Tissue remodelling through branching morphogenesis

Markus Affolter, Rolf Zeller‡ and Emmanuel Caussinus**

Abstract | Branched structures are evident at all levels of organization in living organisms. Many organs, such as the vascular system, lung, kidney and mammary gland, are heavily branched. In each of these cases, equally fascinating questions have been put forward, including those that address the cellular and molecular mechanisms that regulate the branching process itself, such as where the branches are initiated and how they extend and grow in the right direction. Recent experiments suggest that cell competition and cell rearrangements might be conserved key features in branch formation and might be controlled by local cell signalling.

Endothelial tissue
A tissue made up of flattened endothelial cells that forms the interior surface of blood vessels.

E-cadherin
The major Ca^{2+}-dependent cell–cell adhesion molecule involved in the establishment of embryonic epithelium and in ensuring that epithelial cells remain bound together.

Ectoderm
The epithelium that covers the body surface of the early embryo.

**Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.*
‡Developmental Genetics, Department of Biomedicine, University of Basel, Mattenstrasse 28, CH-4058 Basel, Switzerland.
Correspondence to M.A.
e-mail:
markus.affolter@unibas.ch
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Branched structures are evident at all levels of organization in living organisms, ranging from the molecular level to organelles, cells, multicellular organs and entire organisms. In particular, many organs of higher animals, such as the vascular system, lung, kidney and mammary gland, are heavily branched structures. The branching process allows a large increase in the surface area for functional purposes (such as in the lung, kidney and mammary gland) or enables distant places in the organism to be reached (such as with the vasculature and insect tracheal system). What controls where these branches are initiated and what regulates how they extend in the right direction are interesting questions that are being addressed in each of these cases.

In this Review, we focus on the branching of epithelial tissues (specifically, the *Drosophila melanogaster* tracheal system and the mammalian kidney, lung and mammary gland) and the branching of an endothelial tissue (the vasculature). Tremendous progress has recently been made in identifying the molecules involved, and the function of these molecules has been linked to cellular activities that control the branching process. Therefore, the time has come to take a step back and look at the possible conservation of cellular and molecular mechanisms. In all systems that we describe here, cells building the core of the branched organs are either of epithelial or endothelial nature. These cells are, in most cases, polarized along their apical–basal axis throughout the branching process (the apical side being the one that faces the lumen and the basal side the one that faces away from the lumen). Cells adhere to each other through E-cadherin in adherens junctions (BOX 1), but in several instances they have to establish or re-establish polarity to generate a functional epithelium. After the branching process, a luminal network runs through the branched structures to provide space for the transport of gases and nutrients. We do not discuss the large body of published literature dealing with epithelial polarization and lumen formation and refer the reader to recent excellent reviews on these subjects[1–5]. We limit our consideration to the branching process itself, allowing for a better comparison between the different systems.

Branching of the fly tracheal system

Probably the best-characterized multicellular branched organ is the tracheal system of *D. melanogaster*. The tracheal system is a highly ramified epithelial tubular network that allows oxygen to reach every cell in the organism by passive diffusion through its luminal space. Owing to the availability of powerful genetic techniques used in combination with high resolution live imaging and laser ablation (BOX 2), and because of the small number of cells that build the tracheal tree, a comprehensive molecular scenario controlling the branching process has emerged[6–8].

Tracheal branching starts after the invagination of clusters of approximately 80 cells from the surface ectoderm. These cells are fully polarized along the apical–basal axis and are organized as an epithelial sheet around a central luminal space. It is this luminal space that is extensively elaborated in the branching process. Tracheal cells remain polarized during the entire process and do not divide. Branchless (BNL), a fibroblast growth factor (FGF) ligand, initiates the branching process by triggering cell migration and functions at the top of a hierarchy of cell interactions that orchestrate the branching process. Recent studies have attributed new roles to BNL signalling (see below), and it turns out that it is the combined effects of these

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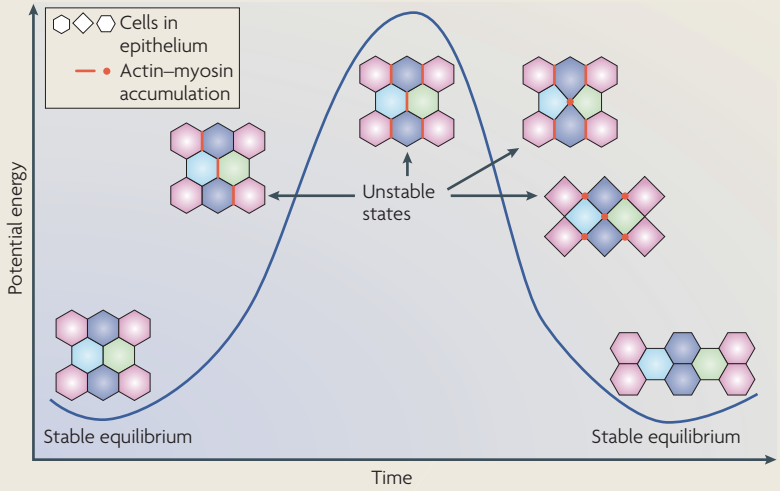
Box 1 | **Quantitative modelling of the forces that shape tissues**



Mechanical forces underlie the morphogenesis of tissues, organs and organisms[96]. Common elegant approaches were recently used to model the forces that shape single layer epithelial sheets[97,98]. Single layer epithelia have been assimilated to two-dimensional networks of edges that meet at vertices (see the figure). Every edge represents the interface between two cells and corresponds to E-cadherin molecules that are engaged in homophilic complexes and interact with subcortical actin–myosin filaments. Intercellular adhesions mediated by E-cadherin generate forces that tend to lengthen cell contacts, whereas cortical tensions controlled by the actin–myosin filaments tend to shorten them. These ideas were formalized to allow computer simulations. The potential energy of single layer epithelial sheets was written as the sum of the energies associated with the length of every edge, the perimeter of every cell and the volume of every cell. Single layer epithelia adopt an organization corresponding to a stable equilibrium, in which potential energy reaches a local minimum. Biologically controlled changes of energies (owing to local accumulation of myosin, for example) destabilize epithelial organization, which evolves to minimize its potential energy and reach a new stable equilibrium. Quantitative modelling of the forces implicated in morphogenesis of more complex structures (single layer epithelial tubes, stratified epithelia and epithelia associated with connective or muscular tissues) will probably benefit from the techniques developed for single layer epithelial sheets. As branching morphogenesis remodels epithelial tissues, the modelling of forces will have to be applied to these morphogenetic processes in the near future to obtain more mechanistic insights into the mechanisms underlying branching.

Actin–myosin filaments
The parallel arrangement of actin and myosin filaments. Using the hydrolysis of ATP, myosin can make the two types of filament slide on each other to shorten the structure as a whole.

Tracheal sac
A cluster of about 80 ectodermal cells that have invaginated within the embryo and formed an epithelial sac.

Filopodium
A thin, dynamic cytoplasmic projection covered with cell membrane that extends from the leading edge of migrating cells. Filopodia contain actin filament bundles and are presumably involved in exploring the cell environment.

BNL-controlled cell behaviours that lie at the heart of tracheal branching morphogenesis (FIG. 1).

BNL determines the tracheal branching pattern. A key role for BNL signalling is the control of cell motility[9,10]. *Bnl* is expressed in a dynamic manner around the invaginated tracheal sac by groups of ectodermal or mesodermal cells[10]. The tracheal cells closest to the source of the BNL ligand sense it through the FGF receptor (FGFR) Breathless (BTL) on their basal side, which induces the formation of numerous filopodia and ultimately the migration of two or three cells away from the sac to generate bud-like extensions[10] (FIG. 1a). During this process, the actively migrating cells remain fully integrated in the tracheal epithelium, and the spatial expression of *Bnl* (or the availability of the BNL ligand) in cells adjacent to the tracheal sac determines the spatial organization of the branches.

During the period in which the branching pattern is specified in the early embryo, it seems that there is no significant feedback on *Bnl* expression between tracheal cells and target tissues, such as the epidermis and muscles, suggesting that tracheal cells might simply follow a 'building plan' instructed by the surrounding tissues. Such exquisite control of the three-dimensional (3D) branching pattern by the cellular environment might seem obvious, as tracheal branches need to be directed to all major sites to provide the oxygen that is vital for larval development.

BNL coordinates cell behaviour during extension. On bud formation, BNL signalling triggers interactions among tracheal cells that implement a hierarchical organization of cells into leading (tip) cells and lagging (stalk) cells[11]. These two cellular phenotypes are not prespecified. Instead, cells compete for branch positions such that cells with the highest BTL receptor activity (triggered by high BNL levels) assume lead positions, whereas cells with lower receptor activity follow these tip cells and ultimately form the stalk. Competition among tracheal cells for the lead position involves Notch-mediated lateral inhibition (FIG. 1b), which prevents additional cells from assuming the lead[11] (see also the vascular branching section).

BNL regulates cell intercalation. When BNL-induced epithelial buds start to elongate, tracheal cells in most branches undergo a fascinating process of intercalation[12]. Cell ablation studies have shown that the forces inducing this exquisite cell intercalation process are generated by BNL-induced tip cell migration[13] (BOX 1; BOX 2). When the tip cells migrate away from the initial sac they elongate the branch stalk and create a tensile stress in the resident cells. This tensile stress is essential for and ultimately triggers cell intercalation, which allows the branches to elongate. Interestingly, tracheal cells in the branches in which extension is accompanied by cell intercalation elongate significantly. Cell ablation studies have established that this particular elongated cell shape is also a consequence of the forces generated by tip cell migration and therefore by BNL signalling (FIG. 1b,c).

BNL regulates cell determination and sprouting. To form a fully functional network of tubes, the independently developed segmental units have to be interconnected through branch fusion events and the terminal cells at the periphery of the network need to connect to target tissues through numerous fine cellular extensions. For these events to occur, two highly specialized types of cells, so-called fusion cells and terminal cells, have to be specified in the right temporal and spatial order.

Whereas fusion cells allow two independent luminal networks to connect through the complex mechanism of fusion cell hollowing, terminal cells extend numerous fine extensions with luminal spaces, which reach virtually all cells in the organism and allow oxygen exchange. BNL signalling controls the activation of genes, such as *escargot* (in fusion cells) and *blistered* (in terminal cells), that determine these cell types[10] (FIG. 1c). However, direct target genes of these transcriptional regulators have not been identified yet and the mechanisms underlying terminal differentiation remain to be determined.

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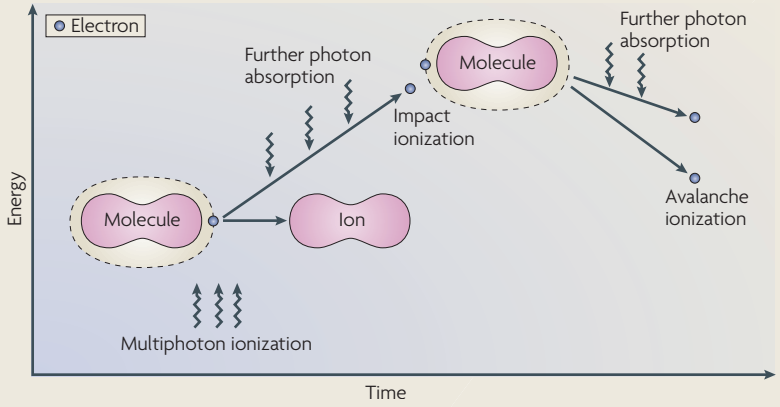
Box 2 | **Techniques to study the mechanics of tissues in intact organisms**



Applying or measuring forces that act on tissues in intact organisms requires methods that do not need contact between a probe and the tissue. Recently, injection of ferrofluid was used to magnetize target cells in *Drosophila melanogaster* embryos. These magnetized cells could be remotely dragged by magnetic tweezers[99]. Laser-induced plasma-mediated ablation was used by several groups to explore tissular tensions[13,100–102]. Tensile stresses can indirectly be determined by observing tissue retraction on laser cutting. Because modern lasers allow the destruction of biological structures with unprecedented precision and selectivity, this technique will probably become a standard experimental protocol. By tightly focusing laser pulses, the irradiated material is locally turned into plasma (partially ionized gas, in which a certain proportion of electrons are free rather than being bound to an atom or molecule). Plasma formation occurs above a well-defined laser intensity threshold in a volume in the region of a micrometer cubed, at which free electrons are released through the interplay of multiphoton and avalanche ionization[103] (see the figure). Plasma formation is usually accompanied by destructive mechanical and thermal side effects that can induce cell lysis by membrane perforation. Thus, the complete ablation of a cluster of two or more cells can be carried out without affecting the surrounding cells. Interestingly, below the plasma formation threshold, low free electron densities can perturb the stability of intracellular structures, such as microtubules or actin fibres, without hampering cell viability[100]. Laser ablation will be important in future studies of epithelial remodelling because only perturbations of a system can give information about local forces underlying the process of morphogenesis (see, for instance, REF. 104).

Intercalation
The process during which cells insert between cells that are already in contact with each other.

Chemoattractant
A molecule with a chemotaxis-inducing effect on motile cells, which migrate towards its source.

Hypoxia
The lack of an adequate oxygen supply to an area of the body.

Angiogenic sprouting
The growth of new blood vessels from pre-existing vessels.

BNL regulates oxygen-dependent branching. In contrast to the events described above, which occur in the developing embryo, the extension of terminal branches is variable and plastic and can be regulated by oxygen during larval development (branching in the embryo does not depend on oxygen). Strikingly, it is again BNL that acts as the chemoattractant to induce and guide the formation of new terminal branches[14]. During larval life, cells experiencing oxygen deprivation induce the expression of *Bnl*, which, in turn, guides new terminal branches towards them. The oxygen delivered by the newly formed terminal branches relieves hypoxia, which decreases *Bnl* expression and aborts further terminal sprouting (in a process analogous to mammalian angiogenesis; see below). The role of BNL might therefore change from developmental control in the embryo to physiological control in larval stages.

Tracheal cells also respond to hypoxia, and they do so by increasing the levels of the BNL receptor BTL[15]. However, to what extent the tracheal branching pattern is shaped by oxygen levels under normal conditions remains to be established. Some results also indicate that the development of the larval pattern during embryogenesis is largely controlled in a developmental manner and is independent of oxygen levels[15].

Although BNL is pivotal for so many of the essential steps that control branching, many other signalling pathways, such as signal transducer and activator of transcription (STAT), epidermal growth factor (EGF), Decapentaplegic (DPP), Wingless (WG), Hedgehog (HH) and slit pathways, also impinge on the branching process (see, for example, REFS 16–20). Particularly interesting in this regard are the slit molecules, as signalling through their roundabout (ROBO) receptors is best known in the control of axonal pathfinding and directly affects cytoplasmic events in responding neuronal cells[21]. However, the BNL signalling pathway is clearly at the core of the control of the tracheal branching process.

Branching of the developing vasculature

For many years, tracheal branching has been compared most often to mammalian lung branching because both organs are involved in oxygen transport and because branching of both organs is controlled by FGF signalling (see below). However, recent studies have unravelled unexpected and stunning similarities in cellular behaviour between tracheal branching in *D. melanogaster* and angiogenic sprouting in vertebrates. Although the fully functional vasculature of, for example, an adult mouse is made up of millions of cells, the elaboration of the complex vascular branching pattern during angiogenesis is in many cases controlled at the single cell level. This means that individual cells take over important functions in establishing the complex 3D outline of the vascular tree (see below).

VEGF induces angiogenic sprouting. Whereas the cells that lead angiogenic sprouts have been shown to form numerous filopodial extensions in several early morphological studies, the eminent functions of such tip cells in the elaboration of the vascular tree during angiogenesis were not fully recognized until more recently. However, the pivotal role of a specific growth factor, namely vascular endothelial growth factor (VEGF), in blood vessel formation was uncovered two decades ago[22]. VEGFA, encoded by one of the four *VEGF* genes in mammals, is key to most, if not all, morphogenetic events during angiogenesis that control migration, proliferation and survival of endothelial cells[23–26].

In a seminal study, Betsholtz and colleagues[27] linked VEGF signalling to tip cell behaviour and directed migration (reviewed in REF. 28). Using the early postnatal mouse retina as a model system, different modes of VEGFA distribution in the extracellular space were shown to regulate endothelial cell behaviour by agonistic activity through VEGF receptor 2. Whereas tip cell migration depends on a gradient of VEGFA, stalk cell proliferation is controlled by VEGFA concentration. VEGFA is secreted by a pre-existing network of astrocytes and stimulates the formation, and guides the outgrowth, of tip cells. Tip cells form numerous filopodia and migrate along astrocytes, but stalk cells divide under the control of VEGFA to allow stalk elongation. Similarly to BNL

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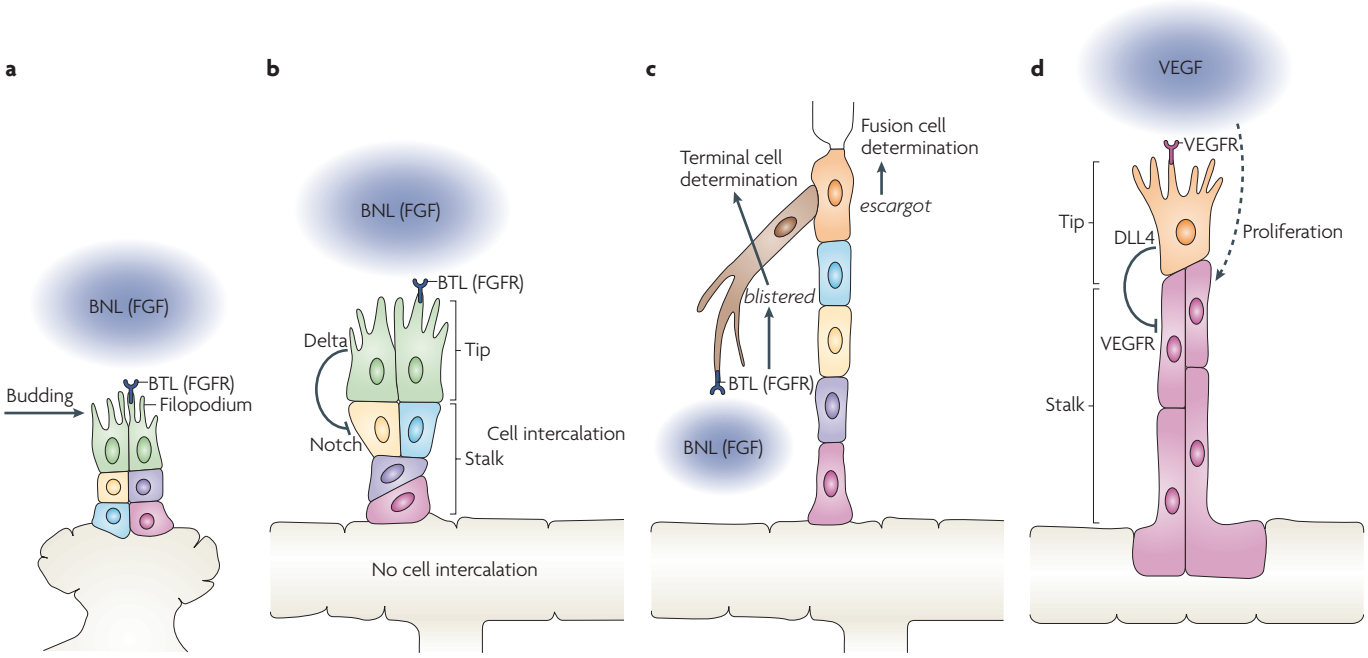

Figure 1 | ***Drosophila melanogaster* trachea and vertebrate vasculature branching.** Branchless (BNL), a fibroblast growth factor (FGF), acts at the top of the hierarchy of cellular events that orchestrate tracheal branching in *Drosophila melanogaster*. **a** | A gradient of BNL controls motility of the tracheal cells close to the BNL source. Tracheal cells responding to BNL (green) through the Breathless (BTL) FGF receptor (FGFR) expand numerous filopodia and migrate actively along the axis of the BNL gradient, generating bud-like tracheal structures. **b** | The newly formed tracheal bud is rapidly organized into a well-defined tip and stalk. This identity determination is mediated by the Delta–Notch signalling pathway. As the tip cells migrate away from the initial structure, they elongate the stalk. The stalk cells intercalate (compare the relative positions of the yellow, purple, blue and pink cells throughout **a**–**c**) and, doing so, release some tensile stress owing to the stalk elongation. **c** | To form a fully functional network of tubes, the tracheal branches have to interconnect their fusion cells and reach every target tissue through numerous fine cellular extensions produced by their terminal cells. Again, BNL is essential for the differentiation of the tip cells into fusion cells (orange; express *escargot*) and terminal cells (brown; express *blistered*). **d** | During vertebrate angiogenesis, vascular endothelial growth factor (VEGF) signalling determines the formation of angiogenic sprouts and controls tip cell (orange) and stalk cell (pink) identity through Delta–Notch signalling. DLL4, Delta-like protein 4.

in the developing trachea, VEGFA thereby guides angiogenic sprouts through endothelial tip cell filopodia (FIG. 1d). However, in the vasculature, VEGF signalling also controls stalk cell proliferation.

VEGFA signalling controls tip and stalk cell formation. How are tip cells selected? And how are neighbouring cells that might sense similar VEGF levels inhibited from becoming tip cells themselves, which would result in the migration of cell clusters or cell sheets rather than organizing new branches into tip and stalk cells? In a series of recently published papers, the selection of tip cells has been linked to the ligand Delta-like 4 (DLL4) and its receptor Notch 1 (REFS 29–34; reviewed in REFS 35,36). DLL4 is a target of VEGF signalling in endothelial cells and is preferentially induced in tip cells owing to their proximity to high levels of VEGF. Interestingly, loss of DLL4–Notch 1 signalling during *in vitro* angiogenesis and in mouse and zebrafish embryos causes ectopic sprouts and increased tip cell numbers (similar to the result of their loss in the *D. melanogaster* tracheal system). In addition, mosaic analysis in the mouse retina and zebrafish intersegmental vessels showed that cells unable to receive Notch signals are more likely to adopt tip cell behaviour than those that do receive Notch signals. These and additional results suggest that individual endothelial cells at branch tips compete for leader positions (that is, the tip cell phenotype) and that these cells suppress the tip cell phenotype in their immediate neighbours by expressing DLL4 and presenting it to the neighbouring cells (FIG. 1d).

Although the details of this competitive inhibition remain to be elucidated (see REF. 28 for a more detailed discussion), it seems that the branching pattern of the vascular tree that is established by sprouting angiogenesis is largely controlled by the VEGF distribution and the Notch-mediated restriction of the tip cell phenotype. VEGF is secreted by many different cell types to induce and attract new vessels. In addition, hypoxia results in the upregulation of VEGF[37], so that endothelial cell migration and sprouting angiogenesis are induced towards the hypoxic area.

Again, in addition to VEGF and Notch signalling, many other major signalling pathways impinge on the angiogenic branching process. Of particular interest are molecules such as the semaphorins, slits and netrins,

Astrocyte
A star-shaped cell that provides support and protection for neurons in the central nervous system.

Intersegmental vessel
A vessel that carries blood from the dorsal aorta between somites to the dorsal side of the neural tube.

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which have attractive and repulsive properties on axonal growth cones and are also required for proper vascular branching (reviewed in REFS 38,39). Several hundred years ago, anatomists observed that nerve fibres and blood vessels often align and/or follow parallel routes, and the identification of similar guiding and repelling cues provides a likely explanation for these morphological observations. In many or possibly most cases, semaphorins, slits and netrins and their respective receptors modulate the response of endothelial cells to VEGF, either directly or indirectly. Although these 'neural' guidance cues might themselves not drive sprouting, they guide angiogenic sprouts towards or away from certain target areas, thus greatly influencing the final branching pattern.

Both the vasculature and the trachea have to reach almost everywhere in an organism, meaning that the developing branches have to be attracted by numerous cells and cell types in the embryo. The genes encoding *D. melanogaster* BNL (in the trachea) and vertebrate VEGF (in angiogenic sprouts) have extremely complex expression patterns, providing attractive cues in virtually every tissue and prefiguring the branching pattern. The expression of both genes is sensitive to hypoxia, which provides a means to re-adjust tissue oxygen levels through stimulating the growth of new vessels into the hypoxic areas.

Branching of the mouse lung

The branched organs discussed in the previous sections (the trachea and vasculature) are conceptually different from the ones we discuss in the following sections (the lung, kidney and mammary gland). The lung, kidney and mammary gland occupy a defined volume in an organism, and the branching process is essentially limited to a 'bag' of mesenchymal tissue. These organs can, to some extent, develop proper branching patterns in organ culture that are controlled by reciprocal feedback interactions between the branching epithelium and the surrounding mesenchyme. In this sense, the budding process might share similarities with epithelial–mesenchymal feedback signalling in vertebrate limb buds. The regulatory feedback loops that control the reciprocal signalling interactions between different cell populations in limb buds involve coordinate regulation from at least three major signalling pathways that control initiation, growth and termination in a largely self-regulatory manner (BOX 3).

Stereotypical branching of the mouse lung bud epithelium. The mammalian lung consists of thousands to millions of airway branches organized in an intricate pattern. In a rather heroic effort, Ross Metzger in Mark Krasnow´s laboratory recently described the 3D branching pattern and lineage of the mouse bronchial tree by reconstructing its developmental history, up to the pseudoglandular stage, from the analysis of hundreds of developmental intermediates in fixed specimens[40]. The outcome of this extensive and careful study is both stunning and enlightening; the branching process seems to be remarkably stereotypical, suggesting that it is hard wired and genetically controlled in time and space — similarly to tracheal branching in flies.

Axonal growth cone
The motile extension of a developing axon. Axonal growth cones use external cues to guide their movements.

The branched tree is generated by three geometrically simple modes of branching: domain branching, planar bifurcation and orthogonal bifurcation (FIG. 2). These three modes are used in three different time orders (but in a predictable manner) throughout lung branching. Domain branching occurs during the onset of each major branch, but once orthogonal branching has started this mode is kept. In between, branches arising by domain branching can undergo planar bifurcation, giving rise to two branches, one of which undergoes domain branching and one that bifurcates again in a planar manner. Thus, for each given point in the developing lung at a given time, the subsequent branching mode is predictable to a large extent, which indicates that branching is tightly organized in space using just three geometrical branching modes (FIG. 2). Whether later branching events are also tightly controlled or are more stochastic remains to be investigated.

Krasnow and colleagues suggest that the three subroutines are controlled by genetic periodicity functions (which determine when side branches form during domain branching), bifurcator functions (which determine when branches bifurcate) and rotator functions (which determine whether bifurcations occur in a planar or an orthogonal manner). Importantly, all of these functions impinge on a branch generator — a key routine that initiates and extends a branch and that might be identical in both cases (analogous to the functions of BNL in the trachea and VEGF in the vasculature). What might this 'branch generator' be in molecular and cellular terms? In other words, how do branches initiate and how do they extend? Does the branch generator also organize branches into tip and stalk structures and does it have molecular similarities with signalling networks that control limb bud organogenesis (BOX 3)?

FGF from the mesenchyme promotes epithelial branching. Pioneering genetic analyses of early mouse lung development, initiated by Brigit Hogan's group, have provided insight into the molecular mechanisms that control the initiation of lung branching and what is most likely to be part of the branch generator. These studies revealed that the FGF, sonic hedgehog (SHH), bone morphogenetic protein (BMP), retinoic acid and Wnt signalling pathways, and many transcription factors (for example, friend of GATA protein 2 (FOG2; also known as ZFPM2) and GATA4 (REF. 41)), are required for normal epithelial lung branching morphogenesis.

Based on genetic loss- and gain-of-function experiments and on the expression patterns of the relevant molecules in the epithelial or the mesenchymal cell layer, numerous molecular scenarios underlying the budding process have been proposed (see REFS 42,43 for more detailed discussions). All of these scenarios attribute a key role to FGF signalling from the mesenchyme to the epithelium, which induces bud formation. Subsequent epithelial–mesenchymal interactions involving FGFs regulate outgrowth and branching and the distal–proximal epithelial interactions that generate tip and stalk cell fates (see below) (FIG. 3a).

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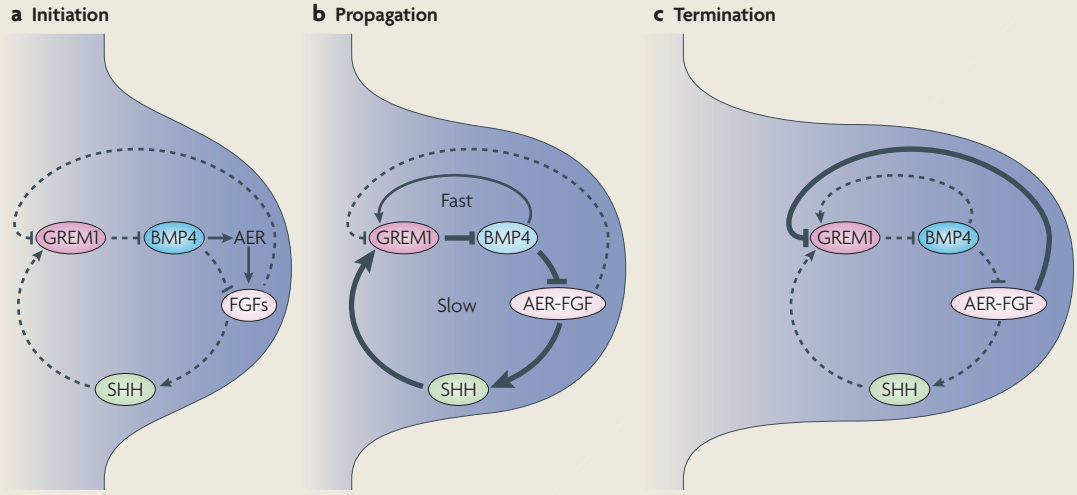
Box 3 | **A self-regulatory signalling system controls limb bud organogenesis**



Two major signalling centres, the mesenchymal limb bud organizer (the polarizing region) in the posterior mesenchyme and the apical ectodermal ridge (AER) at the distal tip, control limb bud morphogenesis. In particular, limb bud organogenesis depends on dynamic and self-regulatory epithelial–mesenchymal feedback interactions that involve sonic hedgehog (SHH), bone morphogenetic protein 4 (BMP4), gremlin 1 (GREM1) and fibroblast growth factor (FGF) signalling from the AER (AER-FGF)[105]. During the initiation of limb bud outgrowth, SHH signalling by the polarizing region and FGF signalling by the AER are activated in parallel with GREM1 (initiation; see the figure, part **a**). In addition, mesenchymal BMP4 is first required for AER formation and rapidly inhibits its own activity by transcriptional upregulation of its antagonist GREM1 (REF. 75). This enables the establishment of the SHH–GREM1–FGF epithelial–mesenchymal feedback loop and a distal progression of limb bud development (propagation; see the figure, part **b**). The fast BMP4–GREM1 and the slower epithelial–mesenchymal SHH–GREM1–FGF modules become interlinked by the BMP4- and SHH-mediated differential regulation of GREM1 expression. These dual-time feedback loops define the core of a robust limb bud patterning system that can buffer variations in a dynamic and self-regulatory manner[75]. During advanced outgrowth, GREM1 expression is progressively inhibited by FGF signalling, which causes the breakdown of the SHH–GREM1–FGF feedback loop and terminates limb bud organogenesis[75,106,107] (termination; see the figure, part **c**). Lung development and ureteric bud outgrowth are also disrupted in *Grem1*-deficient mouse embryos, but are completely restored on genetic reduction of *Bmp4* (REFS 69,70), indicating that these self-regulatory epithelial–mesenchymal feedback signalling interactions also regulate epithelial branching morphogenesis. Indeed, GREM1-mediated reduction of BMP4 activity is required to initiate the glial cell-derived neurotrophic factor (GDNF)–WNT11 epithelial–mesenchymal feedback loop during the onset of ureteric bud outgrowth[69,70], which in turn propagates epithelial branching. Therefore, the BMP4–GREM1 module might fulfil similar roles during the GDNF–WNT11 and SHH–FGF epithelial–mesenchymal feedback signalling pathways that control kidney and limb bud organogenesis, respectively.

Epithelial–mesenchymal feedback and branch extension. *Fgf10* is expressed in a dynamic manner in the distal mesenchyme around the epithelial bud tip and is essential for bud formation and directional outgrowth[44–46]. FGF10 activates the FGF signalling pathway predominantly through FGFR2B, which is expressed by the epithelium and is required for branching (FIG. 3a). FGF signalling activates several genes in the bud tip, some of which are probably involved in the specification of tip versus stalk fates in the growing bud. One of the earliest genes upregulated in response to FGFR2 signalling is sprouty 2 (*Spry2*))[47,48]. SPRY2 negatively regulates FGF signal transduction by inhibiting or dampening the mitogen-activated protein kinase (MAPK) pathway. *Bmp2* and *Bmp4* expression is also induced in the distal epithelium by FGF signalling[49–51] and BMP4 has been shown to have a role in proliferation, survival and the morphogenetic behaviour of distal lung epithelial cells[50]. *Bmp4* is also expressed by the surrounding mesenchyme, but the exact role of BMP4 from this source is unclear.

Another gene specifically expressed in epithelial bud tips is *Shh*. SHH is a secreted signalling molecule and key regulator of limb bud development[44,52] (FIG. 3a; BOX 3). SHH seems to regulate the progression but not the initiation of lung epithelial branching, probably by negatively regulating mesenchymal *Fgf10* expression, which prohibits further bud extension. Several additional signalling pathways have been implicated in regulating proximal–distal polarity during branch outgrowth, including ligands of the Wnt and netrin families[49,53–55]. As these ligands are encoded by members of large gene families, careful genetic dissection of their signalling pathways is required to gain more insights into their functional requirements for normal epithelial lung branching morphogenesis.

From genetics to cell behaviour. Little is currently known about the cellular changes that are induced in the milieu around the nascent lung bud and in the bud itself following FGF10-mediated signalling. *In vitro* studies have

Apical ectodermal ridge
The thickening of the ectoderm rim at the tip of a developing limb bud in a vertebrate embryo.

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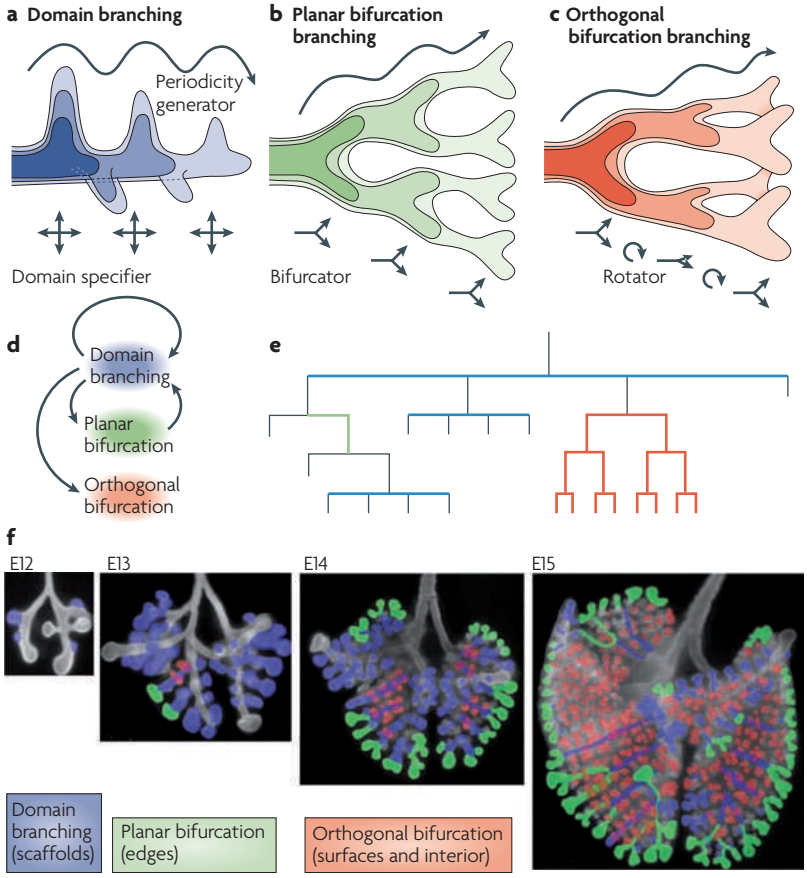

Figure 2 | **Lung patterning by iterative programming.** The pattern of the mouse bronchial tree is generated by different sequences of domain (blue), planar bifurcation (green) and orthogonal bifurcation (red) branching modes. These branching modes seem to arise by the combination of a few components: a periodicity generator, a domain specifier, a bifurcator and a rotator. **a** | During domain branching, daughter branches form in rows or domains at different positions around the circumference of the parent branch. **b** | During planar bifurcation branching, the parent branch forms daughter branches by a series of co-planar bifurcations. **c** | During orthogonal bifurcation branching, the parent branch forms daughter branches by a series of bifurcations occurring at 90° with respect to the preceding ones. **d** | The three branching modes are used concurrently during development, but the transitions from one mode to another seem to be restricted to four routines (arrows). **e** | An arbitrary branch lineage diagram following the branching modes shown in **a**–**d**. **f** | Whole-mount lungs at days 12, 13, 14 and 15 of mouse embryonic development. The branches are pseudo-coloured (following the colour code above) to indicate their branching modes. Domain branching is preferentially used to build the scaffold of the lung, whereas planar branching forms the edges of the pulmonary lobes and orthogonal branching fills the spaces between. Image in part **c** is modified, with permission, from *Nature* REF. 40 © (2008) Macmillan Publishers Ltd. All rights reserved. Image in part **f** is reproduced, with permission, from *Nature* REF.40 © (2008) Macmillan Publishers Ltd. All rights reserved.

revealed a possible chemoattractant function of FGF10, but its precise role at the bud tip remains to be elucidated. FGF10 could control a number of cellular activities such as cell migration, competition, adhesion, sorting and proliferation. The removal of FGFRs in clones of cells, combined with live imaging, could provide many answers, but several technical issues first need to be resolved.

So, what are the cellular events that control branching? How are the periodicity, bifurcator and rotator functions that are predicted to influence or direct the budding programme involved? Do they all work on the emerging bud generator described? It turns out that SPRY2 regulates the site of initiation and the number of branches in specific domains, and thus influences the periodicity generator[40]. The involvement of SPRY2 in the periodicity generator might suggest that feedback regulation controlling the response to FGF is important for this subroutine (FIG. 3a). The gene shifty seems to encode a regulator of the proximal–distal register of entire branching domains[40]. It is essential to identify more molecules that function in the periodicity generator, the domain specifier, the bifurcator and the rotator, because these are the key players that generate the distinct geometries of the branching modes.

Kidney branching morphogenesis

During development of the definitive, so-called metanephric, kidney, branching of the ureteric duct epithelium (which will form the collecting system) is controlled by epithelial–mesenchymal feedback signalling interactions between the ureteric bud epithelium and the surrounding metanephric mesenchyme[56–58] (FIG. 3b). The ureteric bud invades the metanephric mesenchyme as a consequence of stalk elongation and localized proliferation at its distal tip, which leads to a characteristic swelling — the ampulla[59]. The proliferating ampulla cells extend the distal tip bilaterally, which results in symmetrical bifurcation, and the newly formed stalks elongate until branching is initiated again at the distal tips[60]. The subsequent rounds of ureteric epithelial branching occur in a coordinated, but not completely synchronous, manner.

The first bifurcation of the ureteric bud is always symmetrical, whereas subsequent branching events are more diverse[60]. In particular, terminal trifurcation events are often observed between the second and fifth rounds of branching (18% of overall branches). Many of these trifurcated branch points are remodelled into bifurcated branches by differential growth, which shifts one branch to a more proximal position. Furthermore, a large fraction of lateral branches that initiate from elongated stalks rather than terminal ampulla occur during the second and third rounds of branching in mouse embryos (6% of overall branches)[60]. Such lateral branching events might be more common during kidney development in humans than in mice[61].

GDNF from the mesenchyme controls ureteric branching. Initiation of the ureteric bud outgrowth and epithelial branching morphogenesis are controlled by glial cell-derived neurotrophic factor (GDNF) signalling from the metanephric mesenchyme to its cognate receptor rearranged during transfection (RET) in the Wolffian duct — a duct that gives rise to the ureteric bud of the kidney[62]. The nascent ureteric bud and distal tips of the invading and branching epithelium express high levels of RET, whereas the initially low and uniform level of GDNF expression in the metanephric mesenchyme is upregulated specifically in mesenchymal cells adjacent to the ureteric bud[59] (FIG. 3b). Genetic inactivation of *Gdnf*, *Ret* and the RET co-receptor GDNF family receptor α1 (*Gfrα1*) in mice causes renal aplasia (a lack of development of the kidney) in a large fraction of the mutant embryos

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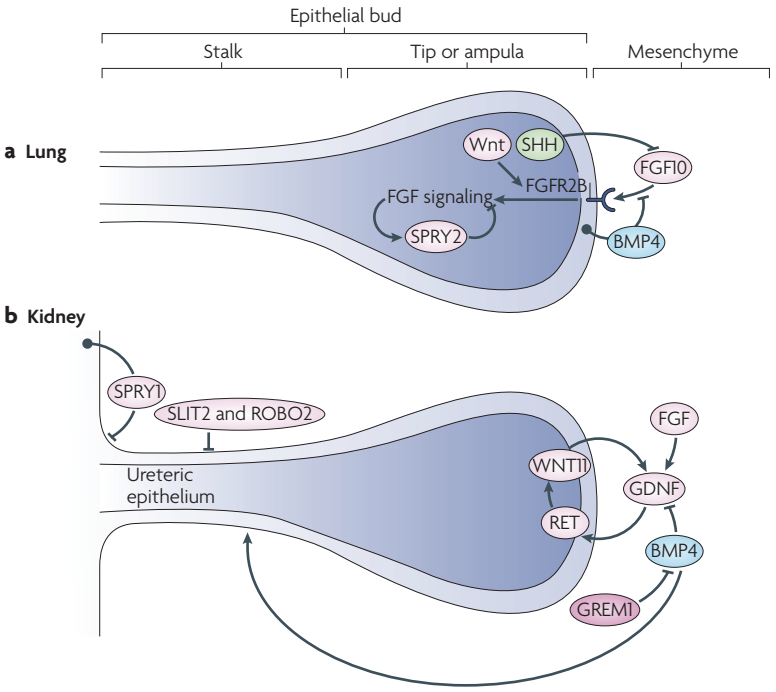

Figure 3 | **Molecular regulation of lung and kidney branching morphogenesis.** **a** | The engine of lung branching morphogenesis in the distal mesenchyme is fibroblast growth factor 10 (FGF10), which signals to FGF receptor 2B (FGFR2B) in the distal epithelium. FGF signal transduction increases sprouty 2 (SPRY2) expression, which, in turn, controls branching by limiting the proliferation and migration of distal tip cells. In addition, sonic hedgehog (SHH) and bone morphogenetic protein 4 (BMP4) signalling by the tip epithelium restrict FGF signal transduction and branching, whereas mesenchymal BMP4 enhances local branching (not shown). These different autocrine and paracrine functions of BMP4 signalling might regulate branch point selection. Canonical Wnt signalling reinforces FGFR2B expression in the epithelium, whereas non-canonical WNT5 signalling inhibits FGF10 expression. **b** | Kidney branching morphogenesis requires signalling of the glial cell-derived neurotrophic factor (GDNF) from the metanephric mesenchyme to its receptor, rearranged during transfection (RET), in the distal ureteric epithelium. Induction of the ureteric bud is restricted to the caudal Wolffian duct by the interaction of SLIT2 with roundabout 2 (ROBO2) in the mesenchyme and SPRY1 in the epithelium. In turn, GDNF signalling induces ureteric bud outgrowth, which is reinforced by WNT11 signalling from the distal epithelium. The establishment of this epithelial–mesenchymal signalling feedback loop requires gremlin 1 (GREM1)-mediated antagonism of BMP4 in the distal mesenchyme, whereas BMP4 signalling in the proximal mesenchyme promotes elongation of the ureteric stalk. FGF signalling in the mesenchyme further enhances GDNF expression.

owing to disrupted branching[62]. GDNF stimulates the proliferation and branching of epithelial tips, and analysis of chimeric mouse embryos reveals that cells lacking RET do not colonize these distal tips[63]. Furthermore, the descendents of distal tip cells contribute descendents to the new tips and to the distal parts of the new stalks.

A recent study reveals that the primary ureteric bud forms as a consequence of extensive cell rearrangements in the caudal Wolffian duct and is stimulated by RET signalling[64]. Chimeric analyses in mouse embryos indicate that primary ureteric tip cells are selected by competition, such that cells with high RET signal transduction activity move to the region of the future ureteric bud. This region forms a pseudostratified epithelium at the time of RET-dependent cell movements, but is itself independent of GDNF–RET signalling[64]. These results, together with the fact that the renal aplasia of *Gdnf*-deficient mouse embryos is rescued by transgene-mediated uniform expression of GDNF in the ureteric epithelium[65], indicate that other factors participate in restricting ureteric bud formation to one site and in controlling the orderly progression of epithelial branching. Indeed, genetic analysis in mice has revealed additional pathways that inhibit the formation of ectopic ureteric buds. In particular, additional ureteric buds form in mouse embryos lacking either the epithelial SLIT2 ligand or the mesenchymal ROBO2 receptor[66]. Analysis showed that SLIT2-mediated ROBO2 signal transduction in the metanephric mesenchyme restricts GDNF expression to the caudal region around the ureteric bud. Ectopic ureteric buds and branching are also observed in mouse embryos lacking the intracellular receptor tyrosine kinase antagonist SPRY1, which normally antagonizes GDNF–RET signal transduction in the Wolffian duct[67].

Epithelial–mesenchymal feedback and branch extension. Following the onset of ureteric bud outgrowth, *Wnt11* expression is activated in epithelial tip cells and WNT11 signalling contributes to the propagation of mesenchymal *Gdnf* expression and branching as part of a self-regulatory epithelial–mesenchymal feedback loop[68]. The establishment of WNT11–GDNF epithelial–mesenchymal feedback signalling and the initiation of branching requires a reduction of BMP4 activity by the BMP antagonist gremlin 1 (GREM1) in the mesenchyme surrounding the ureteric bud[69,70] (FIG. 3b). These interactions are reminiscent of the epithelial–mesenchymal feedback signalling network that controls limb bud development (BOX 3). The initiation of ureteric bud outgrowth is disrupted in *Grem1*-deficient embryos, but restored by additional genetic reduction of *Bmp4* (REF. 69). Treatment of cultured kidneys with the BMP antagonist GREM1 induces supernumerary ureteric buds, whereas increasing BMP activity inhibits tip branching and promotes stalk elongation[71].

In contrast to the other branching systems discussed, the morphoregulatory roles of FGFs during kidney development are still poorly understood. This is probably because of the functional redundancy among FGF ligands, which, in analogy to the FGFs expressed in limb buds, might be indicative of an underlying robust mechanism (BOX 3). Genetic analysis in mice has implicated *Fgf7* and *Fgf10*, both of which are expressed by the metanephric mesenchyme, in the regulation of ureteric branching[72,73]. In agreement, inactivation of *Fgfr2* in the ureteric epithelium reduces the number of tips with ampullas and branching, and elongates stalks[74]. *In vitro* experiments indicate that FGFs might regulate branching by stimulating the proliferation and survival of tip cells[59]. Taken together, these studies begin to reveal how different signalling pathways affect the positioning of the ureteric bud, initiate its outgrowth and coordinate epithelial branching (FIG. 3b).

Whereas the GDNF–RET pathway seems to constitute the 'engine', the other pathways discussed probably ensure the spatially and temporally correct initiation, progression and termination of epithelial branching morphogenesis. In fact, striking molecular and mechanistic

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similarities between the epithelial–mesenchymal signalling interactions controlling limb and kidney morphogenesis have been noted[70]. Therefore, it might be possible to model the interactions of the GDNF, BMP and FGF pathways, as has recently been done for limb buds[75] (BOX 3). The predictive power of such simulations might provide testable clues for a better understanding of how these pathways interact to orchestrate kidney epithelial branching morphogenesis and could also be extended to other branched organs.

Mouse mammary gland branching

The mammary gland is an epidermal appendage consisting of several different cell types, including epithelial cells (luminal epithelial and basal myoepithelial cells) and adipocytes of the fat pad. In developing mouse embryos, mammary placodes form and invaginate to produce buds[76,77]. During mammary gland branching morphogenesis, epithelial cells undergo extensive branching in the fat pad. Subsequently, these epithelial buds sprout and branch to form a rudimentary branched structure.

The most spectacular phase of mammary gland branching morphogenesis, however, occurs postnatally during puberty, when terminal end buds (TEBs; club-shaped structures comprising an outer layer of cap cells and a multilayered inner core of body cells) appear at the duct tips, initiate invasion and fill the fat pads. Proliferation of TEB cells results in ductal elongation, and cleft ing of the TEBs results in bifurcation of the ducts and branch generation. During pregnancy, excessive side branching leads to a further ramification of the mammary tree (reviewed in REFS 78–80).

Stochastic branching of the mammary epithelium. Mammary epithelial branching is also regulated, like lung and kidney branching, by various signals from the epithelium or stroma, such as BMP, Wnt and EGF[76,81–83]. In addition, hormonal control has an important role in mammary gland branching morphogenesis[79,84–87]. However, and in sharp contrast to the other branching processes, no signal has so far been identified that is specifically expressed by the stroma in regions that prefigure branch outgrowth. The mammary epithelial branching process seems to be stochastic and is therefore probably not controlled in a hard-wired manner, as is the case for the branching morphogenesis of the other organs discussed.

Mammary gland branching occurs at the TEBs in a dichotomous manner or through the development of new branches from the duct of pre-existing ones by side (lateral) branching. Another striking difference of branching in the mammary epithelium compared with branching in the kidney and lung is that the branches fill a pre-existing space, the fat pad, which does not grow much to accommodate the developing branching tree. Outgrowing branches might fill the available space most effectively by using self-avoidance mechanisms. Indeed, mammary epithelial cells display such properties; new branches often start off at a sharp angle to existing ones and turn away or stop growing when close to another branch[88]. It has been suggested that transforming growth factor-β (TGFβ) signalling might be involved in such a process[89], which might be somewhat similar to homotypic interactions in limiting arbour territories in developing neurons (see, for example, REF. 90).

Tissue geometry and the extracellular matrix in branching. Because a localized signal directing outgrowth of branches (such as BNL, VEGF, FGF and GDNF signals in the trachea, vasculature, lung and kidney, respectively) has not been identified in mammary glands, different approaches have been taken to try to unravel the branching mechanism. Using a micro-patterning approach to control the initial geometry of tubules formed by mouse mammary epithelial cells in culture, it was observed that the shape of tubules dictates the position of subsequent branches. On stimulation with EGF or hepatocyte growth factor (HGF), branches initiated only from the ends of the tubules and not from their sides, suggesting that positional context was encoded by the pre-existing structure[89]. Because the epithelial cells branch in the absence of mesenchyme in these assays, secreted autocrine inhibitory signalling molecules might determine branch positions at the ends of tubules.

Interestingly, TGFβ inhibits mammary gland branching morphogenesis *in vivo*[91,92] and in the micro-patterning assays[89], possibly by inducing the deposition of extracellular matrix components. Because cells in the engineered tubules produce TGFβ, a local minimum concentration of TGFβ might be generated at tube ends, leading to lower levels of extracellular matrix deposition and increased probability of branching. Although this would be an elegant scenario for the generation of local cues to induce branching, many additional *in vivo* studies will be required to confirm the existence of such a branching mode. Micro-patterning assays, such as those introduced by the Bissell laboratory, will be useful for quantitative analysis.

Cell behaviour during mammary epithelial branching. To visualize the cell behaviour that underlies ductal elongation and branching, Werb and colleagues have recently used primary, organotypic 3D cultures and long-term confocal time-lapse imaging[93]. They showed that cells in elongating ducts reorganize into a multi-layered epithelium, migrate collectively and rearrange dynamically, without forming leading cellular extensions. These observations suggest that branching morphogenesis involves the active motility of both luminal and myoepithelial cells. Whereas epithelial cells advance collectively, myoepithelial cells seem to restrain elongating ducts.

Interestingly, recent inactivation of *Fgfr2* during mammary branching in puberty showed that FGFR2 is required for TEB development[94]. Furthermore, mosaic analyses showed that *Fgfr2*-deficient cells were at a competitive disadvantage to *Fgfr2* heterozygous neighbours in the proliferative part of TEBs, but not in the less proliferative proximal region of the duct[95]. Whether FGFR2 is required solely for cell proliferation in TEBs, or whether it is also essential for cell rearrangements, is currently unknown.

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Box 4 | **Branching in plants**

Wonderful examples of branching patterns can also be observed in the plant kingdom (see the figure). During vegetative development, plants continuously form new leaves that are arranged in regular patterns (phyllotaxis), with defined divergence angles between successive leaves giving rise to different phyllotactic patterns (for example, 180°, alternate, 137.5°, spiral and 90°, dessucate). Note that dessucate patterns resemble the orthogonal bifurcations seen during lung branching (see also FIG. 2). Organ anlagen are laid down in the apical shoot meristem, and all evidence indicates that active transport of the plant hormone auxin (specifically, indole-3-acetic acid), is the key process regulating phyllotaxis[108]. Auxin is transported in a polar manner in plant tissues through the asymmetric localization of the pin-formed family of auxin efflux regulators, which in turn elicit gene regulatory responses by inducing the degradation of inhibitors of the auxin response factors. Accumulation of auxin induces organ formation, which, at the same time, induces depletion and inhibits organ formation in the vicinity of a previously induced organ primordium. Auxin is also involved in the formation of the vasculature in plant appendages and therefore has a pivotal role in their morphogenesis. The quantitative aspects of phyllotaxis and vascularization have stimulated research at the interfaces of molecular biology, physics and mathematics. These studies underscore the importance of computer simulations in formulating quantitative models that can be experimentally verified. The accumulation of ever-increasing amounts of experimental data makes quantitative modelling relevant for a large number of developmental systems[109–112] (see also BOX 3), inclucing organs that arise through branching morphogenesis.

Emerging similarities and differences

The key signalling systems controlling branching in several different species and organs have started to emerge. For example, in *D. melanogaster* trachea and the vertebrate vasculature, FGF and VEGF signalling, respectively, control the branching process at many levels in tip cells (for example, they control cytoplasmic activities, gene expression and cell competition) and they seem to regulate major aspects of the entire branching process. During vascularization VEGF also controls stalk cell division, whereas during tracheal development, stalk elongation is brought about by BNL-induced cell intercalation and shape changes. Similarly, FGF, GDNF and other signalling pathways have various effects on tip and stalk cells during lung and kidney branching morphogenesis. These studies have unravelled an important and general feature of branching morphogenesis: growing branches are polarized through the establishment of a tip and a stalk. At the cellular level, a few cells or a single cell take up the lead position (and only these cells show active migratory behaviour) and are followed by stalk cells in the trachea and vasculature. Epithelial cells in general compete for leading positions, and the molecular mechanisms underlying this competitive behaviour are beginning to emerge. The cell interactions that determine the tip and stalk structures in the trachea and vasculature depend on Notch-dependent lateral inhibition at the single cell level.

In other branching systems, the cellular complexity is much higher as the branching tip is composed of many cells, which makes it unlikely that the Notch pathway is involved in the segregation of tip and stalk cells. In such complex situations, a segregation mechanism based on cell affinity (for example, the ephrin signalling system) might be more likely. In addition, cell proliferation is an important factor contributing to elongation and branching in these complex systems, probably under the influence of mitogenic factors such as FGFs and GDNF. Although research still mainly focuses on the key signalling systems that regulate the initiation and maintenance of branching, recent studies have revealed that directional outgrowth is under the influence of signals that were first identified by their functions in growth cone guidance. This is firmly established and well understood for trachea and vasculature branching, but is beginning to emerge as an equally important feature during lung, kidney and mammary gland morphogenesis. These signalling systems might affect the tip or stalk structures in the lung, kidney and mammary gland.

Outlook

Two of the most burning questions still await clear answers. First, how are branch points defined in time and space? The distributions of VEGF and FGF and the temporal and spatial control of signalling in tip cells (probably regulated by other cues such as neuronal axon guidance systems) probably specify most of the branching patterns in the trachea and vasculature. The molecular and cellular logic of lung, kidney and mammary gland branch point specification remains to be unravelled. At present, it seems that tight spatial control is at work during lung branching, reiterative branching in the kidney and a space-filling mechanism during mammary gland branching morphogenesis. Similar mechanisms might, however, control the branch generator; that is, control the tips of the outgrowing epithelial buds. Second, how is branching morphogenesis correctly terminated? Feedback loops regulating termination of branching morphogenesis have been uncovered for the trachea and vasculature. Hypoxia induces ligands (BNL and VEGF, respectively) to induce new branches or sprouts, which, in turn, cause a reversion of the hypoxia by in-growing vessels that deliver oxygen, and thereby terminating the response. Similar self-regulatory epithelial–mesenchymal feedback loops also induce the termination of limb bud outgrowth (BOX 3), and it will be interesting to determine whether there are great mechanistic similarities between this and the termination of kidney and lung branching morphogenesis.

It will take many more genetic and reverse genetic analyses, combined with live imaging studies, mosaic analyses and cell ablation experiments, to formulate a comprehensive model for lung, kidney and/or mammary gland branching morphogenesis. Helped by complementary analyses in simpler branching systems or budding structures (BOX 4), we expect to soon see the first attempts to model branching in mammalian organs, taking into account the wealth of experimental data gathered. Although this might uncover interesting feed-forward

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and feedback mechanisms, it will take more time to link these signalling interactions to the cell behaviours that control the different aspects of branching morphogenesis discussed here. Mechanical forces will have to be taken into account in the morphogenetic processes, and physical constraints such as growth constraints and physical barriers, exerted by the extracellular matrix or by adjacent organ structures, will undoubtedly constitute key aspects of branching control mechanisms. It is important to realize that branching morphogenesis is integral to the proper formation and growth of all organs, as they all have to be sufficiently vascularized. This is a major unsolved problem of *in vitro* engineered tissues and organs. Therefore, the elucidation of the molecular mechanisms controlling branching morphogenesis will contribute to a much better understanding of organogenesis in general and might unravel mechanisms that are relevant to other, less spectacular morphogenetic processes.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Blistered | *Bmp2* | *Bmp4* | *Escargot* | *Fgf7* | *Fgf10* | sprouty 2 | *Wnt11*
UniProtKB: http://www.uniprot.org
BNL | BTL | DLL4 | GDNF | GREM1 | Notch 1 | RET | ROBO2 | SHH | SLIT2 | SPRY1 | VEGFA

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