Integrated morphodynamic signalling of the mammary gland

Nikolce Gjorevski and Celeste M. Nelson*‡*

Abstract | The mammary gland undergoes a spectacular series of changes as it develops, and maintains a remarkable capacity to remodel and regenerate for several decades. Mammary morphogenesis has been investigated for over 100 years, motivated by the dairy industry and cancer biologists. Over the past decade, the gland has emerged as a major model system in its own right for understanding the cell biology of tissue morphogenesis. Multiple signalling pathways from several cell types are orchestrated together with mechanical cues and cell rearrangements to establish the pattern of the mammary gland. The integrated mechanical and molecular pathways that control mammary morphogenesis have implications for the developmental regulation of other epithelial organs.

Adipocytes
Also known as fat cells. Adipocytes contain one or more lipid droplets and comprise the body's adipose tissue.

Placodes
Epithelial thickenings in the embryo that give rise to an organ.

**Departments of Chemical & Biological Engineering and Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA.*
‡A321 Engineering Quadrangle, Princeton University, Princeton, New Jersey 08544, USA.
Correspondence to C.M.N.
e-mail: celesten@princeton.edu
doi:10.1038/nrm3168
Published online
10 August 2011

The mammary gland produces and delivers milk from mother to newborn. The only organ after which an entire class of animals has been named, the gland is credited for the evolutionary success of mammals, primarily owing to milk's nutritional and antimicrobial content[1]. Lactation, the synthesis and secretion of milk, is made possible by the architecture of the gland. Like other organs used for fluid transport, the mammary epithelium develops into an elaborate network of branched ducts that maximize surface area within a constrained volume. The mature mammary duct consists of an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells that surround a hollow lumen and differentiate into milk-producing alveoli; release of milk through the duct occurs upon hormone-triggered contraction of the myoepithelium[2]. The epithelial ductal tree is enveloped by a basement membrane[3] and embedded within a complex stroma, the mammary fat pad, which contains fibroblasts, adipocytes, blood vessels, nerves and various immune cells, all of which are important for normal mammary development and function[4].

Mammary development occurs in three distinct and differentially regulated stages: embryonic, pubertal and adult (FIG. 1). In mice, embryonic mammary development begins mid-gestation with the formation of five pairs of placodes in the epithelial layer that invaginate into the underlying mesenchyme to form the mammary buds, or anlagen[5,6]. The mammary bud then proliferates and extends 10–20 sprouts[7], thus transforming into a rudimentary ductal structure. After birth, the rudimentary gland enters a phase of morphogenetic quiescence.

Puberty is perhaps the most striking stage of mammary morphogenesis. Prompted by elevated levels of ovarian hormones, including oestrogen, the ends of the rudimentary ducts proliferate and swell into distinct multilayered epithelial structures known as terminal end buds (TEBs)[8]. These ductal structures then undergo successive rounds of elongation, bifurcation and lateral branching until reaching the limit of the fat pad, thus forming a full epithelial tree[9]. During pregnancy, the luminal epithelium proliferates and differentiates into milk-producing secretory alveoli[10,11]. Massive apoptosis then removes up to 80% of the epithelium during post-lactational involution[12–15]. Remarkably, the mammary gland maintains its ability to perform this dramatic remodelling during the pregnancy–lactation–involution cycle for several decades in humans.

The study of mammary morphogenesis during the past century has implicated a long list of signals in its regulation, including hormones, growth factors, receptor tyrosine kinases, extracellular matrix (ECM) molecules and proteases[16]. Over the past 5 years, sophisticated genetic, real-time imaging, computational and culture studies along with large-scale gene profiling have revealed links among the various signals, cellular behaviours and physical phenomena that drive mammary development and have unveiled the integrated nature of these cues. We now understand that the spatial and temporal changes, or 'morphodynamics', that occur during development of the mammary gland are dictated by signalling between several cell types, integrated dynamically over multiple length scales, from cell to tissue, organ and organism.

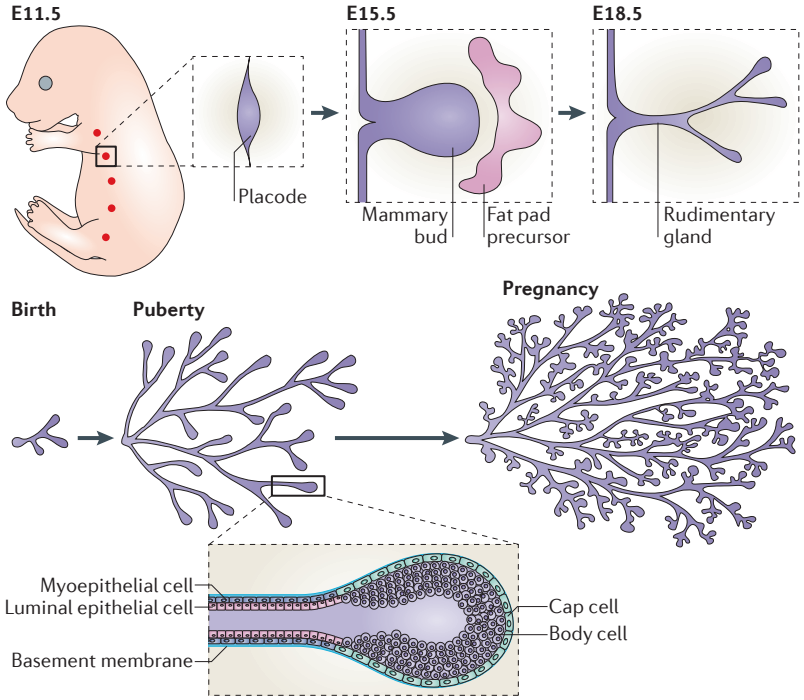


Figure 1 | **The mammary gland undergoes distinct stages of remodelling during development.** In the mouse embryo, mammary development begins when five pairs of placodes form in the epithelium adjacent to the fat pad precursor. These placodes invaginate to form the mammary buds. By embryonic day 18.5 (E18.5), a rudimentary gland has formed that remains morphogenetically quiescent until puberty. During puberty, hormonal cues trigger the formation of the terminal end buds (inset). Through extensive elongation, bifurcation and lateral branching, the full epithelial tree is formed.

Here, we focus on development during puberty, paying close attention to the mechanisms that establish the pattern of the mammary epithelial tree. We direct the reader to excellent reviews on embryonic and post-pubertal development of the mammary gland[5,6,17]. Some of the elegant mechanisms that drive mammary patterning might be conserved across branched epithelia, and others have paved the way for new paradigms in the study of morphogenesis[18–20]. We discuss also the roles of mechanosensing and collective cell migration in branching and how they are integrated together with signalling networks. The principles that have emerged should help us to build comprehensive models for mammary development in particular and organogenesis in general.

Integrated signalling during morphogenesis

Although the process has been difficult to visualize *in vivo*, the transformation of the rudimentary gland into the elaborate network of TEBs during puberty probably involves a series of coordinated cell divisions, rearrangements and shape changes. To understand how a functional mammary tree is formed, a better understanding is needed of the key events in this process. A large number of the signals that direct mammary development overlap with those involved in the morphogenesis of other branched epithelia, but several features make the mammary gland unique. Whereas chemotactic gradients guide extending branches in the trachea of *Drosophila melanogaster*[21,22], the mammalian lung[23] and the ureteric bud that gives rise to the urinary tract and the kidney[24], there exists no evidence for chemotaxis as the guidance mechanism for mammary gland branching[25,26]. Likewise, the large variation observed between mammary glands precludes the possibility for predetermined genetic control of its morphogenesis, as is the case with the largely stereotyped airways of the embryonic lung[27]. The stochastic form of the gland suggests a dynamic control, dictated by microenvironmental context. Indeed, a context-dependent interplay has been unveiled between mechanical factors and molecular signals derived from different cell types that induces the cellular behaviours and matrix remodelling that ultimately drive morphogenesis[28,29].

Hormone-induced paracrine signalling. A plethora of molecular signals cooperate to execute mammary morphogenesis through communication between epithelial and stromal cells (FIG. 2). This process is set in motion by ovarian and pituitary hormones, including oestrogen and growth hormone, which can signal to both types of cell[30]. Knocking out oestrogen receptor-α (ERα) leads to hypoplastic development of the epithelial tree[31,32], whereas exogenous oestrogen can rescue pubertal branching in mice that have had their ovaries surgically removed (ovariectomized mice)[33]. Transplantation experiments have shown that ERα is required in the stroma[34], which, in response to oestrogen, produces hepatocyte growth factor (HGF) to induce epithelial branching[35]. Oestrogen also binds to ERα in the epithelium, thereby inducing the expression of amphiregulin (AREG)[36–40], and its cleavage from the surface by the sheddase ADAM17 (a disintegrin and metalloproteinase domain-containing protein 17)[39]; cleaved AREG can signal back to stromal cells by binding to epidermal growth factor receptor (EGFR) on the stromal membrane. EGFR is required in the stromal compartment[41], and exogenous addition of EGFR ligands can rescue pubertal development of ovariectomized animals[36], which is consistent with an essential role for EGFR-mediated signalling downstream of oestrogen.

Oestrogens, however, are not sufficient, as they fail to rescue mammary branching in animals that have had their pituitary gland surgically removed[42]. Branching is restored by growth hormone or insulin-like growth factor 1 (IGF1)[42]. Transplantation experiments have demonstrated that growth hormone induces expression of IGF1 in stromal cells[43], which signals to its receptor (IGFR1) in the epithelium[42]. Several other receptor tyrosine kinases have profound effects on pubertal mammary development, including RON (also known as MSPR)[44], ephrin type-A receptor 2 (REF. 45) and fibroblast growth factor receptors (FGFRs). Indeed, FGF2 and FGF7 rescue growth and branching of EGFR-null mammary organoids in culture[39], suggesting that FGFR signalling occurs either downstream of, or in parallel to, signalling through EGFR. The EGFR family member ERBB2 has also been implicated in mammary morphogenesis[46,47], although

Chemotactic gradients
Chemical gradients that influence the directional motion of cells in the process of chemotaxis.

Organoids
Multicellular structures that resemble organs in architecture and function.

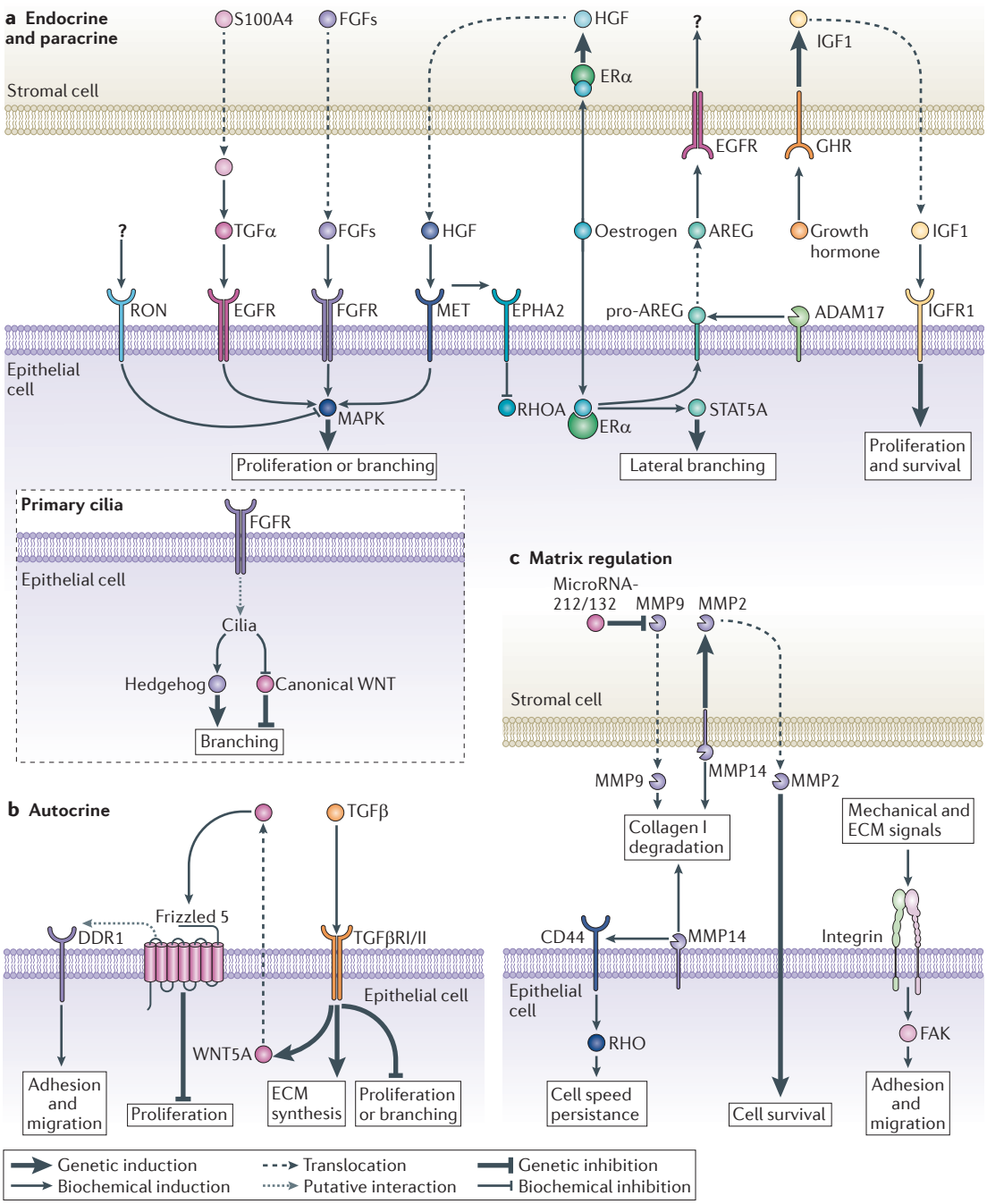


Figure 2 | **Multiple integrated signalling networks regulate mammary morphogenesis during puberty. a** | Global endocrine signals from the ovary[30–35,40] and pituitary gland[30,42,43] activate a plethora of paracrine signalling pathways to initiate mammary morphogenesis. Cellular crosstalk between the epithelial and stromal compartments is mediated by growth factors including insulin-like growth factor 1 (IGF1)[42,43], hepatocyte growth factor (HGF)[35,148] and the epidermal growth factor (EGF)[36–41] and fibroblast growth factor (FGF)[51,52,189] families, which bind to their cognate receptors to induce cell proliferation, survival and branching. Classic pathways such as WNT and Hedgehog, which are activated by signalling through primary cilia, are also emerging as indispensable regulators of the process[62]. **b** | Autocrine cues such as transforming growth factor-β (TGFβ) serve as negative regulators of mammary morphogenesis directly by stimulating extracellular matrix (ECM) synthesis[80] or by activating non-canonical WNT signalling[76] to inhibit proliferation[74] and possibly control cell adhesion and migration[76]. **c** | Mammary gland patterning is directed in part by matrix metalloproteinases (MMPs), which display spatially localized expression and activity during puberty and serve both to control cell migration[122] and survival[65], and to degrade the ECM[65,82]. Integrin-dependent ECM signalling and mechanical cues are emerging as additional regulators of mammary morphogenesis[86,91,92]. ADAM17, a disintegrin and metalloproteinase domain-containing protein 17; AREG, amphiregulin; EGFR, EGF receptor; EPHA2, ephrin type-A receptor 2; ERα, oestrogen receptor-α; FAK, focal adhesion kinase; FGFR, FGF receptor; GHR, growth hormone receptor; MAPK, mitogen-activated protein kinase; TGFβRI/II, TGFβ receptor I and II.

it has no known ligand and its exact role is unclear. ERBB2 is required in the epithelium, but paradoxically the partner proteins that it normally signals with through dimerization, ERBB4 or EGFR, are required only in the stroma[38,39,48]. The compartmental localization and requirement of AREG, ERBB2, EGFR, IGF1 and IGFR1 highlights the crucial importance of integrated paracrine signalling between the epithelium and stroma during pubertal development.

The importance of timing and location. The involvement of many of these molecules has been recognized for nearly two decades. However, it has been difficult to uncouple the individual signals and receptors, given how many downstream effectors they share. For example, EGFR and FGFRs elicit at least part of their effects through mitogen-activated protein kinases (MAPKs), but seem to yield distinct and even antagonistic phenotypic outcomes. The kinetic profile of MAPK activity may determine the final morphogenetic response, at least in culture; sustained MAPK activation downstream of transforming growth factor-α (TGFα) and EGFR induces branching, and transient MAPK activation downstream of FGF7 and FGFR2 induces proliferation[49]. These results suggest that temporal responses may be used by the mammary epithelium to integrate and interpret distinct signals. It is important to underscore, however, that the precise role of MAPKs in mammary morphogenesis *in vivo* is unclear. A combined loss of the MAPK inducers AREG, EGF and TGFα severely impairs branching morphogenesis in mice but has no discernable effect on proliferation, apoptosis or MAPK activation within the TEBs, which suggests that MAPKs may not be sufficient to promote morphogenesis[37].

Sophisticated genetic approaches have recently offered a glimpse into the local roles of inductive signals, notably FGFs, during mammary morphogenesis. Embryonic mammary development is disrupted in FGFR2-knockout animals, preventing investigation of postnatal effects[50]. Two studies have surpassed this problem by using different approaches to modulate FGFR signalling in mice after birth, and they arrived at similar conclusions. In one study, mosaic inactivation was used to investigate the behaviours of FGFR2-null and FGFR2-heterozygous epithelial cells within the same gland[51]. FGFR2-null cells were found to be at a proliferative disadvantage compared with heterozygous cells during pubertal mammary morphogenesis and became progressively depleted from the TEBs. In the second study, transgenic animals were used to inducibly and reversibly attenuate FGFR2 (REF. 52); impairing FGFR2 after birth decreased the proliferation of luminal epithelial cells and resulted in poorly developed glands completely lacking TEBs. The results from these studies suggest that FGFR2 regulates proliferation of luminal epithelial cells and that its function is more important locally within the TEBs than in the subtending ducts. STAT5A also shows a similar spatially restricted response to hormone signalling: it is expressed in response to oestrogen and progesterone in subtending ducts, but not in TEBs[53]. Mice deficient for STAT5A show defects in lateral branching, but not ductal extension or TEB bifurcation[54]. Global paracrine signalling and endocrine signalling can thus have varied local effects depending on whether the responding epithelial cells are in the TEBs or in the ducts.

A possible role for signalling from cilia. The role of other classical signalling pathways in pubertal mammary branching, including WNT and Hedgehog, is controversial. Some studies have suggested that canonical WNT signalling increases branching[55,56], whereas others have reported the opposite finding[57]. Components of the Hedgehog pathway, such as GLI2 and GLI3, are expressed in mammary epithelium during puberty, but transcriptional reporters showed an absence of Hedgehog signalling at this stage in the mammary gland[58]. WNT and Hedgehog signalling are coordinated in part by primary cilia[59–61], which are present on mammary epithelial cells specifically during puberty. Disrupting formation of cilia can block ductal extension and branching morphogenesis *in vivo*[62]. Moreover, blocking the formation of cilia and branching in mammary glands causes increased canonical WNT signalling and decreased Hedgehog signalling, which is consistent with these pathways acting as negative and positive regulators of pubertal branching, respectively. Primary cilia are regulated by FGFs in several epithelial tissues[63], suggesting the tantalizing possibility of a link between such growth factors and WNT and Hedgehog signalling during mammary morphogenesis.

Paracrine control by matrix metalloproteinases. In addition to growth factor receptors, matrix metalloproteinases (MMPs) have emerged as local regulators of mammary branching through their role in signalling and in clearing paths in the surrounding ECM[29]. Distinct spatial patterns of MMPs have been detected in mammary tissue. MMP14 is elevated in and around the TEBs[64,65]; MMP9 is expressed at homogeneously low levels by both the epithelium and the stroma[65]; MMP3 (also known as stromelysin 1) is expressed throughout the stroma[65]; and MMP2 (also known as gelatinase A) is reduced at sites of lateral branching[65]. Consistently, tissue inhibitor of metalloproteinases 3 (TIMP3), an MMP14 inhibitor, is also downregulated in and around the TEBs, whereas TIMP1, which does not inhibit MMP14, is specifically upregulated at these sites[39]. Knockout analyses have revealed age-dependent effects of MMPs on mammary development. MMP2-null mice exhibit delayed ductal invasion during early puberty and increased lateral branching during late puberty[65]. MMP3 does not affect ductal elongation, but instead induces lateral branching during late puberty[65]. Intriguingly, MMP2 and MMP3 seem to contribute to branching via different mechanisms. MMP2 influences branching by promoting cell survival[65], whereas MMP3 induces the local degradation of collagen IV and laminin 111 specifically at sites of lateral branching[65].

Although MMP9-null mice have apparently normal branching[65], this protease may have a redundant inhibitory role in pubertal mammary development[66]. Glands lacking microRNA-212 and -132 show increased expression and accumulation of MMP9 around the ducts, and

Paracrine signalling
A form of cell signalling in which a signal released by one cell elicits an effect within a nearby cell.

Endocrine signalling
A form of cell signalling in which a hormonal signal released by an endocrine gland elicits an effect within a distant cell.

Primary cilia
Long, slender sensory organelles that project from eukaryotic cells and are composed of a microtubule-based cytoskeleton.

MicroRNA
Small non-coding RNA molecules that regulate gene expression at the post-transcriptional level.

Autocrine signalling
A form of cell signalling in which a signal released by a given cell elicits an effect within the same cell.

Morphogen
A chemical signal that forms a concentration gradient and mediates pattern formation during tissue development.

Microfabrication
The process of fabricating micrometre-sized structures. Used in biomedical research to control the size, shape and spatial arrangement of proteins, cells and tissues.

Mechanical stress
A physical quantity defined as force per unit area.

Mechanotransduction
The phenomenon whereby cells interpret mechanical signals and transform them into a biochemical response, such as signalling or changes in gene expression.

a corresponding decrease in the deposition of collagen within the periductal sheath[66]. Again, transplantation experiments have revealed that these microRNAs were required in the stroma to influence the epithelium[66]. MMPs thus provide another example, in addition to that of growth factors discussed above, of highly integrated interactions between the epithelial and stromal compartments. Indeed, MMP3 levels increase during mammary epithelial branching upon treatment with S100A4 (also known as fibroblast-specific protein 1), which is expressed by the stroma during puberty[67,68]. Secreted S100A4 cooperates with TGFα to activate MAPKs and thereby induce MMP3 expression in epithelial cells in culture[67], suggesting substantial crosstalk between MMPs and growth factors during mammary development.

Tissue geometry and physical signals. Although pattern formation during mammary development may be attributed to spatially localized expression and activity of key regulators (including MMPs and signalling through FGFRs, as discussed above; FIG. 3a), the question remains: how do these non-uniformities arise? At the start of puberty, the rudimentary mammary epithelium already has an asymmetric branched geometry and patterning information may be encoded in the shape of this pre-existing non-spherical structure. Tissue geometry can instruct morphogenesis by creating spatial gradients of chemical and mechanical signals (BOX 1). Indeed, computational models of diffusion of the autocrine signalling morphogen TGFβ have shown that its concentration profile is determined by tissue geometry[69]. In microfabricated mammary tissues, branching is inhibited at sites of high TGFβ concentration[69]. Accordingly, TGFβ gradients might specify sites of branch initiation and maintain proper ductal spacing *in vivo*, thus generating the characteristic open architecture of the gland[70]. Indeed, overexpression of TGFβ1 leads to hypoplastic mammary development *in vivo*[71], whereas TGFβ-deficient mice exhibit elevated ductal proliferation and accelerated lateral branching[72–74]. Consistent with these findings, disrupting TGFβ signalling by ectopic expression of its negative regulator SNON leads to enhanced proliferation and lateral branching during puberty[75]. Although the precise mechanism by which TGFβ inhibits branching is still unclear, non-canonical WNT5A acts downstream of TGFβ *in vivo*[76] and downstream of SMADs in culture[77], and WNT5A-null glands phenocopy those of TGFβ-deficient animals[76]. TGFβ and WNT5A may influence branching by modulating cell adhesion by activation of the collagen-binding protein DDR1 (discoidin domain receptor 1)[78]. TGFβ may also affect branching by inhibiting cell proliferation[74,76,79], enhancing ECM deposition[80,81], and modulating MMP expression[82]. There are likely to be multiple feedback loops involved, however, as MMPs can also affect TGFβ activation, both directly and indirectly. For example, signalling downstream of TGFβ was found to be hyperactivated in mammary glands defective for microRNA-212 and -132, suggesting that the MMP9-mediated reduction in collagen led to enhanced activation or bioavailability of TGFβ[66]. Closer examination of how these signalling events are spatially distributed is required to define how the TGFβ gradient directs patterning of the mammary tree.

Microfabrication-based culture models combined with computational approaches have also shown that endogenous mechanical gradients can potentially regulate mammary branching. Within epithelial cells, endogenous mechanical stress arises owing to contraction of the actin cytoskeleton by myosin motors[83,84]. This endogenous mechanical stress is transmitted between adjacent cells in epithelial tissues through cadherin-mediated adhesions[85]. The collective contraction of epithelial cells and transmission of the resulting stress within tissues of non-spherical geometries leads to a local concentration of stress and the formation of mechanical gradients. Such gradients are present within cultured mammary epithelial tissues and branching morphogenesis initiates only from regions of high mechanical stress[86]. Mammary epithelial tissue senses mechanical stress through integrins and focal adhesion kinase (FAK)[87,88], a widely recognized mechanosensory protein[89,90] that is activated specifically at branch sites and is important for mechanotransduction[86]. Inhibiting FAK retards ductal elongation[91] and leads to aberrant branching in culture and *in vivo*[86,92]. Mechanical stress gradients may thus distinguish future branch sites from quiescent ducts.

Several other aspects of mammary epithelial phenotype are influenced by the mechanical environment. Mammary epithelial cells self-organize into tubules when cultured on floating (compliant) collagen gels but fail to form tubules on attached (stiff) gels[88]. Matrix stiffness similarly governs the functional differentiation

Box 1 | **Mechanisms for pattern formation during morphogenesis**

Every tissue and organ within a multicellular organism has a characteristic architecture and specialized function. Complex tissue forms are generated by patterned cellular behaviours including proliferation, apoptosis, shape change and migration, whereas their unique functions are accomplished by patterned differentiation. Although the downstream events that lead to these changes typically include activation of intracellular signalling pathways, the mechanisms that restrict these events in space and time are physical by nature.

Morphogen gradients are a textbook mechanism for pattern formation. The localized production and subsequent diffusion of a soluble signal[154] forms a gradient that can transform a spatially uniform cell population into distinct domains, each defined by the threshold concentration at which they respond to the signal. A notable example of morphogen gradients in development is the anterior–posterior patterning of the *Drosophila melanogaster* embryo by the morphogens Bicoid and Nanos. Importantly, the spatially uniform production of a biochemical signal within an asymmetric geometry can also give rise to a morphogenetically instructive chemical gradient; for example, this is observed during transforming growth factor-β secretion from elongated mammary ducts[69].

Mechanical changes can also drive pattern formation. Mechanical stress triggers both changes in cell behaviour, such as apoptosis[155] and proliferation[156], and changes in cell state, such as differentiation[157,158] and epithelial–mesenchymal transition[159]. Stresses generated by individual cells are transmitted and concentrated into stress gradients that span many cell lengths[86,104,156] and can pattern cell behaviours[156,160]. The mechanical properties of individual cells can also influence pattern formation. Differences in cell adhesion and possibly contractility[161,162] can induce tissue sorting[163]. Intriguingly, even mechanical differences at the subcellular level can result in morphogenetic movements; for example, differential junctional distribution of myosin creates anisotropic cortical forces that drive intercalation and subsequent elongation of the *D. melanogaster* embryo[164].

of mammary epithelial cells, which can synthesize milk proteins in soft but not stiff environments[93]. Drastic perturbations in the normal mechanical environment of mammary tissue can lead to phenotypes that are characteristic of a malignant state. Culturing mammary tissue within matrices of high, tumour-like stiffness disrupts tissue architecture and promotes invasiveness[87,94]; tumorigenesis of the breast *in vivo* is accompanied and possibly driven by ECM crosslinking and stiffening[95].

How might the mechanical environment regulate mammary morphogenesis? Cell-generated forces can unravel proteins and expose otherwise hidden structural motifs (reviewed in reference REF. 96) (FIG. 3c). Given the role of the ECM and its receptors in mammary development (reviewed in REFS 9,97,98), mechanical stress may influence morphogenesis by uncovering cryptic integrin-binding sites within collagens and thereby altering integrin-mediated mechanosensing. Stress-mediated matrix remodelling may also release ECM fragments that have particular biological activities or ECM-bound growth factors (FIG. 3b). Intracellularly, mammary epithelial cells appear to require a coupling between integrins and the actin-binding protein filamin A in order to sense and respond to mechanical stress in the microenvironment[99]; altering this interaction is sufficient to disrupt branching morphogenesis in culture[99]. Mechanical stress also regulates transcription and may thereby alter the synthesis of branching regulators such as MMPs[100,101]. For example, mechanical stress modulates the balance between monomeric and filamentous actin; this balance controls the nuclear localization of transcription factors of the myocardin family[102,103], which induces localized expression of mesenchymal markers within epithelial tissues in culture[104] (FIG. 3d). Intriguingly, neo-expression of mesenchymal markers has also been observed in regions of high stress within mammary tissues in culture[69,105] and TEBs *in vivo*[106]. Whether mechanical stress is involved in the localized induction of these genes and whether they affect mammary development remains to be seen.

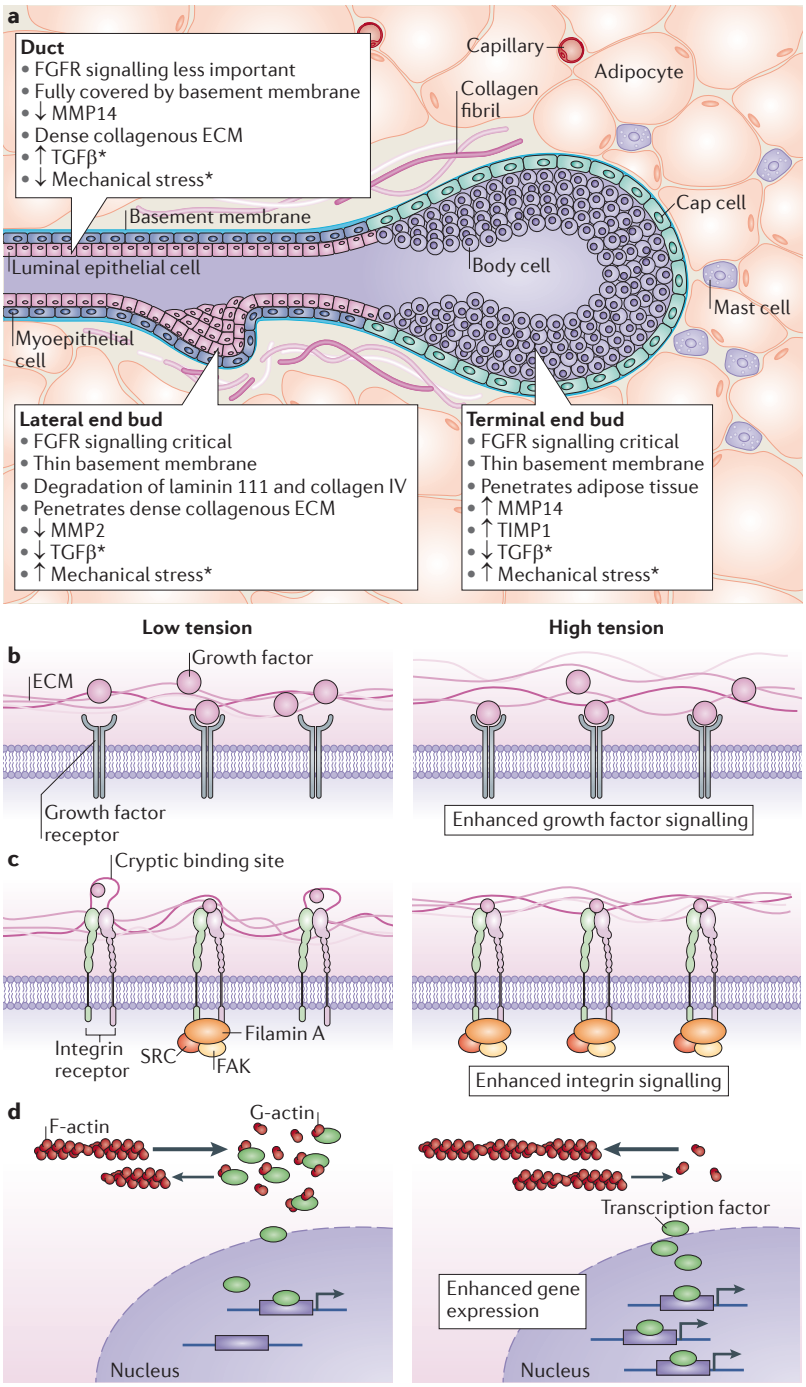
Although culture studies have offered compelling evidence for the role of mechanics in mammary development, homeostasis and disease, the problem is difficult to investigate *in vivo*. Methods are needed to measure spatiotemporal variations in the physical properties and force 'landscape' of the developing mammary gland, as well as to perturb these quantities reproducibly. In the absence of such techniques, mechanical stresses can be inferred indirectly by visualizing mechanically sensitive molecules such as phosphorylated myosin light chain, FAK or SRC[107]. Indeed, immunohistochemical analyses have revealed that tenascin C (secretion of which is enhanced by mechanical tension[108]) is present at high levels around the TEBs[109], which is consistent with computational models predicting increased mechanical stress in these regions[86]. Promising techniques that have been used to directly explore the mechanics involved in the development of other organs could also be adapted for the mammary gland. Morphometric analyses performed by tracking fluorescent markers have quantified mechanical strain and stress during cardiac looping and head-fold formation within the avian embryo[110,111].

Figure 3 | **Potential modes of mechanotransduction in the developing mammary gland. a** | Map of the physical, structural and biochemical features that characterize the terminal end bud (TEB), the subtending duct and the lateral end bud. In the duct, cells are covered by thick basement membrane[3] and are surrounded by dense fibrous extracellular matrix (ECM)[8,81]. Levels of matrix metalloproteinase 14 (MMP14) are low[64,65] and fibroblast growth factor receptor (FGFR) signalling is less important here than elsewhere in the gland[51,52]. Culture studies suggest that mechanical tension is low[86], whereas transforming growth factor-β (TGFβ) signalling is high[69] in the duct. By contrast, FGFR signalling is crucial[51,52] and MMP14 levels are high[64,65] within the TEB. The basement membrane here is thin[3] and the TEB penetrates adipose stroma. Culture studies and computational models suggest that TGFβ concentration is low[69] and mechanical stress[86] is high. The lateral end bud is similar in morphology and organization to the TEB, but penetrates dense collagenous ECM as it extends[8]. Another notable difference is that MMP2 is reduced at sites of lateral branch formation and degradation of laminin 111 and collagen IV is observed[65]. Asterisks indicate studies that were done in cell culture. **b** | Cell-generated mechanical stress can remodel the surrounding matrix, releasing ECM-bound regulatory molecules such as growth factors. Binding of these growth factors to their receptors under conditions of high tension allows increased growth factor signalling. **c** | Cryptic binding sites in the ECM may be unable to access their receptors under conditions of low tension. In response to high tension, remodelling of the ECM may expose these binding sites, allowing them to engage more integrin receptors and trigger enhanced downstream signalling (reviewed in REF. 96). **d** | Mechanical stress can regulate the nuclear localization and activity of transcription factors by modulating the relative levels of globular (G)-actin and filamentous (F)-actin[102,104]. Under high tension, reduced levels of G-actin may allow release of a transcription factor to the nucleus to increase gene expression. TIMP1, tissue inhibitor of metalloproteinases 1.

These techniques could be combined with emerging approaches for intravital imaging of the mammary gland[112–115], including optical coherence tomography, multiphoton microscopy or mammary 'window' imaging[114], which permits long imaging sessions (up to 24 hours) over multiple days. Finally, computational models and engineered tissues are likely to be useful in streamlining *in vivo* investigations of mechanics. The advantages of microfabricated tissue approaches include the ease with which mechanical parameters can be controlled, modulated and interrogated quantitatively[86,104,105]. However, their simplicity, which enables control and tractability, may also be a caveat, as the cellular, chemical and structural complexity of the native mammary environment cannot be fully recapitulated.

Coordinated epithelial motility

Cell migration is crucial for normal development and can be executed by various cells in many contexts; it also has an integral role in mammary morphogenesis. Migration of individual cells typically follows a stereotyped choreography: actin-rich protrusions define



Collective migration
The process in which cells move as a group, without dissolving cell–cell junctions.

Lateral inhibition
The signalling process through which a group of cells reduces the activity of an adjacent group.

Tensile forces
Forces that tend to extend a body.

the cell 'front', and cell–ECM adhesions provide the traction needed for forward propulsion. As the cell generates pulling forces to translocate forward, the rear detaches and retracts from the substratum. A wide range of extracellular signals can induce motility, including growth factors, chemokines and ECM proteins. Spatially localized activation of intracellular signalling components, including phosphoinositide 3-kinase (PI3K) and RHO GTPases (reviewed in REF. 116), confers front–rear cell polarity and controls the individual steps described above. The disassembly of adhesions both at the front and the rear is controlled by signalling pathways, including those occurring through FAK, MAPKs, RHO GTPases and SRC[117,118].

During collective migration, cells remain connected to their neighbours and move as a cohort[119]. Examples of collective migration include epithelial branching morphogenesis, vascular sprouting, border cell migration during *D. melanogaster* oogenesis, epidermal wound closure and the collective invasion of cancer cells. Collective migration may be used widely because migrating cohorts have 'group skills' that are not available to single migrating cells. Specifically, the physical integrity of the collective permits efficient transmission of mechanical and chemical signals. An excellent example of biochemical and mechanical communication during collective migration is the development of the *D. melanogaster* trachea, and the principles that have emerged from this system may help our understanding of mammary morphogenesis. Tracheal branching is triggered by the FGF ligand Branchless (BNL), which signals through the FGFR Breathless (BTL) to induce the formation of actin-rich protrusions, which drive the resulting persistent migration of a subset of cells known as the tip cells. The tip cells are then followed by the lagging or stalk cells. The tip and stalk phenotypes are not pre-specified, but instead depend upon BNL signalling. Specifically, cells with the highest BTL activity become tip cells, whereas those with lower activity form the subtending stalk[21]. Importantly, the leading cells use lateral inhibition through the Notch pathway to suppress the tip phenotype in neighbouring cells[21]. Furthermore, active migration of the tip cell generates tensile forces that drive intercalation of the stalk cells and the subsequent elongation of the branch[120]. Therefore, molecular and mechanical communication within tracheal branches, enabled by the cohesiveness of the group, maintains the hierarchical organization of the participating cells and ultimately drives morphogenesis.

Advances in real-time imaging have identified large-scale coordinated movements of epithelial cells as a key aspect of pubertal mammary development, which is itself a form of collective migration. Time-lapse confocal imaging of primary organoids has shown that the advancing TEBs consist of multi-layered luminal epithelial cells that rearrange dynamically and exhibit reduced apicobasal polarity[121] (FIG. 4). Curiously, a small subpopulation of cells that consistently localize to the leading edge of the TEB is not observed during mammary morphogenesis; this stands in contrast to the tip cells observed during tracheal branching and vascular sprouting. In the developing mammary gland, all cells within the TEBs adopt the tip cell phenotype and are starkly different from the well-organized, polarized and bilayered epithelial cells within the subtending duct. Similar phenotypes have been noted using real-time imaging of the developing kidney and salivary gland (BOX 2).

It is possible that the vigorous cellular rearrangements that occur during mammary development are not random but rather serve to establish the regional differences in gene expression that drive morphogenesis. Indeed, differences in motility are sufficient to induce cell sorting within mammary tissue in culture; in this

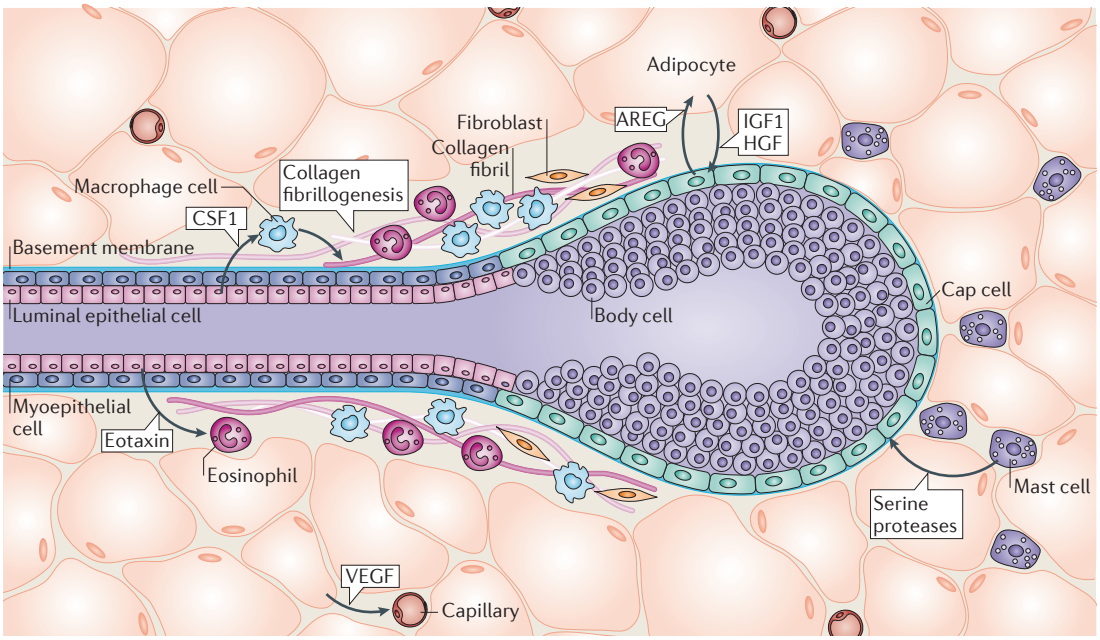


Figure 4 | **Interactions between diverse cell types of the stroma coordinate mammary morphogenesis.** Mature ducts are comprised of luminal epithelial cells surrounded by myoepithelial cells. During puberty, these swell into terminal end buds (TEBs) that consist of multiple layers of body cells (which have reduced polarity and undergo dynamic rearrangements) and a single layer of cap cells at the leading edge. The subtending ducts are surrounded by a sheath of collagen fibrils[81]. Macrophages and eosinophils are recruited to the TEB in part by signals (such as macrophage colony-stimulating factor 1 (CSF1) and eotaxin) released from the epithelium[140,141]. Macrophages enhance the formation of collagen fibrils in the periductal sheath[142]. Mast cells localize to the stroma in front of the invading TEB and secrete serine proteases, which are required for branching and for maintenance of the cap cell layer[145]. Adipocytes form the largest component of the stroma. In response to oestrogen, adipocytes secrete hepatocyte growth factor (HGF), which signals to the epithelium[129], whereas epithelial cells secrete amphiregulin (AREG), which signals to the stroma[39]. Adipocytes also secrete adipokines and vascular endothelial growth factor (VEGF)[135,136]; the latter induces angiogenesis. IGF1, insulin-like growth factor 1.

case, cell populations expressing high levels of MMP14 localize to the tips of branching tubules[122], which is consistent with the increased levels of MMP14 observed in TEBs *in vivo*[65]. Cell motility depends on the level of MMP14; a high level of MMP14 signalling through the CD44 surface receptor and the RHO pathway increases both the speed and directional persistence of migrating cells. These results provide another example of how a conserved set of molecular regulators can perform distinct roles during mammary development depending upon the spatial and temporal context.

Epithelial cells can become motile by undergoing epithelial–mesenchymal transition (EMT), a phenotypic switch wherein epithelia loosen attachments to their neighbours and take on mesenchymal characteristics[123,124]. During morphogenesis in several contexts, EMT is used to increase the collective motility of cell groups while maintaining their connectivity[125]. Might this also be the case with mammary morphogenesis? Although complete dissolution of cell–cell contacts and formation of membrane protrusions are not observed during pubertal mammary development, non-classical epithelial traits are evident, including incomplete polarization, multilayered organization and rapid remodelling of cell–cell junctions to permit cell rearrangements[121,122]. Molecular events that might underlie these cellular phenotypes within the TEBs are starting to emerge. A genome-wide transcript analysis reported that the EMT-related transcription factors SNAI1 (also known as SNAIL1), TWIST1 and TWIST2 are expressed in the TEB microenvironment[106]. Furthermore, expression of the EMT proteome has been observed at the branch-forming regions of mammary tissues in culture[69,105], and many of these genes are required for branch initiation. These genes have also been implicated in the branching of other organs. Notably, ECM-mediated focal upregulation of SNAI2 (also known as SNAIL2) drives cleft formation during branching of the salivary gland *in vivo*[126] and sprouting of kidney epithelial cells in culture[127]. Detailed expression and functional studies are required to fully elucidate the potential role of these factors in collective migration during mammary development.

An ensemble performance

Morphogenesis of the mammary epithelial tree requires integrated interactions among the epithelium and the cells that comprise the stroma[128]. Historically, studies of mammary development have regarded the distinct stromal cell populations as a single compartment, the fat pad. Recent advances in genetic manipulations have allowed the roles of the individual stromal cell types to be dissected and have revealed the distinct functions of each (FIG. 4).

Directional persistence
The tendency of a cell to move in a straight line.

Angiogenesis
The formation of new blood vessels from existing ones.

Macrophages
A type of blood cell that mediates the body's immune response by ingesting foreign material, including pathogens.

Eosinophils
A type of blood cell that mediates the body's immune response by producing chemical agents to combat multicellular pathogens.

Mast cells
A type of cell that is considered to be part of the immune response. Mast cells contain granules rich in histamine and heparin and mediate the body's inflammatory and allergic responses.

Niche
The microenvironment in which stem cells reside, characterized both in terms of location within a tissue and function. The niche is responsible for directing the maintenance, renewal and differentiation of stem cells.

By volume, adipocytes form the largest population of cells within the fat pad. Adipocytes express several of the key ligands and receptors that have been attributed to the stroma, including ERα, IGF1 and HGF[129], and can induce branching in culture[130]. Adipocytes are required for branching, as selective ablation of mammary adipocytes during puberty blocks the formation and branching of TEBs[131] and mice lacking white adipose tissue show mammary branching defects[132]. This effect may be specific to puberty, however, as ablating adipocytes in adult mice leads to enhanced tertiary branching[131]. Nonetheless, pubertal branching is disrupted in obese mice[133], suggesting either systemic or local effects from excess adipose tissue. Adipocytes produce a variety of hormones called adipokines that regulate metabolism, including leptin. Signalling downstream of leptin is disrupted in obese animals, and this interferes with mammary epithelial branching[134]. In addition to signalling directly to the epithelium, adipocytes synthesize and secrete molecules that can regulate the function of other stromal cell types. For example, vascular endothelial growth factor (VEGF) is expressed by mammary adipocytes during puberty[135]. As VEGF is a known inducer of vascular sprouting, adipocytes may thus regulate angiogenesis or vascular barrier function during mammary branching.

Vascularization is important for most solid organs, and the mammary gland is no exception. The mammary fat pad is highly vascularized, which becomes crucial for the transport of fluids and nutrients into milk during lactation. During postnatal development and homeostasis, the vascular tree is remodelled and expanded by angiogenesis, which is regulated by a number of soluble signals, including pro-angiogenic VEGF. The VEGF promoter contains an oestrogen response element[136] that permits transcription of the VEGF gene in cells expressing ERα upon binding of oestrogen[137]. Ovarian oestrogens may thus induce communication between endothelial cells within blood vessels and epithelial cells and adipocytes during puberty.

Cells of the immune system are also required within the stroma of the mammary gland[138,139]. Macrophages and eosinophils are recruited around the TEBs by macrophage colony-stimulating factor 1 (CSF1)[140] and eotaxin[141] secreted locally, and the depletion of either cell type disrupts branching morphogenesis[141]. Macrophages work in part by promoting the formation of long collagen fibres in the ECM around the neck region of the TEB[142], which is thought to promote ductal extension. Mast cells, which are effectors of the innate immune system, also surround the TEBs during puberty[143,144], and mice that lack mast cells have defects in mammary branching[145]. Mast cells induce branching by secreting serine proteases, and mice deficient in an activator of serine proteases also develop hypoplastic glands[145]. Conversely, increasing mast cell accumulation in the mammary gland by feeding mice conjugated linoleic acid increases deposition of collagen in the stroma around the mammary epithelium[146]. Immune cells thus affect branching, in part, by altering ECM synthesis and/or structure around the growing ducts.

The mammary stroma also contains resident fibroblasts, which have received much attention for their role in mammary tumour progression[147]. Mammary fibroblasts secrete HGF and other growth factors and can induce mammary epithelial branching in culture[148,149]. Our understanding of how fibroblasts affect mammary branching morphogenesis is complicated by the fact that these are a heterogeneous cell type: for example, human mammary fibroblasts show substantial differences in protein expression depending on whether the cells are located within fatty or collagenous stroma[150]. Advances in real-time imaging and labelling of specific fibroblast subtypes will probably help to illuminate their distinct roles in the developing epithelium.

Studies of the mammary gland and other branched organs have revealed that one major function of the stromal compartment is to maintain epithelial stem cells (BOX 3). In the salivary gland, the peripheral nervous system within the stroma maintains epithelial progenitor cells in an undifferentiated state[151]. Cholinergic stimulation by parasympathetic innervation also triggers epithelial cells to release heparin-binding EGF, which stimulates branching morphogenesis on binding to EGFR[151]. In the mouse mammary gland, macrophages also appear to be required for stem cell function. The mammary stem cells (MaSCs) of animals depleted of macrophages are unable to repopulate the gland[152], suggesting that macrophages may be an important constituent of the MaSC niche. There is precedence for such involvement, as macrophages have been found to localize to the niche of colon stem cells[153].

Box 2 | **Techniques for visualizing mammalian organogenesis**

Morphogenesis is by nature a dynamic process, but it is difficult to infer morphodynamic events using static images. Recent advances in real-time imaging and cell-labelling techniques have enabled investigation of the dynamic processes that drive morphogenesis. These techniques have been used to study the dynamic interactions between cells and the extracellular matrix, interactions between different cell types, the behaviour of a single cell type within different microenvironments, and the local roles of molecular regulators during the development of mouse mammary and salivary glands and kidney.

Cell–matrix and cell–cell interactions

Cell–matrix interactions and epithelial dynamics during salivary morphogenesis have been studied by visualizing both the epithelial cells and the surrounding extracellular matrix[165]; a subpopulation of epithelial cells was labelled with green fluorescent protein (GFP)-expressing adenovirus, and fluorophore-conjugated fibronectin was used to visualize the matrix. The interactions between luminal and myoepithelial cells in primary mammary organoids has been analysed by taking advantage of differentially expressed markers[121]. Specifically, myoepithelial cells were labelled by keratin 14 promoter-driven expression of actin–GFP, whereas all cells were stained with a commercially available fluorescent dye.

Location-dependent behaviour and local regulators

The contributions made by 'tip' and 'trunk' epithelial cells (as defined by location rather than gene expression) to kidney branching has been studied by monitoring how clusters of GFP-expressing cells and their daughter cells behave in both microenvironments[24]. Mouse embryos were generated that had mosaic expression of RET, a regulator of kidney branching; this was achieved by injecting RET-null GFP-expressing embryonic stem cells into wild-type embryos at the blastocyst stage. Monitoring of the GFP-labelled RET-null cells revealed that they are excluded from the tips of the branches, suggesting a local role for RET signalling. Up- and downmodulation of RET activity showed that cells that are initially randomly dispersed compete for the tip positions of the ureteric bud based on their levels of RET signalling[166].

Box 3 | **Cycles of birth and destruction: the mammary stem cell**

Over 60 years ago, it was demonstrated elegantly that the mammary tree can be completely replenished by transplanting fragments of the mammary epithelium from any stage of postnatal development[167]. We now understand that a single mammary stem cell (MaSC) can repopulate the entire gland[168,169]. MaSCs are important for both pubertal branching and acinar morphogenesis, but different populations of stem cells may complete each function[170–173]. Markers have been identified for MaSCs in mice and women[174]. Bipotent adult mouse MaSCs are enriched in α6-integrin, β1-integrin and CD24, and express low levels of SCA1 (also known as Ly6A.2/Ly6E.1) and lineage surface antigens[168,169,175]. By contrast, luminal progenitor cells express either β3-integrin or low levels of prominin 1 and SCA1 (REFS 176,177). Many of these markers are conserved in human MaSCs[178]. β1-integrin is not only a marker but also regulates MaSC proliferation, self-renewal and the axis of cell division[179]; when deleted of β1-integrin, mammary epithelial cells fail to repopulate the gland but can undergo alveolar differentiation during pregnancy. Conversely, prominin 1 is required for mammary branching but dispensable for repopulation[180]. Self-renewal is also regulated by the Hedgehog[181], Notch[182], WNT and phosphoinositide 3-kinase (PI3K)[183] signalling pathways.

As for all adult stem cells, the MaSC population is maintained by signals from its specialized local microenvironment, or niche. In mice, MaSCs located in the cap region of the terminal end bud are responsible for the growth that drives ductal extension during branching[168,169]; these cells express s-SHIP (SH2-containing inositol 5′-phosphate), a marker of activated stem cells[184]. In humans, multipotent MaSCs reside in nests within the terminal ducts rather than the lobules[185]. Protein microarrays have been used to define niche constituents, which included laminin 111, the Notch ligand jagged 1 and P-cadherin, all of which are present near MaSCs *in vivo*[186]. The niche is both necessary and sufficient for stem cell activity. Indeed, cells other than native MaSCs, including neural stem cells[187] and cells from the seminiferous tubules[188] of male mice, can function as MaSCs when placed within the niche. Further studies are required to define how the integrated signalling within the mammary gland induces maintenance and differentiation of MaSCs during pubertal branching.

Acinar
A berry-shaped cluster of cells.

Bipotent
The ability to give rise to two types of differentiated cells.

Concluding remarks and future directions

The pattern of the mammary epithelial tree is the product of a morphodynamic process integrating molecular and mechanical signals over multiple time and length scales. Information about the reproductive status of the whole organism is conveyed to the organ in the form of endocrine hormones, which signal to the epithelium and multiple cell types in the stroma to produce relatively local paracrine signals. This initiates a dialogue between the epithelium and stroma, with growth factors produced by one compartment binding to cognate receptors in the other compartment. Spatially, these signals are fine-tuned by integrating with medium-range (tissue-scale) information provided by mechanical stress and autocrine morphogen gradients. The multiple paracrine cues thus yield different functional responses depending on where the epithelial cells are located within the mammary gland. The final outcome of this integrated signalling is branching and extension of the TEBs and relative quiescence of the cells in the subtending ducts.

Coincident with this molecular and mechanical signalling, epithelial cells within the duct are moving dynamically. The motions of individual cells are much faster and cover smaller distances than the morphogenesis of the gland itself. Conceptually, it is difficult to integrate these dynamics with the mechanical and chemical signalling that occurs simultaneously across the whole tissue. However, this integration represents one of the major future challenges for our understanding of the development of not only the mammary gland but also other branched organs, such as the lung, kidney and salivary gland, that likewise exhibit complex cellular dynamics. How do these signals affect the coordinated interactions among the epithelial cells? Do cellular rearrangements affect crosstalk with stromal cells? Sophisticated multi-component and interdisciplinary approaches are likely to help uncover the answers to these questions. To understand the molecular regulation of the process fully, the complete spatiotemporal expression and activation profile of every important protein must be defined. Comparing molecular profiles to the changes that are occurring at the level of the cell and the tissue is likely to shed light on the molecular interactions and the cellular and physical mechanisms that drive mammary morphogenesis.

1. Peaker, M. The mammary gland in mammalian evolution: a brief commentary on some of the concepts. *J. Mammary Gland Biol. Neoplasia* **7**, 347–353 (2002).
2. Forsyth, I. A. & Neville, M. C. Introduction: the myoepithelial cell and milk letdown; entrance to the multifunctional role of oxytocin. *J. Mammary Gland Biol. Neoplasia* **14**, 221–222 (2009).
3. Williams, J. M. & Daniel, C. W. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev. Biol.* **97**, 274–290 (1983).
4. Polyak, K. & Kalluri, R. The role of the microenvironment in mammary gland development and cancer. *Cold Spring Harb. Perspect. Biol.* **2**, a003244 (2010).
5. Robinson, G. W. Cooperation of signalling pathways in embryonic mammary gland development. *Nature Rev. Genet.* **8**, 963–972 (2007).
6. Watson, C. J. & Khaled, W. T. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* **135**, 995–1003 (2008).
7. Hens, J. R. & Wysolmerski, J. J. Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland. *Breast Cancer Res.* **7**, 220–224 (2005).
8. Hinck, L. & Silberstein, G. B. Key stages in mammary gland development: the mammary end bud as a motile organ. *Breast Cancer Res.* **7**, 245–251 (2005).
9. Sternlicht, M. D., Kouros-Mehr, H., Lu, P. & Werb, Z. Hormonal and local control of mammary branching morphogenesis. *Differentiation* **74**, 365–381 (2006).
10. Brisken, C. *et al.* Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev. Biol.* **210**, 96–106 (1999).
11. Oakes, S. R., Rogers, R. L., Naylor, M. J. & Ormandy, C. J. Prolactin regulation of mammary gland development. *J. Mammary Gland Biol. Neoplasia* **13**, 13–28 (2008).
12. Walker, N. I., Bennett, R. E. & Kerr, J. F. Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am. J. Anat.* **185**, 19–32 (1989).
13. Lund, L. R. *et al.* Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181–193 (1996).
14. Alexander, C. M., Selvarajan, S., Mudgett, J. & Werb, Z. Stromelysin-1 regulates adipogenesis during mammary gland involution. *J. Cell Biol.* **152**, 693–703 (2001).
15. Watson, C. J. Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. *Breast Cancer Res.* **8**, 203 (2006).
16. McNally, S. & Martin, F. Molecular regulators of pubertal mammary gland development. *Ann. Med.* **43**, 212–234 (2011).
17. Cowin, P. & Wysolmerski, J. Molecular mechanisms guiding embryonic mammary gland development. *Cold Spring Harb. Perspect. Biol.* **2**, a003251 (2010).
18. Lu, P. & Werb, Z. Patterning mechanisms of branched organs. *Science* **322**, 1506–1509 (2008).
19. Davies, J. A. Do different branching epithelia use a conserved developmental mechanism? *Bioessays* **24**, 937–948 (2002).
20. Affolter, M., Zeller, R. & Caussinus, E. Tissue remodelling through branching morphogenesis. *Nature Rev. Mol. Cell Biol.* **10**, 831–842 (2009).
21. Ghabrial, A. S. & Krasnow, M. A. Social interactions among epithelial cells during tracheal branching morphogenesis. *Nature* **441**, 746–749 (2006).
22. Sutherland, D., Samakovlis, C. & Krasnow, M. A. *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091–1101 (1996).
23. Weaver, M., Dunn, N. R. & Hogan, B. L. Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development* **127**, 2695–2704 (2000).
24. Shakya, R., Watanabe, T. & Costantini, F. The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev. Cell* **8**, 65–74 (2005).

25. Affolter, M. *et al.* Tube or not tube: remodeling epithelial tissues by branching morphogenesis. *Dev. Cell* **4**, 11–18 (2003).
26. Silberstein, G. B. & Daniel, C. W. Investigation of mouse mammary ductal growth regulation using slow-release plastic implants. *J. Dairy Sci.* **70**, 1981–1990 (1987).
27. Metzger, R. J., Klein, O. D., Martin, G. R. & Krasnow, M. A. The branching programme of mouse lung development. *Nature* **453**, 745–750 (2008).
28. Schedin, P. & Keely, P. J. Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. *Cold Spring Harb. Perspect. Biol.* **3**, a003228 (2011).
29. Khokha, R. & Werb, Z. Mammary gland reprogramming: metalloproteinases couple form with function. *Cold Spring Harb. Perspect. Biol.* **3**, a004333 (2011).
30. Brisken, C. & O'Malley, B. Hormone action in the mammary gland. *Cold Spring Harb. Perspect. Biol.* **2**, a003178 (2010).
31. Bocchinfuso, W. P. & Korach, K. S. Mammary gland development and tumorigenesis in estrogen receptor knockout mice. *J. Mammary Gland Biol. Neoplasia* **2**, 323–334 (1997).
32. Feng, Y., Manka, D., Wagner, K. U. & Khan, S. A. Estrogen receptor-α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proc. Natl Acad. Sci. USA* **104**, 14718–14723 (2007).
33. Daniel, C. W., Silberstein, G. B. & Strickland, P. Direct action of 17β-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. *Cancer Res.* **47**, 6052–6057 (1987).
34. Cunha, G. R. *et al.* Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *J. Mammary Gland Biol. Neoplasia* **2**, 393–402 (1997).
35. Zhang, H. Z., Bennett, J. M., Smith, K. T., Sunil, N. & Haslam, S. Z. Estrogen mediates mammary epithelial cell proliferation in serum-free culture indirectly via mammary stroma-derived hepatocyte growth factor. *Endocrinology* **143**, 3427–3434 (2002).
36. Coleman, S., Silberstein, G. B. & Daniel, C. W. Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. *Dev. Biol.* **127**, 304–315 (1988).
37. Luetteke, N. C. *et al.* Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* **126**, 2739–2750 (1999).
38. Sebastian, J. *et al.* Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Differ.* **9**, 777–785 (1998).
39. Sternlicht, M. D. *et al.* Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. *Development* **132**, 3923–3933 (2005).
Demonstrated that ADAM17 has a crucial role during mammary branching morphogenesis through cleavage of AREG from the epithelial cell surface to activate EGFR signalling within stromal cells.
40. Ciarloni, L., Mallepell, S. & Brisken, C. Amphiregulin is an essential mediator of estrogen receptor α function in mammary gland development. *Proc. Natl Acad. Sci. USA* **104**, 5455–5460 (2007).
41. Wiesen, J. F., Young, P., Werb, Z. & Cunha, G. R. Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* **126**, 335–344 (1999).
Used knockout mice and mammary gland transplantation experiments to demonstrate that EGFR signalling is required in the stromal compartment.
42. Kleinberg, D. L., Feldman, M. & Ruan, W. IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. *J. Mammary Gland Biol. Neoplasia* **5**, 7–17 (2000).
43. Gallego, M. I. *et al.* Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. *Dev. Biol.* **229**, 163–175 (2001).
44. Meyer, S. E., Zinser, G. M., Stuart, W. D., Pathrose, P. & Waltz, S. E. The Ron receptor tyrosine kinase negatively regulates mammary gland branching morphogenesis. *Dev. Biol.* **333**, 173–185 (2009).
45. Vaught, D., Chen, J. & Brantley-Sieders, D. M. Regulation of mammary gland branching morphogenesis by EphA2 receptor tyrosine kinase. *Mol. Biol. Cell* **20**, 2572–2581 (2009).
46. Andrechek, E. R., White, D. & Muller, W. J. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. *Oncogene* **24**, 932–937 (2005).
47. Jackson-Fisher, A. J. *et al.* ErbB2 is required for ductal morphogenesis of the mammary gland. *Proc. Natl Acad. Sci. USA* **101**, 17138–17143 (2004).
48. Tidcombe, H. *et al.* Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality. *Proc. Natl Acad. Sci. USA* **100**, 8281–8286 (2003).
49. Fata, J. E. *et al.* The $MAPK^{ERK\text{-}1,2}$ pathway integrates distinct and antagonistic signals from TGFα and FGF7 in morphogenesis of mouse mammary epithelium. *Dev. Biol.* **306**, 193–207 (2007).
Showed that although both TGFα and FGF7 signal through MAPKs, they elicit antagonistic phenotypic outcomes in primary mammary organoids owing to differences in the duration of MAPK activation.
50. Xu, X. *et al.* Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* **125**, 753–765 (1998).
51. Lu, P., Ewald, A. J., Martin, G. R. & Werb, Z. Genetic mosaic analysis reveals FGF receptor 2 function in terminal end buds during mammary gland branching morphogenesis. *Dev. Biol.* **321**, 77–87 (2008).
Demonstrated a local role for FGFR signalling in regulating cell proliferation and survival in the TEBs.
52. Parsa, S. *et al.* Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev. Biol.* **317**, 121–131 (2008).
Reported that FGFR signalling was required for TEB maintenance. Loss of FGFR2 impairs proliferation of luminal epithelial cells and results in poorly developed glands that lack TEBs.
53. Liu, X. *et al.* Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.* **11**, 179–186 (1997).
54. Santos, S. J., Haslam, S. Z. & Conrad, S. E. Signal transducer and activator of transcription 5a mediates mammary ductal branching and proliferation in the nulliparous mouse. *Endocrinology* **151**, 2876–2885 (2010).
55. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. & Varmus, H. E. Expression of the *int-1* gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619–625 (1988).
56. Imbert, A., Eelkema, R., Jordan, S., Feiner, H. & Cowin, P. Δn89β-catenin induces precocious development, differentiation, and neoplasia in mammary gland. *J. Cell Biol.* **153**, 555–568 (2001).
57. Gallagher, R. C. *et al.* Inactivation of Apc perturbs mammary development, but only directly results in acanthoma in the context of Tcf-1 deficiency. *Oncogene* **21**, 6446–6457 (2002).
58. Hatsell, S. J. & Cowin, P. Gli3-mediated repression of Hedgehog targets is required for normal mammary development. *Development* **133**, 3661–3670 (2006).
59. Corbit, K. C. *et al.* Vertebrate Smoothened functions at the primary cilium. *Nature* **437**, 1018–1021 (2005).
60. Rohatgi, R., Milenkovic, L. & Scott, M. P. Patched1 regulates hedgehog signaling at the primary cilium. *Science* **317**, 372–376 (2007).
61. Milenkovic, L., Scott, M. P. & Rohatgi, R. Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium. *J. Cell Biol.* **187**, 365–374 (2009).
62. McDermott, K. M., Liu, B. Y., Tlsty, T. D. & Pazour, G. J. Primary cilia regulate branching morphogenesis during mammary gland development. *Curr. Biol.* **20**, 731–737 (2010).
Demonstrated that primary cilia are present on luminal, myoepithelial and stromal cells during mammary branching morphogenesis. The authors also found that ciliary dysfunction impairs ductal elongation, secondary and tertiary branching, and they proposed a role for WNT and Hedgehog signalling in these effects.
63. Neugebauer, J. M., Amack, J. D., Peterson, A. G., Bisgrove, B. W. & Yost, H. J. FGF signalling during embryo development regulates cilia length in diverse epithelia. *Nature* **458**, 651–654 (2009).
64. Szabova, L., Yamada, S. S., Birkedal-Hansen, H. & Holmbeck, K. Expression pattern of four membrane-type matrix metalloproteinases in the normal and diseased mouse mammary gland. *J. Cell Physiol.* **205**, 123–132 (2005).
65. Wiseman, B. S. *et al.* Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *J. Cell Biol.* **162**, 1123–1133 (2003).
Showed that MMP2, MMP3 and MMP14 are non-uniformly expressed throughout the developing mammary gland and that they have distinct roles during branching morphogenesis.
66. Ucar, A. *et al.* miR-212 and miR-132 are required for epithelial stromal interactions necessary for mouse mammary gland development. *Nature Genet.* **42**, 1101–1108 (2010).
Discovered that the microRNA-212/132 family is indispensable during mammary development and functions in the stroma.
67. Andersen, K. *et al.* The metastasis-promoting protein S100A4 regulates mammary branching morphogenesis. *Dev. Biol.* **352**, 181–190 (2011).
68. Cheng, N. *et al.* Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks. *Oncogene* **24**, 5053–5068 (2005).
69. Nelson, C. M., Vanduijn, M. M., Inman, J. L., Fletcher, D. A. & Bissell, M. J. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. *Science* **314**, 298–300 (2006).
Showed that tissue geometry specifies the local concentration of TGFβ and thereby determines sites of branching morphogenesis in engineered mammary tissues.
70. Lee, W. C. & Davies, J. A. Epithelial branching: the power of self-loathing. *Bioessays* **29**, 205–207 (2007).
71. Pierce, D. F. Jr *et al.* Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β 1. *Genes Dev.* **7**, 2308–2317 (1993).
72. Crowley, M. R., Bowtell, D. & Serra, R. TGF-β, c-Cbl, and PDGFR-α the in mammary stroma. *Dev. Biol.* **279**, 58–72 (2005).
73. Joseph, H., Gorska, A. E., Sohn, P., Moses, H. L. & Serra, R. Overexpression of a kinase-deficient transforming growth factor-β type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol. Biol. Cell* **10**, 1221–1234 (1999).
74. Ewan, K. B. *et al.* Latent transforming growth factor-β activation in mammary gland: regulation by ovarian hormones affects ductal and alveolar proliferation. *Am. J. Pathol.* **160**, 2081–2093 (2002).
75. Jahchan, N. S., You, Y. H., Muller, W. J. & Luo, K. Transforming growth factor-β regulator SnoN modulates mammary gland branching morphogenesis, postlactational involution, and mammary tumorigenesis. *Cancer Res.* **70**, 4204–4213 (2010).
76. Roarty, K. & Serra, R. Wnt5a is required for proper mammary gland development and TGF-β-mediated inhibition of ductal growth. *Development* **134**, 3929–3939 (2007).
Showed that WNT5A is required for TGFβ-mediated inhibition of mammary branching morphogenesis and that loss of TGFβ signalling reduces phosphorylation of the collagen receptor DDR1.
77. Pavlovich, A. L., Boghaert, E. & Nelson, C. M. Mammary branch initiation and extension are inhibited by separate pathways downstream of TGFβ in culture. *Exp. Cell Res.* **317**, 1872–1884 (2011).
78. Vogel, W. F., Aszodi, A., Alves, F. & Pawson, T. Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. *Mol. Cell. Biol.* **21**, 2906–2917 (2001).
79. Macias, H. *et al.* SLIT/ROBO1 signaling suppresses mammary branching morphogenesis by limiting basal cell number. *Dev. Cell* **20**, 827–840 (2011).
80. Daniel, C. W., Robinson, S. & Silberstein, G. B. The role of TGF-β in patterning and growth of the mammary ductal tree. *J. Mammary Gland Biol. Neoplasia* **1**, 331–341 (1996).
81. Silberstein, G. B. & Daniel, C. W. Glycosaminoglycans in the basal lamina and extracellular matrix of the developing mouse mammary duct. *Dev. Biol.* **90**, 215–222 (1982).
82. Sternlicht, M. D. & Werb, Z. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* **17**, 463–516 (2001).

83. Chrzanowska-Wodnicka, M. & Burridge, K. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* **133**, 1403–1415 (1996).
84. Landsverk, M. L. & Epstein, H. F. Genetic analysis of myosin II assembly and organization in model organisms. *Cell. Mol. Life Sci.* **62**, 2270–2282 (2005).
85. McNeill, H., Ryan, T. A., Smith, S. J. & Nelson, W. J. Spatial and temporal dissection of immediate and early events following cadherin-mediated epithelial cell adhesion. *J. Cell Biol.* **120**, 1217–1226 (1993).
86. Gjorevski, N. & Nelson, C. M. Endogenous patterns of mechanical stress are required for branching morphogenesis. *Integr. Biol. (Camb.)* **2**, 424–434 (2010).
Demonstrated that mechanical stress is non-uniformly distributed within engineered mammary epithelial tissues and that branching morphogenesis occurs at regions of the tissue where stress is high.
87. Paszek, M. J. *et al.* Tensional homeostasis and the malignant phenotype. *Cancer Cell* **8**, 241–254 (2005).
For the first time, this study related the mechanical environment of mammary tissue to the malignant phenotype. The authors found that elevated tumour-like matrix stiffness impairs mammary tissue architecture and enhances growth through enhanced integrin clustering, extracellular signal-regulated kinase activation and RHO-associated protein kinase-mediated contractility.
88. Wozniak, M. A., Desai, R., Solski, P. A., Der, C. J. & Keely, P. J. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J. Cell Biol.* **163**, 583–595 (2003).
89. Tang, D., Mehta, D. & Gunst, S. J. Mechanosensitive tyrosine phosphorylation of paxillin and focal adhesion kinase in tracheal smooth muscle. *Am. J. Physiol.* **276**, C250–C258 (1999).
90. Yano, Y., Geibel, J. & Sumpio, B. E. Tyrosine phosphorylation of pp125FAK and paxillin in aortic endothelial cells induced by mechanical strain. *Am. J. Physiol.* **271**, C635–C649 (1996).
91. Nagy, T. *et al.* Mammary epithelial-specific deletion of the focal adhesion kinase gene leads to severe lobulo-alveolar hypoplasia and secretory immaturity of the murine mammary gland. *J. Biol. Chem.* **282**, 31766–31776 (2007).
92. van Miltenburg, M. H. *et al.* Complete focal adhesion kinase deficiency in the mammary gland causes ductal dilation and aberrant branching morphogenesis through defects in Rho kinase-dependent cell contractility. *FASEB J.* **23**, 3482–3493 (2009).
93. Alcaraz, J. *et al.* Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. *EMBO J.* **27**, 2829–2838 (2008).
94. Provenzano, P. P., Inman, D. R., Eliceiri, K. W. & Keely, P. J. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene* **28**, 4326–4343 (2009).
95. Levental, K. R. *et al.* Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **139**, 891–906 (2009).
96. Vogel, V. & Sheetz, M. Local force and geometry sensing regulate cell functions. *Nature Rev. Mol. Cell Biol.* **7**, 265–275 (2006).
97. Muschler, J. & Streuli, C. H. Cell-matrix interactions in mammary gland development and breast cancer. *Cold Spring Harb. Perspect. Biol.* **2**, a003202 (2011).
98. Fata, J. E., Werb, Z. & Bissell, M. J. Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res.* **6**, 1–11 (2004).
99. Gehler, S. *et al.* Filamin A-β1 integrin complex tunes epithelial cell response to matrix tension. *Mol. Biol. Cell* **20**, 3224–3238 (2009).
100. Prajapati, R. T., Chavally-Mis, B., Herbage, D., Eastwood, M. & Brown, R. A. Mechanical loading regulates protease production by fibroblasts in three-dimensional collagen substrates. *Wound Repair Regen.* **8**, 226–237 (2000).
101. Ruddy, J. M. *et al.* Differential effects of mechanical and biological stimuli on matrix metalloproteinase promoter activation in the thoracic aorta. *Circulation* **120**, S262–S268 (2009).
102. Connelly, J. T. *et al.* Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. *Nature Cell Biol.* **12**, 711–718 (2010).
Reported that the actin cytoskeleton and transcription factors from the myocardin family transduce physical cues to regulate transcription and ultimately stem cell differentiation.
103. Miralles, F., Posern, G., Zaromytidou, A. I. & Treisman, R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113**, 329–342 (2003).
104. Gomez, E. W., Chen, Q. K., Gjorevski, N. & Nelson, C. M. Tissue geometry patterns epithelial–mesenchymal transition via intercellular mechanotransduction. *J. Cell. Biochem.* **110**, 44–51 (2010).
Showed that endogenous patterns of mechanical stress can induce spatially localized EMT within engineered mammary tissues by controlling the balance of monomeric and filamentous actin and consequently the nuclear translocation of myocardin-related transcription factor A (MRTFA).
105. Lee, K., Gjorevski, N., Boghaert, E., Radisky, D. C. & Nelson, C. M. Snail1, Snail2, and E47 promote mammary epithelial branching morphogenesis. *EMBO J.* **30**, 2662–2674 (2011).
106. Kouros-Mehr, H. & Werb, Z. Candidate regulators of mammary branching morphogenesis identified by genome-wide transcript analysis. *Dev. Dyn.* **235**, 3404–3412 (2006).
107. Chen, C. S. Mechanotransduction — a field pulling together? *J. Cell Sci.* **121**, 3285–3292 (2008).
108. Chiquet-Ehrismann, R. *et al.* Tenascin-C expression by fibroblasts is elevated in stressed collagen gels. *J. Cell Biol.* **127**, 2093–2101 (1994).
109. Inaguma, Y. *et al.* Epithelial induction of stromal tenascin in the mouse mammary gland: from embryogenesis to carcinogenesis. *Dev. Biol.* **128**, 245–255 (1988).
110. Filas, B. A., Efimov, I. R. & Taber, L. A. Optical coherence tomography as a tool for measuring morphogenetic deformation of the looping heart. *Anat. Rec. (Hoboken)* **290**, 1057–1068 (2007).
111. Varner, V. D., Voronov, D. A. & Taber, L. A. Mechanics of head fold formation: investigating tissue-level forces during early development. *Development* **137**, 3801–3811 (2010).
112. Andresen, V. *et al.* Infrared multiphoton microscopy: subcellular-resolved deep tissue imaging. *Curr. Opin. Biotechnol.* **20**, 54–62 (2009).
113. Ewald, A. J., Werb, Z. & Egeblad, M. Dynamic, long-term *in vivo* imaging of tumor-stroma interactions in mouse models of breast cancer using spinning-disk confocal microscopy. *Cold Spring Harb. Protoc* **2011**, pdb.top97 (2011).
114. Kedrin, D. *et al.* Intravital imaging of metastatic behavior through a mammary imaging window. *Nature Methods* **5**, 1019–1021 (2008).
115. Vakoc, B. J. *et al.* Three-dimensional microscopy of the tumor microenvironment *in vivo* using optical frequency domain imaging. *Nature Med.* **15**, 1219–1223 (2009).
116. Ridley, A. J. *et al.* Cell migration: integrating signals from front to back. *Science* **302**, 1704–1709 (2003).
117. Brahmbhatt, A. A. & Klemke, R. L. ERK and RhoA differentially regulate pseudopodia growth and retraction during chemotaxis. *J. Biol. Chem.* **278**, 13016–13025 (2003).
118. Webb, D. J., Parsons, J. T. & Horwitz, A. F. Adhesion assembly, disassembly and turnover in migrating cells — over and over and over again. *Nature Cell Biol.* **4**, e97–e100 (2002).
119. Friedl, P. & Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nature Rev. Mol. Cell Biol.* **10**, 445–457 (2009).
120. Caussinus, E., Colombelli, J. & Affolter, M. Tip-cell migration controls stalk-cell intercalation during *Drosophila* tracheal tube elongation. *Curr. Biol.* **18**, 1727–1734 (2008).
Showed that tensile forces generated by tip cells in *D. melanogaster* tracheal branches drive the intercalation of the remaining cells within the branch.
121. Ewald, A. J., Brenot, A., Duong, M., Chan, B. S. & Werb, Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Dev. Cell* **14**, 570–581 (2008).
Used long-term time-lapse imaging to visualize the dynamics of epithelial cells during branching morphogenesis of mammary organoids and observed that branching occurs through a multilayered, partially polarized epithelium, which featured large-scale coordinated cell movements.
122. Mori, H., Gjorevski, N., Inman, J. L., Bissell, M. J. & Nelson, C. M. Self-organization of engineered epithelial tubules by differential cellular motility. *Proc. Natl Acad. Sci. USA* **106**, 14890–14895 (2009).
Demonstrated that differential motility is sufficient to drive cell sorting within engineered mammary tissues. Cells expressing high levels of MMP14 exhibit high directional persistence, which promotes their localization to the leading edge of the tissue.
123. Thiery, J. P., Acloque, H., Huang, R. Y. & Nieto, M. A. Epithelial–mesenchymal transitions in development and disease. *Cell* **139**, 871–890 (2009).
124. Kalluri, R. & Weinberg, R. A. The basics of epithelial–mesenchymal transition. *J. Clin. Invest.* **119**, 1420–1428 (2009).
125. Revenu, C. & Gilmour, D. EMT 2.0: shaping epithelia through collective migration. *Curr. Opin. Genet. Dev.* **19**, 338–342 (2009).
126. Onodera, T. *et al.* Btbd7 regulates epithelial cell dynamics and branching morphogenesis. *Science* **329**, 562–565 (2010).
127. Leroy, P. & Mostov, K. E. Slug is required for cell survival during partial epithelial–mesenchymal transition of HGF-induced tubulogenesis. *Mol. Biol. Cell* **18**, 1943–1952 (2007).
128. Arendt, L. M., Rudnick, J. A., Keller, P. J. & Kuperwasser, C. Stroma in breast development and disease. *Semin. Cell Dev. Biol.* **21**, 11–18 (2010).
129. Hovey, R. C. & Aimo, L. Diverse and active roles for adipocytes during mammary gland growth and function. *J. Mammary Gland Biol. Neoplasia* **15**, 279–290 (2010).
130. Pavlovich, A. L., Manivannan, S. & Nelson, C. M. Adipose stroma induces branching morphogenesis of engineered epithelial tubules. *Tissue Eng. Part A* **16**, 3719–3726 (2010).
131. Landskroner-Eiger, S., Park, J., Israel, D., Pollard, J. W. & Scherer, P. E. Morphogenesis of the developing mammary gland: stage-dependent impact of adipocytes. *Dev. Biol.* **344**, 968–978 (2010).
132. Couldrey, C. *et al.* Adipose tissue: a vital *in vivo* role in mammary gland development but not differentiation. *Dev. Dyn.* **223**, 459–468 (2002).
Showed that an absence of white adipose tissue prevents pubertal mammary morphogenesis but not alveolar differentiation during pregnancy.
133. Kamikawa, A. *et al.* Diet-induced obesity disrupts ductal development in the mammary glands of nonpregnant mice. *Dev. Dyn.* **238**, 1092–1099 (2009).
134. Thorn, S. R., Giesy, S. L., Myers, M. G. Jr & Boisclair, Y. R. Mammary ductal growth is impaired in mice lacking leptin-dependent signal transducer and activator of transcription 3 signaling. *Endocrinology* **151**, 3985–3995 (2010).
135. Hovey, R. C., Goldhar, A. S., Baffi, J. & Vonderhaar, B. K. Transcriptional regulation of vascular endothelial growth factor expression in epithelial and stromal cells during mouse mammary gland development. *Mol. Endocrinol.* **15**, 819–831 (2001).
136. Liang, Y., Brekken, R. A. & Hyder, S. M. Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones. *Endocr. Relat. Cancer* **13**, 905–919 (2006).
137. Dabrosin, C., Margetts, P. J. & Gauldie, J. Estradiol increases extracellular levels of vascular endothelial growth factor *in vivo* in murine mammary cancer. *Int. J. Cancer* **107**, 535–540 (2003).
138. Reed, J. R. & Schwertfeger, K. L. Immune cell location and function during post-natal mammary gland development. *J. Mammary Gland Biol. Neoplasia* **15**, 329–339 (2010).
139. Coussens, L. M. & Pollard, J. W. Leukocytes in mammary development and cancer. *Cold Spring Harb. Perspect. Biol.* **3**, a003285 (2011).
140. Van Nguyen, A. & Pollard, J. W. Colony stimulating factor-1 is required to recruit macrophages into the mammary gland to facilitate mammary ductal outgrowth. *Dev. Biol.* **247**, 11–25 (2002).
141. Gouon-Evans, V., Rothenberg, M. E. & Pollard, J. W. Postnatal mammary gland development requires macrophages and eosinophils. *Development* **127**, 2269–2282 (2000).
Demonstrated an essential role for macrophages and eosinophils in postnatal mammary morphogenesis.

142. Ingman, W. V., Wyckoff, J., Gouon-Evans, V., Condeelis, J. & Pollard, J. W. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev. Dyn.* **235**, 3222–3229 (2006).
143. Lilla, J. N., Joshi, R. V., Craik, C. S. & Werb, Z. Active plasma kallikrein localizes to mast cells and regulates epithelial cell apoptosis, adipocyte differentiation, and stromal remodeling during mammary gland involution. *J. Biol. Chem.* **284**, 13792–13803 (2009).
144. Atabai, K., Sheppard, D. & Werb, Z. Roles of the innate immune system in mammary gland remodeling during involution. *J. Mammary Gland Biol. Neoplasia* **12**, 37–45 (2007).
145. Lilla, J. N. & Werb, Z. Mast cells contribute to the stromal microenvironment in mammary gland branching morphogenesis. *Dev. Biol.* **337**, 124–133 (2010).
146. Russell, J. S., McGee, S. O., Ip, M. M., Kuhlmann, D. & Masso-Welch, P. A. Conjugated linoleic acid induces mast cell recruitment during mouse mammary gland stromal remodeling. *J. Nutr.* **137**, 1200–1207 (2007).
147. Orimo, A. *et al.* Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**, 335–348 (2005).
148. Montesano, R., Matsumoto, K., Nakamura, T. & Orci, L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**, 901–908 (1991).
149. Simian, M. *et al.* The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development* **128**, 3117–3131 (2001).
150. Fleming, J. M. *et al.* Interlobular and intralobular mammary stroma: genotype may not reflect phenotype. *BMC Cell Biol.* **9**, 46 (2008).
151. Knox, S. M. *et al.* Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis. *Science* **329**, 1645–1647 (2010).
152. Gyorki, D. E., Asselin-Labat, M. L., van Rooijen, N., Lindeman, G. J. & Visvader, J. E. Resident macrophages influence stem cell activity in the mammary gland. *Breast Cancer Res.* **11**, R62 (2009).
153. Pull, S. L., Doherty, J. M., Mills, J. C., Gordon, J. I. & Stappenbeck, T. S. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc. Natl Acad. Sci. USA* **102**, 99–104 (2005).
154. Crick, F. Diffusion in embryogenesis. *Nature* **225**, 420–422 (1970).
155. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M. & Ingber, D. E. Geometric control of cell life and death. *Science* **276**, 1425–1428 (1997).
156. Nelson, C. M. *et al.* Emergent patterns of growth controlled by multicellular form and mechanics. *Proc. Natl Acad. Sci. USA* **102**, 11594–11599 (2005).
157. McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K. & Chen, C. S. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* **6**, 483–495 (2004).
158. Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677–689 (2006).
159. Nelson, C. M., Khauv, D., Bissell, M. J. & Radisky, D. C. Change in cell shape is required for matrix metalloproteinase-induced epithelial–mesenchymal transition of mammary epithelial cells. *J. Cell. Biochem.* **105**, 25–33 (2008).
160. Ruiz, S. A. & Chen, C. S. Emergence of patterned stem cell differentiation within multicellular structures. *Stem Cells* **26**, 2921–2927 (2008).
161. Harris, A. K. Is cell sorting caused by differences in the work of intercellular adhesion? A critique of the Steinberg hypothesis. *J. Theor. Biol.* **61**, 267–285 (1976).
162. Schotz, E.-M. *et al.* Quantitative differences in tissue surface tension influence zebrafish germ layer positioning. *HFSP J.* **2**, 1–56 (2008).
163. Foty, R. A. & Steinberg, M. S. The differential adhesion hypothesis: a direct evaluation. *Dev. Biol.* **278**, 255–263 (2005).
164. Rauzi, M., Verant, P., Lecuit, T. & Lenne, P. F. Nature and anisotropy of cortical forces orienting *Drosophila* tissue morphogenesis. *Nature Cell Biol.* **10**, 1401–1410 (2008).
165. Larsen, M., Wei, C. & Yamada, K. M. Cell and fibronectin dynamics during branching morphogenesis. *J. Cell Sci.* **119**, 3376–3384 (2006).
166. Chi, X. *et al.* Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis. *Dev. Cell* **17**, 199–209 (2009).
167. DeOme, K. B., Faulkin, L. J. Jr, Bern, H. A. & Blair, P. B. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.* **19**, 515–520 (1959).
168. Shackleton, M. *et al.* Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84–88 (2006).
169. Stingl, J. *et al.* Purification and unique properties of mammary epithelial stem cells. *Nature* **439**, 993–997 (2006).
170. Kim, N. D., Oberley, T. D. & Clifton, K. H. Primary culture of flow cytometry-sorted rat mammary epithelial cell (RMEC) subpopulations in a reconstituted basement membrane, Matrigel. *Exp. Cell Res.* **209**, 6–20 (1993).
171. Kim, N. D. & Clifton, K. H. Characterization of rat mammary epithelial cell subpopulations by peanut lectin and anti-Thy-1.1 antibody and study of flow-sorted cells *in vivo*. *Exp. Cell Res.* **207**, 74–85 (1993).
172. Kamiya, K., Gould, M. N. & Clifton, K. H. Quantitative studies of ductal versus alveolar differentiation from rat mammary clonogens. *Proc. Soc. Exp. Biol. Med.* **219**, 217–225 (1998).
173. Kordon, E. C. & Smith, G. H. An entire functional mammary gland may comprise the progeny from a single cell. *Development* **125**, 1921–1930 (1998).
174. Visvader, J. E. & Smith, G. H. Murine mammary epithelial stem cells: discovery, function, and current status. *Cold Spring Harb. Perspect. Biol.* **3**, a004879 (2011).
175. Sleeman, K. E., Kendrick, H., Ashworth, A., Isacke, C. M. & Smalley, M. J. CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. *Breast Cancer Res.* **8**, R7 (2006).
176. Sleeman, K. E. *et al.* Dissociation of estrogen receptor expression and *in vivo* stem cell activity in the mammary gland. *J. Cell Biol.* **176**, 19–26 (2007).
177. Asselin-Labat, M. L. *et al.* Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nature Cell Biol.* **9**, 201–209 (2007).
178. Lim, E. *et al.* Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast Cancer Res.* **12**, R21 (2010).
179. Taddei, I. *et al.* β1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. *Nature Cell Biol.* **10**, 716–722 (2008).
180. Anderson, L. H., Boulanger, C. A., Smith, G. H., Carmeliet, P. & Watson, C. J. Stem cell marker prominin-1 regulates branching morphogenesis, but not regenerative capacity, in the mammary gland. *Dev. Dyn.* **240**, 674–681 (2011).
181. Liu, S. *et al.* Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.* **66**, 6063–6071 (2006).
182. Dontu, G. *et al.* Role of notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res.* **6**, R605–R615 (2004).
183. Korkaya, H. *et al.* Regulation of mammary stem/progenitor cells by PTEN/Akt/β-catenin signaling. *PLoS Biol.* **7**, e1000121 (2009).
184. Bai, L. & Rohrschneider, L. R. s-SHIP promoter expression marks activated stem cells in developing mouse mammary tissue. *Genes Dev.* **24**, 1882–1892 (2010).
185. Villadsen, R. *et al.* Evidence for a stem cell hierarchy in the adult human breast. *J. Cell Biol.* **177**, 87–101 (2007).
186. LaBarge, M. A. *et al.* Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. *Integr. Biol. (Camb.)* **1**, 70–79 (2009).
187. Booth, B. W. *et al.* The mammary microenvironment alters the differentiation repertoire of neural stem cells. *Proc. Natl Acad. Sci. USA* **105**, 14891–14896 (2008).
188. Boulanger, C. A., Mack, D. L., Booth, B. W. & Smith, G. H. Interaction with the mammary microenvironment redirects spermatogenic cell fate *in vivo*. *Proc. Natl Acad. Sci. USA* **104**, 3871–3876 (2007).
189. Sternlicht, M. D. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. *Breast Cancer Res.* **8**, 201 (2006).

Acknowledgements
We apologize to colleagues whose work could not be discussed owing to space limitations. The authors are supported by grants from the US National Institutes of Health (CA128660, HL110335 and GM083997), Susan G. Komen for the Cure (FAS0703855), the David & Lucile Packard Foundation and the Alfred P. Sloan Foundation. C.M.N. holds a Career Award at the Scientific Interface from the Burroughs Wellcome Fund.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Authors' homepage: http://www.princeton.edu/~cmngroup

ALL LINKS ARE ACTIVE IN THE ONLINE PDF