that a number of common factors are responsible. Understanding the mechanisms regulat-

ing normal developmental processes in the mammary gland may help us to understand how tumours can adopt and corrupt these normal mechanisms to promote their own growth and invasion.

## Systemic and local regulators of mammary epithelial cell behaviour

Growth and development of the mammary gland at puberty is regulated at the systemic level by ovarian and pituitary hormones and, at the local level, by paracrine interactions between ductal epithelial cells and their surrounding stroma. The systemic hormones that regulate this process were initially identified by hormone depletion and replacement studies. Estrogen and growth hormone (GH), produced by the ovaries and the pituitary, respectively, are known to play a central role in mammary ductal morphogenesis at puberty. Estrogens were shown to restore TEB formation and normal mammary development in ovariectomised mice [10], and GH, but not prolactin, was found to rescue mammary gland development in hypophysectomised mice [11]. Later studies on mice with mutated or deleted estrogen receptor α (ERα) or GHR confirmed a critical role for these hormones in mammary branching morphogenesis [12, 13], whereas mammary glands in mice lacking progesterone receptor (PR), ERβ or prolactin receptor developed normally at the pubertal stage [4, 14, 15]. However, PR and prolactin receptor knockout studies revealed that these hormones were necessary in the later development of the mammary gland, for the ductal side branching and alveologenesis that occur during pregnancy [14, 15].

Although the above endocrine compounds are essential for normal development, increased exposure to these hormones is known to raise breast cancer risk. Risk factors include exposure to high levels of estrogens before birth, longer cumulative exposure over the reproductive lifetime, high serum estrogen levels and exposure to exogenous hormones such as those in oral contraceptives [16]. However, hormones can also have a protective effect: a recent study has shown that the pregnancy hormone human chorionic gonadotropin induces differentiation of the mammary gland and downregulates ER levels via CpG island methylation, leading to a long-term protective effect against breast cancer [17]. Chemical disruption of endocrine signalling pathways at crucial developmental timepoints can also increase the risk of developing breast cancer in later life. The crucial exposure periods are during phases of rapid growth, particularly neonatal growth of the rudimentary gland, pubertal development and functional differentiation during pregnancy. Exposure to endocrine-disrupting compounds at these developmental stages can induce lasting alterations in gland morphology and sensitivity to certain carcinogens [18].

These systemic hormones are very powerful regulators of development; however, with the advent of knockout mice and tissue transplantation experiments, it has become clear that the wide-ranging effects of these agents are not due to direct hormone action but rather to the actions of multiple secondary paracrine effectors. At the local level, the branching pattern of epithelial cells seems to be controlled by signals not only from the epithelial cells themselves, but also from the stroma. This was first illustrated by tissue transplantation experiments in mice, in which embryonic mammary epithelium was combined with salivary mesenchyme [19]. The mammary epithelium developed in a morphologically similar pattern to that of the salivary gland, while retaining the functional capacity of mammary epithelium: ability to respond to hormonal stimulation and secrete milk proteins. Therefore, the morphological structure of the epithelium is dependent on the mesenchyme it contacts, whereas functional differentiation is dependent on the epithelial component. Seminal experiments by Cunha and colleagues, in which ER knockout (ERKO) epithelium from neonatal mice was recombined with wild-type mammary stroma and *vice versa*, revealed that epithelial ER was not required for hormonal regulation of ductal development – the process was instead regulated by paracrine signalling from ER-positive stromal cells [12]. However, similar tissue recombination studies which used adult ERKO and wild-type mice concluded that both epithelial and stromal ER was necessary for complete mammary gland development in adult mice [20]. This research was complicated by the fact that the ERKO mice still expressed a truncated form of ER, which was later shown to possess significant transactivating capability [21].

Further knockout studies have suggested that epithelial cells are the primary target for estradiol during puberty, and that ERα may exert its effects in a paracrine fashion on neighbouring mammary epithelial cells (MECs) [22]. This is supported by analysis of human tissue showing that ERα is expressed in only a subset of MECs, distributed throughout the ducts, and proliferation of adjacent ER-negative cells is controlled by paracrine factors released by ER-positive cells. A similar paracrine mechanism exists for PR-induced proliferation. This hierarchical system may be disrupted in tumours, which commonly have a high proportion of ER-positive proliferating cells [23]. These studies demonstrate that ERα expression in the mammary gland and its responsiveness to estrogen is

highly dependent on the developmental stage of the gland. A deeper knowledge of ER action in the mammary gland is essential in order to understand its role in breast cancer, as both a regulator of epithelial cell growth and a key therapeutic target. Endocrine therapies such as tamoxifen are one of the most effective breast cancer treatments available for ER-positive tumours, but *de novo* and acquired resistance are still major problems. ER signalling is also closely integrated with other growth factor signalling pathways, and the EGFR/Her2 pathway has been implicated in tamoxifen resistance [24]. Understanding how molecular crosstalk between ER and other growth factor-signalling pathways combine to stimulate the many downstream targets of ER and promote growth is crucial in order to combat resistance to endocrine therapy.

The role of pituitary GH in pubertal development of the mammary gland was demonstrated by tissue recombination experiments on GHR null and wild-type mice. Virgin GHR null mice exhibit severe defects in ductal outgrowth and branching during puberty. GHR null epithelium transplanted into wild-type cleared fat pads grew normally, whereas wild-type epithelia in GHR null fat pads exhibit stunted growth, consistent with stromally mediated GH signalling [25]. The effects of GH in mammary gland development have indeed been shown to be regulated by a stromal intermediary: insulin-like growth factor 1 (IGF-1). A study on IGF-1 knockout mice, which displayed impaired pubertal mammary development, showed that treatment of the mice with IGF-1 and estrogen restored normal development, whereas treatment with GH and estrogen did not, suggesting that IGF-1 is the downstream effector of GH in the mammary gland [11]. In addition, tissue recombination studies with IGF-1R knockout mice have demonstrated the importance of IGF-1R in epithelial rather than stromal cells [26]. These data strengthen the hypothesis that systemic GH binds to its stromal receptor to induce secretion of IGF-1, which in turn binds to epithelial IGF-1R to exert an effect on TEB elongation and ductal branching. Studies in mice show that GH and estrogen pathways can also synergise in the stromal compartment, with GH capable of inducing ER expression and estrogen-stimulating IGF-1 expression [2, 11].

The systemic hormones progesterone and prolactin also act on the mammary gland, particularly in the adult gland during pregnancy and lactation. Progesterone and its receptor are required for ductal side branching and alveologenesis, and PR null mice exhibit defective lactation. Prolactin is essential for normal lobuloalveolar differentiation and lactation. These systemic hormones are thought to act synergistically in order to activate the 'alveolar switch', a genetic programme induced in response to pregnancy which results in altered expression of transcription factors and paracrine effectors. This programme orchestrates the massive tissue remodelling that occurs in preparation for lactation, expanding the epithelial cell population and inducing differentiation of alveolar cells, resulting in a functional secretory gland [27]. These hormones also play a role in breast cancer. The role of progesterone receptor in breast cancer is complex, but it is associated with tumour initiation and cell proliferation [28]. High levels of serum prolactin in women are associated with an increased risk of breast cancer [29]. Studies have shown that prolactin acts both directly through its receptor and also exerts an indirect effect which modulates levels of other signalling molecules such as estrogen and progesterone [14]. The role of these hormones in development and cancer have been reviewed in depth [28, 29].

## Localised regulation of branching and invasion

The above hormones and growth factors have been shown to stimulate mammary epithelial cell growth, but what controls the precise growth and inhibition needed to form the complicated, intricately branched, regularly spaced structure of the mature mammary gland? Even more importantly, what drives the tips of the gland to invade the mammary fat pad? Similar mechanisms may also drive invasion in cancer, and the molecular basis of this invasion is discussed in detail in another review in this series (McSherry et al.). During puberty, the high rate of proliferation in the TEB, and a thickening of the BM and ECM around the neck of the bud by increased secretion of sulphated glycosaminoglycans and type I collagen, are thought to combine to create a pressure within the bud which is channelled forward by the constricting ECM girdle [30]. The mechanism of side branching in mature ducts is different: lateral branches must invade through a barrier of myoepithelial cells, BM and stromal ECM, and this process may therefore be under the control of distinct mechanisms, many of which are currently unknown [6]. However, it is clear that the systemic hormones discussed previously cannot directly regulate formation of the intricate structure of the mammary gland, and instead must drive development via a complex web of locally interacting paracrine effectors (Fig. 2). Mechanisms like apoptosis are also important for sculpting the internal lumen of the mammary gland. Recent studies by Brugge and colleagues showed that knocking down certain BH3-only proteins such as Bim and Bmf in

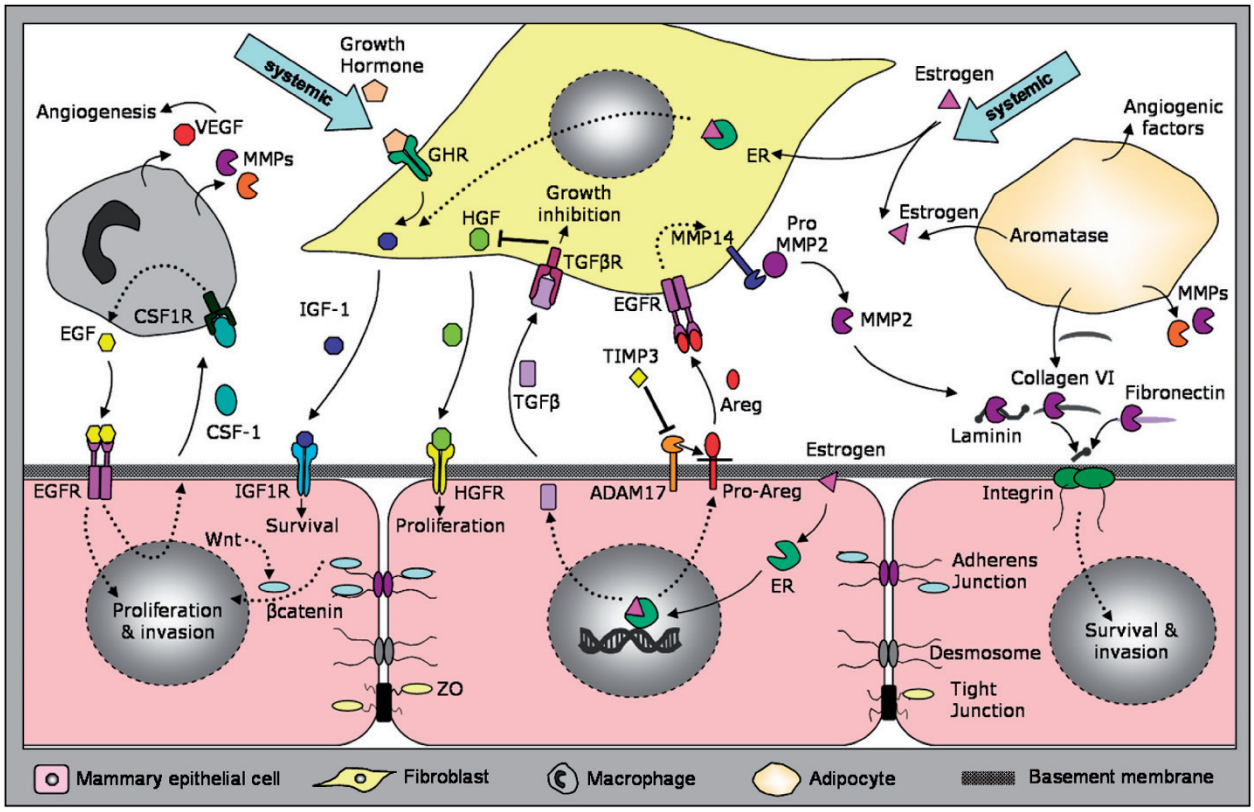


**Figure 2.** Outline of the current hypothesis of interacting endocrine, paracrine and adhesion signalling pathways which influence epithelial and stromal cell behaviour in the mammary gland during development and cancer. Some of the pathways depicted are not exclusive to one type of stromal cell. Dotted arrows indicate indirect interactions.

mice delays luminal clearance of the mammary ducts during puberty [31, 32]. Although these ducts are eventually cleared by caspase-independent death mechanisms, the filling of the ducts in these knockout mice, and in the analogous *in vitro* MEC acini, is similar to the ductal carcinoma *in situ* (DCIS) cancer phenotype. This raises the possibility that these caspase-independent mechanisms of cell death may be important tumour suppressor mechanisms during luminal filling in the early stages of breast cancer.

### *The multifaceted role of TGFβ*

The precise pattern of the mature ductal structure in the mammary gland would suggest a need for localised growth inhibitors. One such molecule, which is known to play a significant role in mammary morphogenesis, is transforming growth factor beta (TGFβ), secreted in a latent form from ductal epithelium, activated extracellularly and acting on its receptor to inhibit lateral branching and ductal growth [33, 34]. An *in vitro* branching assay carried out by Bissell and colleagues has demonstrated the importance of TGFβ in specifying branch points [34]. Branching in MEC tubules was found to be determined by tubule geometry and the local concentration of inhibitory factors such as TGFβ – the concentration was lowest at tubule ends where branches formed, and branches would not form at close proximity to other tubules, explaining the precise spacing of branches in the mammary tree. Since it is known that TGFβ acts primarily on stromal receptors, its growth inhibitory function must be mediated by stimulation or inhibition of diffusible paracrine factors. One promising candidate is hepatocyte growth factor (HGF), a mitogenic cytokine secreted by fibroblasts which promotes cell proliferation, survival and motility by binding to its tyrosine kinase receptor, c-met, and has been shown to induce tubule formation in many epithelial cell lines [35]. It is known to be negatively regulated by TGFβ [36]. HGF also binds to heparan sulphate proteoglycan, a component of the ECM, and this binding enhances its signalling through c-met [37]. In human breast, HGF is secreted by stromal fibroblasts, and c-met is present exclusively in the epithelium [38]. Conditional knockout of the TGFβ type II receptor in murine stromal fibroblasts has been shown to stimulate tumour cell growth and motility via upregulation of expression of HGF, TGFα, and macrophage-stimulating protein [36]. Another possible factor responsible for the downstream effects of

TGFβ is parathyroid hormone-related protein, which is known to be positively regulated by TGFβ; overexpression of this protein in mice inhibits ductal extension during puberty [39].

Substantial evidence supports a role for TGFβ in lateral branching inhibition: in mice, TGFβ has been shown to localise to the periductal stroma in areas where lateral budding is suppressed, and this ECM-associated TGFβ is selectively lost where lateral buds are forming [40]. Interestingly, the luminal and myoepithelial cells of the mouse mammary duct respond differently to HGF, the putative downstream factor for TGFβ. HGF appears to have a mitogenic effect on luminal cells while exerting a morphogenic effect on myoepithelial cells, inducing them to extend tubular structures [35]. Since TGFβ is thought to suppress HGF, the loss of TGFβ at lateral branch points would therefore promote lateral branching. HGF/c-met signalling is also a factor in cancer, and HGF is overexpressed in invasive human breast cancer [38]. *In vitro*, increased HGF/c-met signalling has been shown to promote epithelial cell migration and invasion [41], and activation of c-met by HGF can induce anchorage-independent growth via β4-integrin phosphorylation [42]. HGF has also been shown to promote angiogenesis via positive regulation of VEGF and negative regulation of TSP1 [43]. The pleiotropic effects of HGF in cancer make it a promising therapeutic target, and monoclonal antibodies to HGF have shown therapeutic potential [44], with clinical trials currently under way.

TGFβ released from epithelial cells is also crucial for the control of ductal elongation: in TGFβ heterozygous knockout mice, which have less than 10% of the wild-type TGFβ levels, both ductal and alveolar development were increased 2- to 4-fold, consistent with its role as a growth inhibitor [33]. Interestingly, upon exposure to ovarian hormones, proliferation was 15-fold greater in $TGF\beta^{+/-}$ mice compared to $TGF\beta^{+/+}$ controls, suggesting that TGFβ acts to regulate proliferation in response to hormonal stimulation. This relationship between estrogen and TGFβ was examined further, and it was found that, in TGFβ-deficient mice, colocalisation of ERα with markers of proliferation was significantly increased [45]. This colocalisation rarely occurs in normal human breast cells, and studies have shown that increased proliferation in the ERα-positive cell population increases breast cancer risk [46]. Expression of constitutively active TGFβ in the epithelial cells of these mice via the MMTV promoter suppressed proliferation of ERα-positive cells, further supporting a restraining role for TGFβ to counteract the growth-promoting effect of estrogen [45]. It would follow that TGFβ dysregulation might promote proliferation of the ERα-positive cell population and increase breast cancer risk.

However, the role of TGFβ in breast cancer seems to be much more complex: it appears to play a bipolar role and is capable of both promoting and suppressing tumour growth, earning it the description 'the molecular Jekyll and Hyde of cancer' [47]. In normal epithelial cells, TGFβ acts via its receptor and the Smad transcription factors to exert cytostatic and apoptotic effects and thus inhibit tumour formation. On the other hand, if a tumour is initiated, the tumour cells often lose their ability to respond to anti-proliferative TGFβ signals, and this molecule becomes tumour-promoting. This altered response is thought to be due to disrupted downstream responses to TGFβ stimulation. During tumour progression, TGFβ has been shown to promote tumour cell invasion and metastasis in a number of ways, including interactions with growth factor signalling networks, suppression of immune responses, promotion of epithelial-to-mesechymal transition (EMT) and stimulation of angiogenesis [47, 48]. In light of the unpredictable nature of TGFβ signalling, it is clear that multiple cell- and context-dependent factors influence the effect of TGFβ on cell behaviour in the tumour situation. Despite these uncertainties, it has become clear that TGFβ gains a growth-promoting role in advanced cancer, and treatments which block TGFβ signalling have shown some efficacy in clinical trials [49].

### *The ErbB family of receptors and their ligands*

An important localised regulator in the mammary gland is the epidermal growth factor receptor (EGFR/ErbB1) pathway, a ligand-activated tyrosine kinase receptor, in combination with one of its ligands, amphiregulin. Amphiregulin expression has been shown to be upregulated during puberty, and to be an essential growth factor for ductal development. In the mouse, expression of amphiregulin is upregulated in developing epithelial ducts [50], whereas its receptors, members of the ErbB family, only need to be present in stromal cells for normal mammary development [51]. Expression of this ErbB ligand is also upregulated in breast tumours [52]. Amphiregulin is created as a transmembrane precursor, which must be cleaved in order to bind to its receptor on adjacent stromal cells. The transmembrane metalloproteinase ADAM17 (A disintegrin and metalloproteinase 17), also known as TACE (TNFα converting enzyme), has been proposed to carry out this cleavage: studies have shown it to be expressed in epithelial cells, and its expression is upregulated in concert with amphiregulin [53]. The defective ductal growth seen in ADAM17 null mice can be rescued by amphiregulin treatment, suggesting that this metalloproteinase may indeed

cleave and release amphiregulin. Furthermore, expression of the only known inhibitor of ADAM17, tissue inhibitor of metalloproteinases 3 (TIMP3), is known to be locally downregulated in TEBs, which would promote a focused release of amphiregulin and increased growth, providing a mechanistic solution to the precise growth promotion occurring at the tip of end buds [53]. However, since the EGFR can bind up to seven different ligands and dimerise with itself or any of three related receptors (ErbB2-B4), there exists a huge diversity in EGFR signalling in the mammary gland, much of which is poorly understood. A deeper understanding of this signalling network is not just important for studies of mammary gland development – these signalling factors also play a central role in breast cancer. Dysregulated ErbB signalling, as a result of mutations, receptor overexpression or ligand overexpression, can result in tumour initiation or increased growth and migration of existing tumour cells. Each of the four ErbB receptors is known to be overexpressed in breast cancer. For example, ErbB1/EGFR is commonly overexpressed or amplified in steroid receptor-negative tumours, suggesting that EGFR-directed therapies may be effective in this subset of tumours [54]. A mechanism for constitutive EGFR activation in the absence of genetic mutations was recently described by Kenny et al. [55], via a TACE/ADAM17-dependent autocrine loop which can confer growth factor independence to tumour cells. Inhibition of ADAM17 activity by small molecule inhibitors or small interfering RNA (siRNA) reverts the malignant phenotype in breast cancer cell lines by preventing cleavage and release of the EGFR ligands TNFα and amphiregulin. ADAM17 and TNFα expression was also found to be closely correlated in published breast cancer gene expression datasets, and predictive of poor prognosis, suggesting that ADAM17 inhibition may be an effective therapeutic strategy for the treatment of breast cancer [55]. The gene encoding ErbB2 or Her2 is also amplified in 20–30 % of breast cancers and has been shown to associate with increased proliferation, metastatic potential, risk of relapse and a poor prognosis [54]. Targeting this receptor with a specific monoclonal antibody, known as Herceptin (trastuzumab), can elicit a response in around a third of patients with Her2-overexpressing tumours. However, redundancy and crosstalk between different ErbB receptors are thought to contribute to resistance, and new therapies based on monoclonal antibody or tyrosine kinase inhibitor strategies, which target both EGFR and Her2, have shown clinical benefit [56]. Clarification of these mechanisms of resistance and the types of crosstalk in the ErbB network is crucial for the success of future breast cancer therapies.

***The degrading role of matrix metalloproteinases***

A family of ECM proteins known as matrix metalloproteinases (MMPs), which can degrade many components of the ECM, have also been shown to regulate localised growth and inhibition during mammary development, as well as promoting tumour invasion. Functions of MMPs include cell signalling as a result of alteration of the microenvironment, release of bioactive components from the ECM such as fibronectin and laminin, activation of growth factors, and cleavage of cell-cell and cell-matrix adhesion proteins [57]. MMPs are also proposed to 'clear a path' for invading ducts in the mammary gland during puberty due to their matrix remodelling activities. This remodelling process is an essential part of ductal development – removal of MMP activity in a primary MEC culture model by addition of inhibitors reduced branching stimulated by a variety of growth factors, including EGF, fibroblast growth factor-7 (FGF7), FGF2 and HGF [58]. A study by Wiseman and colleagues [59] showed that mice treated with a broad-spectrum MMP inhibitor displayed severely limited ductal invasion, but also an increased amount of lateral budding, suggesting differential effects of MMPs depending on the location. Focusing on MMP-2, which is highly expressed in the stroma in front of advancing TEBs, this group found that MMP-2 null mice exhibited retarded ductal invasion and increased lateral branching, suggesting a differential effect depending on its location – thus, MMP-2 promotes ductal elongation while repressing lateral budding. Surprisingly, in looking for a mechanism to explain the retarded invasion, this group found no evidence of an altered ECM in MMP-2 null mice, but instead attributed the effect to reduced epithelial cell survival due to a lack of MMP-2, contradicting the 'path clearing' theory. This pro-survival effect was thought to be due to the cleavage and release of survival factors sequestered by the ECM, or activation of other substrates of MMP-2, resulting in altered signalling within the TEB [59]. Previous studies in cell lines have shown that cleavage of the ECM protein laminin-5 by MMP-2 produces a bioactive laminin fragment, which in turn induces mammary epithelial cells to migrate [60], implying that a combination of both cell migration and 'pushing' mechanisms may be driving ductal invasion.

Different MMPs may regulate other aspects of ductal development, such as MMP-3 (Stromelysin-1), which is thought to regulate ductal side branching [59]. Lateral branching in the mammary gland requires degradation of BM, as well as proliferation and invasion of epithelial cells into the surrounding stroma, with MMP-3 being known to cleave BM components, including laminin, collagen IV and

nidogen. MMP-3 null mice also displayed fewer sites of ECM degradation along the ducts. This research suggests that MMP-3 is involved in both branch-site specification and induction of branch formation in dormant stem/progenitor cells along the mammary ducts [59]. The invasion-promoting abilities of MMPs also have a well-established role in tumours, and current evidence suggests that these proteases can promote tumour invasion, angiogenesis and metastasis by means of their ECM-degrading activities [57]. In fact, without these ECM-degrading proteases, cancer cells may be unable to break through the BM barrier blocking their invasion. The putative pro-survival properties of MMP-2 may also play a role in promoting tumour progression [59]. Studies on transgenic mice expressing an autoactivated form of MMP-3 showed that, apart from promoting invasion of existing tumours, increased MMP-3 activity can lead to initiation of premalignant and malignant growths in the mouse mammary gland, possibly by virtue of its ability to cleave cell surface proteins such as integrins and alter cell signalling [61].

The importance of both ErbB and MMP signalling in tumour progression was recently illustrated by the lung metastasis studies of the Massagué lab [62, 63]. This study implicates a panel of four genes: the pan-ErbB ligand epiregulin, Cyclooxygenase-2 (COX2), and MMP-1 and -2, which cooperate to facilitate lung metastasis. Using mice inoculated with a lung-metastatic subpopulation of the breast cancer cell line MDA-MB-231, this group showed that individual silencing of these genes moderately inhibited tumour progression. However, silencing of all four genes simultaneously produced a synergistic effect which almost completely suppressed primary tumour growth, inhibited angiogenesis, and prevented the spread of tumour cells from the primary site and their intravasation into the lung. This outcome could also be achieved by treatment with a combination of existing drugs: the EGFR antibody cetuximab, the COX2 inhibitor celecoxib, and the broad-spectrum MMP inhibitor GM6001 [62]. This study shows that inhibition of multiple steps of tumour progression, from growth and angiogenesis to dissemination and extravasation, is necessary for the effective suppression of tumour metastasis.

### *An abundance of transcription factors*

A fundamental component of all of the above signalling pathways is the transcriptional machinery of the cell, which can modulate the cellular response to a specific signalling pathway via recruitment of transcriptional activators and co-activators. This level of interaction can account for tissue-specific responses, and also provides a mechanism for integrating the multiple signals entering the nucleus. Steroid receptor coactivators, or SRCs, are transcriptional co-activators which interact with a number of DNA-binding transcription factors, and are involved in recruiting and organizing members of the coactivating complex [64]. Differential requirements for SRCs in the mammary gland and uterus have been shown to account for tissue-specific responses to progesterone signalling – SRC-3 modulates progesterone-stimulated transcription in the mouse mammary gland, while SRC-1 is responsible in the uterus, resulting in altered expression of specific target genes [65]. The transcriptional co-activator CITED1 has also been shown to modulate responses to hormonal stimulation, in this case, by estrogen – CITED1 null mice display defective growth and branching at puberty [66], and CITED1 has been shown to act as a selective coactivator for estrogen-dependent transcription of a range of genes, by enhancing the transcriptional activity of ERα [67, 68]. CITED1 is also known to act as a cofactor for Smad-4 downstream of TGFβ [69], thus providing a mechanism for integrating these two important signalling pathways in mammary gland development.

The transcription factor GATA-3 was also recently found to be an essential regulator of luminal epithelial cell differentiation in both the developing and mature mammary gland [70, 71]. This gene was identified as being highly upregulated during puberty in the mouse, and was found to localise to the luminal epithelial cell population. Targeted deletion of GATA-3 in the mammary gland of pubertal mice prevented TEB formation, and inducible deletion in adult mouse mammary gland caused expansion of an undifferentiated luminal cell population, suggesting a key role for GATA-3 in maintenance of the differentiated luminal phenotype in the mammary gland. The central role of these transcription factors in development and maintenance of normal breast tissue suggests that their disruption could be involved in tumour development. Indeed, re-analysis of a human breast cancer DNA microarray dataset has shown that CITED1 expression positively correlates with ERα expression, and high CITED1 expression associates with longer survival, suggesting that perpetuation of this signalling pathway in breast tumours may signify good prognosis [68]. Similarly for GATA-3, higher expression levels of this gene associate with ERα positivity in breast cancer, and expression is highest in well-differentiated breast tumours with good prognosis [72]. These studies highlight how valuable new prognostic biomarkers can be in order to more accurately predict disease outcome in breast cancer.

This is by no means a comprehensive account of all of the signalling pathways in the mammary gland, and

this subject has been reviewed in detail [4]. Yet it is clear even from this brief overview that in order to form the complex branched structure of the mature mammary gland, mammary epithelial and stromal cells need to be able to respond to a plethora of stimulatory and inhibitory signals which precisely specify the location and growth rate of new branches. As targeted overexpression and inhibition studies show, disruption of even one of these pathways can have a knock-on effect in the hundreds of interconnected pathways regulating growth and differentiation of the mammary gland, and if an imbalance is not corrected by regulatory mechanisms, such as apoptosis of the aberrant cells, this may lead to cancer.

## Adhesion within the mammary gland

Adhesions between cells, as well as between cells and the matrix, are critical for formation of a complex structure such as the mammary gland. Adhesion molecules control the organisation of groups of cells, and also communicate signals from neighbouring cells and the stroma in order to give each cell an accurate picture of its surroundings and regulate cell behaviour accordingly. The purpose of this communication is to promote cell proliferation and differentiation only when the cell is in the proper microenvironment and in close contact with neighbouring cells, consequently sending aberrant cells apoptotic signals. One of the primary functions of adhesion molecules in the ductal structure of the mammary gland is in generating and maintaining cell polarisation. Adhesion molecules on different cell surfaces are linked via the cytoskeleton to facilitate integration of diverse signals and propagation of correct polarity. Both cell-cell and cell-matrix adhesions are often disrupted in cancer. Cells interact with each other and the matrix via a wide range of mechanisms, which can be separated into two distinct groups: junctional complexes, which are composed of multiple proteins, and simple receptor-ligand pairs [73]. These can be further divided into cell-cell and cell-matrix adhesions.

## Cell-cell adhesions

The main cell-cell junctional complexes in epithelial structures are adherens junctions, desmosomes, tight junctions and gap junctions. Each of these junctions has a distinctive morphology, location and function. The central components in adherens junctions and desmosomes are cell-cell adhesion molecules called cadherins. These are type I transmembrane glycoproteins which interact with identical partners in neighbouring cells via their amino-terminal ends. The cadherin superfamily includes cadherins, protocadherins, desmogleins and desmocollins. Structurally, they share an extracellular $Ca^{2+}$-binding domain and are dependent on $Ca^{2+}$ ions to function [74, 75]. In the mammary ducts, E-cadherin is present in the luminal epithelial cells, whereas P-cadherin is present in the myoepithelial and cap cells. The role of these cadherins in ductal integrity during mammary gland development was investigated by inserting pellets containing function-perturbing E- and P-cadherin antibodies into mouse pubertal mammary glands [74]. Blocking of E-cadherin caused disruption of the luminal epithelial cell layer, with detached cells floating in the lumen. DNA synthesis and growth were also inhibited, but all of these effects were reversible, and normal development resumed upon removal of the antibody. These results imply that the high rate of proliferation seen in the TEB at puberty is dependent on cadherin-mediated cell-cell adhesions. Blocking of P-cadherin disrupted the cap cell layer but had no effect on luminal cells. A study which overexpressed P-cadherin in the luminal cells of mouse mammary glands reported no abnormalities in TEB structure; however, precocious alveolar differentiation was seen in virgin mice, and in older mice, mammary gland hyperplasia and dysplasia were detected [76]. Given the role of these cell adhesion molecules in maintaining tissue architecture in the normal mammary gland, it is not surprising that altered expression of both E- and P-cadherins can occur in breast cancer. E-cadherin-mediated cell-cell adhesion is generally lost as tumours progress towards malignancy, and loss of E-cadherin is thought to be a requirement, in many cases, for tumour cell invasion and metastasis [77]. The lost E-cadherin is often replaced by mesenchymal cadherins as a tumour progresses – this is known as the 'cadherin switch'. This switch in cadherins has prognostic significance – E-cadherin expression is associated with good prognosis [77], whereas upregulation of P- or N-cadherin in breast tumours has been strongly associated with poor prognosis [78, 79].

The cadherins are not just involved in cell adhesion, but can affect cell behaviour via its cell signalling machinery. The cytoplasmic tail of cadherin is connected to the actin cytoskeleton via a complex containing α-catenin, β-catenin, γ-catenin (plakoglobin) and p120-catenin. Interaction with both the catenins and the actin cytoskeleton is essential for cadherin adhesive activity [80]. β-Catenin is an important transcription factor which can interact with a number of other signalling pathways, most notably the Wnt pathway, which has a major role in many developmental processes and cancer. Under normal conditions, β-catenin is bound to E-cadherin at

the cell surface. If E-cadherin is lost, β-catenin is released into the cytoplasm, phosphorylated and targeted for degradation. Wnt signals stabilise free β-catenin, which translocates to the nucleus and acts as a cofactor to the TCF/LEF transcription factors, controlling transcription of target genes involved in regulation of cell growth, including c-myc and cyclin D1, and thus stimulating cell proliferation [81]. Consequently, there is a delicate balance between the Wnt and cadherin pathways which, if disrupted, can alter tissue integrity and lead to cancer progression. Other members of the cadherin superfamily such as desmogleins and desmocollins, components of desmosomes, are also known to be involved in epithelial morphogenesis and cell type-specific positioning [75].

***Tight junctions***

Tight junctions are the most apically located cell-cell junctional complexes, forming an intramembrane barrier that prevents diffusion of proteins and phospholipids between the apical and basolateral compartments of the plasma membrane, thus maintaining cell polarity. Tight junctions also create a variable barrier regulating paracellular transport of ions and small molecules, which has variable permeability and selectivity in molecular size and ion type depending on the cell type involved [82]. In the mammary gland, this barrier can also vary in response to events such as puberty, pregnancy and lactation [83]. Tight junctions are composed of fibrillary strands which tightly seal intercellular spaces, cytoplasmic proteins which connect the strands to the actin filaments of the cytoskeleton, and signalling proteins which can regulate junction composition and gene transcription. The main integral components of tight junctions are three membrane proteins: occludin, claudins and junctional adhesional molecules, which in turn are linked to cytoplasmic proteins such as the Zona Occludens (ZO) family members, which communicate with numerous signalling molecules [82, 83]. Tight junctions are particularly important in the secretory epithelial cells of the lactating mammary gland, to ensure properly polarised secretion. Several studies have investigated the effect of lactogenic hormones on tight junctional sealing. Nguyen and colleagues ovariectomised pregnant mice and found that tight junctional sealing followed ovariectomy [84]. Progesterone withdrawal was implicated as the trigger for closure, as injection of progesterone within 4 h of ovariectomy delayed closure. *In vitro* studies have suggested a similar role for both glucocorticoids and prolactin in stimulating junctional sealing, measured in epithelial sheets by transepithelial electrical resistance. Both glucocorticoids and prolactin appeared to exert this junctional sealing effect by upregulating expression of the tight junction components occludin and ZO-1 [85]. A later study found that treatment of rat mammary epithelial cells with the synthetic glucocorticoid dexamethasone induced the reorganisation of both tight and adherens junctions via downregulation of the small GTPase RhoA [86]. This downregulation stimulated relocalisation of ZO-1 and β-catenin to sites of cell-cell contact, thus linking the network of adhesive interactions within the cell. Recent work in the mouse found a differential regulation of claudin protein expression during pregnancy, lactation and involution, suggesting that altered claudin levels in the mammary gland regulate the tight junction remodelling needed for lactation [87].

Alteration of tight junction components has been reported in a variety of cancers. Modified cell-cell interactions allow cancer cells to disregard the normal restrictions and grow in a range of environments without the requisite cell-cell contact and adhesion signals. Various members of the claudin family of integral junction proteins have been reported to be altered in invasive cancers. Immunohistochemical analyses on a panel of breast tumours found that Claudin-1 was present in normal breast and DCIS, but lost in invasive ductal carcinoma (IDC) [88]. In support of this, *in vitro* studies found that re-expression of Claudin-1 in breast tumour spheroids increased the rate of apoptosis, suggesting that loss of this protein in tumour cells may contribute to cell survival, possibly by virtue of the ability of tight junctions to control nutrient and growth factor supplies to the cell [89]. Claudin-7 is also thought to be lost in breast tumours, and its loss was shown to correspond to histological grade [90]. On the other hand, studies on other members of the claudin family have drawn contradictory conclusions: that Claudin-3 and -4 are overexpressed in primary breast carcinomas [91]; Claudin-2, -3, -4 and -5 are not associated with histological grade in breast tumours [92]; and Claudin-4 expression is lost in grade 1 invasive tumours, but expression increases with tumour grade [88]. Analysis of other cancer types reveals that Claudin-3 and -4 are overexpressed in ovarian cancer [93], possibly due to promoter hypomethylation [94]; Claudin-1 is upregulated in colon cancer [95]; and Claudin-4 is downregulated in bladder cancer, associated with promoter hypermethylation [96]. These data imply that claudin expression in cancers depends on the tumour type, and different members of the claudin family are differentially regulated and perhaps have separate functions in tight junction activity. Interestingly, Claudin-3 and -4 were found to be specific receptors for *Clostridium perfringens* enterotoxin. This has been

proposed as a treatment for claudin-overexpressing tumours, as binding of the toxin induces rapid and specific cytolysis of claudin-expressing breast carcinoma cells [91].

Other components of tight junctions are also known to be disrupted in the transition from localised to invasive breast cancer. For example, expression of ZO proteins is thought to be lost or altered as tumours become more invasive, in particular ZO-1, the loss of which has been associated with poor prognosis in breast tumours [97]. Altered localisation of this protein has also been correlated with tumour invasiveness – ZO-1 appears to dissociate from the membrane and become cytoplasmically localised in more invasive tumours, correlating with increased levels of MMP-14, a protease known to be involved in tumour invasion [98].

***Axonal guidance proteins in the mammary gland***

One interesting type of cell-cell adhesion in the mammary gland is the interaction between neuronal receptors and their membrane-bound ligands. These were previously known to regulate neuronal guidance, and are now proposed to mediate adhesive interactions between body cells and cap cells in the TEB. One example of this is the Netrin-Neogenin system. Loss of either gene in mice results in disordered TEBs with breaks in the BM, cap cells breaking away and migrating ahead of the TEB, or into the preluminal compartment. Netrin-1 (Ntn-1) is a secreted factor which acts through its receptor, Neogenin (Neo1). Localisation studies in the mouse mammary gland show that Ntn-1 and Neo1 are expressed within close range of each other at the body cell/cap cell boundary, and Ntn-1 is secreted by the body cell layer and binds to Neo1 on the neighbouring cap cells, acting to restrain their highly motile behaviour. This interaction seems to be required for TEB adhesion and integrity rather than guidance, although the localisation of Neo1 expression could in theory determine the direction of TEB elongation [99]. The likelihood that mutations in either of these genes might lead to cancer was raised by the authors of this paper, as disruption of the cap cells, putative stem cells, could have consequences during the lifetime of the gland, eventually leading to tumour formation. There is, thus far, no strong evidence for this, although one study suggests a tumour-suppressing role for Ntn-1 via suppression of a protein called Cripto-1, which has been shown to stimulate invasion and migration of mammary epithelial cells both *in vitro* and *in vivo*, and has been proposed to induce EMT [100]. Neogenin has also been shown to bind to a number of different ligands, and its role in development and possibly cancer is yet to be clarified [101]. Subsequent proteomic-based studies have revealed a role for other neuronal guidance proteins in the developing mammary gland, such as semaphorins and their receptors, plexins and neuropilins, which were found to be highly upregulated in TEBs in comparison to ducts [102]. In mammary gland development, these guidance proteins are thought to regulate direction of growth of TEBs. Semaphorins and neuropilins have also been implicated in breast cancer, and are thought to promote cell migration, invasion and angiogenesis, both directly and indirectly [103, 104]. The importance of these neurotrophic factors in mammary gland development and breast cancer has yet to be fully understood.

## Cell-matrix adhesions

Moving from the cell-cell to the cell-matrix boundary, adhesion proteins called integrins are the main receptors for the ECM and transmit contextual signals between the extracellular stroma and the intracellular cytoskeleton. The luminal epithelial cells are surrounded first by a layer of myoepithelial cells, which secrete a BM consisting mainly of type IV collagen, laminin, proteoglycans and nidogen. The myoepithelial cell layer promotes organisation of the luminal cell layer, as it deposits BM, maintains cell polarity by secreting laminin-1, and modulates stromal-epithelial signalling. This stabilising influence can often be disrupted in tumours, leading to lack of luminal cell organisation and polarity, and alteration of cell-cell adhesions [105]. Outside the BM layer lies the interstitial ECM, largely composed of collagen type I and III, and numerous ECM proteins such as fibronectin, laminin and tenascin [106]. Due to the presence of the BM, integrins do not usually contact the interstitial ECM directly. This can change in invasive cancers, as the BM is degraded, thus affecting intracellular signalling. Integrins can signal in either direction, transmitting signals to the cell about its surroundings, or to the surroundings from the cell. Adhesion of these receptors to ECM substrates is essential for cell survival, known as anchorage dependence, and can affect almost any cell behaviour, including cell proliferation, differentiation, polarisation, and migration. Integrins are $\alpha$-$\beta$ heterodimers of transmembrane glycoproteins: different subunits are present in different cell and tissue types, and the subunit composition can change during developmental processes or cancer progression, altering the binding specificity and signalling properties of the integrin [107].

Knockout studies of various integrin subunits in mice have revealed a particularly important *in vivo* role for

the α2 and β1 subunits which, when combined, form a collagen/laminin receptor [107]. α2 integrin null mice are viable, fertile and capable of lactation, but have significantly lowered branching levels during puberty [108]. Function-perturbing β1 integrin antibody pellets implanted into pubertal mammary glands in mice revealed reduction in end bud number and ductal growth. Similar results were seen with anti-laminin antibodies, since laminin signals primarily through β1 integrins. HGF signalling was also shown to be dependent on functional β1 integrins, which would further affect stromal-epithelial communications [109]. β1 integrins are also involved in post-pubertal mammary gland remodelling, since conditional deletion of β1 integrin in the luminal epithelia of mice resulted in impaired alveologenesis and lactation. This was due in part to defective prolactin signalling through β1 integrin to the transcriptional activator Stat5 [110, 111]. Interestingly, the phenotype for integrin subunit knockouts in collagen gels in culture is much more severe than in mice, suggesting that other adhesion receptors may compensate for lack of integrins in the *in vivo* situation [108]. Indeed, other ECM receptors have been shown to be essential for normal mammary ductal development, such as the laminin receptor, dystroglycan, involved in epithelial polarisation [112], or the collagen receptor discoidin domain receptor 1 (DDR1), needed for normal growth, branching and lactation in the mammary gland [113].

Integrin signalling is particularly significant in tumour invasion, as these adhesion molecules mediate the majority of epithelial-stromal interactions, and if dysregulated can allow tumour cells to survive in an unpolarised state in an unfamiliar microenvironment. Altered integrin expression is found in both the early and late stages of tumour progression, from the loss of polarity and growth control to invasion and metastasis. Tumour cells are thought to switch integrins during the metastatic process, downregulating integrins such as $\alpha_2\beta_1$ that mediate adhesion to BM and maintain epithelial cells in a quiescent state, and upregulating integrins such as $\alpha_v\beta_6$, which promote growth, survival and invasion [114]. Considering the above evidence, it is clear that the integrity of the end bud is paramount to successful ductal outgrowth, and mutations which disrupt cell adhesions can cause aberrant growth during puberty. Similar mutations in the ductal epithelial cells of the adult gland can disrupt tissue architecture, leading to altered cell-cell contacts, polarity, survival, motility and eventually resulting in malignancy.

## Microenvironmental influences in development and cancer

The mammary gland is composed of epithelial and stromal cells which communicate with each other via the ECM. Although studies of breast development and cancer have traditionally focused on the epithelial cell component of the mammary gland, the stromal component has since been recognised as an important regulator of epithelial cell behaviour, rather than simply a structural support [6, 115]. The stroma associated with the normal mammary gland is vastly different to the tumour stroma. The normal gland has little connective tissue, mainly concentrated around the ducts, and is mainly composed of adipose tissue. The tumour stroma contains abundant connective tissue, probably due to increased secretion of growth factors, and this can affect the behaviour of epithelial cells both mechanically due to stromal stiffness [116] and biochemically due to altered signalling [6]. In the normal gland, MECs receive positional cues from their microenvironment, which allow the cells to orientate themselves into regular structures with apical and basal surfaces, surrounded by a BM. This is disrupted in the cancerous gland [117]. The mammary stroma is a heterogenous and structurally complex tissue made up of numerous components including fibroblasts, inflammatory cells, adipocytes, blood vessels, ECM and BM. Each of these components is essential for normal development of the mammary gland during puberty [118–120], and each can be exploited during tumour formation and progression to allow the epithelial cells to invade the surrounding stroma [121–123].

### *Fibroblasts*

Fibroblasts are the principal cellular component of the connective tissue, lying embedded within the matrix and largely responsible for its synthesis. These spindle-shaped cells secrete ECM components and growth factors, regulate inflammation and wound healing, and regulate differentiation and morphogenesis of epithelial cells. Fibroblasts are important in maintaining the composition of the ECM by secreting collagen types I, III and V, fibronectin and matrix metalloproteinases (MMPs), and are also involved in BM formation by secretion of laminin and type IV collagen [124]. In addition to their role in ECM maintenance, fibroblasts are essential for the differentiation and homeostasis of many epithelial tissues [124, 125]. Several recent studies illustrate the importance of fibroblasts in both development and tumourigenesis in the mammary gland. A technique widely used to analyse stromal-epithelial interactions in the mouse mammary gland is the cleared fat pad

transplantation system, where the undeveloped gland is removed from the fat pad prior to puberty. This leaves a 'cleared' fat pad into which mammary epithelial cells can be engrafted and grow to form a functional mammary gland [126]. Kuperwasser et al. exploited this system by developing a mouse model in which both the stromal and epithelial components of the reconstructed mammary gland are of human origin [120]. Previous attempts to colonise murine fat pads with human MECs had failed to produce functional structures, as the MECs did not proliferate [127]. However, when the stroma was 'humanized' with immortalised human breast fibroblasts, and subsequently coinjected with a mixture of primary human MECs and fibroblasts, the cells grew to form functional ductal and lobuloalveolar structures. This suggests that the role of human fibroblasts in mammary gland development is species-specific, and also indicates the importance of fibroblasts for MEC growth and invasion in both development and tumourigenesis [120].

*In vitro* co-culture models have also been utilised to demonstrate the importance of fibroblasts in MEC behaviour. Shekhar et al. examined the abilities of different stromal cell types to stimulate growth and morphogenesis of the phenotypically 'normal' MCF10a and preneoplastic MCF10AT1-EIII8 cell lines [123]. Co-culture of either of these cell lines with normal primary breast fibroblasts in a three-dimensional system results in retarded growth and morphogenesis of both cell lines. Co-culture with tumour-derived fibroblasts in the same system induces growth of ductal and alveolar structures in both cell lines, demonstrating the dominant role of the stroma in regulating epithelial cell behaviour. Addition of endothelial cells to these co-cultures resulted in a significant increase in epithelial cell proliferation, morphogenesis, invasion and ECM degradation, further illustrating the influence of stromal-epithelial interactions on MEC behaviour. How are the properties of fibroblasts altered in tumours to form an environment that supports tumour progression? Fibroblasts and other stromal cells closely associated with tumours are thought to acquire a modified phenotype similar to that seen temporarily in wound healing and developmental remodelling such as branching morphogenesis [123] and involution [9]. These carcinoma-activated fibroblasts (CAFs) may be induced by the growth factors TGFβ and platelet derived growth factor (PDGF), both of which are secreted by tumour cells [128], and can aid tumour progression in a number of ways. They are known to induce expression of serine proteases and MMPs which degrade and remodel the ECM [129], upregulate expression of pro-migratory factors in the ECM such as tenascin-C [130], secrete growth factors and cytokines to promote tumour growth and invasion, and release VEGF (vascular endothelial growth factor) which recruits endothelial cells and stimulates angiogenesis [124]. The profound effect of activated fibroblasts on epithelial cells was demonstrated when mammary fibroblasts engineered to overexpress HGF or TGFβ, both of which are known to be overexpressed in human breast cancers [38], induced development of poorly differentiated and invasive carcinomas in adjacent mammary epithelia, while wild-type fibroblasts had no effect [120]. Interestingly, considerable evidence also exists that mutations can occur in stromal fibroblasts prior to the onset of epithelial cancer [131, 132], raising the possibility of a causative role for fibroblasts in the initiation and progression of a subset of tumours.

### *Immune cells*

Another important component of mammary stroma are the migratory cells of the immune system, including macrophages, eosinophils, neutrophils and mast cells, many of which are recruited to the stroma during developmental remodelling processes and tumour progression. Macrophages, in particular, are known to be involved in the morphogenesis of many tissues [133], and are recruited in large numbers to the TEBs of developing mouse mammary glands, as well as to growing tumours. An essential role for macrophages in mammary gland development was demonstrated in studies of γ-irradiated mice. These mice have a depleted leucocyte population, which results in restricted formation of the mammary ductal tree [119]. Bone marrow transplantation restored normal mammary development and renewed the macrophage population around the growing ducts. Macrophages were generally located around the neck of the TEBs and not at the tip, and interestingly were also detected inside the buds, where a role in phagocytosis of apoptotic epithelial cells was proposed. Macrophages can be recruited to sites of epithelial invasion by a range of growth factors and chemokines, often produced by epithelial cells [122]. Colony stimulating factor 1 (CSF-1) is the main regulator of macrophage recruitment. Studies using mice homozygous for a null mutation in the gene for CSF-1 ($Csfm^{op}/Csfm^{op}$) show that, in the absence of CSF-1, the macrophage population within the mammary gland is severely diminished. This impairs the normal development of the mammary gland during puberty, with reduced formation of TEBs and restricted outgrowth and branching of the ductal tree [119]. When mice with the same CSF1 null mutation were crossed with transgenic mice predisposed to mammary cancer (PyMT-MMTV model), the absence of CSF-1 did not influence

primary tumour incidence or proliferation rates, but delayed advancement to invasion and metastasis. Transgenic expression of CSF-1 targeted to the mammary epithelium of both ($Csfm^{op}/Csfm^{op}$) and tumour-susceptible mice accelerated progression to invasion and metastasis, and enhanced infiltration of macrophages in the primary tumor [134].

Clinical studies support these data, and a study of CSF-1 overexpression in breast cancer revealed a correlation with high-grade tumours and poor prognosis, as well as with high rates of leukocytic infiltration [135]. In mice, ductal cells have been shown to produce CSF-1, and expression of the CSF-1 receptor primarily on macrophages may explain the close association of macrophages with epithelial tissues [134]. Despite the abundance of data advocating a central role for macrophages in development and tumourigenesis of the mammary gland, it remains unclear how exactly macrophages influence these processes. During pubertal development in the mammary gland, macrophages adopt a trophic role towards epithelial cells by supplying them with substances to facilitate ductal invasion, such as growth factors, cytokines, angiogenic factors and proteases [136]. In the tumour situation, these properties can be exploited by tumour cells to sustain growth and invasion. For example, in co-culture experiments, increased expression of CSF-1 by tumour cells induces macrophages to express increased levels of EGF, which in turn promotes invasion and upregulated CSF-1 expression in tumour cells, forming a paracrine feedback loop. Blocking either CSF-1 or EGF signalling inhibits invasion [137]. Tumour-associated macrophages (TAMs) also produce large amounts of proteases, such as uPA (urokinase plasminogen activator) and MMPs, and are known to be present as leukocytic infiltrates of tumours which coincide with areas of BM breakdown, leading to the hypothesis that TAMs secrete proteases which allow tumour cells to enter the stroma [119]. In addition, TAMs are known regulators of tumour angiogenesis, an essential requirement of invasion. They secrete VEGF, a key angiogenic regulator [138], and have been shown to gather in avascular or hypoxic areas of tumours, where they are thought to stimulate angiogenesis by releasing a wide range of angiogenic factors [122, 139].

Analysis of other immune cells recruited in the mammary gland during puberty have revealed that eosinophils, recruited by their chemokine eotaxin, localise around the head of TEBs and play an essential role in branching morphogenesis. The mechanism for this is unknown, but eosinophils are known to secrete the chemokine C10, which is a macrophage recruitment factor, pointing to an interactive relationship between these two immune cell populations [119].

### *Adipocytes*

Adipocytes are one of the most abundant stromal cell types in the mammary gland, yet are the least studied in relation to stromal-epithelial interactions, and are often viewed as inert energy-storing cells rather than the highly active endocrine cells they are. Proteomic studies on the mammary gland have revealed that adipocytes secrete a range of hormones, cytokines and growth factors [140], and also have the ability to metabolise androgen to estrogen via an aromatase pathway, thus exerting a paracrine effect on estrogen-responsive epithelial cells [141]. The role of adipocytes in mammary gland development and differentiation was examined in a study of transgenic mice lacking white adipose tissue [142]. In the absence of adipocytes, rudimentary mammary trees are formed prior to birth, but TEBs do not reappear at puberty, and ductal elongation and branching are severely curtailed, resulting in formation of a few short swollen ducts. It is not clear whether this is a hormonal/paracrine effect or simply due to the physical absence of the mammary fat pad. However, during pregnancy, epithelial cell division and alveolar cell formation occur, demonstrating that adipocytes are not required for epithelial cell differentiation, and that development and pregnancy are regulated by discrete stromal-epithelial interactions in the mammary gland. The growth-promoting properties of adipocytes indicate a possible role in tumour progression, which a number of studies have investigated in recent years. One comprehensive study by Iyengar and colleagues used a DNA microarray-based gene expression profiling approach to determine the paracrine effect of adipocytes on MCF-7 cells [121]. MCF-7 cells treated with conditioned media from the 3T3-L1 adipocyte cell line showed induction of numerous pathways involved in tumour progression, including increased cell proliferation, invasive potential and angiogenesis. Cell migration and angiogenesis assays using conditioned media supported this data. A subcutaneous *in vivo* coinjection system using adipocyte and breast cancer cell lines further demonstrated this paracrine effect: in mice, tumour cells coinjected with adipocytes grew faster and formed larger tumours than tumour cells injected alone or with fibroblasts [121]. This research suggests that adipocytes may exert a powerful influence on breast tumour progression.

### *The extracellular matrix*

Taking the above information into account, it is clear that stromal cells exert a considerable influence on the behaviour of epithelial cells in the mammary gland. However, the stromal cells are embedded in the interstitial ECM, and must signal through this and the BM before reaching the ductal cells. The ECM is not

simply an anchor for cells; it plays an active role in cell signalling and is capable of binding and sequestering numerous signalling molecules. This influence on epithelial cell behaviour is not solely exerted via signalling molecules – altered ECM characteristics can physically affect adjacent epithelial cells. This was demonstrated by Weaver and colleagues by culturing MEC acini in matrix of varying stiffness, and examining their morphology and localisation of signalling molecules [116]. Increasing the matrix stiffness caused increased cell growth, inhibition of lumen formation, alteration of adhesion proteins and loss of polarity. This was found to be due to increased cytoskeletal tension inducing integrin aggregation and thus enhancing ERK activation and focal adhesion formation, showing that the stiffness of tumour stroma can mechanically influence tumour cell behaviour. Remodelling of ECM and BM by enzymes such as MMPs does more than just remove physical barriers to ductal growth or tumour invasion, it actively controls and coordinates signalling by releasing sequestered molecules [115]. The role of MMP signalling in mammary epithelial branching and invasion during development and cancer has been discussed, but other factors also possess ECM remodelling activity, such as uPA. uPa is a serine protease which specifically converts plasminogen into active plasmin. This protease activity is mainly dependent on the ratio of uPA to its primary inhibitor, plasminogen activator inhibitor-1 (PAI-1). Activation of plasmin has wide-ranging effects on the ECM – plasmin is known to cleave several ECM components such as laminin and fibronectin, and can also activate latent MMPs, leading to indirect stimulation of ECM remodelling [143]. Plasminogen has been shown to be essential for normal mammary development and recurring differentiation of secretory epithelium during pregnancies, whereas uPA-deficient mice have no evident mammary abnormalities during development, pregnancy and involution, suggesting that plasminogen can be activated by multiple mechanisms [144, 145]. uPA and PAI-1 are also independent prognostic factors in breast cancer, correlating with poor prognosis [143, 146]. uPA is thought to mediate its pro-metastatic effect not just by ECM remodelling, but by promoting cell proliferation and migration, altering cell adhesion, stimulating angiogenesis and inhibiting apoptosis [143].

The effects of proteases like MMPs, uPA and plasmin are mediated by ECM components and growth factors which can be cleaved, sequestered or degraded in order to affect epithelial cell behaviour directly or indirectly, such as fibronectin and tenascin-C. Fibronectin is required during branching morphogenesis, particularly during cleft formation, where it builds up and is involved in conversion of cell-cell to cell-matrix adhesions by binding to integrins [147]. This mechanism is thought to be dysregulated in cancer, leading to disruption of cell adhesions, which contributes to tumour cell invasion [148]. Tenascin-C is an ECM glycoprotein which is suppressed in normal tissues but expressed in the stroma and periductal region as a marker for pre-invasive and invasive cancers. This protein is upregulated concurrently with MMP-3-mediated BM degradation and is also known to stimulate MMP expression by fibroblasts, resulting in a positive feedback loop which promotes tenascin-C activation and cell [130, 149].

In addition to cleavage of proteins, the ECM can regulate epithelial cell behaviour by binding and sequestering a variety of growth factors and signalling molecules such as amphiregulin, IGF (insulin-like growth factor) binding proteins, TGFβ, Wnts and FGFs [53]. The composition of the ECM varies depending on the developmental stage of the gland, and changes in the organisation of the ECM at different stages can influence metastatic potential. This is particularly apparent during involution, a process which involves apoptosis of secretory cells and massive tissue remodelling, leading to formation of a mammary microenvironment similar to that present during inflammation, with increased levels of fibroblasts and immune cells, high MMP activity, and cleavage of ECM proteins such as collagen and laminin. This activated stroma has been shown to promote tumour invasion: when nulliparous matrix isolated from rats was mixed with MDA-MB-231 tumour cells, migration and invasion of these cells in a transwell filter assay was curtailed, whereas matrix isolated from rats undergoing involution stimulated their invasion [9]. *In vivo* studies supported these data and pointed to a role in metastasis: MDA-MB-231 cells mixed with rat nulliparous or involution matrix were injected into the cleared mammary fat pads of nude mice. The cells mixed with involution matrix formed more metastases with higher rates of angiogenesis. This study provides evidence to support the phenomenon of pregnancy-associated breast cancer, whereby a short-term increase in breast cancer risk occurs with pregnancy, followed by a long-term protective effect. The risk of pregnancy-associated breast cancer is greater in older first-time mothers, and there is a high mortality rate because metastasis is common. This was previously thought to be due to the promotional effect of pregnancy hormones, but from the above evidence it is clear that the altered state of the ECM during involution also plays a key role. This is a significant finding, linking the developmental state of the mammary gland to the development of cancer, and highlighting the importance of the microenvironment in tumour growth and metastasis [150].

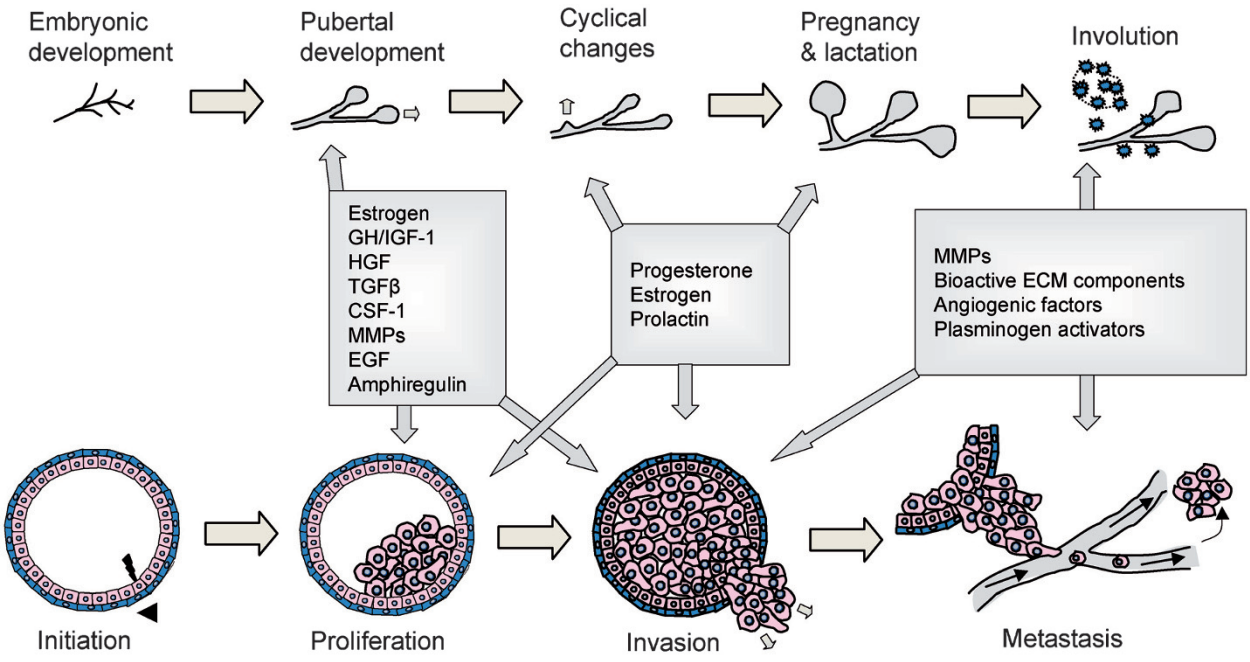


**Figure 3.** Examples of some signalling molecules which are involved in different stages of both mammary gland development and breast cancer.

***Myoepithelial cells and the basement membrane***

Although not a component of the mammary stroma, myoepithelial cells could be constituted as part of the microenvironment of luminal epithelial cells, since these cells and the BM they secrete form the main barrier between epithelial cells and the stroma. Apart from their contractile function during lactation, physical and paracrine interactions between luminal and myoepithelial cells are crucial for establishing and maintaining luminal cell polarity, regulating proliferation and apoptosis, and suppressing invasion. Myoepithelial cells are thus thought to exert a tumour-suppressing effect both physically and biochemically. During the various stages of development, frequent changes can be seen in the myoepithelial and BM layers: for example, the myoepithelial layer usually forms a continuous barrier between luminal epithelial cells and stroma, but in the alveoli during pregnancy this layer is stretched and many luminal cells contact the BM, directly altering cell adhesion and signalling [151]. The BM is also susceptible to developmental changes, particularly during puberty, when the BM at the tip of mouse TEBs is significantly thinner than normal ductal BM, and that around the neck of TEBs can build up to 14 times the normal thickness. These alterations may have a functional role in permitting TEB invasion [3]. One mechanism by which myoepithelial cells exert their tumour-suppressor activity is via downregulation of MMP activity in adjacent tumour cells and fibroblasts in co-culture experiments, thus inhibiting invasion [152]. This is important in DCIS lesions, preventing BM breakdown and EMT of tumour cells, but is eventually overcome, and invasive tumours no longer contain a myoepithelial component. The importance of the myoepithelial layer in imparting positional signals to neighbouring cells can be seen in 3D culture, where isolated luminal epithelial cells cultured in Collagen I gels form lumen-less structures with reverse polarity, until myoepithelial cells are added, stimulating proper polarisation and formation of hollow bilayered acini [105]. This organising influence was found to be directly related to the ability of the myoepithelial cells to produce the α1 chain of laminin, a component of the BM. Tumour-derived myoepithelial cells often lose this polarising ability, and *in vivo* examination of laminin-1 in tumours revealed greatly reduced expression of this protein in breast tumours, suggesting a strong causal link between loss of laminin-1 and breast cancer. Loss of this protein in tumour-derived myoepithelial cells could also lead to a weakening of the BM, allowing tumour cells to overcome this barrier and become invasive.

Mammary stroma is therefore a heterogenous environment consisting of multiple cell types. An important component of this environment is the endothelial cell population which forms the complex network of blood vessels in the mammary gland. These blood vessels undergo cycles of angiogenesis in tandem with the normal developmental cycle of the mammary gland, and can be harnessed in tumorigenic processes to feed the abnormal cells [118]. Numerous signalling

pathways within the mammary gland interact with endothelial cells to affect angiogenesis, and some of these have been targeted in breast cancer therapies. Angiogenesis in breast cancer has been reviewed in detail [153, 154].

## Stem cells: enabling mammary gland remodelling and cancer?

The definition of a stem cell is an undifferentiated cell with very high growth potential, capable of the production of the entire lineage [155]. Thus far, there has been no definitive identification of a mammary epithelial stem cell, although much experimental evidence seems to point towards the existence of such a cell, and many researchers argue that only stem cells could support the rapid expansion of the epithelial cell population that occurs during pregnancy. Mammary stem cells are also proposed to be the cells of origin of breast cancer, due to their ability to accumulate mutations over long lifetimes and their capacity to produce unlimited progeny. The constant remodelling of the mammary gland during oestrus cycles and pregnancies requires an immense growth capacity. This quality might also explain in part the higher risk of breast cancer in nulliparous glands, and the protective effect of pregnancies: the virgin gland is proposed to possess a higher percentage of undifferentiated stem cells, leading to a greater growth capacity and therefore cancerous potential [156].

During mammary gland development, the cap cells at the tip of the TEB are thought to be the main site of stem/progenitor cell division and differentiation. These putative stem cells are thought to undergo asymmetric cell division to generate one identical cap cell and one progenitor cell [157]. Cap cells need to generate progenitor cells for both the myoepithelial and the luminal epithelial lineages to facilitate ductal elongation [3]. However, how does this explain outgrowth of lateral branches in the mammary tree? One theory is that lobuloalveolar progenitor cells present along the ducts are stimulated hormonally and produce an invasive transit cell that can break down ECM, resist inhibitory signals, invade the stroma and then differentiate to form a lateral duct [158]. There is so far little direct evidence for this, although putative stem cells have been detected along the epithelial ducts [157].

In mice, serial transplantation studies have revealed that cells from a single clonal origin or possibly even a single cell could reconstitute an entire mammary tree [159, 160]. This is unlikely to occur in the normal mammary gland, and it has been suggested that the clonal units are in fact the TEBs, or TDLUs in humans. X-linked inactivation analysis of human TDLU and DCIS samples reveals that cells in each microdissected sample were derived from a monoclonal origin, due to the fact that random X-chromosome inactivation was found to be non-random within each TDLU [161]. This suggests that the mammary gland is organised into distinct stem cell-derived monoclonal sections, and thus implies that any tumour arising within a clonal unit would therefore be monoclonal in origin. Stem cells have proven difficult to identify due to a lack of definitive markers, but the search for experimental evidence of the existence of mammary epithelial stem cells has utilised many different techniques, including histological analysis, 5-bromo-2-deoxy-uridine (BrdU) retention, efflux of Hoechst dye, expression of various 'stem cell' markers such as Sca-1, CD49f and CD24, and non-adherent mammosphere culture. These techniques and their use in the isolation of putative stem cells are covered in detail in Molyneux et al. in this review series. The longevity, proliferative ability, and capacity for differentiation of these tumour-initating cells may account for the ability of tumours to recur, sometimes many years after the removal of the primary tumour, and often with chemoresistant properties. Their identification and characterisation is crucial for the development of cancer stem-cell targeted therapeutics, some of which are already being developed [162].

## Modelling the normal and neoplastic gland

Numerous methods exist for modelling mammary gland development and cancer *in vivo* and *in vitro*. One of the earliest models used to simulate normal mammary gland development and tumour growth was the cleared fat pad transplantation system described previously [126]. Transgenic mouse models have also provided significant insight into the genes and pathways regulating normal growth in the mammary gland, and how overexpression or knockdown of specific genes can disrupt normal developmental processes and sometimes lead to aberrant growth and cancer. However, these models are relatively inflexible when it comes to studying the precise biochemical pathways controlling the normal and neoplastic state in mammary epithelial cells. Cell culture-based studies are much more flexible and accessible to manipulation, but early studies were extremely limited, being restricted to monolayer culture which failed to recapitulate the complex cellular organisation of the *in vivo* ductal structure. The advent of three-dimensional culture systems allowed more accurate *in vitro* modelling of the ductal structure, when it was found that epithelial cells

cultured in certain types of collagen gels or reconstituted BM from Engelbeth-Holm-Swarm murine tumours (Matrigel) formed hollow polarised growth-arrested structures [163, 164]. These 'acini' or 'mammospheres' retain both the structural and functional characteristics of mammary ducts, communicating through normal cell-cell and cell-matrix adhesions, and secreting milk proteins into their lumens upon stimulation by prolactin [163]. Cell lines commonly used for this model are the phenotypically 'normal' MCF10a cell line, and the progressively more invasive HMT-3522 cell line series [165]. The mammosphere model has since been invaluable in delineating numerous signalling pathways necessary for the polarisation and organisation of epithelial cells. Genetic manipulation of the MECs has identified numerous genes which, when overexpressed or silenced, disrupt the organised phenotype, indicating a possible role in cancer progression. For example, function-blocking β1 integrin antibodies were shown to inhibit the formation and function of MEC acini, showing that contact with the ECM is essential for cellular organisation and polarity, and supporting *in vivo* studies on β1 integrin [166]. Cell-cell adhesions are also critical for acinus formation, as determined by the disruptive influence of function-blocking antibodies against cadherins from both adherens and desmosomal junctions [75]. Numerous signalling requirements for polarisation and organisation have been elucidated by this model, including the need for insulin signaling mediated by IGF-1 [167], and glucocorticoid signaling mediated by JNK [168].

Primary MECs cultured in Matrigel can also be induced to form acinar structures. This provides a useful model for pre-invasive and invasive cancers such as DCIS and IDC *in vitro*, as the progression towards invasion can be followed step by step, from the filling of the lumen, to BM degradation and tumour cell invasion. Heterologous co-culture models of MECs with myoepithelial or stromal cells increases the complexity of this model system, and manipulation of these models has contributed to our knowledge of the intricate network of interconnected paracrine pathways in the mammary gland. A three-dimensional (3D) model of epithelial tubules has also been developed to study ductal formation *in vitro,* by collagen gel overlay [169]. This method was recently used in a previously mentioned study of the regulatory mechanisms controlling spacing of lateral branches in the mammary gland, which found that tubule geometry and local concentrations of inhibitors dictate branch position [34]. These examples demonstrate the power and flexibility of 3D cell culture models for the study of ductal architecture in both the normal and malignant context.

Despite the advantages of cell culture models for human breast cancer, an *in vitro* assay can never replicate the complex interactions that occur in the *in vivo* situation, and much of the research referenced in this review draws on the most commonly used breast cancer model – the mouse. Studies on the comparative biology of the mouse and human mammary gland show organs with surprisingly similar structure and function, with some important caveats. The functional unit of the human breast is the TDLU, compared with the lobuloalveolar unit or TEB in the mouse. Both of these structures are hormone-responsive, and it is from here that the majority of mammary cancers are derived. However, there are some morphological differences between these functional units: the mouse TEB consists of a single bulbous structure which can be stimulated during pregnancy to develop into a more complex lobuloalveolar structure, whereas the human TDLU consists of multiple bulbous ends known as acini, which expand to become functional during pregnancy (see Fig. 4). Differences in stromal composition, and relative ratios of hormone-dependent and -independent cancers, are also important when drawing comparisons between these species. Consideration of these issues is vital when determining the implications of in vivo studies, and this subject has been reviewed in detail [66, 170–172].

## Prospects for new cancer targets?

Despite the rapid advances being made in the field of mammary gland biology, many of the precise details of how the mammary gland develops and how breast cancer progresses remain unclear. A recent genome-wide study on the expression profiles of TEBs and their microenvironment has revealed previously uncharacterised genes, enriched for within or around TEBs, with potential roles in mammary morphogenesis [173]. Analogous proteome-wide approaches to both pubertal and involuting glands have also shed light on differentially expressed proteins with possible roles in the developmentally regulated cycling of the mammary gland [102, 174]. These putative novel factors may fit into the current knowledge of signalling and growth regulation in the mammary gland, or might constitute entirely new pathways, which could perhaps shed new light on mechanisms involved in breast cancer progression. Indeed, the rapid growth and invasion of TEBs make these TEB-associated genes perfect candidates for investigating possible associations with breast cancer. Numerous genes discovered as regulators of morphogenesis have been translated into the cancer field, and vice versa, and developmental pathways such as estrogen recep-

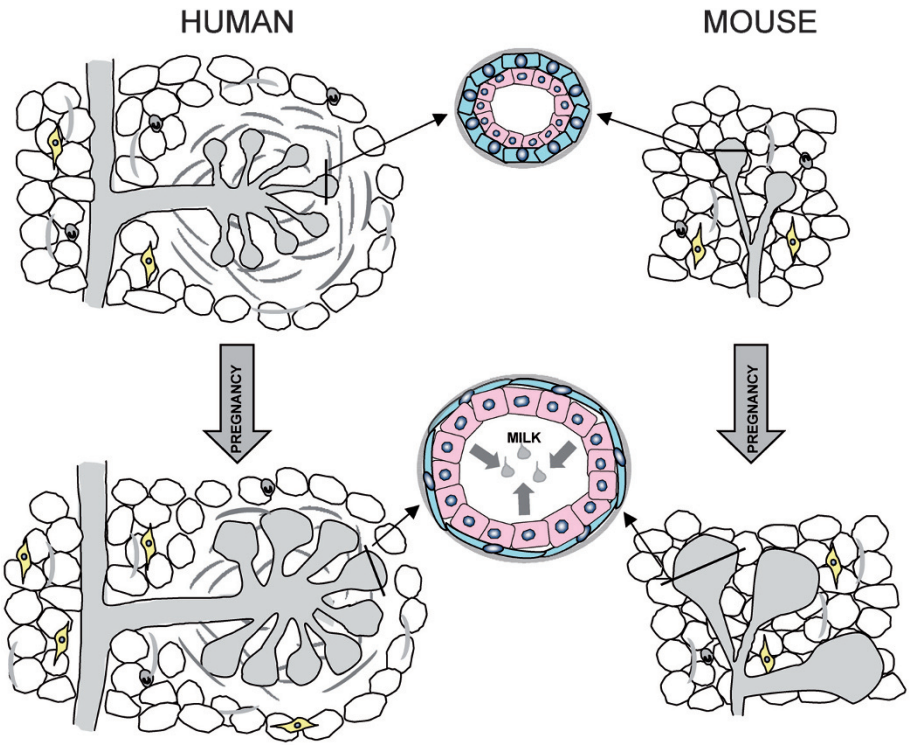


**Figure 4.** Comparative biology of the human and mouse mammary glands. The human TDLU consists of a number of structures known as 'acini' within a loose intralobular stroma, surrounded by the dense extralobular stroma. The acini expand and become functionally differentiated during pregnancy. The mouse TEB consists of a single bulbous structure which can be stimulated during pregnancy to develop into a more complex lobuloalveolar structure. There does not seem to be any specialized stroma surrounding the mouse TEB, but this may be due to differences in the relative size of the glands [172]. Despite the morphological differences, the cellular organisation and function of individual acini/TEBs is strikingly similar.

tor signalling and ErbB signalling are important targets for current breast cancer treatments.

There is an urgent need for effective prognostic and predictive biomarkers in breast cancer in order to anticipate clinical outcome and treatment response. The rise in -omic techniques has allowed identification of cancer-related gene or protein 'signatures', which are discussed in another review in this series (Culhane and Howlin). These signatures have allowed classification of new breast cancer subtypes, one of which is the 'basal-like' tumour negative for ER, PR and Her2 – the so-called triple-negative tumour. The implications this technology holds for breast cancer diagnosis and treatment is reviewed in this issue (Mullan and Millikan). The potential of hypoxia and hypoxia-regulated proteins as prognostic and predictive biomarkers and treatment targets is also considered (Lundgren et al.).

Recent crossover studies such as Brugge et al.'s $Bim^{-}Bim^{-}$ mice [31], and studies on molecules such as CITED1 [68] and GATA-3 [70, 71], demonstrate the value of developmental models, both *in vivo* and *in vitro,* in understanding the mechanisms of growth and invasion in the developing gland, and their relevance to malignancy. As our knowledge of the cues regulating mammary gland development broadens, it is becoming apparent that many common themes are evident in breast cancer, from rapid proliferation to invasion, adhesion and apoptosis, and indeed almost all of the signalling pathways that influence branching have also been associated with cancer to some extent. Thus, a clearer understanding of the mechanisms involved in regulation of TEB invasion and branching can aid our insight into how these mechanisms may be harnessed by aberrant cells in breast tumours to promote their own growth, survival and invasion.

*Acknowledgements.* Funding is acknowledged from the Irish Research Council for Science, Engineering and Technology, Science Foundation Ireland, and the Health Research Board of Ireland, the latter under the auspices of the 'Breast Cancer Metastasis: Biomarkers and Functional Mediators' Research Programme. The UCD Conway Institute is funded by the Programme for Third Level Institutions (PRTLI), as administered by the Higher Education Authority (HEA) of Ireland.

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