

Mammary development in the embryo and adult: a journey of morphogenesis and commitment

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Mammary gland development occurs through distinctive stages throughout embryonic and pubertal development and reproductive life. At each stage, different signals are required to induce changes in both the epithelium and the surrounding mesenchyme/stroma. Recent studies have provided new insights into the origin, specification and fate of mammary stem and progenitor cells and into how the differentiated lineages that comprise the functional mammary gland are determined. The development of new tools and culture techniques has also enabled the factors that influence branching morphogenesis in the embryonic and pubertal gland to be identified. A surprising recent discovery has been that mammary epithelial cells commit to differentiated lineages using the same signalling pathways that regulate lineage determination in T helper cells.

Introduction

Mammary glands are epidermal appendages that possibly evolved from ancient apocrine glands that were associated with the skin (Oftedal, 2002). The primary function of the mammary gland is to provide nutrition for the young in the form of milk protein and fat. However, there are other benefits that are provided by lactation, such as the provision of immune factors that are secreted into the milk, which provide protection from infection, and also the close contact that occurs between mother and infant during nursing, which might have developmental benefits (Peaker et al., 2002). The mammary gland is a complex secretory organ that consists of a number of different cell types: epithelial cells that form the ductal network of the gland; adipocytes, which constitute the fat pad and in which the ductal network is embedded; vascular endothelial cells, which make up the blood vessels; stromal cells, including fibroblasts; and a variety of immune cells. There are two main types of epithelium in the mammary gland: luminal and basal. The luminal epithelium forms the ducts and the secretory alveoli, whereas the basal epithelium consists essentially of myoepithelial cells. These two types of epithelium form a bi-layered structure of simple epithelium that is embedded within the fatty stroma.

There are three main stages of mammary gland development both in rodents and humans: embryonic, pubertal and adult. Hormones and growth factors play a role in these different stages of mammary development and are also implicated in breast cancer. The mammary gland is an ideal tissue in which to study a range of developmental processes, as discussed below. In the embryo, the signals that induce the formation of mammary placodes from the skin are beginning to be elucidated. Similar processes are involved in the formation of other appendages, such as teeth and feathers (Wu et al., 2004). After birth, mammary development is arrested until puberty, when extensive elongation of the ducts, accompanied by secondary

branching, takes place, thus providing a readily accessible system in which to study branching morphogenesis. The hallmarks of development during pregnancy are the formation of tertiary branches, which terminate in alveolar buds, and the rapid proliferation of the luminal epithelium accompanied by differentiation and commitment to the secretory alveolar lineage. A lactogenic switch occurs during late pregnancy that is accompanied by the expression of the milk proteins, whey acidic protein (WAP) and α -lactalbumin, and by the formation of lipid droplets. Finally, following lactation, removal of the now surplus alveolar cells is accomplished by cell death (apoptosis). Post-lactational regression, or involution, is the most dramatic example of physiologically regulated apoptosis in an adult tissue. In a tightly coordinated series of events, ~80% of the epithelium is removed within a few days. The mouse mammary gland provides, therefore, a model that can be genetically manipulated to provide insights into a variety of normal developmental processes. Mouse models have also been used extensively to study the development of breast cancer.

In this review, we discuss recent studies in mice on the morphogenesis and lineage commitment events that occur during all three stages of mouse mammary gland development. Important new insights have been obtained from these studies, including the unanticipated involvement of signalling pathways previously associated with T lymphocyte lineage decisions, in mammary epithelial lineage choice. Tools have been developed that allow the enrichment of mammary stem cells from the adult gland, and it can be only a matter of time before we can prospectively identify mammary stem cells and the factors required for their self renewal. Importantly, this will allow the hierarchy of progenitors and their inter-relationship to be determined. This will be a major step forward, not just for developmental biology, but also for breast cancer research. The ability to genetically modify the mouse has made it the model of choice and this review therefore focuses on studies in the mouse. Although there are some differences in the architecture and hormonal control of mammary glands between mice and other rodents and between mice and humans, similar developmental processes are shared between them.

Embryonic mammary gland development

Mammary development is not evident in the mouse until mid-gestation. The first distinct feature is the formation of the milk lines from overlying ectoderm (as discussed in more detail below), followed by the formation of five pairs of placodes that invaginate to form buds (see Fig. 1A,B). These induce the formation of the mammary mesenchyme. The buds then sprout and branch to form a rudimentary structure that has approximately five ductules that embed in the subdermal fat pad (see Fig. 1C). Development is arrested from embryonic day (E) 18 until puberty. Mammary development in the male differs between mouse and man with regression of the rudimentary tissue in mice being induced in response to androgens, whereas human males retain a connection to the nipple.

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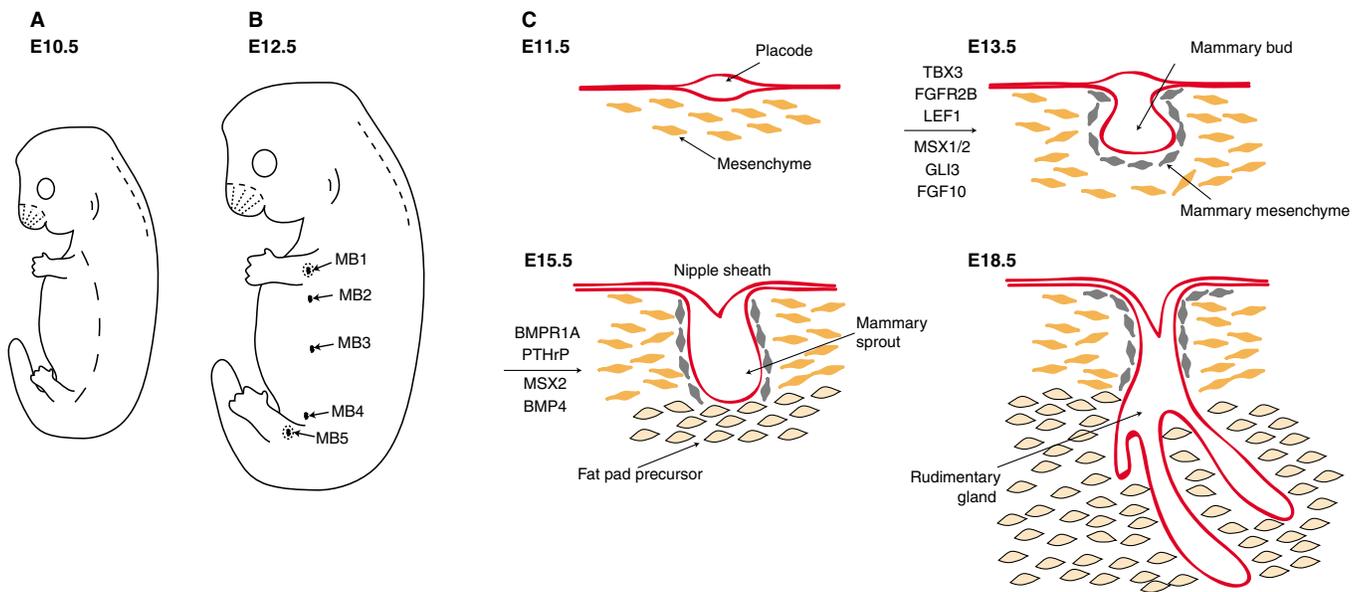


Fig. 1. Embryonic mammary gland development. (A,B) Diagram of an E10.5 mouse embryo (A) showing the position of the milk line (dashed line between limbs), and of an E12.5 mouse embryo (B) showing the positions of the five pairs of mammary placodes, which become mammary buds (MB1-5) along the anteroposterior axis (MB1 and MB5 are hidden by the limb buds and only one flank is shown). (C) Overview of mouse embryonic mammary gland development. Placodes, which are visible at E11.5, transform into bulbs of epithelial cells, which sink into the underlying mesenchyme at E13.5 to become the mammary buds. The mesenchymal cells (orange) that surround the buds condense to become the mammary mesenchyme (grey). By E15.5, these buds elongate to form sprouts, which develop a lumen with an opening to the skin, marked by the formation of the nipple sheath. As the end of pregnancy approaches, at E18.5, the sprouts become small arborized glands that invade what has now become the fat pad (buff). Development is essentially arrested at this stage until puberty.

Patterning and placode formation

Mammary gland development in the mouse is first observed on E10.5 with the appearance of the mammary lines (milk lines) in both male and female embryos. These are two ridges of multilayered ectoderm that arise from the embryonic skin and run in an anteroposterior (AP) direction from the fore- to the hindlimb buds on the ventral surface of the embryo. The mammary line can be observed by in situ staining for the wingless gene *Wnt10b* (Veltmaat et al., 2004). In the TOP-Gal Wnt reporter transgenic line, β -galactosidase expression is detected as a thin line between fore- and hindlimb buds (Chu et al., 2004). It has been proposed that ectodermal cells migrate along the mammary line and coalesce to form epithelial placodes (Veltmaat et al., 2004). Parathyroid hormone-related protein (PTHrP; also known as PTHLH – Mouse Genome Informatics) is expressed by E11.0 and the growth factors, fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 4 (BMP4) are also expressed in the placodes. Recently, the expression of a *lacZ* reporter from a *Bmp4* promoter has shown that BMP4 is expressed in both mammary epithelium and mesenchyme between E11.5 and E14.5 (Hens et al., 2007). An interaction between BMP4 and T-box 3 (*Tbx3*; a gene associated with ulnar-mammary syndrome, which is characterised by deficiencies in the ulnar ray in the upper limb and hypoplasia of the mammary glands) has been proposed to determine the dorsoventral (DV) boundary of the body, where the formation of mammary buds is initiated (Cho et al., 2006).

At E11.5, five pairs of symmetrically positioned mammary placodes form at reproducible locations (see Fig. 1B). However, each placode is not identically determined, as different signals are required for the development of each pair, and pairs of placodes appear in a specific order: number 3 before 4, which develop before

1 and 5 (which appear simultaneously), and finally number 2. This last pair develops from streaks of WNT10B-positive cells that extend from placodes 1 and 3. The absence of particular placodes has been observed in mice with specific genetic mutations, such as the extratoes (*xt*) mutant that has a deletion in the *Gli3* gene (*Gli3^{xt/xt}*) (Veltmaat et al., 2003). Similarly, loss of *TBX3* in humans produces mammary hypoplasia and nipple loss, whereas mice lacking *Tbx3* lose all mammary buds, although they occasionally retain pair number 2 (Eblaghie et al., 2004). By contrast, *Lef1*-null mice (LEF1 is a nuclear target of the canonical Wnt signalling pathway) form small mammary placodes that degenerate, although bud-pair 4 is sometimes retained (van Genderen et al., 1994), whereas mice that lack *Fgf10*, or its receptor *Fgfr2b*, have only bud-pair 4 (Mailleux et al., 2002). In contrast to bud loss, supernumerary glands have been observed in mice and humans (Grossl, 2000). The scaramanga (*ska*) locus is associated with aberrant bud formation in mice and results in absent (frequently loss of placode 3), supernumerary or misplaced buds (Howard and Gusterson, 2000). The *ska* gene was later identified as that which encodes neuregulin 3 (NRG3), a ligand for the tyrosine kinase receptor ERBB4 (HER4). In mouse embryo explant culture, NRG3-soaked beads induce the expression of LEF1 and bud formation (Howard et al., 2005).

A component of the hedgehog (Hh) signalling pathway, GLI3, has recently been shown to regulate bud formation (Hatsell and Cowin, 2006). GLI3 is one of three homologues of the *Drosophila melanogaster* gene *cubitus interruptus* (*ci*), which encodes a microtubule-bound transcription factor that can be phosphorylated to generate a transcriptional activator (Ci^A), or proteolytically cleaved to generate a repressor (Ci^R). The Hh signalling network regulates pattern formation and stem/progenitor cell fate in many organs (Ingham and McMahon, 2001). Complex interactions exist between

the various components of the mammalian Hh pathway that can result in positive or negative signalling via one of three secreted ligands [sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH)], which bind to either of two patched-family Hh receptors (PTCH1 and PTCH2), inducing target gene transcription via activator forms of the Gli family members GLI1, GLI2 and GLI3. In the absence of ligand, PTCH1 interacts with a transmembrane effector protein called smoothened (SMO); binding of Hh to PTCH1 releases its inhibition of SMO and induces Hh target genes via the activator forms of the Gli factors (Hooper and Scott, 2005).

In order to clarify the role of Hh signalling in embryonic mammary gland development, Hatsell and Cowin (Hatsell and Cowin, 2006) examined the expression of *Gli1*, *Gli2* and *Gli3* using a combination of *lacZ* reporter expression and in situ hybridisation. Since GLI1 is a direct transcriptional target of positive Hh signalling, *Gli1-lacZ* expression in transgenic mice reports Hh pathway activity. *lacZ* expression is absent in mammary buds (although it is present in hair follicles), indicating that positive Hh signalling is not required for mammary development. Furthermore, embryos null for either *Gli1* or *Gli2* have no obvious defects in mammary bud formation. However, removal of *Gli3* abrogates TOP-Gal expression in the mammary line in the region of placode 3, and results in the loss or misplacement of bud-pairs 3 and 5. Replacing GLI2 with GLI1 in *Gli3*-heterozygous mice, by driving expression of the constitutive *Gli1* activator under the control of the *Gli2* promoter, also results in loss of buds 3 and 5. This suggests that GLI3 is required to suppress Hh target genes that are involved in patterning and bud formation. Overall, this study shows that the Gli^A/Gli^R ratio (Gli activator forms to Gli repressor forms) provides a crucial developmental signal threshold for buds 3 and 5, further emphasizing the susceptibility of different placodes to different developmental signals.

In E11 *Gli3^{xt/xt}* (extratoes mutant) mice, the lack of expression of the Wnt signalling reporter TOP-Gal in the central region of the mammary line demonstrates that the GLI3-mediated repression of the Hh pathway is required prior to the early patterning events that precede mammary placode formation (Hatsell and Cowin, 2006). Thus, although the Hh pathway is active in epidermal appendages, such as in hair follicles, it is either inactive or repressed throughout embryonic mammary development. Interestingly, GLI3 is also involved in FGF10 signalling because recombinant FGF10 can rescue mammogenesis in *Gli3^{xt/xt}* mutants (Veltmaat et al., 2006). It has been suggested that the intra-somatic FGF10 gradient, in concert with the ventral elongation of the somites (as suggested by the phenotype of *Pax3*-deficient mouse mutants), determines the correct DV position of the mammary epithelium (Veltmaat et al., 2006). As there is no evidence for positive Hh signalling in the embryonic mammary gland (Hatsell and Cowin, 2006), this function of GLI3 must be mediated by another mechanism.

Another signalling pathway has recently been implicated in early embryonic mammary gland development. GATA3 is a transcription factor that controls the differentiation of T lymphocytes in response to parasitic infections. In transgenic mice in which a modified β -galactosidase gene was knocked-into the *Gata3* locus, blue staining was observed as early as E12.5 in mammary buds (Asselin-Labat et al., 2007). Moreover, the conditional deletion of *Gata3* in mammary placodes using the keratin 14 (*Krt14*) promoter-driven expression of Cre recombinase resulted in a variable loss of placodes and a failure to develop the nipple sheath (Asselin-Labat et al., 2007).

By E13.5, morphologically distinct epithelial buds can be distinguished in mouse embryos, and by E14.5 these buds have sunk into the underlying dermis. Expression of dickkopf 1, an

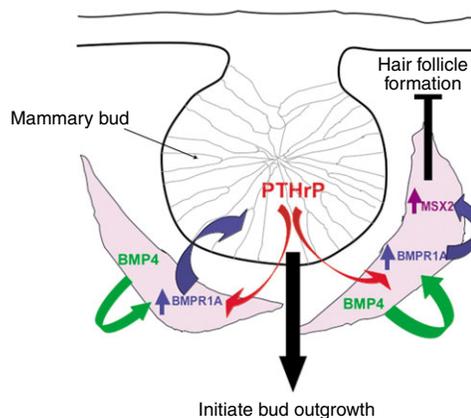


Fig. 2. Induction of bud outgrowth. Cross-section of an embryonic mouse mammary bud at E13.5-15.5. PTHrP and BMP signalling interact to initiate mammary bud outgrowth and nipple formation. PTHrP, which is secreted from mammary epithelial cells of the mammary bud, increases BMPR1A expression in the mammary mesenchymal cells (purple shading), which can now respond to BMP4. This triggers epithelial outgrowth, elevates MSX2 expression, and inhibits hair follicle formation within the nipple sheath. Modified with permission from Hens et al. (Hens et al., 2007).

inhibitor of Wnt signalling, under the control of the *Krt14* promoter can abolish bud formation (Chu et al., 2004). In male mouse embryos, the activation of androgen receptors causes the buds to degenerate and disappear by E15.5. At this stage in female embryos, each bud begins to elongate to form a so-called mammary sprout that invades the precursor of the fat pad, into which the sprout will grow after birth. PTHrP signals to the mesenchyme to initiate the formation of mammary-specific dense mesenchyme (Hens et al., 2007), which is essential for determining epithelial cell fate. A hollow lumen is then formed that opens onto the surface of the skin and gives rise to the nipple (see Fig. 1C). PTHrP is important also for the development of the nipple sheath, and the overexpression of PTHrP in basal keratinocytes converts dermis to mammary mesenchyme and suppresses hair follicle formation (Foley et al., 2001).

Branching morphogenesis and ductal elongation

Around E16, mammary sprouts begin to ramify into a small number of ductules, and by E18.5 they have developed into small tree-like glands that bear between 10 and 15 small branches. The branching morphogenesis of the mammary sprout requires soluble factors that are supplied by the mammary fat pad precursor. Recently, elegant work from John Wysolmerski's laboratory, in which embryonic mouse mammary buds were explanted and cultured, has shown that PTHrP, which is secreted by mammary epithelial cells, sensitises mammary mesenchymal cells (which develop around E13.5) to BMP signalling by upregulating the expression of BMP receptor 1A (BMPR1A) in the mammary mesenchyme but not the epithelium (Hens et al., 2007) (see Fig. 2). Importantly, the addition of BMP4 to cultures of dissected embryonic mammary buds from *Pthrp*-null mice rescued the phenotype (lack of sprouting from the buds and branching morphogenesis) seen in the absence of exogenous BMP4. Conversely, the addition of the secreted BMP inhibitor noggin to wild-type buds reduced bud sprouting by 50%. These data indicate that BMP4 is downstream of PTHrP in its role as a regulator of embryonic mammary ductal branching morphogenesis. Hens et al.

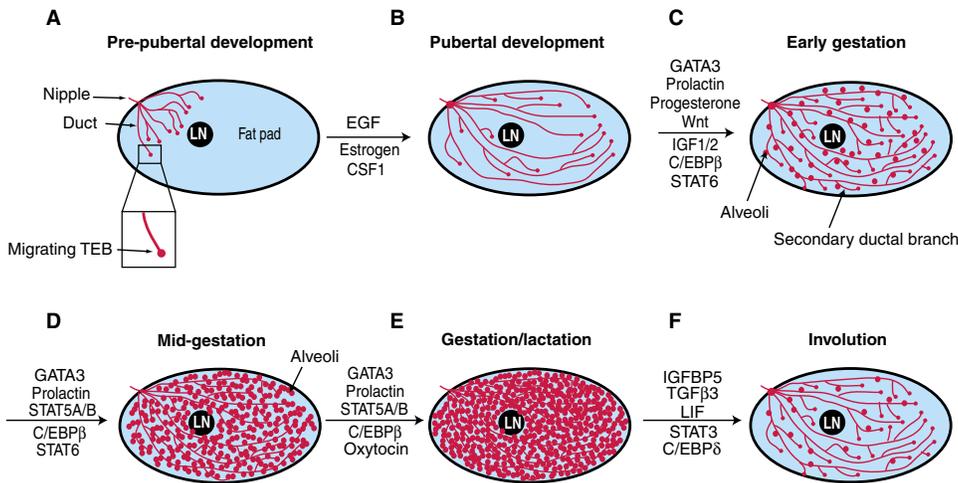


Fig. 3. Adult mouse mammary gland development. A schematic of the stages (A-F) of mammary gland development in the adult mouse, from pre-puberty through to pregnancy, lactation and involution. Some of the important factors in these developmental processes are highlighted. LN, lymph node; TEB, terminal end bud.

hypothesised that *MSX2*, a homeodomain transcription factor, could mediate the signals from PTHrP and BMP4, as *Msx2*-null mice have been reported to have a similar arrested bud development phenotype to *Pthrp*-deficient mice (Satokata et al., 2000). This hypothesis is supported by results obtained by culturing mouse C3H10T1/2 mesenchymal cells, which show that PTHrP and BMP4 synergistically induce *Msx2* expression, and by the fact that loss of *Msx2* can rescue the loss of hair follicles seen in KRT14-PTHrP overexpressing mice (Hens and Wysolmerski, 2006). Taken together, these results demonstrate that PTHrP and BMP4 induce the expression of *MSX2* in mammary mesenchyme to mediate the PTHrP-regulated suppression of hair follicle formation around the bud and nipple (Fig. 2). Use of embryonic bud organ culture should enable further insights into the factors produced by the epithelium and/or mesenchyme that promote the outgrowth of the mammary bud.

These studies have revealed that complex interactions exist between signalling pathways, have clarified the role of Hh pathway components and have revealed new molecular players in embryonic mammary development, such as *GATA3*. The demonstration that gradients of FGF10 function in the positioning of the placodes is an important finding and might provide insights into the different requirements for placode formation at different locations. Finally, the elegant embryonic bud culture studies of Hens and Wysolmerski will provide an impetus for further work on embryonic branching morphogenesis.

It is also interesting that hair follicle formation is suppressed in favour of mammary gland development. It was demonstrated recently in mice that the ablation of epithelial SHH signalling results in the transformation of some hair follicles to a strikingly mammary-gland-like fate (Gritli-Linde et al., 2007). It is also worth noting that in the absence of white adipose tissue (WAT), which starts to accumulate around the developing mammary ductal system at E18.0 (Sakakura, 1987), branching is arrested at this stage and that only 3-4 ducts develop by birth in mice genetically modified to lack all WAT (Couldrey et al., 2002). It is not known whether this is owing to a defect in paracrine signalling or altered physical interactions.

The mammary gland is unusual in that development arrests at E18.5 and does not commence again until puberty, when much of its development takes place. Thus, we now turn to the events that occur during adult mammary gland development and to the recent discoveries that have shed light on this process.

Adult mammary development

The major events that occur during adult mammary gland development, including the developmental cycle of pregnancy, lactation and involution are depicted in Fig. 3.

Mammary development at puberty

At birth, the mouse mammary gland is competent to produce milk (as it is in humans, in whom it is sometimes referred to as witch's milk). In the first few weeks after birth, growth of the mammary tree is commensurate with body growth (allometric growth) (Fig. 3A). Terminal end buds (TEBs), which are club-shaped structures comprising an outer layer of cap cells and a multilayered inner core of cells called body cells, appear at the tips of the ducts and start to invade the fat pad. Allometric growth ceases when serum levels of estrogen start to rise at puberty. Proliferation within the TEBs results in ductal elongation, and clefting of the TEBs results in bifurcation of the ducts to generate branches (Fig. 3B). Apoptosis has been detected in the body cells and could be the mechanism for lumen formation (Humphreys et al., 1996). By ~10-12 weeks of age, the TEBs have disappeared, the limits of the fat pad are reached and growth ceases. The appearance of TEBs is not observed in A-ZIP/F-1 mutant mice that lack WAT and in which ductal development is severely disrupted (Couldrey et al., 2002) (A-ZIP/F-1 is a dominant-negative protein that inhibits the DNA-binding and function of B-ZIP proteins in both the C/EBP and AP1 families of transcription factors), implicating the fat pad in TEB formation and ductal elongation, either physically or as a source of secreted factors. It is worth noting that cycles of side-branching followed by apoptosis occur with each oestrus cycle and that, in some mouse strains, this branching can be extensive. Thus, cell death is a crucial homeostatic event in the mature virgin mammary gland, although this has received little attention until recently.

The expression of *Gata3* in the body cells (but not the cap cells) of TEBs has hinted at a role in post-natal ductal branching and elongation, as have experiments in which MMTV-Cre has been used to delete *Gata3* in luminal epithelium. In the absence of *Gata3*, TEBs fail to develop and a drastic reduction in ductal outgrowth occurs. This reduced outgrowth was even seen in heterozygous *Gata3* mice in one study (Asselin-Labat et al., 2007), but not another (Kouros-Mehr et al., 2006) (this discrepancy might reflect the use of different MMTV-Cre transgenic lines in the two studies). Loss of *Gata3* is associated with a decrease in the proportion of cells positive for estrogen receptor α (ER α ; also known as ESR1 – Mouse

Genome Informatics), suggesting that GATA3 is involved in either the expression of ER α or the commitment to the ER α -expressing lineage. Using a bioinformatics approach, Kouros-Mehr et al. (Kouros-Mehr et al., 2006) identified FOXA1 as a possible component of the GATA3 regulatory network. The correlation between *Gata3* and *Foxa1* expression, the presence of a GATA3-binding site in the *Foxa1* promoter, and the role of FOXA1 in estrogen signalling and the binding of ER to chromatin, led these authors to suggest that FOXA1 mediates cross-talk between GATA3 and ER α signalling, as discussed further in the following section.

Branching morphogenesis

Branching morphogenesis is a complex process that is regulated by a wide range of factors expressed in the epithelium or stroma, including hormones and growth factors, extracellular matrix molecules and matrix metalloproteases, morphogens and immune cells (Sternlicht et al., 2006). These factors provide both global and positional cues. However, the question remains as to the mechanism by which the initiation and formation of secondary ductal branches is determined.

A crucial regulator of branching in the virgin gland is estrogen, which has two receptors, ER α and ER β , with ER α being the more important for development. The original knockout model of ER α (the ERKO mouse) established that estrogen is important for pubertal development (Bocchinfuso et al., 2000). However, this phenotype is partly due to reduced prolactin (PRL) levels. A complete knockout of ER α subsequently showed that TEBs are absent in the mammary glands of *Er α* -null mice and that the ducts failed to invade the fat pad (Mallepell et al., 2006). A more refined version of this knockout has recently been developed with the aim of removing ER α only in the epithelium, at different stages of development. By conditionally deleting ER α , Feng et al. (Feng et al., 2007) have shown that ER α is required for both prepubertal development and during late pregnancy for alveologenesis and lactation.

Recently, Mina Bissell's laboratory has utilised a novel mammary cell culture system to address the role of tissue geometry and morphogenetic gradients in mammary gland branching morphogenesis (Nelson et al., 2006). Using mouse mammary epithelial cells and a three-dimensional micropatterned collagen gel assay to control the geometry of the initially formed tubules, these researchers demonstrated that the addition of epidermal growth factor (EGF) or hepatocyte growth factor (HGF) to this culture system induced the formation of multicellular branches from the central tubule that invaded the surrounding collagen. Using real-time imaging of the expression of green fluorescent protein (GFP) under the control of the vimentin mesenchymal gene promoter, branches were found to form at locations of previous GFP expression, supporting the idea that an epithelial-mesenchymal transition-like event is required at branch points for branching to occur. Branching, but not the expression of vimentin/GFP, could be blocked by inhibiting the activity of the growth factor epimorphin (also known as syntaxin 2 – Mouse Genome Informatics), which has previously been shown to be important for branching during puberty. Furthermore, changing the geometry of the tubule to either a curved or a bifurcated structure changed the position of the branches. This suggests that locally secreted inhibitory morphogens, such as transforming growth factor (TGF) β 1, could influence branching. The researchers then tested this hypothesis by constructing 3D computer-generated models of diffusion gradients of morphogens that correctly predicted the sites of branching. Thus, their findings suggest that the geometry of ducts and their position relative to neighbouring ducts can control the sites of branching. This interesting study lays

the foundation for further work that could incorporate other components of the mammary gland, such as adipocytes and isolated TEBs. Using a different 3D culture model (organoids in Matrigel culture), the Bissell laboratory have also shown that TGF α is sufficient to induce branching morphogenesis and that the duration of an active ERK1/2 (also known as MAPK3/1 – Mouse Genome Informatics) signal is crucial to this process (Fata et al., 2007). Thus, signal intensity and duration are also of crucial importance for morphogenesis.

The secreted protein, milk fat globule-EGF factor 8 (MFGE8), which is composed of two EGF repeats and two discoidin domains, is required for the efficient removal of apoptotic mammary epithelial cells during post-lactational regression. A role for MFGE8 in branching morphogenesis has now recently been suggested because of the severely reduced branching and thin, poorly developed TEBs that are observed in *Mfge8*-null mice (Ensslin and Shur, 2007). Interestingly, MFGE8 is expressed by both luminal and myoepithelial cells.

Lumen formation

Lumen formation is an essential process in embryogenesis. It is first required for blastocyst formation and subsequently for ductal and tubule development in a variety of organs, including the kidney and lung. A hollow lumen can be formed in several ways. In the blastocyst, for example, cells die to produce the luminal space. A similar mechanism has been proposed to produce the ductal lumen during ductal morphogenesis of the mammary gland, as there is evidence that apoptosis occurs within the body cells of the TEB (Humphreys et al., 1996). The apoptosis of these cells can be reduced by the overexpression of the pro-survival BCL2 factor. More recently, studies using mice null for *Bim* (also known as *Bcl2l1*), a BH3-only-domain regulator of apoptosis, have revealed that BIM is essential for the removal of the surplus epithelium in the duct (Mailleux et al., 2007). In the absence of BIM-mediated cell death, these cells switch to a more squamous cell type and subsequently die via a caspase-3-independent mechanism. It will be interesting to determine whether cell death and lumen formation is a caspase-regulated process or whether alternative cell death mechanisms are utilised.

The occlusion of the mammary duct lumen occurs in mice deficient in the axonal guidance molecules ROBO1 and SLIT2 (Strickland et al., 2006). TEBs in *Slit2*^{-/-} or *Robo1*^{-/-} mice display spaces between the cap and luminal/body cell layers, a phenotype similar to that seen in netrin 1 (*Ntn1*)-null mice. Mammary glands in *Slit2*^{-/-}; *Ntn1*^{-/-} mutant mice show not only defects in TEB structure, but also severe ductal abnormalities that suggest a peeling apart has occurred of the luminal epithelial and myoepithelial cell layers. This notion is supported by in vitro assays that show that *Slit2*^{-/-}; *Ntn1*^{-/-} double-deficient mammary cells are severely compromised in their ability to form bi-layered organoids. Furthermore, this deficiency is rescued by the addition of purified SLIT2. These axonal guidance molecules might thus be of crucial importance during the rapid growth and morphogenesis that occur during puberty to maintain the integrity of the bi-layer.

The size of the lumen can also be affected by a variety of factors, including the transcription factor CCAAT/enhancer binding protein (C/EBP) β (also known as CEBP β) because *Cebpb*-null virgin mice exhibit cystic, enlarged mammary ducts with decreased secondary branching (Seagroves et al., 1998).

These studies highlight the importance of using sophisticated genetically engineered models to address the role of growth/signalling factors in specific cell types and at specific

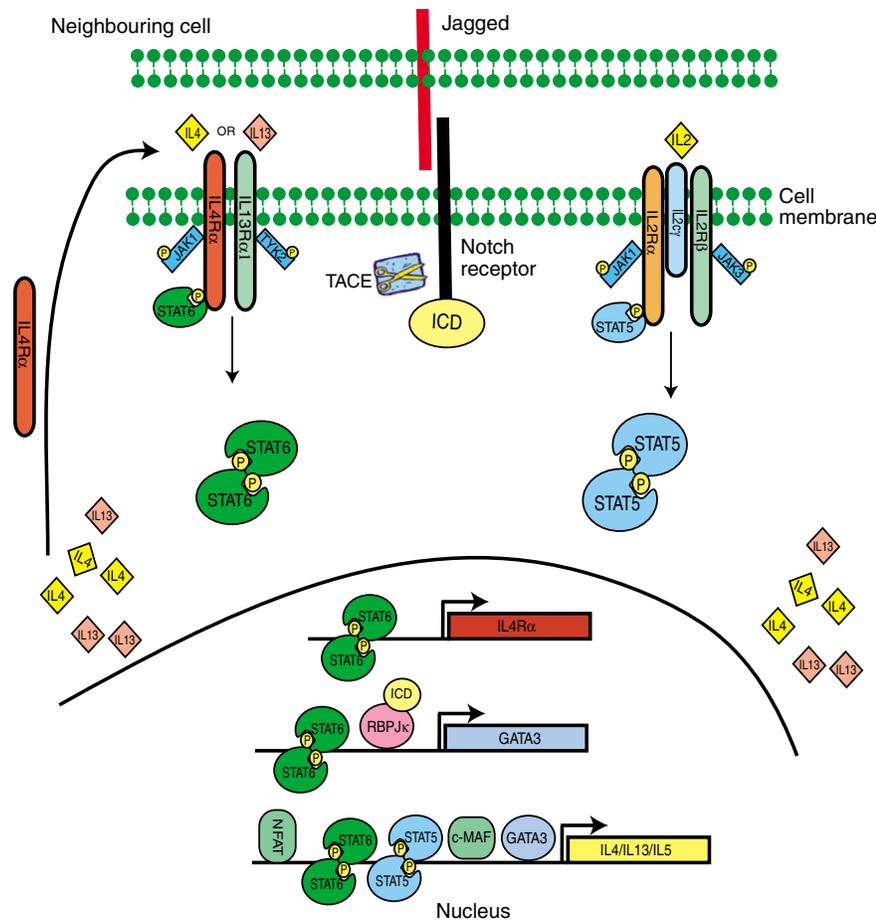


Fig. 4. Signaling in T cell lineage commitment. Multiple pathways are implicated in T helper type 2 (Th2) cell differentiation. Importantly, IL4/13 signalling through STAT6 induces the transcription of IL4R α and GATA3, which is the master regulator of Th2 differentiation. GATA3, along with other transcription factors, STAT6, STAT5 and c-MAF, induce the transcription of IL4, IL5 and IL13. GATA3 is also activated by the Notch pathway independently of STAT6 activity, providing an explanation for GATA3 expression in *Stat6*-null mice. The balance between different members of the nuclear factor of activated T-cells (NFAT) family of transcription factors also plays a role in the transcription of IL4 and Th2 differentiation. P, phosphorylation; TACE (ADAM17); ICD, intracellular domain.

developmental stages during mammary development. TEB structure is important also for mammary gland morphogenesis, a process that is also controlled by morphogenetic gradients of secreted factors.

Mammary development during pregnancy

During pregnancy, the mammary gland has to undergo further development and morphological change to prepare for lactation (Fig. 3D-F). Lactation requires the production of specific cells that can synthesize and secrete copious amounts of milk. The hormone progesterone (P) induces extensive side-branching and alveologenesis and, in combination with PRL, promotes the differentiation of the alveoli, which are the structures that synthesize and secrete milk during lactation. In the absence of the P receptor (PR; also known as PGR – Mouse Genome Informatics), side-branches and alveoli do not form (Brisken et al., 1998). The PRL receptor is also essential for alveolar differentiation (Ormandy et al., 1997). The alveolar luminal cells, together with the surrounding myoepithelial cells, probably arise from bi-potent ductal progenitors, although there is evidence that distinct duct-limited and lobule-limited progenitor cells exist in both mouse and rat (Smith and Boulanger, 2003). There are at least two populations of slowly dividing (label-retaining) cells in the ductal epithelium, which are either ER-positive or ER-negative (Booth and Smith, 2006). In normal mammary glands of both mice and women, ER α -positive cells are not normally proliferative, but this association is lost in breast cancer. It is likely that there are discrete factors that specify and maintain differentiated alveolar cells during pregnancy.

Recent studies into mammary gland development have provided new insights into the signals that are necessary for progenitor cells to commit to the luminal lineage and for the maintenance of the differentiated state of luminal cells. By analogy with the differentiation paradigm of T helper (Th) cells, these findings have led to a model in which uncommitted transit-amplifying (TA) or progenitor cells are directed down either of two lineages based on the expression and response to Th1 or Th2 cell cytokines (as discussed in more detail below).

Progenitor cell regulation and lineage commitment during pregnancy

PR function is mediated through several factors, including the Wnt pathway. Canonical Wnt signalling requires β -catenin and the suppression of this pathway results in impaired alveolar development, suggesting that the cross-talk that occurs between these pathways is important for mammary gland development during pregnancy. This hypothesis was tested recently by crossing a transgenic mouse model of constitutive β -catenin activity onto a background of PR deficiency (Hiremath et al., 2007). Surprising results were obtained from this study that suggest that cells at the tips of ducts respond differently to the absence of PR than do cells along the ducts. Precocious development of alveoli at the ends of ducts was observed in response to constitutive β -catenin expression in the complete absence of PR. However, in mice heterozygous for *Pr*, precocious development was also seen along the lateral borders of the ducts. The authors concluded that although PR signalling is required for β -catenin responsiveness in the ducts, it is not required at the ductal tips.

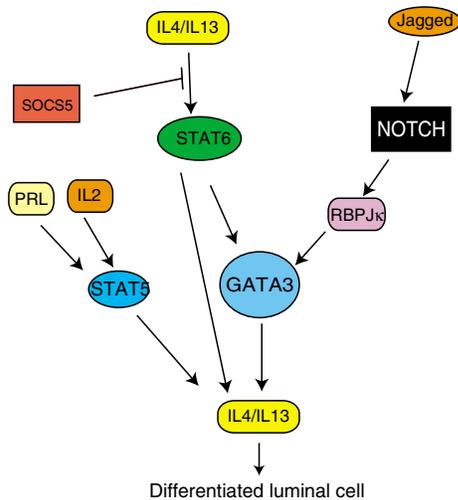


Fig. 5. The IL4/13-STAT6-GATA-3 pathway in mammary gland development. A summary of recent findings that identify the IL4/13-STAT6-GATA3 pathway as being an important regulator of mammary gland development and lineage commitment.

The prevailing paradigm, that steroid hormones and PRL are the principal temporal regulators of mammary gland differentiation (Hennighausen and Robinson, 2005), has been given an added perspective with the publication of several papers demonstrating that signalling pathways that are normally associated with lineage commitment in Th cells (Khaled et al., 2007; Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006) also function in mammary lineage commitment.

The polarization of Th cells into either Th1 or Th2 is regulated by cytokines: interleukin (IL) 12 activates STAT4 and commits naïve T cells to the Th1 lineage, whereas IL4 and IL13 activate STAT6 and promote the Th2 lineage (Ansel et al., 2006). Th2 cells are required for the response to parasitic infections, and once Th2 cells have been generated, commitment to this lineage is reinforced by the secretion of the type-2 cytokines IL4, IL5 and IL13. This is associated with changes in chromatin structure and with the transcriptional upregulation of the Th2 transcription factors GATA3 and c-MAF. Conversely, Th1 cells are produced in response to viral infections, and these cells secrete the type-1 cytokines IFN γ and TNF, again reinforcing commitment to this lineage and inducing the expression of the transcription factor T-Bet (also known as TBX21 – Mouse Genome Informatics) (Fig. 4).

A role for STAT6 and its upstream cytokines IL4 and IL13 in the expansion of the luminal lineage has recently been demonstrated in mice deficient for these pathway components (Khaled et al., 2007). STAT6 phosphorylation in wild-type mice increases by day 5 of gestation, and this increase correlates with the expression of IL4R α and GATA3 in the epithelium followed by c-MAF induction later in pregnancy. In the absence of STAT6, a 70% decrease in the number of alveoli is seen at day 5 of gestation and correlates with diminished epithelial cell proliferation. Similar phenotypic results are seen in *Il4^{-/-}/Il13^{-/-}* double-mutant mice (Khaled et al., 2007), whereas deletion of SOCS5, a negative regulator of STAT6, results in precocious alveolar development. Importantly, mammary epithelial cells in culture secrete the type-1 cytokines IL12a, IFN γ and TNF in the undifferentiated state, but when induced to differentiate by a lactogenic hormone cocktail (PRL, dexamethasone and insulin), they switch to secreting type-2

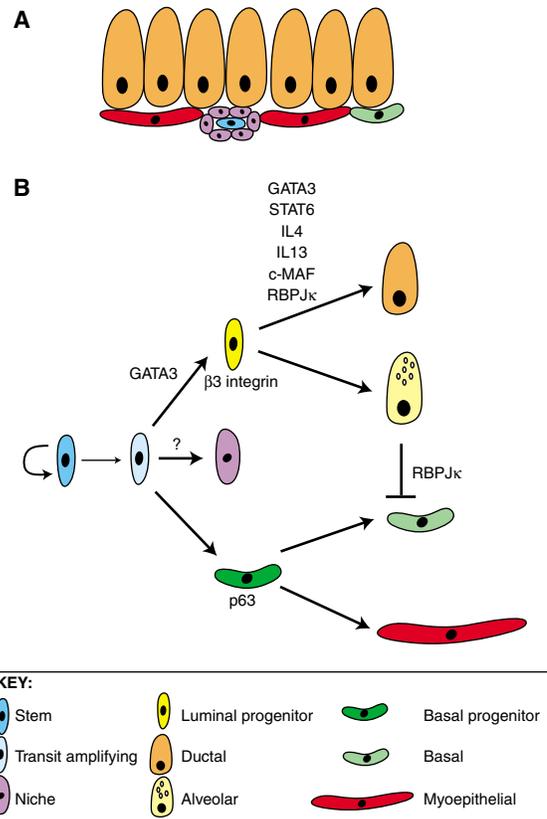


Fig. 6. Lineage commitment in mammary progenitor cells. (A) A mammary stem cell (blue) in its niche (purple). (B) Stem cells self-renew in their niche in a process that might require GATA3. Asymmetrically dividing stem cells produce transit-amplifying (light blue) daughter cells that commit to either the basal or luminal progenitor lineages. A common progenitor for ductal and alveolar cells is depicted, although there might be two different progenitors. Induction of STAT6 in response to IL4 and IL13 induces further expression of GATA3 and possibly of c-MAF, which are required for differentiation to alveolar cells. RBPJk is required to maintain the alveolar phenotype and suppresses differentiation along the basal lineage.

cytokines (IL4, IL13, IL5). This unexpected discovery demonstrates a role for these immune cell cytokines in epithelial cell fate and raises interesting questions about the evolutionary origins of mammary and immune cells and the role of T cell cytokines in the regulation of mammary progenitor cells.

The role for Th2 signalling factors in mammary development is further highlighted in two similar studies in which *Gata3* was conditionally deleted at different stages of mammary development (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Deletion of *Gata3* in alveolar cells during pregnancy using the *Wap* promoter to drive Cre expression in the epithelium during pregnancy revealed that *Gata3* deficiency leads to a block in alveolar differentiation and to failed lactogenesis (Asselin-Labat et al., 2007). Similar results were obtained using a doxycycline-inducible Cre line WAP-rtTA-Cre (Kouros-Mehr et al., 2006), which allows *Gata3* to be deleted at specific times in the epithelium from late pregnancy onwards. The observation that the apparently *Gata3*-null outgrowths contain a non-deleted *Gata3* allele indicates that a selective pressure retains a functional *Gata3* allele in the surviving outgrowths. The long-term administration of doxycycline (14 days) results in additional defects

in the luminal epithelium, including the disruption of the ductal architecture and a marked detachment of cells into the lumen that is associated with cell death.

Gata3 deficiency also results in the expansion of undifferentiated mammary epithelial cells, as revealed by immunostaining. This suggests that GATA3 might be required to maintain the quiescent state of differentiated luminal cells. This idea is supported by the observation that levels of GATA3 and proliferative behaviour are inversely correlated. Alternatively, it could be that GATA3 is required primarily to maintain the differentiated state and thus is not directly involved in cell cycle control.

Using FACS to isolate an epithelial subpopulation of cells (CD29^{lo} CD24⁺ CD61⁺), it was shown that this luminal progenitor pool increases significantly in size in *Gata3*-deficient mice, further supporting the notion that loss of GATA3 blocks the differentiation of luminal progenitors (Asselin-Labat et al., 2007). The subsequent overexpression of GATA3 in these cells showed that expression of the milk proteins β -casein and WAP could be induced even in the absence of lactogenic hormone stimulation. This indicates that GATA3 promotes the differentiation of lineage-restricted progenitor cells. Interestingly, haploinsufficiency of *Gata3* suggests that absolute levels of protein are important for mammary gland development; indeed, humans with only one functional copy of *GATA3* have reduced levels of GATA3 protein and deficiencies in Th2 responses, serum IgE levels, and often abnormalities of the kidneys, thyroid gland and in hearing.

In Th2 cells, *Gata3* is required for the continued expression of *Il4*, *Il5* and *Il13*, as their promoters have GATA3-binding sites. It is interesting that phosphorylated STAT5 levels are reduced in STAT6-deficient mammary glands, perhaps suggesting that levels of IL5, which is known to activate STAT5, are reduced in the absence of STAT6. A hierarchy of signalling from IL4/IL13 through STAT6 and GATA3 is thus an important constituent of commitment to the alveolar luminal lineage (see Fig. 5). The cytokine signals that lie upstream of GATA3 in embryonic and pubertal mammary gland are yet to be determined. It will also be important to identify and compare the signals that lie downstream of GATA3 at different stages of mammary gland development, as these findings could have important implications for breast cancer, not least because GATA3 is highly expressed in breast cancers of the luminal A subtype, which also express ER α (Sorlie et al., 2003).

An interesting counterpoint to the STAT6 and GATA3 stories is the role of Notch signalling in lineage commitment. The Notch pathway is crucial also in Th1/Th2 determination (Ansel et al., 2006). Notch signalling is mediated by the DNA-binding protein RBPJ κ (in the absence of Notch, RBPJ κ represses Notch target genes through the recruitment of a co-repressor complex). The binding of the Notch intracellular domain (NICD) to RBPJ κ displaces these co-repressors from RBPJ κ , resulting in the derepression of promoters that contain RBPJ κ -binding sites. NICD then recruits several coactivators, including mastermind-like protein (MAML) and CBP/p300 (CREBBP), and results in the transcription of target genes, such as *Hey1*. The conditional deletion of *Rbpjk* in mouse mammary epithelium during pregnancy results in the expression and accumulation of p63 (also known as TRP63 – Mouse Genome Informatics) in luminal cells, suppressing their luminal characteristics and inducing more basal-like features, including the expression of keratin 5 (Buono et al., 2006). This phenotype was also associated with increased proliferation rates, but growth of the virgin mammary gland was not affected. Interestingly, there was a transient amplification of keratin 6-positive luminal cells in these mice that could reflect either a block in differentiation or a slower

progression through the lineage to fully differentiated luminal cells. Thus, the canonical Notch pathway is required for the maintenance, but not for the establishment, of luminal cells and is particularly important for the proliferation of these cells during pregnancy. These studies provide new insights into the signals necessary for the commitment to, and maintenance/differentiation of, the luminal lineage, and are summarised in Fig. 6.

Finally, a role for both the Hh and Wnt pathways in stem cell self-renewal/maintenance has been suggested by two recent studies. Using the MMTV promoter to overexpress a constitutively active form of SMO (MMTV-SmoM2) in limiting-dilution transplantation studies (in which a maximum of one stem cell is transplanted per gland), Moraes et al. showed that there is a decrease in the frequency of regenerative stem cells in MMTV-SmoM2 epithelium compared with wild-type (Moraes et al., 2007). In a similar limiting-dilution study, and based on the expression of the putative stem cell markers keratin 6 and p21 (also known as CDKN1A – Mouse Genome Informatics), ductal cells from mice deficient for the Wnt coreceptor LRP5 have also been shown to exhibit little to no stem cell activity (Lindvall et al., 2006), suggesting that LRP5-mediated canonical signalling is required for mammary stem cell activity.

Concluding remarks

This is an exciting time for mammary gland biologists. The identification of novel signalling pathways that regulate lineage commitment, the refinement of genetically modified mouse models, the development of stem cell enrichment procedures, and the ability to culture embryonic mammary glands and follow stem cells as they commit to different lineages will bring important new insights. The development of improved humanised mouse models and three-dimensional culture models will be important for future work.

C.J.W. is supported by a BBSRC Research Development Fellowship and W.T.K. is funded by a BBSRC project grant.

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