

Review

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Building branched tissue structures:
from single cell guidance to coordinated
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Branched networks are ubiquitous throughout nature, particularly found in tissues that require large surface area within a restricted volume. Many tissues with a branched architecture, such as the vasculature, kidney, mammary gland, lung and nervous system, function to exchange fluids, gases and information throughout the body of an organism. The generation of branched tissues requires regulation of branch site specification, initiation and elongation. Branching events often require the coordination of many cells to build a tissue network for material exchange. Recent evidence has emerged suggesting that cell cooperativity scales with the number of cells actively contributing to branching events. Here, we compare mechanisms that regulate branching, focusing on how cell cohorts behave in a coordinated manner to build branched tissues.

This article is part of the themed issue 'Systems morphodynamics: understanding the development of tissue hardware'.

1. Introduction

When an organism reaches equilibrium with its environment, it ceases to live. Therefore, life requires a constant and dynamic transfer of energy and matter between an individual and its surroundings. For an organism to reach homeostasis throughout its body, there must be a balance between absorption, trafficking and expulsion of materials used in life. As the complexity of organisms increases, the mechanisms to achieve this fundamental objective of robust material transfer also tend to scale in intricacy. One mechanism to increase the transfer rate between an organism and its surrounding environment is to increase the surface area for exchange. Owing to spatial constraints within the organism, tissues that require large surface area for proper function often assume higher-order geometries to maximize area within a given volume. Branched tissue architecture is universally conserved in systems that demand a rapid and dense expansion of surface area for transport. Organs such as the lung, vascular network, kidney and mammary gland all use branched architectures to achieve the goal of importing, transporting or secreting materials to or from the organism. Other tissue systems, such as the neuronal network, use branching to increase the robustness of intercellular connectivity, which facilitates rapid, long-range communication between cells throughout the organism.

Branched tissue networks are built by several morphometric mechanisms. Branch generation can be directed by just a few cells or, in many cases, can involve a coordinated morphogenetic action of cell cohorts to collectively construct a branch (figure 1). In general, as the mechanisms of branching include a greater number of cells that contribute to branch formation, the regulation of collective movements of cellular cohorts increases. For example, axon branching within the neuronal network requires the morphogenetic action of a single cell responding to guidance cues [1]. This action guides axons to appropriately innervate multiple target tissues (figure 1). However, many branched tissues use some form of collective migration. Endothelial cells in blood vessel branches

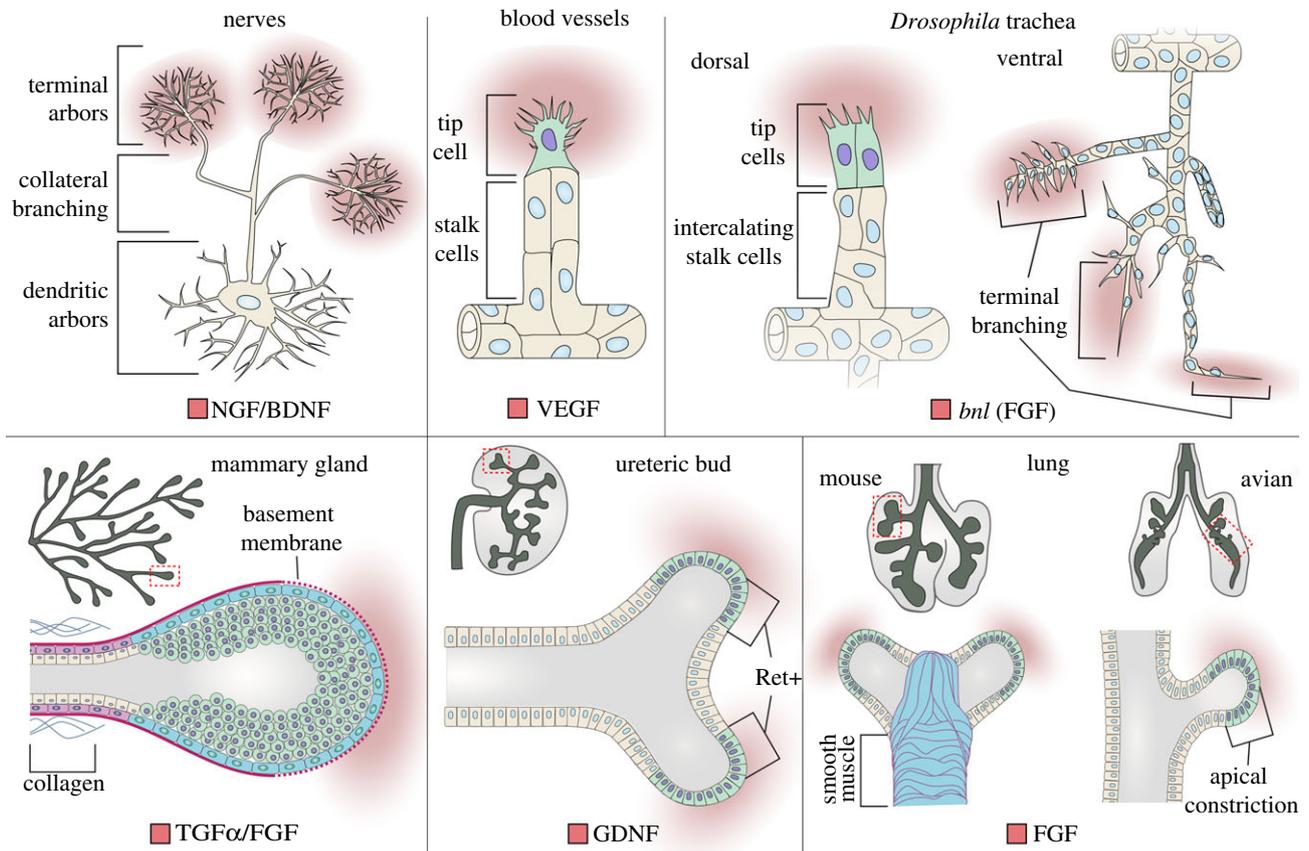


Figure 1. Branching morphogenesis drives the development of multiple organs. Some examples include the nervous system, vascular network, *Drosophila* trachea, mammary gland, ureteric network in the developing kidney and the airway epithelium in the lung. Diffusible signalling factors direct branching events; however, these factors are often specific to those particular tissues. These signals can regulate terminal branch locations and densities, as observed in nerve arborization and *Drosophila* trachea terminal tip cells. Additionally, these signalling pathways work to specify cells within cohorts undergoing branching, such as blood vessel and *Drosophila* dorsal branch tip cell selection, as well as highly proliferative Ret⁺ cells in ureteric branch tips. Mesenchyme-derived growth factors also facilitate collective migration of mammary epithelial cells to the terminal end bud (TEB). Growth factors found in the pulmonary mesenchyme promote airway epithelial branching, which requires airway smooth muscle differentiation and changes in epithelial cell shape. Top row: tissues that form branches by the action of single cells (nerves and *Drosophila* trachea terminal cells), or few cells within a cohort undergoing branching. Bottom row: branching systems that coordinate the behaviour of many cells within a cohort to drive branching. Cells that direct branching within a cohort are highlighted in green.

and cells within the branched *Drosophila* tracheal network both use similar mechanisms of specifying a population of migratory leader cells, while follower cells incorporate into the stalk of the growing branch [2] (figure 1). Other branching systems, such as the mammary gland and kidney, require multiple contributing factors for branch generation, including remodelling of extracellular matrix (ECM) components and the accumulation of highly proliferative cells in the migratory front of the branch [3–7] (figure 1). While many branched tissues are generated by focal proliferation and collective migration of the branch tip, some tissues branch independently of these mechanisms. Epithelial branching in the lung, for instance, occurs via epithelial expansion through proliferation, but there are no obvious patterns of proliferation or migration [8]. Instead, airway smooth muscle constricts growing branches to drive branch tip bifurcation [9]. Additionally, changes in epithelial cell shape, via apical constriction, drive branch initiation in the airways [8] (figure 1). Here, we compare the mechanisms that regulate the generation of branched networks as the number of cells contributing to the morphogenetic movements of the branching programme increases. In general, actomyosin dynamics serve as a conserved driving force for branching among different systems, although the signalling that regulates cytoskeletal rearrangements can

vary. When the number of cells contributing to the branch is small, actomyosin dynamics directs branching by regulating cell migration. As tissue complexity increases, actomyosin-dependent regulation of other cell behaviours, such as tissue contractility, begins to play a role.

2. Branching driven by single cell extension

(a) Nerve branching

Complex branched tissue structures can be constructed by the activity of one or very few cells within the organ system. For example, neurons can bifurcate at the axonal tip or generate collateral branches from primary axons to facilitate the innervation of various tissue targets throughout the body plan of the organism [1,10–12] (figure 1). Similar to the branching morphology of dendrites at the nerve cell body, axons often branch into arborized structures at locations of terminal innervation to increase robustness of neuronal signal transduction [1,13]. While terminal arborization and dendrite branches are morphologically distinct from the branching patterns observed along the axonal shaft, the signalling cues that specify the locations and density of branches are conserved. Therefore, understanding the spatio-temporal regulation of various

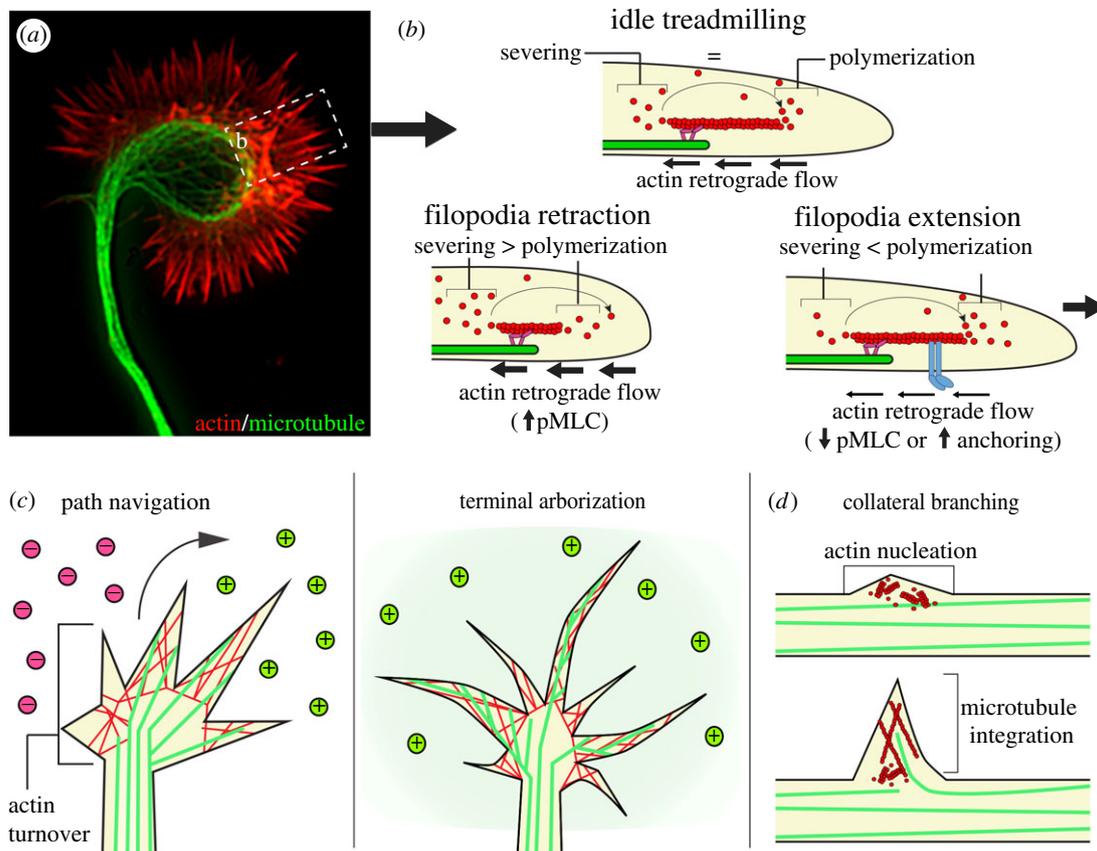


Figure 2. Actomyosin dynamics in axon branching. (a) Growth cone of a migrating axon. Actin is prominent throughout the growth cone lamellipodia and filopodia. Microtubules are characteristically found more proximal to the axon shaft, integrating into filopodia to influence actomyosin dynamics. Adapted from [15]. (b) Actomyosin dynamics found in the growth cone. When actin polymerization equals severing, F-actin maintains a homeostatic length with myosin II-mediated retrograde flow of actin (referred to as actin treadmilling). Filopodia rapidly retract when F-actin is severed and actin monomers are prevented from being added to the plus end of F-actin. Also, increased phosphorylated myosin light chain (pMLC) promotes retraction of F-actin and filopodial extensions. Conversely, increased actin polymerization, decreased pMLC or anchoring F-actin to transmembrane proteins slows actin treadmilling and promotes filopodial extensions. (c) Distribution of chemoattractive or repulsive cues directs growth cone turning and arborization. (d) Collateral branching occurs in the axon shaft, and therefore a growth cone must be constructed de novo. Actin polymers first nucleate at the incipient branch site, which is then stabilized by microtubules reorganizing into the new branch. This results in the formation of a new growth cone.

nerve guidance cues and the molecular mechanisms that alter localized cytoskeletal dynamics to initiate nerve branching have been of great interest to neurobiologists.

The terminal ends of migrating axons possess growth cones, which dynamically extend and retract filopodia from lamellipodia to explore the microenvironment for cues that direct guidance [14] (figure 2). Signals that stabilize axonal cellular extensions cause regions within the growth cone to persist preferentially in the direction of the guidance molecule. This causes the terminal ends of axons to turn towards chemoattractive cues [12,16]. Conversely, chemorepulsive cues shift the growth cone to locally retract, leading to a regional inhibition of migratory capacity [17]. The balance between attractive and repulsive cues fine-tunes the guidance of axon terminals, which ultimately establishes the architectural foundation of the branched neural network required to properly innervate the organism.

Depending on the ligand/receptor combination, guidance cues such as neurotrophins, netrins, slits and semaphorins (Semas) can act as either chemoattractive or repulsive cues. These families of molecules direct the growth of the axon by modulating actomyosin dynamics within filopodia or lamellipodia in the growth cone (figure 2a). The leading edge of growth cones of migrating axons exhibits a myosin II-mediated retrograde flow of F-actin, known as actin

'treadmilling' [14] (figure 2b). The rate of actin treadmilling opposes that of microtubule extension into the leading edge of the growth cone, which is required for stabilization of extended filopodia [17]. Additionally, the rate of actin treadmilling is inversely proportional to the advancement of the leading edge of the cell [18]. Slowing this retrograde flow of actin by either anchoring actin filaments to transmembrane receptors or shifting the balance of actin depolymerization to polymerization promotes the forward extension of filaments towards the leading edge [19–22]. This directional actin polymerization enhances filopodial elongation, which is required for axon migration and branching [23–25]. Chemoattractive cues promote local actin polymerization within the growth cone, while repulsive cues promote actin depolymerization [12] (figure 2c). When axon terminals reach tissue regions that express high levels of chemoattractive cues, such as neurotrophins (NT), terminal arborization can ensue [26]. Therefore, understanding the signalling pathways that regulate microtubule integration into axon growth cone extensions and modulate the retrograde flow rate of actin at the axonal leading edge will allow development of mechanistic models to describe axonal pathfinding and branching, including terminal bifurcations and arborization. Here, we describe major ligand/receptor combinations that alter actomyosin dynamics to facilitate chemoattractive and

repulsive axonal responses, which give rise to a branched axonal network.

Neurotrophins are a class of chemoattractive proteins that promote axonal survival and branching. Ligands within this family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4, which are secreted factors that alter actin dynamics needed for filopodial extensions during axonal pathfinding. Secreted NTs have two classes of receptors, tropomyosin receptor kinase (Trk) receptors A, B and C [27], and p75 [28]. NGF and BDNF both promote axonal filopodial extension and lamellipodial stabilization [29,30]. While these two molecules promote chemoattraction of axons, they have different effects on actomyosin dynamics. For example, NGF reduces F-actin retrograde flow by decreasing myosin light chain (MLC) phosphorylation [31] and by increasing the coupling between F-actin and focal adhesions, which shifts actin dynamics in the growth cone from a steady state of actin recycling to one of polymerization [21,31]. This allows for regional extensions of actin-rich filopodia in the direction of NGF. Filopodial extensions are also stabilized because NGF promotes the recruitment of microtubules into the newly formed filopodia [32]. As retrograde flow of actin is reduced, microtubules can preferentially polymerize in the direction of filopodial extensions [31]. Moreover, NGF activates the microtubule stabilization protein adenomatous polyposis coli [32], therefore simultaneously promoting F-actin plus-end extension and microtubule incorporation into growing filopodia to direct axonal pathfinding and terminal bifurcations.

BDNF affects growth cone dynamics by altering the relative rates of polymerization and depolymerization affecting the minus end of actin filaments. Unlike NGF, growth cone responses to BDNF appear to be independent of myosin II [33]. BDNF stimulation of axon growth cones promotes the activation of actin depolymerizing factor (ADP) and cofilin, which are both required to generate actin monomers at the minus end of filaments [33]. Although inhibiting myosin II enhances growth cone responses to BDNF, loss of myosin II activity is not required. This suggests that BDNF drives the extension of F-actin by increasing the rate of minus end depolymerization, which increases the local concentration of actin monomers that can be incorporated into the plus end required for filament extension. Similarly to NGF, BDNF also promotes microtubule integration into filopodia [34], which coupled with actin extension promotes growth cone guidance. Another line of evidence that BDNF and NGF affect actin dynamics differently is the differential responses of these two NTs to repulsive cues. Stimulation with NGF followed by a subsequent exposure to the repulsive cue Sema3A prevents growth cone collapse. However, stimulation with BDNF followed by Sema3A leads to enhanced breakdown of the growth cone [35]. Sema3A signalling promotes growth cone collapse by simultaneously activating cofilin/ADP to depolymerize F-actin [36] and endocytic absorption of growth cone plasma membrane [37,38]. Therefore, BDNF may enhance axonal sensitivity to Sema3A-mediated axon repulsion by increasing depolymerization of F-actin while Sema3A is promoting endocytic uptake of the growth cone membrane, which results in rapid retraction of filopodia and growth cone collapse.

Netrins are another class of secreted ligands that can be either chemoattractive or repulsive cues depending on the receptor expressed by the axon. Vertebrate netrin protein

members include secreted proteins netrin 1–4 [39] and glycosyl phosphatidylinositol (GPI)-anchored netrinG1 and G2 [40,41]. Here, we focus on netrins 1–3, as netrin-4 and netrinG proteins are not diffusible guidance cues. In mammals, secreted netrins include netrins 1 and 3, while birds and zebrafish use netrins 1 and 2 [42]. The mammalian orthologue of netrin 1, uncoordinated 6 (UNC6), was first identified in *Caenorhabditis elegans*, where UNC6 is critical for proper axon guidance during embryogenesis [43]. Since then, netrins 1–3 have been described as guidance cues for nerves in many vertebrate models [44–46]. Netrin ligands provide chemoattractive and repulsive signals by binding receptors of the deleted in colorectal cancer (DCC) family or UNC5, respectively [42]. During chemoattractive netrin signalling, netrin binds DCC homodimers and activates the actin-remodelling proteins Rac, Cdc42 and WASP (Wiskott–Aldrich syndrome protein)/Arp2/3 (actin-related protein) through the cytosolic adapter protein NCK1 (non-catalytic region of tyrosine kinase adaptor protein) [47,48]. This promotes growth cone expansion and filopodial stabilization in netrin-stimulated axons [49,50]. Netrin/DCC can also activate Rac and Cdc42 through focal adhesion kinase (FAK) signalling [51–55]. After netrin ligand binds DCC, FAK becomes phosphorylated, subsequently activating p130^{CAS}, which activates Rac and Cdc42 and induces chemoattraction of migrating axons [56]. Therefore, FAK and NCK1 both promote localized actin polymerization within the growth cone. Although these upstream signalling components are specific to netrin, the activation of Rac and Cdc42 results in actin dynamics that drive directional growth cone expansion similar to NGF [21]. As mentioned above, netrins can act as chemorepulsive cues, which are transduced through netrin receptors UNC-5 or DCC/UNC-5 heterodimers [57]. While the cytoplasmic domain of UNC-5 is known to be required to activate netrin-mediated chemorepulsion [58], the downstream signalling mechanism underlying the cascade remains unclear. However, netrin-1 signaling activates RhoA/ROCK (rho-associated, coiled-coil-containing protein kinase), which in turn promotes myosin light chain kinase (MLCK) phosphorylation and myosin II-mediated growth cone collapse. Dysregulation of any of these signalling components results in misguided axons *in vivo* [59].

Another class of guidance cues that possess dual functions of promoting axonal branching and chemorepulsion are the secreted Slit ligands (Slit-1, -2 and -3) that signal through roundabout (Robo) receptors [60–62]. Slit was originally identified as a chemorepulsive cue in axons during *Drosophila* embryogenesis [63]. When signalling through Robo, Slit promotes axonal guidance by modulating the activity of cytoskeletal remodelling proteins. For example, Slit inactivates Cdc42 [64–66] and promotes the activation of RhoA [59]. This probably prevents actin filament extension in growth cones as well as myosin-mediated retraction of filopodia [62]. Interestingly, Slit-mediated axon repulsion also occurs when activating Rac [65,67]. Similar to netrin/DCC signalling, NCK1 is recruited to the cytoplasmic domain of Slit-activated Robo receptor [67], which facilitates the activation of Rac. However, the signalling cascade to promote Slit-mediated Rac activation does not appear to affect Rho [67], which suggests a strong role for Rho in directing axon repulsion during Slit/Robo signalling. Slit also directs terminal arborization and collateral branching of axons. Addition of recombinant Slit2 fragments results in enhanced lateral branching of the dorsal root ganglion cells [62], and

while loss of Slit ligands or Robo receptors completely abrogates collateral axonal branch induction *in vivo*, the nascent branches are highly misguided [61]. Moreover, branching is highly abrogated in terminal sensory axonal arbors when Slit/Robo signalling is lost [61]. The seemingly opposing functions of Slit to direct axon repulsion and branch initiation could be explained by Slit's ability to dually regulate the state of Rac activation and actin polymerization.

Semas are a family of proteins that are involved with axon guidance and branching, most notably known for promoting axon repulsion [68]. Semas comprise eight classes of proteins, seven found in vertebrates and invertebrates, and one found in viruses [69]. Most of the classes of Semas proteins found in animals are membrane-bound and therefore cannot participate in long-range axon guidance. However, the invertebrate Sema class 2 and vertebrate class 3 are secreted ligands, and both are critical for proper axon guidance and direct branching [68]. Sema ligands activate signal transduction by binding plexin receptors [70], and in most cases signal transduction requires that the bound Sema ligand is stabilized to plexin through co-receptor neuropilin (Nrp) [71]. As a rapid inducer of growth cone collapse, Sema3A is one of the most highly studied Sema proteins. Sema3A drives robust actin remodeling in axon growth cones by modulating the activity of the actin-severing protein, cofilin [36]. Sema3A activates the p21-activated kinase/LIM domain kinase (PAK/LIMK) signalling pathway in a Rac-dependent manner [72]. This in turn drives cofilin phosphorylation and inactivation. Within minutes after Sema3A stimulation, phosphorylated cofilin associates with actin throughout the growth cone [36]. By an unknown mechanism, cofilin within the growth cone is subsequently dephosphorylated (activated) and actin filaments are rapidly severed, resulting in growth cone collapse [36]. Sema3A signalling through PlexinA also promotes localized translation of RhoA directly in the growth cone [73], which increases the concentration of RhoA within regions of the growth cone. Moreover, RhoA-mediated retraction during Sema3A stimulation depends on MLCII, implicating myosin contractility in collapsing the growth cone [74]. The invertebrate semaphorin isoform, Sema2A, also elicits a conserved function of axon repulsion; however, the signal transduction regulating the actomyosin dynamics within growth cones has yet to be fully elucidated [75,76]. Sema3A also directs the branch complexity of dendritic arbors around the cell body of axons, which facilitates the maturation of neural connectivity of axonal pathways. In dendritic bodies, Sema3A signalling through plexinA/Nrp stimulates FERM/RhoGEF (ARHGEF)/pleckstrin domain protein-1 (Farp1), which is sufficient and required to drive dendritic branching [77]. While Farp1 is a known activator of Rac [78], it is unclear how actin dynamics are specifically affected during Sema3A-mediated dendritic branching.

Many of the factors described above influence actomyosin dynamics along the stalk of a migrating axon to stimulate collateral branching, albeit via slightly different mechanisms (figure 2*d*). Collateral branches extend from the stalk of the axon, where no growth cone currently exists, and therefore must generate a new growth cone *de novo* prior to branch elongation [79]. Collateral branches form through the nucleation of actin filaments at putative branch sites [80], which is stimulated by growth factors such as NGF [81,82]. Actin initially accumulates in highly dynamic filopodia [83]. Microtubule bundles in the stalk of the axon are then fragmented and reorganized into a putative collateral extension [84,85].

Stabilizing parallel microtubule bundles in the filopodial extension prevents the filopodium from retracting, which is required for collateral branch formation [86]. Continued polymerization of actomyosin in the filopodia allows the new stalk of the collateral branch to extend, eventually leading to the formation of a new growth cone [1].

3. Branching driven by collective migration

Nerves provide an example of how guidance cues can influence the actomyosin machinery to initiate branches at the single cell level. There are many branched organs that require cohorts of cells to cooperatively orchestrate branching events. This can be achieved by collective migration, differential proliferation or asymmetric constriction within the cohort. Here, we review various mechanisms of branch generation driven by coordinated collective migration.

(a) Blood vessel branching

Blood vessels and nerves often follow similar migratory tracks in the body, which can be explained by conserved expression of receptors for critical guidance cues [10]. For example, both nerves and blood vessels use netrin-mediated chemoattraction and repulsion through DCC or UNC5 receptors, respectively [87,88]. While nerve and blood vessel branching networks appear similar in some regions of the body, there are other regions where differential guidance of nerves and blood vessels is required for proper tissue function [89]. To achieve this, blood vessels respond to secreted factors differently than nerves. Blood vessel branching is driven by actomyosin dynamics within filopodial extensions similar to axon growth cones. However, branching in vasculature is fundamentally different from that in nerves because cells must maintain luminal wall structure and vessel integrity during branch generation.

Blood vessels can form by two distinct mechanisms, vasculogenesis and angiogenesis (figure 3). Vasculogenesis is the formation of a vascular network *de novo* [90], whereas angiogenesis is the generation of new branches from a pre-existing vascular network [91]. During embryogenesis, mesodermal cells differentiate into angioblasts, which are precursors to vascular endothelial (VE) cells, via the simultaneous activation of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) signalling [92,93]. Clusters of angioblasts coalesce to form blood islands throughout the mesoderm [94] and stabilize cell–cell contacts through junctional proteins such as VE-cadherin and platelet endothelial cell adhesion molecule (PECAM)-1 [95]. As angioblasts proliferate, the size of the blood island increases and cells extend projections towards high levels of growth factors, such as VEGF [96]. This results in neighbouring blood islands extending towards one another, and ultimately fusing the tips of cellular extensions between cell clusters. This morphogenetic movement builds the initial vascular plexus in the embryonic mesoderm [96].

Angiogenesis can be driven by growth factor expression adjacent to an existing blood vessel or by a neighbouring tissue becoming hypoxic [91,97]. Endothelial cells throughout the vascular network express receptors for many branch-inducing growth factors, such as VEGF-receptor 2 (VEGFR2) [98]. In addition, endothelial cells can also robustly activate hypoxia-inducible factor (HIF) signalling under hypoxic

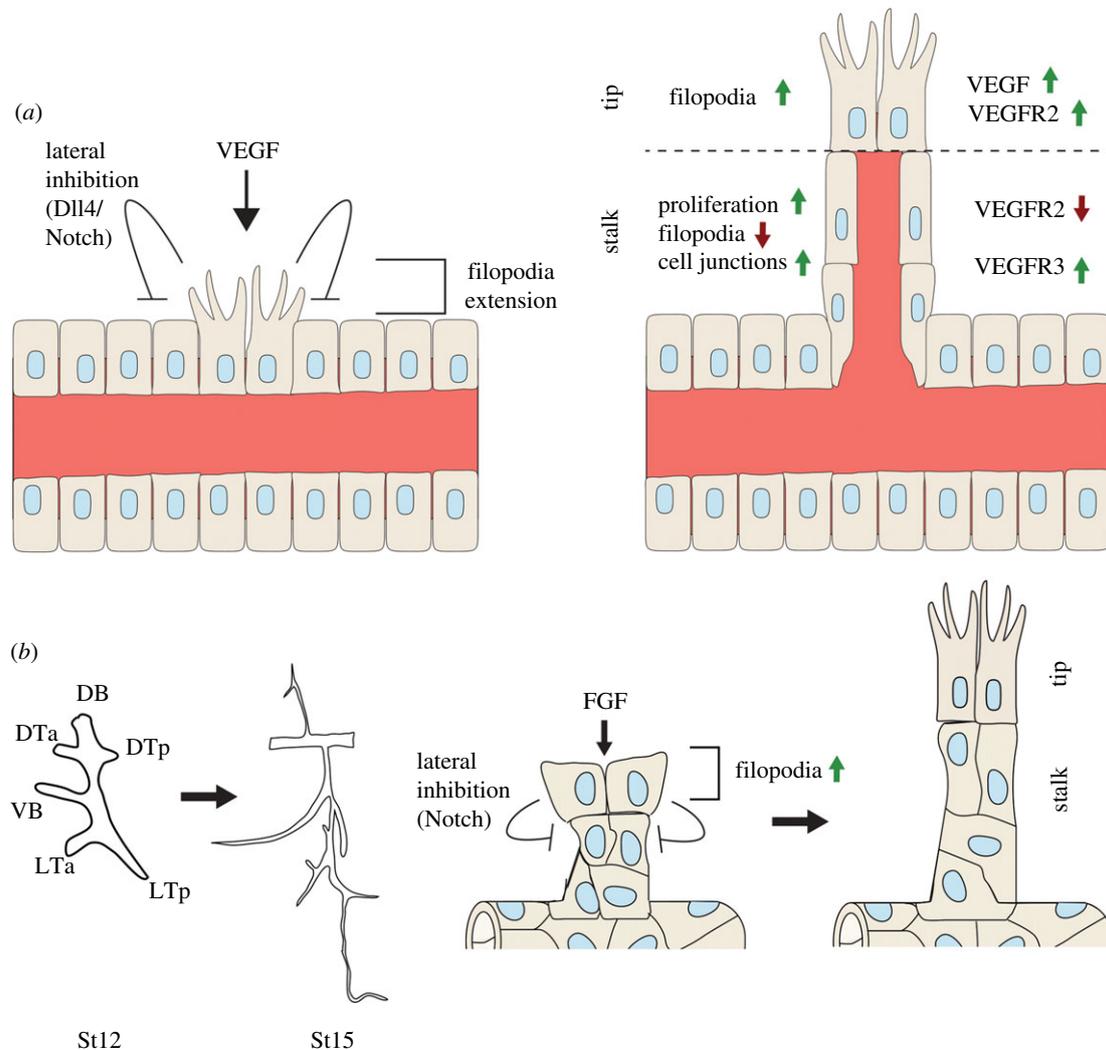


Figure 3. Contact inhibition specifies tip cells from stalk cells in blood vessels and *Drosophila* trachea. (a) When an existing blood vessel is stimulated with VEGF, cells experiencing the highest concentration of VEGF extend filopodia into the surrounding mesenchyme. Activated endothelial cells simultaneously initiate lateral inhibition of neighbouring cells, through Dll4/Notch signalling. This specifies vessel stalk cells to express VEGFR3, which drives increased proliferation and strong cell junctions. (b) Critical stages of *Drosophila* trachea branching also uses Notch-mediated lateral inhibition to specify tip cells.

conditions [99–101], which promotes vascular branching. Endothelial cells also integrate physical cues, such as fluid forces, to enhance responses to biochemical induction of vessel branching [102,103]. In VEGF-mediated induction of vascular branching, endothelial cells closest to the source of VEGF preferentially extend filopodia towards the ligand pool [104] (figure 3a). The direction of these filopodial extensions is further guided by interstitial fluid flow, which probably promotes revascularization of damaged tissue [105]. Interestingly, many of the actin-reorganizing factors used in nerve branching are conserved in VEGF/VEGFR2 signal transduction. For example, after VEGF stimulation, NCK associates with the cytoplasmic domain of VEGFR2, which in turn activates Arp2/3 and F-actin assembly [104]. Moreover, VEGF stimulates the activation of PI3 K/LIMK and p38/MK2/LIMK signalling cascades, thereby reducing cofilin activity and actin turnover [104,106]. In addition, microtubule incorporation into filopodia is required for the stabilization of endothelial cell extensions [107,108]. VEGF also stimulates the formation of focal adhesion complexes in a NCK/PAK-dependent manner [109]. These focal adhesion complexes are necessary for endothelial cells to generate the traction required for migration [110]. Under hypoxia, endothelial cells activate HIF, thus increasing VEGF expression,

which in turn promotes autocrine stimulation of VEGF signalling within patches of endothelial cells [99,101,111]. Other signals, such as platelet-derived growth factor (PDGF), promote VEGF expression in perivascular cells, therefore activating VEGF signalling locally in endothelial cells [112]. FGFs can directly enhance angiogenic branching, presumably by regulating actin filament dynamics in a similar fashion as VEGF [113,114]. Taken together, the extension, exploration and stabilization of endothelial filopodia in the direction of angiogenic cues are critical for branch formation during angiogenesis.

As mentioned above, vascular integrity is maintained during branch elongation, which requires the preservation of contacts between neighbouring endothelial cells as they migrate. Endothelial cells achieve this by specifying 'tip' cells, which extend filopodia in the direction of migration [115] while maintaining cell–cell contacts with 'stalk' cells that integrate into the wall of the new branch [2] (figure 3a). This is orchestrated by cell contact-mediated lateral inhibition, wherein tip cells provide juxtacrine signalling cues to stalk cells that suppress filopodial extensions and stabilize cell–cell contacts [116]. Lateral inhibition is mediated by Notch and is induced as tip cells begin to migrate towards VEGF (figure 3a). The tip cell expresses the transmembrane Notch ligand delta-like 4 (Dll4) [117], presenting it to neighbouring

endothelial cells that express Notch receptors [118]. Upon activation of Notch signalling, the frequency of filopodial extension by stalk cells is inhibited, partly because of down-regulation of VEGFR2 in stalk cells [119]. Stalk cells increase their expression of VEGFR3 [119], which promotes VEGF-mediated proliferation that is required for branch elongation [120]. VEGF thus specifies tip cells, which in turn specify stalk cells to facilitate vascular branching during angiogenesis. Tip cells functionally resemble the growth cone of branching axons. However, unlike axonal branching, lateral inhibition directs stalk cells to maintain contact with tip cells and facilitates collective migration of the cohort.

(b) *Drosophila* tracheal branching

Drosophila tracheal development uses some conserved mechanisms found in vascular branching. For example, endothelial cells use VEGF to specify a tip cell population that explores local microenvironments, which directs stalk cells to follow by maintaining intercellular contacts. Similarly, *Drosophila* tracheal branches use FGF signalling to select a population of cells to become migratory leaders, which maintain intercellular contacts with other neighbours in the cohort, thus facilitating collective migration during branch formation [121]. The *Drosophila* tracheal network forms between stages 11 and 16 during embryogenesis in three sequential and morphologically distinct phases [122]. By stage 11, ten placodes consisting of roughly 80 ectodermal cells form along the lateral sides of the embryo [123]. These placodes undergo invagination to form a sac, and by stage 12 six primordial tracheal branches can be identified at highly stereotypic positions [122]. These branch primordia have been divided into three dorsal branches (dorsal, dorsal trunk anterior and dorsal trunk posterior), and three ventral branches (visceral, lateral trunk anterior and lateral trunk posterior) (figure 3b). These primordia extend from the placode, giving rise to the primary branches of the tracheal network by stage 13 [122]. By stage 14, the dorsal trunk anterior (DTa) branch extends and fuses with the dorsal trunk posterior (DTp) branch growing from a neighbouring tracheal segment, creating the main tracheal branch [124]. As the remaining dorsal and ventral branches extend, single cells within these branches extend from the primary branch to form the secondary branch network (figure 3b). Depending on local gene expression in the surrounding tissues, secondary branches will either extend and fuse with secondary branches from neighbouring tracheal segments, or will generate cytoplasmic extensions to form the terminal branches used for gas exchange [125].

Interestingly, this elaborate branched structure is formed from the geometrically simple ectodermal placodes without cell proliferation. For this reason, many studies have investigated how these cells collectively migrate and rearrange during branch generation. As mentioned above, DTa and DTp are two of the three primary dorsal branches. The DTa and DTp branches extend towards neighbouring tracheal cell clusters, until fusing with the next closest DT branch to form a single dorsal trunk tube [124]. The DB cells undergo 'tip' cell-mediated migration that resembles migratory patterns observed in vascular angiogenesis [121]. These tracheal cells compete for the position in the cohort that is surrounded by the highest concentration of branchless proteins (*bnl*), the *Drosophila* homologue of FGF [125]. Cell position within the extending branch is mediated by signalling through Notch,

echoing mechanisms in angiogenic sprouting. In fact, Notch is capable of specifying cells within the dorsal branch of the trachea to become fusion cells to link the tubular network between the extending DBs [126].

The ventral branches of the trachea undergo extensive secondary branching. Similar to dorsal branch extension, ventral branches exhibit collective migration with tip cells extending highly dynamic filopodia towards regions of high *bnl* (FGF) expression [127]. Continual *bnl*/FGF stimulation of tracheal cells promotes activation of the transcription factor *pointed-1*, which drives the differentiation of primary branches into secondary branches [122]. The secondary branches again extend similarly to migratory patterns observed in endothelial cells. As secondary branches elongate, cells activate *terminal-1* and branched protrusions from individual cells extend in a non-stereotyped manner [122]. An explanation for the highly variable branching patterns of terminal cellular branch extensions could be that terminal branches sense target tissue where oxygen is needed. As expected, *bnl*/FGF-mediated terminal branching is greatly enhanced under hypoxia [128–130]. This shows that the tracheal tubular network is initially highly stereotyped during branching morphogenesis, but the densities of terminal branches are refined to tissue regions in need of greater oxygen supply.

(c) Mammary gland branching

Like branching in the *Drosophila* trachea and vertebrate blood vessels, mammary gland epithelia branch through concerted collective migration; however, this group of cells promotes branching without extending filopodia directly into the surrounding mesenchyme [131]. Instead, mammary epithelial cells within the terminal end bud (TEB) constantly compete for a position at the leading edge of the epithelial cohort. This collective migration, coupled with proliferation at the tip of the branch, generates a highly branched epithelial network [4].

In the mouse, mammary gland development is initiated at embryonic day (E) 11.5, when epithelial cells form a stratified placode and invaginate into the underlying mesenchyme [132]. By E14.5, the primary mammary branch has formed, with epithelial cells condensed into a single stalk and terminal bulb [132]. At birth, mice have a rudimentary gland with approximately ten branches, each containing a multi-cell-layered TEB [4]. Throughout puberty, the mouse mammary gland progressively expands into a ductal tree [133] via duct elongation and non-stereotypic dichotomous and lateral branching [134] (figure 4). Branching of the mammary ductal tree requires coordinated morphogenesis by luminal epithelial cells, as well as the myoepithelial cap cells that encase the TEB [136]. The mechanisms that promote branching morphogenesis in mammary epithelial cells can be summarized into three major categories. First, localized proliferation within the TEB drives directional branch extension [137]. Second, remodelling of the ECM by either proteolytic turnover or localized expression promotes or constrains epithelial branch extension, respectively [138]. Last, collective migration within the TEB cohort can drive extension or bifurcation of the TEB [131].

Growth factors and hormones both stimulate proliferation and branching in the TEB, as well as the formation of lateral branches along the subtending duct [136]. Growth factors including epidermal growth factor (EGF), transforming growth factor alpha (TGF α) [139,140], TGF β [141,142], FGF

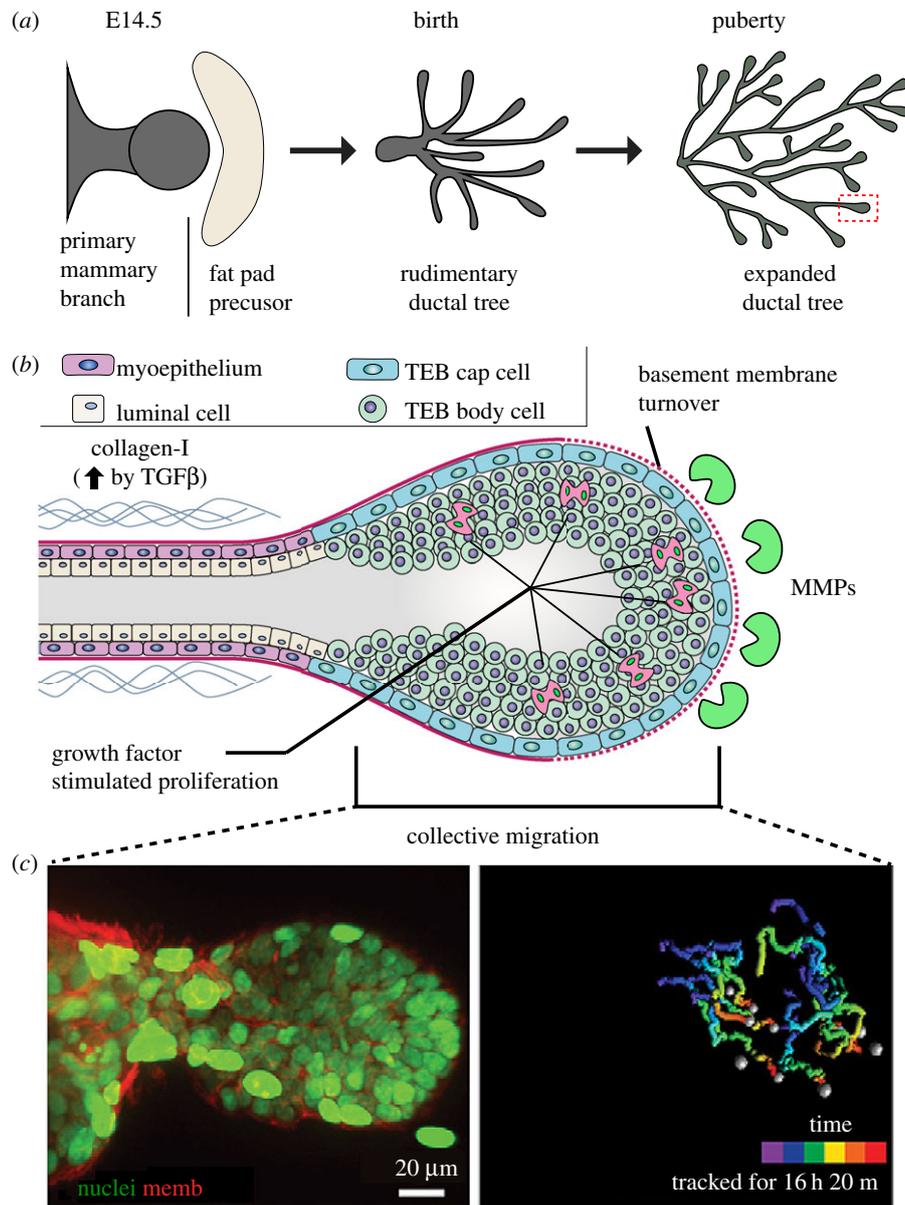


Figure 4. Mammary gland development and regulation of branching at the TEB. (a) Critical stages of mammary gland development, including embryonic, pre-pubertal and pubertal. (b) Major contributing factors that regulate mammary TEB branching involve ECM growth factor-stimulated cell proliferation, ECM remodelling and (c) collective cell migration within the TEB. Adapted from [135].

[137], insulin-like growth factor (IGF) [143,144], hepatocyte growth factor (HGF) [145,146] and hormones such as oestrogen [147] have all been found to influence branching morphogenesis in the mammary gland. Interestingly, loss-of-function experiments of all the above growth factors, excluding TGFβ, result in reduced proliferation, as well as abrogated ductal elongation and lateral branching. These data clearly indicate the importance of proliferation throughout the ductal network to promote branching morphogenesis. Loss of TGFβ signalling, however, increases lateral branching [141,142]. TGFβ is a known suppressor of growth factors that promote mammary branching morphogenesis, such as IGF [148] and HGF [149]. When kinase-deficient TGFβ receptor is expressed in the mammary gland stroma, TGFβ signalling is lost and lateral branching of the mammary gland is increased. This change in branching morphogenesis occurs with the concomitant upregulation of HGF and IGF [142], indicating that the balance of growth factors within the mammary gland stroma directs the density of branches of the mammary epithelium. TGFβ

also regulates ECM synthesis in other epithelial branching systems [150], and aberrant ECM may promote lateral branching in the mammary gland.

The distribution of ECM around mammary epithelial branches appears to be differentially regulated [151], and it is likely that spatio-temporal regulation of ECM remodelling helps drive branching morphogenesis. To understand the importance of ECM remodelling in mammary branching, several mouse models have been generated with dysregulated matrix metalloproteinase (MMP) expression. Mice that overexpress MMP3 [152] or MMP14 [138] exhibit increased lateral branching in the mammary gland, indicating that spatial remodelling of ECM components may facilitate branch outgrowth. The available data suggest that a partially degraded ECM is compliant enough to facilitate branch extension [153], therefore regulating branching density.

Mammary epithelial cells migrate collectively within the TEB using mechanisms distinct from those described above [131]. Because luminal epithelial cells are surrounded by

cap cells, they do not extend filopodia directly into the surrounding mesenchyme, as observed during branching of the vasculature and *Drosophila* trachea [154]. Mammary epithelial cells instead constantly rearrange and compete for a front position in the TEB [131]. As epithelial cells migrate closer to the TEB, cells increase the migration rate and directional filopodial extensions towards the elongating front of the TEB in a pERK/Rac1-dependent manner [135]. Therefore, regulation of mammary branching morphogenesis probably couples a coherent migratory pattern within the TEB with growth factor stimulated-proliferation and regional ECM remodelling to fine-tune ductal elongation and lateral branching (figure 4*b,c*).

4. Branching driven by non-migratory mechanisms

Branching morphogenesis can also be directed by mechanisms other than directed cell migration. For example, branching can result from localized proliferation within a cohort of cells or by localized changes in cell shape. Here, we review examples that demonstrate how branching is driven via mechanisms independent of collective migration.

(a) Kidney branching

Branching of the ureteric bud in the kidney is largely driven by localized proliferation in the tips of epithelial branches [6,155]. The initiation of the Wolffian stalk, which gives rise to the stalk of the ureteric network, is driven by collective migration and rearrangement of epithelial cells to the location of branch extension in a glial cell line-derived neurotrophic factor (GDNF)/Ret-dependent manner [156]. After formation of the Wolffian duct, the ureteric bud undergoes rapid and iterative branching that depends on reciprocal signalling between the mesenchyme and ureteric branch epithelium [157–159]. GDNF expressed in the proximal mesenchyme signals through its receptor, Ret, to specify epithelial branch tip cells that contribute to new branches [7]. In the presence of GDNF, Ret-positive epithelial cells proliferate to preferentially exclude Ret-negative cells from the growing branch tip [7]. In addition to GDNF/Ret signalling, FGF signalling through FGFR2 is also required for robust branching of the ureteric epithelium [160]. Interestingly, loss of FGFR2 expression in the ureteric bud results in reduced proliferation and significantly smaller branched networks within the kidney [160]. The distribution of these growth factors throughout the metanephric mesenchyme is probably regulated by ECM sequestering. Sulfated proteoglycans influence the distribution of active growth factors in many tissues [161]. Proper O-sulfation of heparan sulfate proteoglycan (HSPG) is required for robust ureteric branching [162]; therefore ECM control of the spatio-temporal distribution of growth factors facilitates proper ureteric branching. Collectively, these data show that growth factor-driven proliferation can give rise to stereotyped branched networks.

(b) Lung branching

Unlike the other branching programmes described here, the vertebrate lung does not employ collective migration or localized proliferation to generate new branches. Instead, the airway epithelium uses global proliferation to extend the

epithelial surface, but the branches themselves are generated by several other contributing mechanisms, including epithelial shape change and localized smooth muscle cell differentiation.

Starting at E4 in the chick, E9 in the mouse and E26 in human, the primary bronchi of the lung extend from the gut primordium [163–166]. Avian lungs undergo monopodial branching, where secondary branches form laterally from the primary bronchus [167]. As these secondary branches elongate, projecting towards the lung mesothelium, monopodial branching generates tertiary bronchi, which give rise to the parabronchi [168]. The parabronchi from the dorsal secondary bronchi extend and approach the elongating tips of parabronchi derived from ventral secondary bronchi. At this stage of avian lung development, parabronchial tubes fuse via anastomosis, which establishes a luminal network that connects the dorso and ventrobronchi. This ultimately gives rise to a continuous, unidirectional flow of gas from the dorsobronchi to the ventrobronchi in the avian lung [169].

In mammals, the first set of branches from the primary bronchus, or domain branches, also form in a monopodial manner. In the mouse, domain branching from the right primary bronchus gives rise to the cranial, middle and accessory lobes of the lung, while the right caudal and left lobes originate from the primary bronchus itself [170]. As the middle and accessory lobes elongate, another round of domain branching generates the secondary branches of these lobes. Secondary branches of the right caudal and left lobes are also generated by domain branching. As these branches elongate, the complexity of the tree is rapidly increased by recursive dichotomous branching, wherein the tip of the elongating branch bifurcates into two new daughter branches [170]. These bifurcation events require a specific spatio-temporal accumulation of smooth muscle cells at the cleft of the bifurcation site [9], suggesting that smooth muscle contractility constrains the growing epithelium (figure 5*a*).

Extensive work has been devoted to elucidating the molecular basis of branch generation in vertebrate lungs. Interactions between the airway epithelium and mesenchyme direct the branching programme in the lung. For example, transplanting bronchial mesoderm to the trachea induces ectopic branching events, whereas tracheal mesoderm prevents bronchial branching [171]. This suggests that local secretion of growth factors could drive branching in the lung. FGF10 has been implicated as a critical signal that regulates branching of airway epithelial cells. FGF10 transcript appears to be localized adjacent to epithelial branches in both avian [172] and mouse [173] lungs. Moreover, FGF10 increases epithelial proliferation and branch extension in cultured lung explants [173,174] and FGF10-deficient mice fail to form primary bronchi [175]. These data clearly indicate that mesenchymal FGF10 is sufficient to drive chemotaxis and proliferation of lung epithelial cells and is required for proper lung development. Therefore, FGF10 was hypothesized to promote differential proliferation to specify sites of branch formation in the lung. To address this hypothesis, investigators have employed mesenchyme-free cultures of lung epithelium to test the role of growth factors to induce differential proliferation as a mechanism to promote branch formation. Interestingly, FGF stimulation of mesenchyme-free airway epithelium promotes a homogeneous increase in proliferation, with no correlation between branching pattern and focal epithelial proliferation [176]. In fact, these culture systems revealed that epithelial branching precedes the establishment

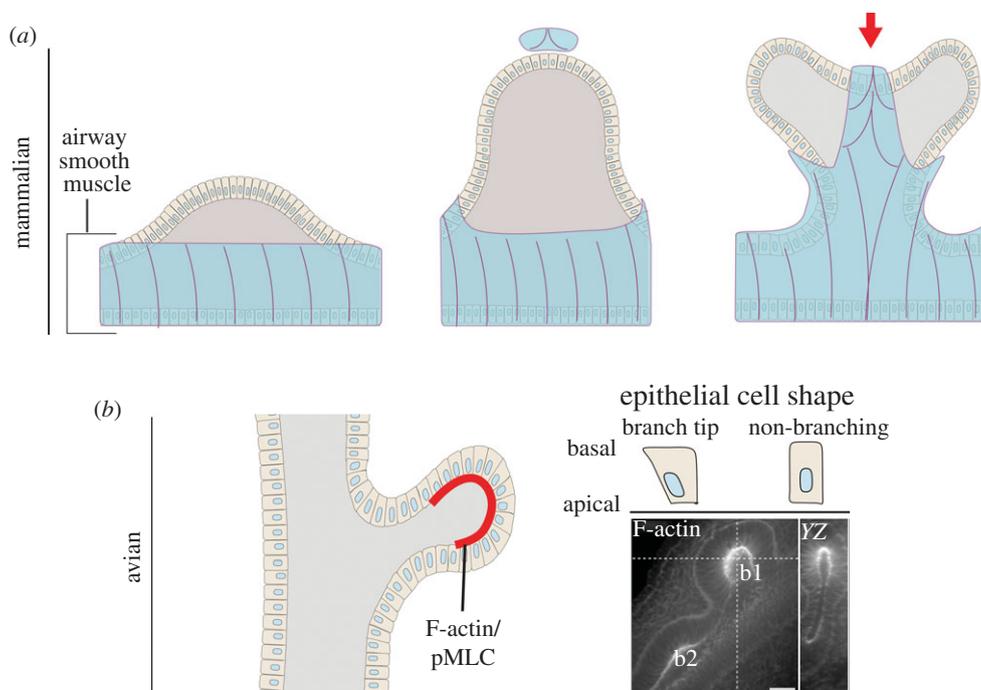


Figure 5. Comparison between branching mechanisms in the mammalian and avian lung. (a) In the mammalian lung, the epithelium bifurcates; branch extensions bifurcate to generate two daughter branches. Bifurcations require the differentiation of airway smooth muscle at the tip of the branch. (b) Airway branching morphogenesis in the avian lungs occurs by epithelial cells changing shape. Epithelial cells within the branch accumulate apically localized F-actin and pMLC. Cell contraction of the apical surface causes epithelial cells to become trapezoidal and is required for branch formation. This contraction causes branches to assume a figure-eight-shaped cross-section. Confocal image was adapted from [8].

of differential proliferation patterns, suggesting that local proliferation is not required for branch initiation.

To further understand the role of proliferation in airway epithelial branching, embryonic chicken lungs were cultured in the presence of the cell cycle inhibitor, aphidicolin. Despite inhibiting cell proliferation, new branches still formed in these explants, suggesting that proliferation is not required for branch initiation in the chicken lung [8]. Instead, the airway epithelial cells were found to constrict their apical surfaces, a cell shape change that was sufficient to generate a branch [8] (figure 5b). As FGF family members can modulate actomyosin dynamics in several tissues [177,178], FGF may act to initiate branching in avian lungs by locally inducing apical constriction of the epithelium. Consistent with this hypothesis, embryonic chicken lungs cultured in the presence of the FGFR inhibitor, SU5402, fail to undergo apical constriction or to initiate branching [8]. It remains unclear if domain branching in the mammalian lung also proceeds via apical constriction. Collectively, these data suggest that FGF secreted from the mesenchyme is critical for branch growth, but does not initiate branches via differential proliferation. Instead, branches form through local changes in epithelial cell contractility, which are probably mediated by FGF signalling.

5. Conclusion

When comparing mechanisms of branching morphogenesis across multiple tissues, there are several principles that are conserved in all models. First, branching seems to be influenced by diffusible factors that are spatio-temporally regulated by populations of cells proximal to branch sites. Therefore, communication between different tissues is critical for proper branch site specification and initiation. Second,

many branching events involve cytoskeletal rearrangement, which affects cellular behaviours such as migration and contraction. Although various models use different signalling cascades to direct branching, actomyosin dynamics is often at the core of the morphogenetic action. A third conserved theme observed across branching models is that as the number of cells within the tissue undergoing morphogenesis increases, so must the cooperativity between cells actively contributing to the branching event. This includes mechanisms such as lateral inhibition in blood vessels and *Drosophila* trachea, collective migration in mammary gland TEB, specification of highly proliferative Ret-positive cells in ureteric branch tips and changes in epithelial cell shapes in the branching airway epithelium. In contrast to the organogenesis of non-branched tissues, such as skin, tissues undergoing branching morphogenesis exhibit dynamic changes in geometry. In addition, these branched structures must integrate into surrounding tissues, a process that is often regulated by factors secreted by cells in neighbouring tissue layers. This further illustrates the importance of communication between tissue layers during the formation of branched networks.

As we further understand the cooperative nature of these branching mechanisms, additional questions emerge. For instance, collective migration of mammary epithelial cells is important for branching, but how these migratory patterns generate forces required for branch extension is not clear [131]. It is possible that differential migratory rates could preferentially push slower cells towards the stalk of the branch [135]; however, the role of this particular characteristic of migration dynamics within mammary TEB has yet to be investigated. Additionally, changes in cell geometry via asymmetric cell contractility can initiate branching, as in the lung branching programme [8]. It is not clear, however, how regions in the epithelium are specified to initiate changes in cell shape.

To address these types of complex questions, future studies will probably need to consider coordinated and cooperative mechanisms within cell populations to fully understand how branch initiation and elongation are regulated.

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