TOPICAL REVIEW

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TOPICAL REVIEW

How to build an epithelial tree

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Abstract

Nature has evolved a variety of mechanisms to build epithelial trees of diverse architectures within different organs and across species. Epithelial trees are elaborated through branch initiation and extension, and their morphogenesis ends with branch termination. Each of these steps of the branching process can be driven by the actions of epithelial cells themselves (epithelial-intrinsic mechanisms) or by the cells of their surrounding tissues (epithelial-extrinsic mechanisms). Here, we describe examples of how these mechanisms drive each stage of branching morphogenesis, drawing primarily from studies of the lung, kidney, salivary gland, mammary gland, and pancreas, all of which contain epithelial trees that form through collective cell behaviors. Much of our understanding of epithelial branching comes from experiments using mice, but we also include examples here from avian and reptilian models. Throughout, we highlight how distinct mechanisms are employed in different organs and species to build epithelial trees. We also highlight how similar morphogenetic motifs are used to carry out conserved developmental programs or repurposed to support novel ones. Understanding the unique strategies used by nature to build branched epithelia from across the tree of life can help to inspire creative solutions to problems in tissue engineering and regenerative medicine.

Abbreviations

BARW	branching-and-annihilating random walk
BM	basement membrane
CDH	congenital diaphragmatic hernia
CE	convergent extension
ECM	extracellular matrix
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GDNF	glial cell line-derived neurotrophic factor
MMP	matrix metalloproteinase
OCD	oriented cell division
PCP	planar cell polarity
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

1. Introduction

Branched epithelial trees are found within several organs in our bodies. Their specialized structures maximize surface area and the number of branch tips (usually the site of functional units in the organ), allowing for the flow and exchange of fluids [1–3]. Branching morphogenesis, the process that builds these epithelial trees, requires both biochemical [3] and mechanical signals [2]. Despite the common general purpose of branched organs, nature has evolved a beautiful diversity of branched architectures and strategies for branching morphogenesis across the tree of life. Conceptually, branching morphogenesis can be broken down into simple and discrete steps: branch initiation, branch extension, and branch termination. Here, we examine each of these

steps and, for each, review examples of how epithelial trees sculpt themselves (intrinsic mechanisms) and examples of how the epithelium is shaped by surrounding tissues (extrinsic mechanisms).

Each branched organ has a unique epithelial organization, the properties of which dictate the type of branching that occurs (figure 1) [1]. Epithelial trees can be made up of single-layered sheets of cells that are folded into tubes, like in the kidney and lung, or as multi-layered masses of cells that eventually resolve into ducts, like in the salivary gland, mammary gland, and the pancreas. Terminal buds may contain dozens or hundreds of epithelial cells, which may be cuboidal, columnar, pseudostratified, or stratified. The epithelial tree has long taken center stage in studies of branching morphogenesis, partly because it is itself branched, but also because of the remarkable ability of isolated epithelia to form three-dimensional, branched-like structures in culture[4–6]. The epithelial-intrinsic mechanisms that drive these morphogenetic events in culture may play roles *in vivo*, but there are many differences between the branching that occurs in isolated epithelia and in intact organs.

Epithelial trees are surrounded by mesenchymal cells that influence the specification, initiation, extension, and shaping of epithelial branches. Classic tissue-recombination studies uncovered the importance of epithelial–mesenchymal interactions in branching morphogenesis [7, 8]. For example, the epithelium of the quail anterior sub-maxillary gland (also known as the submandibular gland) [9] normally elongates and is surrounded by dense BM and circumferentially aligned mesenchymal cells. However, epithelium isolated from the sub-maxillary gland can be induced to branch when transplanted into mesenchyme isolated from the quail sub-lingual gland or murine submandibular gland [10], both of which support branching of their resident epithelia. These experiments revealed that the mesenchymal tissues surrounding epithelia are powerful drivers of branching morphogenesis. The host mesenchymes of branched organs are as diverse in their organization as their resident epithelial trees. Each mesenchyme is comprised of or differentiates into several different cell types, produces a suite of growth factors, and provides specific mechanical signals that influence the shape of the adjacent epithelium (figure 2). Branched epithelia are also surrounded by ECM, including the aforementioned BM and the interstitial matrix, to which cells adhere via cell–ECM adhesions. The microenvironment surrounding the epithelium provides extrinsic control over branching, adding to the diversity of morphogenetic motifs that are used to build epithelial trees.

While not the main focus of this review, it is important to note that several types of conceptual models have been constructed to provide frameworks for understanding the process of branching

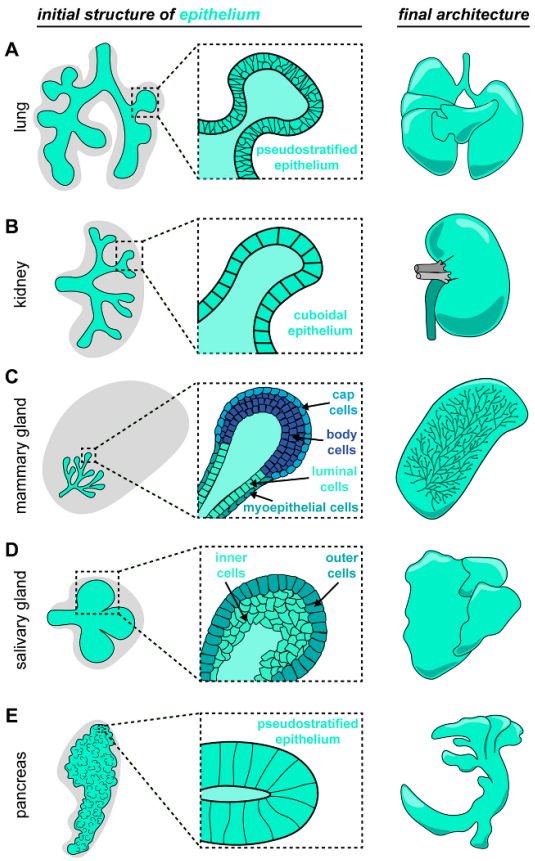

Figure 1. Schematics of branched organs in the mouse. (A) The embryonic lung epithelium is pseudostratified during branching morphogenesis. (B) The kidney epithelium consists of a simple tube of cuboidal cells as the uretic bud undergoes branching. In the postnatal and adult kidney, the epithelia of the collecting ducts and the proximal tubule of the nephron remain cuboidal. (C) In the branching mammary gland, the ductal epithelium consists of a single layer of luminal epithelial cells surrounded by myoepithelial cells. In contrast, cap cells surround body cells in the TEBs. (D) The tip epithelium of the branching salivary gland consists of outer and inner cell layers. (E) The pancreas begins as a multi-layered epithelium that resolves into a pseudostratified epithelium as branching occurs.

morphogenesis. These models fall broadly into three categories: network-scale models, morphogen diffusion models, and mechanical models (reviewed in [11]). Network-scale models are used to predict the overall patterns of branching and are agnostic to the cells that comprise the tree or the biochemical or physical factors involved in its morphogenesis [12–16]. Morphogen diffusion models include reaction-diffusion models and diffusion-limited growth models. Reaction-diffusion models assume that branching morphogenesis is templated by patterns of diffusible morphogens, and are used to predict regions of high morphogen concentration based on the expression of inhibitors that operate in feedback loops [17]; these models can be solved using simple toy geometries [18, 19] or geometries obtained from real organs [20, 21]. In diffusion-limited growth models, the simulated epithelium grows in response to and depletes a morphogen, resulting in a strong dependence on epithelial shape: protruding regions

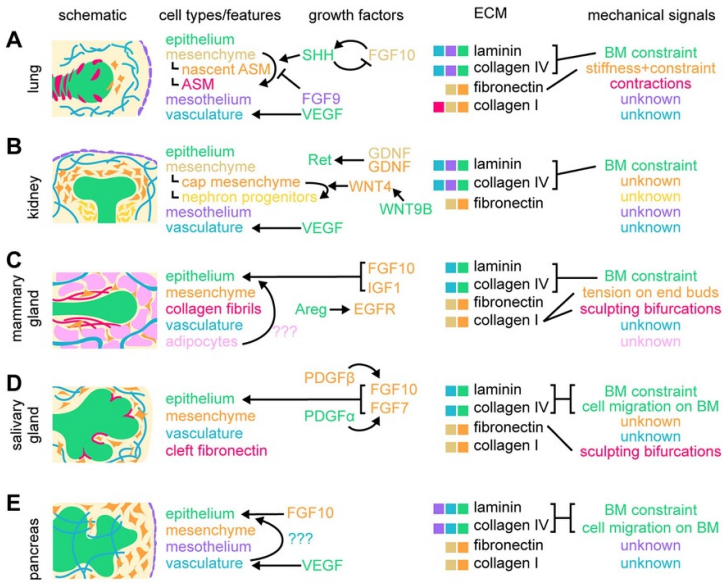

Figure 2. Composition of the mesenchyme in branched organs of the mouse. (A) The embryonic lung contains the airway epithelium, the surrounding mesenchyme (including progenitor cells that differentiate into ASM), and vasculature, and is surrounded by the mesothelium. An FGF10-SHH signaling loop regulates branching and smooth muscle differentiation. Mesothelial FGF9 blocks smooth muscle differentiation and epithelial VEGF induces formation of the vasculature. The epithelium, mesothelium, and vasculature each contact a BM containing laminin and collagen IV (color-coded boxes indicate the cell types that interact with each ECM component). The mesenchyme contains interstitial ECM proteins, including fibronectin and collagen I. Differentiating smooth muscle and interstitial fibronectin constrain the epithelium. (B) The embryonic kidney contains the ureteric epithelium, the surrounding mesenchyme (including cap mesenchyme and nephron progenitors), and vasculature, and is surrounded by the mesothelium. Mesenchymal GDNF signals via epithelial Ret to regulate branching. Epithelial WNT9B signals through mesenchymal WNT4 to specify nephron progenitors from the cap mesenchyme. The BM of the epithelium, mesothelium, and vasculature contain laminin and collagen IV. The mesenchyme contains fibronectin. The epithelium is thought to be constrained by its BM. (C) The pubertal mammary gland contains an epithelium, mesenchyme, vasculature, and adipocytes. Mesenchymal FGF10 and IGF1 signal to the epithelium, and epithelial Areg signals to the mesenchyme via EGFR. Adipocytes communicate with the epithelium via unknown signals. The BM of the epithelium and vasculature contain laminin and collagen IV. Collagen fibrils sculpt epithelial bifurcations and may generate tension on branch tips. (D) The embryonic salivary gland contains the epithelium, mesenchyme, and vasculature. Mesenchymal FGF7 (downstream of epithelial PDGFα) and FGF10 (downstream of mesenchymal PDGFβ) signal to the epithelium. The BM of the epithelium and vasculature contain laminin and collagen IV. The mesenchyme contains interstitial ECM proteins, including fibronectin and collagen I. The BM provides constraint and promotes epithelial cell migration. Fibronectin accumulates in clefts and sculpts bifurcations. (E) The embryonic pancreas contains an epithelium, mesenchyme, and vasculature, and is surrounded by the mesothelium. Mesenchymal FGF10 signals to the epithelium. Epithelial VEGF signals to the vasculature, and the vasculature regulates morphogenesis of the epithelium through unknown signals. The BM of the epithelium, mesothelium, and vasculature contain laminin and collagen IV. The mesenchyme contains interstitial ECM proteins, including fibronectin and collagen I. The BM provides constraint and promotes epithelial cell migration.

(or branches) are exposed to higher concentrations of morphogen and therefore grow more [22–24]. Morphogen diffusion-based models also typically assume the epithelium somehow 'grows' towards regions of high morphogen concentration without defining whether this growth results from cell proliferation, rearrangements, collective migration, or some other mechanism. Mechanical models are used to elucidate how the physical properties (e.g. mechanical stiffness) and growth rates of simulated tissues generate different morphologies [25–29]. Models of different types can be combined to build multiscale simulations, as has been used to model branching morphogenesis of the kidney [30]. Network-scale models, reaction-diffusion models, and mechanical models each have useful features (and oversimplifications) and, when used to test predictions experimentally, can provide insight into how epithelial trees are constructed.

2. Mechanisms for branch initiation

2.1. Epithelial-intrinsic mechanisms

Invasion, which drives the initiation of branches in the *Drosophila* trachea and in the vertebrate vasculature, is one morphogenetic motif that has yet to be observed in branched epithelia of vertebrate organs [31]. In vertebrates, epithelia instead form new branches by shaping cell collectives through several different non-invasive morphogenetic motifs.

2.1.1. Apical constriction

In many morphogenetic processes, epithelial cells actively fold locally into wedged shapes that generate global changes in tissue shape. For example, during gastrulation in *Drosophila*, the presumptive endoderm undergoes apical constriction, which leads to tissue folding and begins the process of invagination [32]. Thus, one attractive hypothesis for the initiation

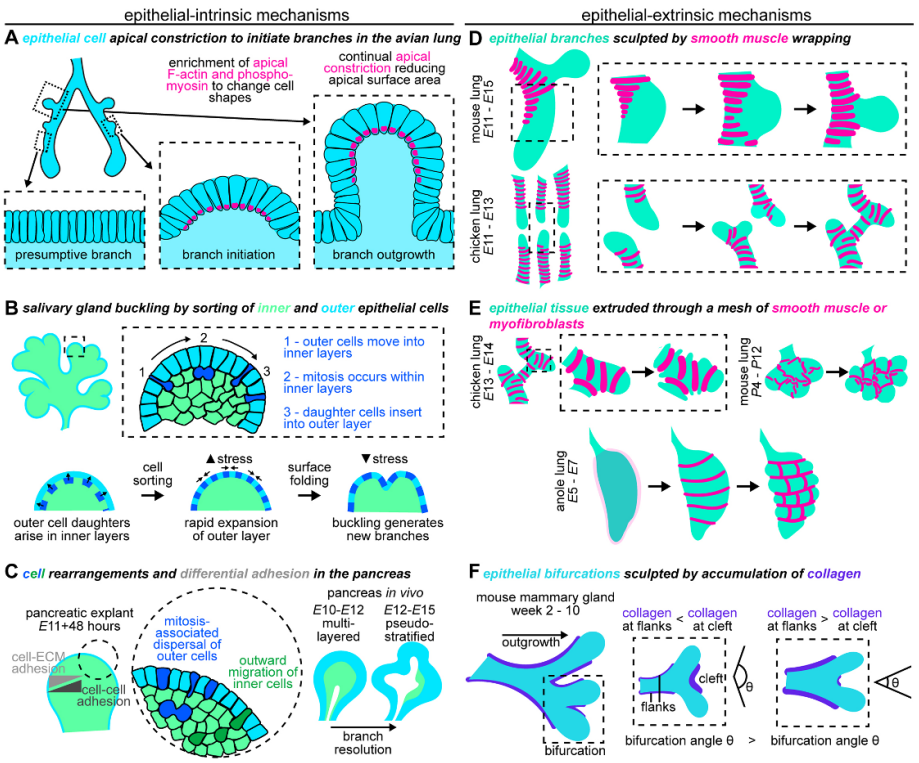

Figure 3. Intrinsic and extrinsic mechanisms that drive initiation of epithelial branches. (A) Branches initiate in the avian lung by apical constriction of the columnar epithelial cells at specific sites along the primary bronchus. Apical constriction is characterized by the enrichment of F-actin and phospho-myosin at the apical side of the cell. (B) Branches initiate in the salivary gland epithelium by expansion and then folding of the outer cell layer. This expansion and the subsequent buildup of stress in the surface plane of the tissue arises because outer cells divide within the inner layers and their daughter cells are then sorted back into the outer layer. (C) In pancreatic explants, outer cells divide within the inner layers and then return to the outer layer, and inner cells migrate into the outer layer. Cell–ECM adhesion dominates outer cell behavior, while differences in cell–cell adhesion sort inner cells away from outer cells. *In vivo*, inner and outer cell layers resolve into a single-layered pseudostratified epithelium as branching progresses. (D) Smooth muscle wrapping constrains the epithelium to initiate new branches in early mouse lungs and anastomosis-stage chicken lungs. (E) Branches and atria extrude through a smooth muscle mesh around the epithelium in the early anole lung and late chicken lung, respectively. Alveoli extrude through a mesh of myofibroblasts that surrounds the epithelium during alveologenesis in the mouse lung. (F) The angle of bifurcation (θ) in the mammary gland is tuned by the relative accumulation of collagen in the cleft versus along the flanks.

of new branches is that localized activation of the actomyosin cytoskeleton constricts the apical surfaces of cells at presumptive branch sites, inducing localized folding. This morphogenetic motif appears to drive branch initiation in the embryonic chicken lung, where lateral branches emerge from the primary bronchi in stereotyped locations [33, 34]. The highly columnar epithelium at these locations undergoes a myosin-dependent apical constriction (figure 3(A)), which is sufficient to establish the initial geometry of the branch [35]. However, apical constriction alone is not sufficient to drive branch extension, indicating that other forces are required to refine the epithelium after this initial change in cell and tissue shape [35].

Apical constriction has also been suggested to drive branch initiation in the embryonic mouse lung. Prior to forming branches, mouse airway epithelial cells have been observed to thicken along their apical-basal axes in a *Fzd2*-dependent manner [36]. Epithelium that lacks *Fzd2* fails to thicken, forms branches at a reduced rate, and eventually becomes swollen and cystic. However, apical constriction has not been reported to occur in the mouse lung epithelium prior to the emergence of a presumptive branch, so the extent to which this physical mechanism is conserved remains unclear. β-catenin has been hypothesized to play an important role in changes in epithelial cell shape during branching in the mouse lung. Loss of epithelial β-catenin results in severe, lung-wide defects, including an increase in airway diameter and a loss of distal airways [37]. Pharmacologically inhibiting β-catenin leads to a cystic morphology in lungs cultured as explants [38]. When the mesenchyme is surgically removed from the embryonic airway epithelium, inhibiting β-catenin leads to a decrease in the number of branches that form in culture whereas activating β-catenin leads to an increase [38]. However, these studies did not report the shapes of individual epithelial cells before and after branch initiation. Furthermore, genetically deleting β-catenin from the lung epithelium has no effect on the formation of the initial lobes and primary bronchi [37]. The evidence for apical constriction in the mouse lung is therefore dubious at best.

During branching morphogenesis of the ureteric bud, newly formed branches contain wedge-shaped

epithelial cells, in striking contrast to the columnar cells that populate the rest of the kidney epithelium [39]. The apical surfaces of these wedge-shaped cells are enriched in F-actin and phosphorylated myosin, consistent with localized contraction of the actomyosin cytoskeleton [39, 40]. Inhibiting the activities of myosin and Rho kinase (ROCK) disrupts branching in cultured kidney explants, suggesting that apical constriction initiates branching in the kidney. Inhibiting signaling through GDNF, which is required for branching morphogenesis of the ureteric bud [41], results in a loss of F-actin at the apical surface of cells located in the tips of epithelial branches [40]. These data suggest the enticing possibility that localized actomyosin contraction is downstream of biochemical signals supplied by the mesenchyme adjacent to the ureteric bud epithelium [40]. Further work is needed to uncover how biochemical signaling downstream of a soluble factor, such as GDNF, induces apical constriction in only the subset of epithelial cells that form the nascent branch.

2.1.2. Localized proliferation

Branch initiation has also been hypothesized to result from the proliferation of epithelial cells located at sites of presumptive branches. This local increase in proliferation would lead to an increase in the number of cells, which could in principle initiate a branch by inducing outgrowth. Indeed, a high rate of proliferation has been observed in nascent branches and branch tips in organs such as the lung [42–44], kidney [45], and mammary gland [46]. These observations led to multiple studies investigating whether this higher rate of proliferation causes branch initiation.

In the highly proliferative developing epithelium of the embryonic mouse lung, globally inhibiting DNA synthesis by treating cultured explants with aphidicolin reduces branch initiation [47]. Using mesenchyme-free lung epithelia in culture, BrdU-incorporation experiments revealed that epithelial cells in branch tips have an increased rate of proliferation relative to those in branch stalks, but only after new branches have already formed [43]. These results are consistent with those using live-imaging and EdU-incorporation in intact lungs, which revealed that proliferation increases at the tips of lateral branches and bifurcations, but only after the branches have already formed [26, 42]. These data suggest that localized proliferation does not drive branch initiation in the embryonic mouse lung.

Localized proliferation may, however, play a role in branching morphogenesis of the ureteric bud epithelium. In the very first branching event during kidney development, the ureteric bud evaginates from the Wolffian duct. Intriguingly, the epithelial cells located at the site of the presumptive ureteric bud proliferate at higher rates than their neighbors prior to bud initiation [45]. Inhibiting the cell cycle by treating explants with methotrexate prevents initiation of the ureteric bud, suggesting a role for localized proliferation [45]. Consistently, increased proliferation is observed at the tips of branches in both kidney explants and mesenchyme-free ureteric bud epithelium [39, 45]. These patterns of proliferation are not observed when branching is induced in kidney explants by adding GDNF to the culture medium. However, proliferation is increased in nascent branches when GDNF-soaked beads are placed adjacent to epithelial tips [45]. The authors of that study proposed that localized proliferation may either require a locally high concentration of GDNF, or a gradient of GDNF adjacent to the epithelium [45].

2.1.3. Buckling

An alternative epithelial-intrinsic mechanism to initiate branches is buckling morphogenesis [48], which results from uniform rather than patterned proliferation of the epithelium. When an epithelial sheet or tube grows uniformly within a surrounding viscoelastic material, such as a mesenchyme or gel, the epithelium will experience a gradual accumulation of in-plane compressive stresses. Once the mechanical load from the compressive stresses reaches a critical level, the epithelium will spontaneously buckle, producing a wave-like morphology. The wavelength increases as the stiffness of the epithelium increases (producing fewer 'branches' per unit area) and decreases as the rate of epithelial growth increases (producing more 'branches' per unit area) [49]. Buckling therefore results spontaneously from tissue-wide behaviors, as opposed to apical constriction, which is driven locally by spatially patterned behaviors. As a result, buckling can result in branch initiation in the absence of local sources of a branch-inducing signal. Murine airway epithelial rudiments denuded of mesenchyme and embedded in Matrigel (a viscoelastic foundation) undergo buckling morphogenesis [49]. These isolated epithelia first form cysts and then grow uniformly in response to growth factors within the culture medium. Eventually, multiple branches form simultaneously, with a wavelength that depends on the rate of epithelial growth. Several embryonic epithelial tissues generate the same morphology when denuded of mesenchyme and embedded in Matrigel [7], suggesting that buckling morphogenesis is a general response of growth under confinement in culture, and not a recapitulation of morphogenetic processes that occur *in vivo*.

Buckling of a different nature has been observed during development of the mouse embryonic salivary gland [29, 50] (figure 3(B)). Unlike the single-layered pseudostratified airway epithelium of the murine lung, the salivary gland epithelium is a multi-layered mass of cells (figure 1), which can be thought of as an 'outer cell' surface surrounding 'inner cell' layers. In salivary gland explants, outer cells move into the inner layers to divide, and then daughter cells

insert into the outer layer, leading to rapid growth of the outer layer and a buildup of in-plane stress. To relieve these stresses, the outer layer buckles and thus initiates new branches. The sorting of inner and outer cells is driven by differential cell adhesion: inner cells have stronger cell–cell adhesions and outer cells have stronger cell–ECM adhesions, which enhance their affinity for the BM that envelops the epithelium [29]. As a result, cells that insert themselves into the outer layer adhere to the BM and drive expansion of the outer surface. If the BM is degraded by treating salivary gland explants with enzymes, outer cells continue to ingress and divide but their daughters remain within the inner layers. As a result, there is a build-up of 'branching potential' within the gland. Restoring the BM allows these daughter cells to incorporate into the outer layer and leads to even more rapid surface expansion and buckling [29]. While cell adhesions drive the cell-sorting phase of the process, the cytoskeleton is important for translating surface expansion into buckling. Pharmacologically disrupting actomyosin contractility affects the wavelength of buckling in a dose-dependent manner without affecting cell proliferation [50], presumably by decreasing the elastic modulus (stiffness) of the epithelial layer. Therefore, the morphogenetic motif that drives salivary gland development is buckling resulting from growth that is brought about by cell sorting.

Similar to the salivary gland, the epithelium of the embryonic pancreas is initially a multi-layered mass of cells (figure 1). The pancreatic epithelial tree is comprised of branches at two completely different length scales—large, lateral branches (>100 μm in length) that extend continuously from *E*12.5 to *E*15.5 and smaller 'tips' (<100 μm) that stud the surface of the epithelium beginning at *E*12.5, which branch to form new tips over developmental time [51, 52]. 'Tips' may form via a buckling mechanism similar to that observed in the salivary gland [29]. Live imaging of pancreatic explants suggests that cells of the inner and outer layers largely remain within their respective compartments at short timescales [53], but that cells of the inner layer move into the outer layer at longer timescales [54] (figure 3(C)). Additionally, cells of the outer layer undergo mitosis within the inner compartment and then reinsert into the outer layer, exactly as in the salivary gland [53] (figure 3(C)). In principle, the combination of these cellular behaviors could cause an expansion of the outer layer that is sufficient to induce buckling in explants. The dynamics of outer cells depend on cell–ECM adhesion via integrins [53], whereas the sorting of the two populations depends on differential cell–cell adhesion mediated in part by p120-catenin [54] (figure 3(C)). As branching morphogenesis proceeds *in vivo*, the multi-layered pancreatic epithelium resolves into a pseudostratified layer [51] (figure 3(C)). This process could generate in-plane compressive forces similar to those that result from the migration of inner cells into the outer cell layer, thus leading to buckling. Mutant phenotypes suggest that the physical mechanisms of branching morphogenesis of pancreatic explants are conserved *in vivo*: branching fails in β1-integrin mutants [53] and slows in p120-catenin mutants [55]. The salivary gland and the pancreas may therefore use slightly different molecular and cellular mechanisms to generate in-plane compressive stresses, which drive the buckling that initiates branches.

2.1.4. Cell sorting

Rearrangements of epithelial cells have also been observed during development of the embryonic kidney, where they are biased by growth factor signaling. Mesenchymal cells produce GDNF that binds to the receptor tyrosine kinase Ret in the epithelium to promote branching morphogenesis. Genetic mosaic experiments revealed that the expression level of Ret in a given cell is associated with the position that cell adopts within the developing ureteric tree. Cells lacking Ret are excluded from branch tips and populate branch stalks [56], whereas cells with constitutively high Ret remain in branch tips [57]. However, it is not immediately obvious how this cell sorting contributes to branching morphogenesis. In mesenchyme-free explants, epithelial branches form in a random pattern that does not resemble the orderly bifurcations observed *in vivo* or in whole-organ explants. In genetic mosaic experiments using explants, cells with constitutive expression of Ret come together and initiate branches [57], suggesting that high Ret activity promotes a form of epithelial-intrinsic branching, albeit one with currently unknown cellular mechanisms. Sorting of cells with different levels of Ret signaling *in vivo* might help to restrict this 'branch-initiating activity' to branch tips. In line with this possibility, genetically deleting *Mek1/2* to prevent signaling downstream of GDNF dysregulates cell–cell adhesions, prevents cell rearrangements (and presumably sorting of cells based on levels of Ret activity), and inhibits branch initiation *in vivo* [58]. Overall, epithelial-intrinsic mechanisms of branch initiation rely on the ability of epithelial cells to change shape and rearrange with respect to their neighbors.

2.2. Epithelial-extrinsic mechanisms

Epithelial tissues can also be shaped into branches by epithelial-extrinsic mechanisms. The exact role played by extrinsic signals depends on the composition of the mesenchyme that surrounds the epithelium (figure 2). In some organs, the mesenchyme differentiates into visceral smooth muscle cells that shape the epithelial tree. In other organs, ECM dynamics specify the locations of branch initiation and clefting.

2.2.1. Mesenchymal cell-guided folding

Airway smooth muscle (ASM) shapes branches in the mouse lung [26, 59, 60] and is thought to play a similar role in human lungs [61, 62]. Branching morphogenesis in the mouse lung occurs via repeated implementation of two branching modes: domain branches, which form laterally off the side of existing branches, and bifurcations, which split the tip of a parent branch into two daughter branches [63]. ASM influences the initiation of both types of branches, and the timing and location of smooth muscle differentiation are specific to the branching mode [26, 59]. Domain branches are gradually sculpted by a continuous layer of smooth muscle that differentiates in the proximal-to-distal direction along the parent epithelial tube (figure 3(D)) [26]. In contrast, bifurcations are split by a new population of smooth muscle cells that differentiates at, and thus specifies, the cleft at the future bifurcation site [59]. As discussed above, when denuded of mesenchyme the growing airway epithelium undergoes buckling morphogenesis to fold itself into a morphology that resembles branches [5, 49]. However, in the absence of smooth muscle, the epithelium buckles randomly, rather than generating stereotyped patterns of branches. Sculpting forces from smooth muscle differentiation are required in order for the growing airway epithelium to initiate branches at stereotyped locations and times [26]. Branch initiation in the mouse lung therefore appears to result from an externally constrained buckling mechanism [1].

Smooth muscle differentiates in unique patterns and at different developmental stages in the lungs of species that represent different classes of vertebrates: mouse [60], chicken [64, 65], lizard [66, 67], and frog [68, 69]. In the chicken, ASM does not appear until embryonic day (*E*) 7, after the underlying tree has already branched extensively [65]. Consistently, branches in the embryonic chicken lung do not initiate via constrained buckling, but instead by apical constriction, as described above [35]. Smooth muscle begins to differentiate around the epithelium during the intermediate stages of chicken lung development, but it remains unclear whether this tissue plays a role in epithelial morphogenesis. The mechanisms of avian lung branching therefore appear to be quite different from those in other vertebrates. Furthermore, the chicken lung undergoes two types of epithelial morphogenesis: branching to form the airways and cyst-like expansion to form the air sacs. Inductive signals from the mesenchyme dictate which mode of morphogenesis occurs: transplantation of dorsal lung mesenchyme onto denuded tracheal epithelium leads to the formation of branches, whereas transplantation of ventral lung mesenchyme leads to the formation of cysts [70]. The mechanisms underlying these different epithelial responses to dorsal and ventral mesenchyme are still being elucidated. Several signaling molecules are expressed at different levels in the dorsal and ventral mesenchyme [70], and there is evidence that the diffusivity of FGF10 differs in each region due to chemical and structural differences in the mesenchyme [23]. Understanding how these signals lead to different cell behaviors and morphogenetic motifs will help us to understand the physical mechanisms of branching in the avian lung.

During the later stages of chicken lung development, from *E*11 to *E*13, the terminal ends of branches extend towards one another and fuse, a process called anastomosis [64, 71]. Throughout the process of extension and anastomosis, the airways are wrapped by mesh-like smooth muscle; as the tips of branches approach each other, the smooth muscle-wrapped epithelium bifurcates into two daughter branches (figure 3(D)), in a pattern similar to that observed during bifurcation in the developing mouse lung [26, 59]. The similarity in tissue organization between these two morphogenetic events suggests that perhaps smooth muscle-guided branching is a conserved developmental motif that can be adapted to different contexts, when the correct cell types are present. Consistently, branches of the mouse prostate epithelium are surrounded by a smooth muscle layer that also appears to physically influence branching [72].

The timing as well as the physical appearance of smooth muscle differs between the early mouse lung and the late-stage, anastomosing avian lung. Whereas murine ASM forms a continuous layer that covers the entire epithelial tube, avian ASM forms a more net-like wrapping. Strikingly, small epithelial protrusions known as atria, which eventually give rise to air capillaries that interweave with the vasculature to form the avian gas-exchange unit, appear in the gaps between smooth muscle fibers, suggesting that smooth muscle may physically guide their morphogenesis (figure 3(E)). Similar patterns of contractile cells are observed in the later stages of mouse lung development, when myofibroblasts differentiate and form a contractile mesh that templates local bulging of the alveolar epithelium (figure 3(E)) [73–75].

Compared to mammalian and avian lungs, much less is known about the development of amphibian or reptilian lungs. Mature frog and lizard lungs, for example, have vastly different final morphologies from those of the mouse or chicken [66, 69]. These organs lack a tree-like epithelium and instead maximize surface area by using a series of corrugations known as faveolae that serve as the gas-exchange surface of the sac-shaped epithelium. Similar to the mouse, smooth muscle differentiates in the early stages of development of the lungs of the frog (*Xenopus laevis*) and the brown anole lizard (*Anolis sagrei*). However, instead of remaining as a continuous layer, this contractile tissue rearranges into a lattice-like meshwork (figure 3(E)). In the anole, the corrugations of the epithelial surface emerge through the gaps in the meshwork as the pressure of the fluid within the lumen of the lung

increases, akin to the deformations that appear in a stress-ball when squeezed [67]. Smooth muscle initially differentiates uniformly around the epithelium, and then is refined first into horizontal bundles by the expansion of the underlying epithelium, and then into connecting bundles by contraction of the horizontal bundles. The stress-ball mechanism is strikingly similar to that observed during alveologenesis of the mouse lung (figure 3(E)) [73] and to that proposed to drive the formation of air capillaries in avian lungs (figure 3(E)) [64]. Stress ball morphogenesis thus represents another example of a morphogenetic motif repurposed in different species and developmental stages to achieve similar changes in overall epithelial architecture.

Transcriptomic and biophysical analyses of the embryonic pulmonary mesenchyme of the mouse reveal that remodeling of the cytoskeleton and adhesions occurs early during the process of mesenchymal differentiation into ASM. The undifferentiated mesenchyme immediately adjacent to the tips of epithelial branches (the sub-epithelial mesenchyme) has elevated levels of filamentous actin, exhibits increased migration, and may experience greater cortical tension than the mesenchyme further away [76]. These findings suggest that undifferentiated mesenchyme and/or immature smooth muscle can physically sculpt the airway. Consistently, treatments that stimulate the early stages of ASM differentiation and expand the compartment of sub-epithelial mesenchyme constrain the adjacent epithelium and inhibit airway branching [76]. Mutants that achieve the opposite effect on the mesenchyme develop epithelia with unconstrained, cystic morphology [76]. Further, knockout of myocardin (*Myocd*), which promotes maturation into contractile smooth muscle, has no effect on epithelial branching [76, 77]. A similar role for mesenchymal cells in patterning adjacent epithelial tissues has also been described during morphogenesis of the avian skin [78]. Stiffening and condensation of the mesenchyme has been exploited to drive predictable patterns of folding to generate three-dimensional engineered tissues [79]. Experiments using isolated mesenchymal cells cultured in collagen in a ring-shape show that the mesenchyme can spontaneously self-organize into a periodic pattern, providing evidence for mesenchyme-intrinsic behaviors in dictating tissue architecture [80]. Future experiments will shed light on whether and how mesenchymal-self organization participates in the stereotyped patterning of epithelial trees.

2.2.2. Matrix-guided folding

The ECM acts as a passive sculptor of epithelial trees, influencing branching morphogenesis through stiffness gradients or constraints, rather than though active contraction [81]. Early work demonstrated a role for collagen in salivary gland branching morphogenesis by treating explants with collagenase and collagenase inhibitors, which prevented and stimulated clefting, respectively [82]. Additionally, fibronectin accumulates within clefts that form between adjacent epithelial buds [83]. As the levels of fibronectin increase, epithelial cells decrease their adhesions to their neighbors in favor of cell–ECM adhesions and, eventually, mesenchymal cells migrate into the cleft [83]. Throughout branch growth and clefting, the salivary gland epithelium is surrounded by a BM. High-magnification imaging revealed that the BM is highly perforated, particularly in regions of active outgrowth [84]. Perforations depend on MMP activity, and MMP-mediated turnover of the ECM permits branch outgrowth [84]. Additionally, epithelial protrusions were observed poking through the perforations, possibly expanding them, in a manner dependent on actomyosin contractility [84]. Branching of the salivary gland epithelium therefore relies on a balance between constraint imposed by this specialized ECM and the essential role of adhesion to the BM in regulating the cellular rearrangements that drive buckling [29].

During puberty, the epithelial tree of the mammary gland is elaborated by extension of branches, bifurcation of branch tips, and lateral branching off of existing ducts [85]. The exact mechanisms by which the terminal end buds (TEBs) of the mouse mammary gland bifurcate remain unclear, but recent work has revealed a role for ECM in sculpting these bifurcations *in vivo*. Collagen accumulates in the mesenchyme around epithelial ducts and TEBs, and is typically thickest around non-branching regions and thinnest at the tips of branches. Collagen first appears within the cleft site at nascent bifurcations, and collagen fibers accumulate between the daughter branches as bifurcation proceeds (figure 3(F)) [27]. Comparing the patterns of collagen distribution at fixed time points to computational simulations (network-scale models) revealed that this parameter controls the angle of bifurcation: high levels of collagen at the cleft compared to the flanks yields more obtuse angles, whereas high levels of collagen at the flanks yields more acute angles. To date, the ECM appears predominantly to refine or support the shaping of branches rather than their initiation—epithelial-intrinsic or active mesenchymal mechanisms produce new branches, and ECM deposition helps to cement the new tissue morphology.

3. Mechanisms for branch extension and widening

3.1. Epithelial-intrinsic mechanisms

After their initial formation, epithelial branches lengthen and widen over developmental time. Dysregulation of the size of epithelial tubes may have profound implications for organ function and can lead to disease. Many studies have focused on the extension that occurs concomitant with branch initiation.

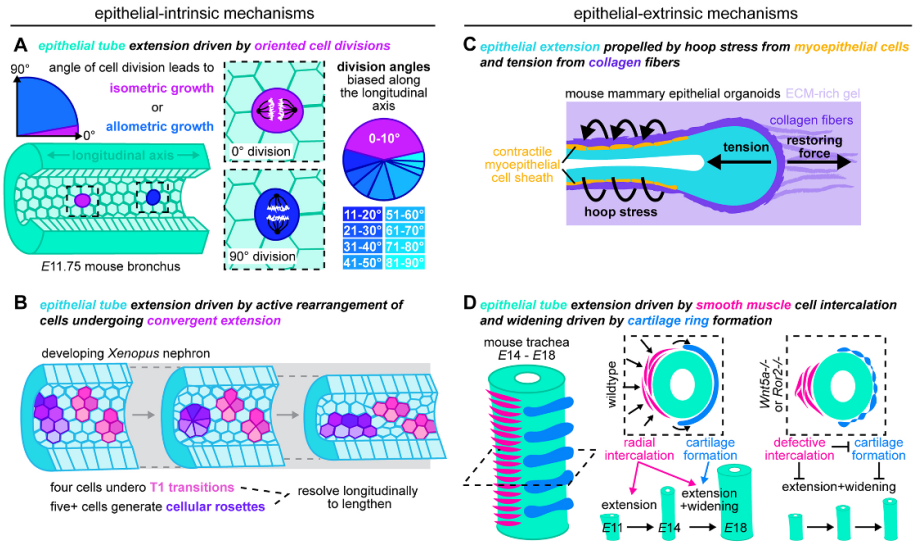

Figure 4. Intrinsic and extrinsic mechanisms that drive extension and widening of epithelial branches. (A) In the mouse lung, the angle at which epithelial cells divide causes the tube to grow either longitudinally (axially) or circumferentially. Branches extend when there is an enrichment in cells dividing in the axial direction (0°). (B) *Xenopus* kidney tubules lengthen by undergoing CE movements. Four cells can undergo a T1 transition while larger groups of cells form clusters termed rosettes. In both cases, circumferential junctions shrink to bring cells closer together, and then new junctions are formed longitudinally. Kidney tubules extend when cells undergo recursive rounds of intercalation. (C) Myoepithelial cells generate hoop stress to push the tips of branches forward in mammary epithelial organoids. At the same time, epithelial cells at branch tips pull on collagen fibrils in the surrounding matrix, which generates tension that promotes branch extension. (D) Smooth muscle wraps around the circumference of the tracheal epithelium, which helps to lengthen the epithelial tube and to assemble half-rings of cartilage. Together, smooth muscle and cartilage then support the extension and widening of the trachea. Wnt5a and Ror2 regulate the intercalation of smooth muscle cells around the epithelial tube.

However, in several organs, including the lung and the kidney, the epithelium continues to extend long after branching ceases, which ensures that the organ continues to grow with the embryo. Epithelial tubes predominately lengthen or widen using two epithelial-intrinsic mechanisms: OCD or CE.

3.1.1. Oriented cell divisions (OCDs)

Cell divisions that orient along the length of a branch bias the epithelium to grow in the axial direction, thus extending the branch while maintaining its diameter (figure 4(A)). In the developing kidney, OCDs are required for elongation of both proximal tubules and the collecting duct [86–88]. Loss of OCDs leads to shorter and wider kidney tubules [86–88]. At the molecular level, the genes that regulate OCDs are mainly implicated in regulating planar polarity or ciliogenesis. Planar polarity refers to the orientation of cellular structures or behaviors along the plane of the tissue (or in the axial direction in the case of epithelial tubes). The genes implicated in planar polarity are often broadly referred to as PCP or non-canonical Wnt signaling genes [89]. Here, for clarity, we refer to PCP genes as those that comprise the core PCP complex (in mice, the transmembrane proteins Celsr1-3, Frizzled3/6, and Vangl1/2), whereas we refer to non-canonical *Wnt* genes as the Wnt ligands implicated in regulating planar polarity. Planar polarity was initially connected to the regulation of kidney tubules when lineage-tracing experiments revealed mitotic clones aligned in the axial direction within postnatal murine kidney tubules [88]. Subsequent work revealed that OCDs are at least partially under the control of signaling through non-canonical Wnt9b, as loss of *Wnt9b* leads to a reduction in OCDs [87]. Similarly, loss of the core PCP complex leads to a reduction in OCDs in the postnatal kidney [86]. However, the molecular mechanisms that connect the angle of cell division to the core PCP complex or non-canonical Wnt signaling in the kidney remain to be elucidated.

Primary cilia function as signaling centers within the cell and are particularly important in Hedgehog signaling, though they regulate other pathways as well [90–92]. However, there also appears to be a role for primary cilia in regulating morphogenesis of epithelial tubes. Specifically, loss of cilia from the kidney epithelium via *Kif3a* deletion causes an increase in the diameter of collecting duct tubules due to loss of planar polarized behaviors including OCDs [86]. Due to the similarity in phenotype between cilia and PCP mutants, the authors investigated whether the core PCP complex was disrupted in epithelia lacking *Kif3a*. However, core PCP asymmetry remained intact in these mutants, suggesting that OCDs and other planar polarized cellular behaviors are governed by cilia either downstream of core PCP or that cilia regulate these behaviors in an orthogonal manner [86]. The question of whether and how cilia and the core PCP complex regulate each other has

been investigated in various tissues ranging from the ependyma to the inner ear; the current consensus is that though cilia clearly regulate planar polarized behaviors, it remains to be determined whether this is independent from or directly downstream of PCP [93]. Further work is required to identify how cilia regulate the changes in cell morphology required for OCDs.

OCDs have also been implicated in the regulation of branch extension in the embryonic mouse lung [42, 94, 95]. Specifically, cells have been observed to divide preferentially in the axial direction of the epithelial tube in both newly emerging branches and established airways [42, 94, 95]. However, the mechanisms that regulate OCDs in the lung appear to be distinct from those in the kidney. The PCP complex does not become asymmetric in the mouse lung until *E*14.5, long after the start of epithelial branching [96]. Asymmetric localization is required for PCP proteins to relay polarity information, so it is unlikely that the PCP pathway regulates the orientation of cell divisions during branching in the lung. Instead, global tissue mechanics have been suggested to regulate the angle of cell divisions by signaling through Erk1/2. Over-expression of a constitutively active form of Ras or Braf causes cells within the embryonic airway epithelium to divide randomly, leading to isometric growth of the airways rather than branch extension [94, 95]. Surprisingly, mosaic overexpression of BRAF does not affect elongation at the tissue level, as wildtype cells compensate for the randomly oriented divisions of their neighbors by shifting their division axis to maintain axial growth [95]. This observation led to the hypothesis that cells adjust their division angles in response to mechanical forces from their neighbors; indeed, stretching of whole-lung explants leads to a bias in cell division in the direction of stretch [95]. Thus, while OCDs broadly regulate branch extension, the upstream signals that feed into the cell-intrinsic decision to undergo an axial division vary depending on tissue context.

3.1.2. Convergent extension (CE)

Epithelial cells can also promote branch extension through the formation and biased resolution of multicellular rosettes, leading to CE of the tube (figure 4(B)). Rosettes have been observed within the epithelium of mouse and frog kidneys [97], suggesting that this epithelial-intrinsic mechanism is conserved in vertebrate kidney development. Like OCDs in the kidney, CE is regulated by both the core PCP complex as well as non-canonical Wnt signaling [86–88]. In the developing *Xenopus* nephron, live-imaging analyses revealed that epithelial cells converge into multicellular rosettes that resolve along the axial direction of the tissue, leading to CE of kidney tubules [97]. Similar rosettes were observed in fixed samples of embryonic mouse kidneys [97]. To determine whether PCP regulates CE in *Xenopus*, a mutant form of Disheveled that perturbs PCP signaling was introduced into developing nephrons, which disrupted the bias in rosette resolution and resulted in wider kidney tubules [97]. Similarly, loss of genes that encode for components of the core PCP complex ablates changes in cell shape associated with the formation of rosettes and leads to wider tubules in the mouse kidney [86]. Loss of *Wnt9b* leads to similar defects in cell shape and tubule width [87], but a direct link between Wnt9b and core PCP signaling has not been shown.

Loss of core PCP components has been implicated in the morphogenesis of other branched epithelia, but the underlying cellular behaviors remain unclear. For example, loss of the PCP gene *Vangl2* in the mouse mammary epithelium leads to a narrowing of the ducts, potentially from changes in cell proliferation or apoptosis [98]. In the pancreas, PCP components are localized asymmetrically within the epithelium and ectopic expression of Vangl2 leads to pancreatic hypoplasia [99]. However, it remains unknown if this phenotype reflects a mechanistic role for Vangl2 in morphogenesis of the pancreas or if overexpression of Vangl2 introduces new defects. Future studies are needed to determine whether PCP proteins also regulate OCD and CE in these tissues.

3.2. Epithelial-extrinsic mechanisms

The extension of epithelial branches is aided by molecular and physical signals from the surrounding mesenchyme. Extension can be driven passively by the surrounding ECM or actively by contractile mesenchymal cells. The physical properties and cellular composition of the mesenchyme therefore help to define the lengths of branches within epithelial trees.

3.2.1. ECM-guided extension

The ECM appears to play an important role in branch extension in the epithelium of the pubertal mammary gland. The mammary gland epithelium branches within the dense and optically-inaccessible mammary fat pad, making it challenging to study the dynamics of branch extension in the intact organ. To circumvent this issue, organoids have been derived from mammary epithelial cells and embedded in Matrigel, where they undergo morphological changes reminiscent of branch initiation and extension (figure 4(C)) [100]. Contractile forces exerted by mammary epithelial cells assemble the collagen that surrounds the organoids into fibers. The tip of the tissue exerts tension on these fibers, which is balanced by a restoring force [100]. Treating the organoids with cytochalasin D to impair actomyosin contractility relaxes the epithelial cells and allows the restoring force to pull the tip forward. Conversely, laser ablation of the collagen fibers within this culture model leads to retraction of the tensed epithelial tip. However, the microenvironment in the intact gland is vastly different from this simplified culture model (figure 2(C)),

raising important caveats about conclusions made from experiments with organoids [1, 6, 85]. Notably, investigations of the developing gland *in vivo* have alternately revealed the presence and absence of aligned collagen fibers [27, 101]; it is likely that the adipocyte-rich mesenchyme observed *in vivo* affects both ECM organization and epithelial morphogenesis in a manner that cannot be recapitulated by culture in Matrigel (figure 2(C)).

In the embryonic avian lung, branch extension requires remodeling of the BM that wraps the airway epithelium [65]. As epithelial branches form, the BM thins locally, presumably to facilitate branch extension. Mesenchymal cells near branches express MMP2, which is required for remodeling the BM and allowing branch extension [65]. A similar role for the BM has been proposed to regulate branching of the embryonic mouse lung [102]. The 'run in a stocking' model posits that local thinning creates a weakness in the BM, and that the growing epithelium imposes tension on this weakened region that further thins and disrupts the BM [102]. However, this conceptual model has not been definitively demonstrated in the embryonic mouse lung. Remodeling of the interstitial ECM also appears to play a role in branch extension in the embryonic avian lung [65]. Epithelial cells at branch tips express tenascin-C, a large non-adhesive ECM protein that is then transported by migrating mesenchymal cells to distances several cell diameters away from the epithelium. Inhibiting FAK prevents tenascin-C expression and transport and inhibits branch extension [65]. Cell–ECM adhesion-mediated signaling through FAK may therefore participate in a mechanosensitive feedback loop that remodels the ECM and promotes branch extension. Different ECM components, both within the BM and the surrounding mesenchyme, therefore appear to have specific effects on the development of the epithelial tree.

3.2.2. Mesenchymal cell-guided extension

The epithelium of the mammary gland is stratified into basal, luminal, and myoepithelial layers. Smooth muscle actin (*Acta2*)-expressing myoepithelial cells wrap the entire luminal epithelium of the gland *in vivo* and do so in a sparser pattern in organoids, where they may promote branch extension (figure 4(C)). While these cells are epithelial, here we classify their actions as epithelial-extrinsic since they behave independently of the rest of the epithelium and function more like smooth muscle cells or myofibroblasts. Simulations have suggested that extension of mammary epithelial branches in cultured organoids requires stresses to accumulate within the epithelium immediately behind the tip of the branch [103]. Contractile myoepithelial cells that wrap circumferentially around the luminal epithelium within organoids have been proposed to provide hoop stresses that are sufficient to push the tip of the branch forward into the surrounding Matrigel (figure 4(C)). It remains unclear whether myoepithelial cell-induced hoop stress would be sufficient to promote branch extension within the complex stromal microenvironment of the fat pad *in vivo*. Nonetheless, the myoepithelium shows higher levels of phosphorylated myosin light chain, indicative of increased contractility, compared to the luminal epithelium in the pubertal mouse mammary gland *in vivo* [103].

Smooth muscle cells appear to play a similar role to myoepithelial cells during extension of the mouse embryonic trachea. In mammals, smooth muscle only wraps halfway around the tracheal epithelium—the other half is wrapped by periodic half-rings of cartilage that condense from the surrounding mesenchyme (figure 4(D)). The tracheal tube grows in two phases: during the first phase, the tube elongates, smooth muscle differentiates, and the cartilage half-rings are assembled; during the second phase, the tube increases in both diameter and length with support from the growing cartilage [104]. Morphometric analyses and genetic experiments revealed that radial intercalation of smooth muscle cells downstream of the PCP pathway components *Wnt5a* and *Ror2* is required to properly organize the tracheal smooth muscle layer [104]. Failure of smooth muscle cell intercalation prevents elongation of the tracheal tube and proper formation of the cartilage half-rings (figure 4(D)). It is possible that smooth muscle plays a similar role in the extension of branches of the lung or of other organs. The lungs of *Wnt5a*$^{-/-}$ and *Ror1/2*$^{-/-}$ mice appear smaller and perhaps more stunted than control lungs [104], suggestive of a possible defect in branch extension. Overall, contractile mesenchymal cells that surround epithelial branches play an important role in branch extension—the identity of these cells depends on the organ, but they have similar mechanical effects.

4. Branch termination

4.1. Epithelial-intrinsic mechanisms

Termination of branches is an essential, but relatively understudied, aspect of tree building. Here, we highlight three examples that provide hints about the epithelial-intrinsic mechanisms of branch termination. However, it is important to note that in most cases the endogenous signals that terminate branching are unknown—these signals may be regulated spatially, to prevent branches from colliding with each other, or regulated temporally, to cease branching morphogenesis at a specific stage of development.

4.1.1. Collision avoidance

In a BARW model, the tips of simulated branches can extend or bifurcate with some probability, or terminate based on their proximity to neighboring branches as a way to avoid collision (figure 5(A)) [14].

This BARW model reproduces essential features of the tree-like epithelium observed in the mammary gland, where a stochastic branching process generates a ramified network without any branches colliding or crossing each other. The signal thought to inhibit branching and prevent collisions in the mammary gland is transforming growth factor β1 (TGFβ1). Beads soaked in TGFβ1 and implanted into the mammary fat pad prevent epithelial branching [105], and the geometry of the branching epithelium defines patterns of TGFβ1 concentration [106]. Branches that reach regions with high levels of TGFβ1 near neighboring branches may cease to grow, preventing collisions. The BARW framework was also used to model the developing kidney, where branch termination was proposed to be induced by nephron formation. However, no correlation has been found between branch termination and nephron formation *in vivo* [107]. While branch termination is an important event in building these networks, the biological mechanisms by which branches terminate remain unclear. Inhibitory factors capable of stopping branch extension can be identified by embedding developing tissues with beads soaked in diffusible molecules; this approach can also shed light on how branches avoid colliding with one another as they extend into their surrounding mesenchyme [14].

4.1.2. Tip differentiation

In the lung, the tips of epithelial branches eventually differentiate into alveoli, specialized multicellular units that perform gas exchange, while the epithelium within the branch stalks differentiates into a variety of airway cell types. The developing lung epithelium is therefore thought to be composed of two compartments: distal (branch tips) and proximal (branch stalks). The expression of fate markers for these two compartments closely follows branching, with *Sox9* expressed distally and *Sox2* expressed proximally. Around *E*16, the domain of cells expressing the proximal marker (*Sox2*) ceases to expand, despite the fact that the distal *Sox9*-expressing tips continue to branch, suggesting that the boundary between the two compartments is set at this developmental stage [108]. The levels of glucocorticoid hormone increase during later gestation, and stimulation with a glucocorticoid agonist leads to premature boundary formation, with early expression of alveolar markers just distal to the Sox2^{+} domain [108]. Mutants for the glucocorticoid receptor exhibit a delay in boundary formation and have enlarged Sox9^{+} branch tips and decreased expression of alveolar markers, supporting the hypothesis that glucocorticoid signaling sets the boundary between proximal and distal fates [108]. Glucocorticoid signaling may therefore regulate the formation of the compartment boundary by promoting alveolar differentiation. These findings raise the possibility that differentiation can serve as a signal to terminate branching.

4.1.3. Lumen formation

In epithelia that branch as a solid mass of cells or a multi-layered tissue, one of the final steps of tree building is the formation of a continuous lumen. The formation of a lumen is usually accompanied by the resolution of the epithelium into a bi-layered or pseudostratified layer. In the mammary gland, a continuous lumen extends as TEBs propel forward and ducts elongate, aided by apoptosis of inner cells of the TEB nearest the bi-layered ducts [109]. In the salivary gland, the lumen does not form by apoptosis [110]. Instead, inner cells form a network of small lumens at *E*12.5–13.5 by locally recruiting F-actin, polarity proteins, and myosin [111]. These small lumens are stabilized by cell–cell adhesions [112]. Signals from local parasympathetic neurons then appear to induce the small lumens to join into a single continuous lumen that extends through the ducts of the salivary gland [110]. However, the physical mechanisms of lumen coalescence in the developing salivary gland remain unclear. In contrast, the pancreas exhibits a striking form of lumen morphogenesis in which the epithelium harbors an elaborate network of microlumens before any branches or 'tips' are detected on the surface of the tissue [51, 52]. Continuous lumens form by *E*12.5 [52]. Lumen coalescence occurs in a two-step process: lumens first join stochastically, then they are refined into a hierarchical tree-like network [113]. Simulations suggest that fluid flow through the ducts helps to drive the transition from stochastic connections to a continuous and ordered lumen [113]. Therefore, lumen formation, while necessary for the final function of the organ and considered to be a final step in some cases, can occur at any stage of the branching process.

4.2. Epithelial-extrinsic mechanisms

Similar to epithelial-intrinsic mechanisms, epithelial-extrinsic mechanisms to terminate branching are also poorly understood. These physical or molecular signals may come from the boundary of the organ itself, or from non-epithelial cells types closer to the developing tree.

4.2.1. Branch termination at the organ boundary

Branched epithelia are subject to the physical constraints of the organ in which they reside, and their growth must therefore be regulated by the space that the organ occupies and the shape of the organ itself. In CDH, the visceral organs herniate into the fetal chest cavity, altering the local mechanical environment and limiting the space within which the lungs can develop [114]. CDH patients exhibit under-branched lungs, perhaps due to the lack of space. Consistently, Wilm's tumor 1$^{-/-}$ (*Wt1*$^{-/-}$) mutant mice accumulate fluid in the ventral chest cavity that confines the organs to the dorsal chest cavity, effectively compressing the lungs and decreasing the number of branches that form (figure 5(B)) [115]. Further, physically constraining

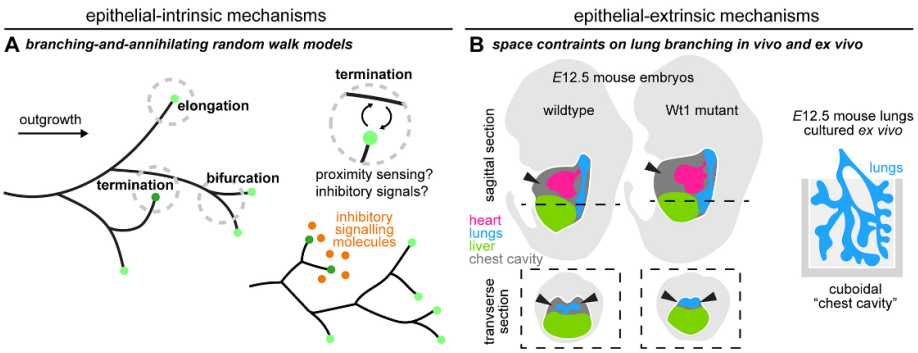

Figure 5. Intrinsic and extrinsic mechanisms that regulate termination of epithelial branches. (A) BARW models use three simple behaviors (elongation, bifurcation, and termination) to simulate the architecture of branched networks. In these models, termination is usually caused by proximity to a neighboring branch or the presence of inhibitory molecules. The biological mechanisms that induce branching to stop remain unclear. (B) Spatial constraints limit the development of the lung of *Wt1*$^{-/-}$ mice *in vivo*, a genetic model of CDH, where expansion of the ventral chest cavity compresses the lungs nestled in the dorsal chest cavity, and in lungs cultured *ex vivo* within microfabricated chest cavities of different shapes (e.g. cuboidal).

embryonic lungs *ex vivo* in artificial chest cavities of different shapes halts branching morphogenesis and results in lungs of the same size and shape as the cavity (figure 5(B)) [116].

The final number of epithelial branches depends on the overall size of the organ as well as how the epithelial tips are packed together. In the kidney, the tips of epithelial branches are confined to the surface of the organ [117]. At early stages of development, the tips of branches are organized into H-shaped patterns on the surface of the kidney. At later stages of development, the tips of branches take on a more vertical configuration, such that the distance between branch points and the surface of the organ increases. A computational model was used to predict that an external force pulls the branch points inward to generate the final configuration of tip packing [117]. Disrupting the mechanical anchoring of epithelial tips to the mesenchyme causes the tips to retract from the surface of the organ, consistent with the prediction of the model. Conversely, dissecting the inner portion of the kidney away from the surface layer eliminates the inward pulling force and results in abnormally packed tips. This computational model and accompanying experimental observations suggest that the architecture of an epithelial tree is refined in order to maximize branch number.

4.2.2. Vascular endothelial cell-mediated termination

The stroma surrounding branched epithelia harbors vascular endothelial cells, which typically form a plexus that is closely associated with the epithelium. Apart from their role in delivering oxygen and other nutrients, the vasculature has also been shown to play so-called 'perfusion-independent' roles in the development of several organs [118]. These roles have been typically revealed by culturing organs *ex vivo*, where the vascular network is disconnected from the heart and therefore ceases to deliver blood. The vasculature in the developing pancreas suppresses epithelial branching both *in vivo* and *ex vivo*: overexpression of VEGF leads to hypervascularization and stunts branching of the pancreatic epithelium in embryos and in explants, whereas inhibition of VEGFR increases branching of the epithelium [119]. Endothelial cells have an opposite effect on epithelial branching in the developing lung; treating lung explants with VEGF leads to hypervascularization and enhanced epithelial branching [120]. The perfusion-independent roles of blood vessels are therefore organ-specific and may function primarily by altering signaling in the local microenvironment. In some cases, such as in the pancreas, these signals lead to termination of epithelial branching.

5. Outlook: a diverse toolbox for building epithelial trees

The examples highlighted above emphasize that there are many different cellular and physical mechanisms used to build epithelial trees across organs and species. These examples include morphogenetic motifs conserved between organs (e.g. buckling of multilayered epithelia in the mouse salivary gland and pancreas) and species (e.g. smooth muscle sculpting of the epithelia in mouse and lizard lungs). We also highlighted differences in the morphogenetic motifs used to build branched epithelia (e.g. ordered bifurcations in the mouse lung vs stochastic branching in the mammary gland) and species (e.g. apical constriction in the chicken lung vs smooth muscle sculpting in the mouse lung). Amazingly, we still lack a complete understanding of the physical mechanisms that drive epithelial branching morphogenesis across organs in a single model organism, including the mouse. Venturing beyond the conventional and established model systems will undoubtedly reveal surprising new mechanisms that build epithelial trees.

Morphogenetic motifs have been proposed to lie on a spectrum between two modes: programmed

and self-organized [121]. Branching by invasive cell migration, as in the *Drosophila* tracheal system [122], typically follows a pre-pattern of morphogen sources, suggesting that it employs the programmed mode. Conversely, branching of cell collectives by growth and tissue folding may employ the self-organized mode, leading to stochastic branching patterns such as those observed in the mammary gland [123]. Realistically, most morphogenetic motifs likely employ a combination of both modes, as has recently been shown for neuronal branching [124, 125]. In the mammalian lung, branching of the epithelium is highly stereotyped, suggesting reliance on a pre-pattern of morphogens [63]. However, the physical mechanisms of epithelial branching in the lung involve self-organized behaviors, such as buckling and constraint from surrounding tissues [26, 49, 59]. Branched epithelia may therefore serve as an interesting model with which to understand the interplay between these two conceptually distinct modes of morphogenesis.

One of the clear gaps in our understanding of the development of branched organs concerns how established epithelial trees grow through late gestation and postnatally. During the early stages of branching in many organs, the stalks of branches appear uniformly sized. However, adult organs show a remarkable diversity in tube structure; for example, the bronchial tree of the adult mammalian lung is fractal, with newer generations of conducting airways of smaller caliber than their parent branches [126, 127], whereas the adult mammary gland has ducts of a far more uniform diameter [128]. The final form of adult organs is likely optimized for the ultimate physiological function of the tissue. For example, the fractal pattern of the mammalian lung allows for efficient airflow but is not so efficient as to introduce problems such as bronchial constriction [129]. Branched tissues may employ distinct strategies to elongate and dilate their constituent branches, but the underlying molecular and cellular mechanisms remain unclear. With advances in high-resolution imaging of cleared whole organs and computational analysis of the resulting datasets, the field now has the tools to address some of these long-standing questions.

In this review, we described differences in the mechanisms that drive epithelial morphogenesis in the lungs of different terrestrial vertebrates. Other taxa use different organs for gas exchange, including gills, which are themselves divergent across species, from the internal gills of zebrafish to the external gills of axolotl [130]. Zebrafish gill filaments arise from the same embryonic tissues as the parathyroid gland in mammals, and their specification and budding may be controlled by similar factors [131]. However, little is known about the morphogenesis of gill filaments. It is therefore unclear whether the mechanisms that drive morphogenesis of gills resemble those that drive morphogenesis in the murine parathyroid gland or the lung (or neither). Axolotls are especially remarkable because they can have both external gills and lungs—while understudied, there is some evidence that the lungs of axolotls may be similar to those of *Xenopus*, in terms of epithelial structure and smooth muscle wrapping [132]. Future work in these model organisms is expected to uncover both novel as well as conserved cellular and physical mechanisms that drive morphogenesis.

Morphogenesis of the salivary gland has been studied extensively in mouse [29, 83, 84, 133, 134] and to a lesser extent in birds [9, 10]. A somewhat similar class of organs are the venom glands. Snake venom glands (of which there are a variety of types) are understudied, but may contain myoepithelial cells [135, 136] and/or smooth muscle cells [137], and are coupled to skeletal muscles important for rapid expulsion of venom [138]. Organoids derived from snake venom gland epithelial cells form spheres, whereas the epithelium appears more branched *in vivo* [137], perhaps due to the presence of sculpting forces from its adjacent smooth muscle or mesenchyme. Scorpion venom glands also contain branched epithelia and muscle populations—either smooth or striated—that presumably support rapid expulsion of venom [139, 140]. As with those of the snake, virtually nothing is known about how these glands develop and whether the differentiation of myoepithelial or muscle cells influences branching of their epithelia. Given the similarities and differences in their function (secretion of a liquid, but at very different speeds), it will be interesting to determine whether venom glands employ similar ECM-driven clefting and growth-induced buckling as observed in the salivary gland of the mouse.

Amongst all of this biological diversity, there exist conserved morphogenetic motifs that are repurposed in different contexts to build epithelial trees. We believe that understanding the mechanisms that comprise the biological toolbox of branching morphogenesis can inspire unique strategies to engineer and repair tissues. As such, biodiversity and the investigation of strange and surprising biological processes have direct implications for our creativity and success in the fields of regenerative medicine and tissue engineering.

Data availability statement

No new data were created or analyzed in this study.

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