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Comparative Mechanisms of Branching Morphogenesis in Diverse Systems

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Abstract

Much progress has been made in recent years toward understanding mechanisms controlling branching morphogenesis, a fundamental aspect of development in a variety of invertebrate and vertebrate organs. To gain a deeper understanding of how branching morphogenesis occurs in the mammary gland, we compare and contrast the cellular and molecular events underlying this process in both invertebrate and vertebrate organs. Thus, in this review, we focus on the common themes that have emerged from such comparative analyses and discuss how they are implemented via a battery of signaling pathways to ensure proper branching morphogenesis in diverse systems.

Keywords

Drosophila trachea; Air sacs; Lung; Kidney; Submandibular gland; Mammary gland; Prostate; Epithelial-mesenchymal crosstalk

Introduction

A major impediment to development of large and complex animals is the requirement for efficient exchange of gases, nutrients, metabolites and wastes with the environment. This problem was elegantly solved during metazoan evolution by packing a large surface area of epithelium into a relatively small volume via a process called branching morphogenesis, whereby epithelial tubules are expanded and remodeled during embryonic and postnatal development. Indeed, branching morphogenesis is a fundamental developmental process involved in formation of a variety of organs, from fly trachea and air sacs, to mammalian lungs, kidneys, vasculature, pancreas, as well as many exocrine glands, including mammary and salivary glands [1]. As a result, a deeper understanding of branching morphogenesis is not only crucial to our understanding of how these organs develop, but to understanding the molecular etiology of many human diseases, such as polycystic kidney disease, kidney and lung agenesis, and many malignancies including breast and prostate cancer [2,3].

Given their structural simplicity and genetic accessibility, the *Drosophila* tracheal and air sac systems have been used to identify cellular and molecular events involved in branching. Many of the molecules and processes involved in fly branching morphogenesis are now known to be highly related to those involved in the development of complex vertebrate organs [4–6]. From these studies, it has become clear that a small number of related pathways fulfill a similar role in branching of various systems, whereas molecular differences between distinct branched structures or organs will help explain how each organ becomes unique [2,7,8].

Our goal in this review is to consider the cellular and molecular mechanisms governing mammary branching in the context of recent advances concerning regulation of branching morphogenesis in other organ systems, including invertebrate systems. For this reason, the hormonal control of branching morphogenesis, which is clearly important but unique to the mammary gland [9–11], is not considered here. Nor do we discuss mechanisms controlling tubulogenesis as it is beyond the scope of this review, even though branching morphogenesis is one of several ways to generate and remodel epithelial tubes [12–14]. We will first discuss various modes of branching and their underlying cellular mechanisms in diverse branched organs. In addition, we focus on the molecular basis of epithelial–mesenchymal crosstalk in branching morphogenesis and how different molecules are deployed to execute similar functions. Finally, we will discuss the versatile roles of the extracellular matrix (ECM) in regulating this process and point out interesting directions for future studies. Thus, in the current review we assemble and analyze some common themes that are shared by a variety of organs to ensure their proper branching morphogenesis in the face of molecular differences that render each of these organs unique.

Branching Patterns in the Mammary Gland

A Brief Description of Mammary Ductal Development

The “tree-like” ductal epithelium observed in the mature mammary gland is derived from a single mammary bud that forms in mice at around embryonic day 11 [15]. Unlike the primary budding that occurs in the lung, or the ureteric buds that form in the kidney, both of which arise from a hollow tube, the mammary sprout starts out as a solid cord when the mammary bud invaginates into the primary mammary mesenchyme [15–17]. The process whereby the lumen initially forms in the mammary sprout has not been carefully examined, however, it is thought to be similar to the process that occurs in the salivary gland and pancreas, where lumina form by an apoptosis-mediated cavitation process [12].

By birth, a rudimentary ductal tree consisting of ~ 10–20 branches can be seen in each of the ten mammary glands in the mouse [15]. Interestingly, with the exception of the lung, where the initial orders of branching are stereotypical, the branch patterns of other vertebrate branched organs, including the mammary gland, are stochastic. In fact, branch patterns seem to differ even between contralateral mammary glands at the same position along the anterior-posterior axis of the body [15]. Thus, an early global patterning event that is thought to occur to ‘hard-wire’ the branching pattern of the lung [5], does not seem to function in the mammary gland or in most other vertebrate branching systems. After birth, the primitive ductal network remains relatively quiescent until puberty, growing just enough to keep pace with enlargement of the mammary fat pad associated with normal body growth [18,19]. The surging female hormones at puberty then set the “branching machinery” in motion and, for the subsequent 4–5 weeks, a rigorous epithelial invasion by ductal elongation and branching takes place until the fat pad becomes entirely filled [18,19].

Modes of Branching

Two major modes of branch-point formation, namely primary branching and secondary branching, are thought to occur concomitantly with ductal elongation in the stromal fat pad [10,11,16,18,20]. Primary branching occurs when the tip of invading mammary epithelium, a bulbous terminal end bud (TEB), splits dichotomously at a sharp angle to give rise to two TEBs, each of which continues to invade the stroma and give rise to two subtending primary ducts. This dichotomous splitting or bifurcation of the TEB takes place at fairly regular intervals and together with ductal elongation are responsible for the formation of a simple, primary ductal system in the mature gland [20]. Secondary branching, which is sometimes called side-branching or lateral branching, also occurs during post-pubertal mammary development and

denotes the budding and elongation of perpendicular secondary ducts from the already formed portion of the primary ducts behind the invading TEBs. In addition, a third mode of branching called tertiary branching takes place during recurrent estrous cycles and early pregnancy and denotes the initial formation of alveolar buds from the primary and secondary ducts [19,20]. During late pregnancy, the surface area of the alveolar buds expands tremendously due to a significant increase of cell proliferation and differentiation to prepare for milk production for the mother's offspring [9–11,16,18–20].

Similar modes of branching are thought to occur in other vertebrate branched organs, for example lung and kidney [21,22]. However, the proposed modes of branching in these tissues are based on evidence from analyses on fixed tissues. The most serious caveat, therefore, is that important details of the highly dynamic branching process may have been missed by deducing its various aspects from a final structure. Recent technical advances in the time-lapse imaging of organ cultures *in vitro* have shown that this is indeed the case [23]. Using time-lapse video recording of cultured embryonic mouse kidney, in which the epithelium is labeled with green-fluorescent protein (GFP), Watanabe and Costantini [23] found that ~75% of the renal tubular branches form by bifurcation and that ~7% form by lateral branching. However, they also discovered that ~18% of branches form by a third mode of branching: trifurcation, in which the tips of the tubules (the ampulae) split in three different directions simultaneously. Remarkably, a remodeling event of the branch-point was observed, such that what was previously assumed by end-point analysis to be two separate bifurcation events could actually be the product of one trifurcation event followed by unequal elongation of the ductal epithelium [22,23]. Similar events may also occur in other vertebrate branching systems. Thus, as a result of recent advances in long-term *in vivo* and cell culture-based imaging techniques as well as organotypic culture methods, we may learn surprisingly important new details of how branching actually proceeds in the mammary gland in real time.

Identity and Fate of the Functional Units of Mammary Branching

One question regarding functional units of the branched mammary epithelium (the TEBs and the ducts), is what genes specify their identities. Although we do not yet know the answer to this question, there is evidence that tips and trailing ducts of the branches are specified by different genes. In the kidney, for example, expression of Ret and its co-receptor Gfr α 1, as well as expression of Wnt11, Crlf1, and CXCL14 are specifically restricted to ampulae [24–26], whereas Wnt7b and collagen XVIII are only expressed in trunks [26]. Likewise, markers that distinguish TEBs from trailing ducts have been identified in the mammary gland. For example, Wnt5a, Wnt7b, Sprr1a, MT1-MMP and numerous other genes are specifically expressed in TEBs, whereas caseins and many other genes appear to be expressed exclusively in more proximal ducts [141]. Future studies should yield important insights concerning molecular mechanisms that determine the identities of these two structures.

Another under-explored aspect of mammary gland biology regards the fates and lineages of TEBs and ducts. There are at least three models to explain the relative contribution of TEBs and ducts to the final branching network. The first model argues that TEBs and ducts are two separate lineages and that the only function of TEBs is to invade stroma and bifurcate. Thus, according to this model, it is the elongation of ducts that gives rise to the entire ductal epithelial network. The alternative model argues that ducts are passive entities, and it is the TEBs that, by giving rise to the trailing ducts, exclusively contribute to the final ductal tree. The observation that most mammary epithelial proliferation occurs in TEBs seems to support the latter hypothesis [27]. The third model takes the middle ground and proposes that TEBs and ducts are not separate lineages and they both contribute to the final branching network.

Important progress has recently been made in the field of kidney branching concerning respective fates of ampulae and ducts. Using chimeric analyses generated by injecting Ret

mutant cells into wild-type blastocysts, Shakya et al. [25] created mosaic kidneys in which the ducts, but not the ampulae, were labeled with Ret mutant cells expressing GFP. Mutant cells are excluded from ampulae because Ret function is required for formation of the ureteric bud (UB), the tip of which gives rise to future ampulae [25]. Consequently, mutant cells only contribute to the trunk of ureteric bud and ducts, which normally do not express Ret protein, in subsequent branching events. Because ampulae and ducts were differentially marked, their fates could be followed through live imaging. In addition to confirming that ampulae actively branch and give rise to trailing ducts, the authors found that the ducts are not passive entities, but that they, too, actively participate in ductal elongation and, most surprisingly, in remodeling of branch-points. Together, these data demonstrate that both ampulae and ducts contribute to ductal elongation and branching, and thus determine branch patterns (Fig. 1). Considering the aforementioned similarities between the branching modes seen in both kidney and mammary gland, it will be interesting to see whether mammary TEBs and ducts behave in a similar manner to their renal counterparts.

Cellular Events of Branching Morphogenesis

Migration, Cell Rearrangement and Change of Cell Shape

The cellular events leading to distinct steps in branching morphogenesis are best understood in the *Drosophila* tracheal system [4,6,28]. In brief, the onset of fly tracheal development starts at 5 h after egg-laying (AEL) with formation of ten tracheal sacs on each side of the embryo by invagination of the surface ectoderm. During the next 2 h, tracheal sacs separate from the ectoderm, elongate and undergo two rounds of cell division to give rise to epithelial sacs consisting of ~80 cells. In subsequent branching events, there is no cell proliferation, apoptosis or change in cell number in the tracheal system [29]. At 7 h AEL, primary branches start to form by invaginating or budding in six different but stereotypical directions. At each bud site, two cells migrate out of the sac, followed by a small number [3–20] of cells. As they migrate, cells of the primary branch elongate and intercalate such that their initial side-by-side positioning is rearranged to an end-to-end configuration, with the lumen of resultant tubes enveloped by one single cell. At 11 h AEL, the lead cell of the primary branch extends and separates from neighboring cells to form a secondary branch. At 13 h AEL, the lead cell also elaborates fine cytoplasmic extensions, or terminal branches. Importantly, the pattern of terminal branches is variable and is determined by physiological oxygen concentration of the surrounding tissue. In essence, tracheal branching creates morphologically distinct tubes, which form apical junctions through a variety of mechanisms [28]. Together, tracheal branching is characterized by three main cellular processes: cell migration, cell rearrangement and change of cell shape [4,6,28].

In contrast, cellular events that occur during branching morphogenesis in vertebrates are poorly understood. At first glance, vertebrate branching is a relatively simple process, involving reiterative events of ductal elongation and branch-point formation through invagination or budding of a group of cells in an epithelial sheet, or splitting the tips of epithelial tubes. As opposed to the process that takes place in the tracheal system, cell proliferation is concomitant with all stages of branching morphogenesis in vertebrate organs. The increase in cell number, along with current limitations in organ culture and extended live animal imaging techniques have made characterization of cellular events in vertebrate systems extremely challenging. Using an *in vitro* lung culture model, Weaver et al. [30] showed that FGF10 beads can attract epithelial cells to migrate toward the source of ligand. Likewise, beads soaked in glial-cell-line derived neurotrophic factor (GDNF) induce and attract ectopic ureteric buds from the Wolffian duct [31–33], thus also suggesting that cell migration may contribute to vertebrate branching. However, filopodia and/or lamelliopodia have yet to be documented in vertebrate systems to argue definitively that the cells are indeed migrating, rather than undergoing directional proliferation. However, epithelial cells in some systems are very motile, constantly

changing their positions in trunks and ampulae of the developing kidney [25]. Comparable observations have been made in organotypic mammary cultures and live mammary gland imaging *in vivo* (A. Ewald and Z. Werb, unpublished results). Such cell rearrangements may play a role in convergent extension of epithelial cells and thus participate in ductal elongation and control of tube-size in renal branching [25]. Together, cell migration, rearrangement and change of cell shape are likely to be conserved cellular events underlying vertebrate branching in general.

Epithelial–mesenchymal Transition and Cell Proliferation in Branching Morphogenesis

The unique tubulogenic process of branching and the lack of cell proliferation in the fly trachea have also limited its usefulness as a developmental paradigm to study vertebrate branching morphogenesis. In this regard, recent characterization of fly air sac development may provide insights that help us understand vertebrate branching processes. Briefly, the thoracic air sac develops during remodeling of the tracheal system, when the increase in body-size demands higher oxygen supplies in the early third instar larva at around 70 h AEL [34]. The thoracic air sac starts out as a group of cells migrating out from an existing tracheal branch, the transverse connective of the wing imaginal disc, toward a stereotypical direction. Significantly, cell proliferation accompanies the outgrowth process of air sacs, making it similar to the budding events seen in vertebrate systems [34,35]. In addition, as in the tracheal system, both cell rearrangement and change in cell shape occur in developing air sacs of the fly.

It has been proposed that cells at the leading edge of branching epithelium in vertebrate organ systems may undergo a transient epithelial–mesenchymal transition (EMT) [36]. Evidence for this hypothesis derives from the observation that, in MDCK cell cultures, branches form by initial depolarization and cell migration, followed by repolarization and lumen formation [37,38]. A similar process accounts for sprouting angiogenesis [39]. In air sacs, however, migrating cells at the leading edge, as evident by their filopodial and lamellipodial extensions, seem to remain embedded within the epithelium, contact the lumen and form apical junctions with neighboring cells, thus arguing against the premise that EMT plays a role in branching morphogenesis [35]. Indeed, there is no real evidence that a transient EMT takes place during mammary branching in organotypic cultures *in vitro* (A. Ewald and Z. Werb, unpublished results).

A long-standing question regarding cell proliferation has been whether it plays a permissive or instructive role in branching morphogenesis in vertebrate organs. Intuitively, an increase in cell number is necessary to add more ‘building-blocks’ to branching epithelium. On the other hand, directional cell proliferation may be a powerful force to generate forward movement during ductal elongation and invasion into the mesenchyme. This question has now been addressed in fly air sacs. Using loss-of-function studies based on mitotic recombination, in which mutant cells are generated in a heterozygous environment, Cabernard and Affolter [35] examined the role of several major signaling pathways, including Dpp/BMP, Hedgehog (HH), VEGF, Frizzled, EGFR and Breathless (Btl)/FGFR signaling, in the outgrowth of fly air sacs. They found that Btl/Fgfr mutant cells are unable to take the leading edge position during migration, although they can proliferate and occupy stem and trunk regions. These data suggest that Btl/Fgfr signaling is essential for cell migration but is unnecessary for cell proliferation in air sacs. On the other hand, EGFR seems to be important for cell proliferation and survival because mutant clones are much smaller than normal. Remarkably, however, mutant cells can contribute to the trunk region and to the leading edge. These data demonstrate, therefore, that cell proliferation *per se* is unlikely to be the driving force for branching morphogenesis, at least within fly air sacs (Fig. 2).

That cell proliferation plays a permissive role has yet to be established in vertebrate branching systems, although there is evidence to suggest that cell proliferation and branch-point formation

are differentially regulated. For example, pharmacological attenuation of Erk/MAP-kinase activities by PD98059 leads to a potent inhibition of branch-point formation in kidney organ culture [23,40]. However, the overall length of renal tubules formed in treated cultures is barely affected. As a result, the average fragment is significantly longer than normal [23,40]. The overall production of renal tubules is essentially a net measurement of the amount of cell proliferation that has occurred in culture. Thus, consistent with the aforementioned relationship between cell proliferation and migration in the fly air sacs, these data demonstrate that cell proliferation and branch-point formation are independent events at the molecular level in kidney branching.

Interestingly, inhibition of PI3 kinase activities blocks both ductal elongation and branch-point formation in kidney organ culture [41]. Likewise, loss of Ras function abrogates both cell proliferation and migration in the fly air sacs, a phenotype resembling that seen in the absence of both Egfr and Btl/Fgfr function [35]. Thus, cell proliferation and branch-point formation in vertebrate tissues, or cell proliferation and migration in the fly air sac, seem to be governed by similar as well as distinct molecular events. Taken together, these data highlight the complexity of molecular mechanisms responsible for branching morphogenesis and the challenges we face to fully understanding them.

Molecular Basis of Epithelial–Mesenchymal/Stromal Crosstalk in Branching Morphogenesis

Epithelial–mesenchymal crosstalk is one of the central themes in developmental biology for its essential role in specification, patterning, outgrowth and differentiation of a variety of vertebrate organs, including the limb, pharyngeal arches, and intestines to name just a few. Not surprisingly, interactions between epithelium and embryonic mesenchyme, or stroma in the case of the post-natal mammary gland, are required for branching morphogenesis to occur properly in different vertebrate systems. We discuss in the following sections molecular mechanisms governing epithelial–mesenchymal crosstalk: how the mesenchyme signals epithelium, and how the epithelium responds, and vice versa, in branching morphogenesis.

Role of Mesenchymal/Stromal Cues in Epithelial Branching Morphogenesis

Studies in the fly suggest that mesenchymal cues in vertebrate systems can serve at least two functions. The first function is to guide the direction of epithelial outgrowth and the second is to promote cell proliferation and/or survival in the epithelium. In the fly trachea, one of the most insightful observations is that Branchless (Bnl/Fgf), and its receptor Breathless (Btl/Fgfr), determine the stereotypical branch pattern [42,43]. Bnl/Fgf, which is normally restricted to distinct cell clusters adjacent to the epithelium [4,5], binds to and activates Btl/Fgfr located on tracheal epithelium, and thus acts as a chemoattractant to guide epithelial migration [42,43]. Remarkably, although all tracheal epithelial cells express Btl/Fgfr and can respond to Bnl/Fgf, they compete for the ligand, and the cell with highest Bnl/Fgf signaling activities becomes the lead cell [44]. Once the lead cell has been determined, it is the only cell in the primary branch to require Bnl/Fgf signaling as a guidance cue. Remaining tracheal cells follow the lead cell in a Bnl/Fgf-independent manner [44]. As noted earlier, the cell number does not change during fly tracheal branching and specific survival cues are not required. On the other hand, in air sacs where cell proliferation accompanies epithelial out-pouching, EGF signaling is required to function as an epithelial survival factor.

Similar themes may function in vertebrate organ systems as well. Generally speaking, however, the mesenchymal environment in the vertebrate is more complicated than the fly systems and a myriad of factors, many of which are ligands of receptor tyrosine kinases (RTKs), are required for vertebrate branching morphogenesis [3,19,45]. Without an accurate description of cell

behaviors in vertebrate branching as mentioned, one of the most challenging questions regarding mesenchymal factors is whether they function as guidance cues or survival factors for branching epithelium.

Among the mesenchymal factors, the FGFs are the most often documented factors to play a role in the epithelial branching of vertebrate organs. For example, in the lung a loss of Fgf10 expression in the mesenchyme, or of Fgfr2 in the epithelium prevents primary budding and, as a result, the organ fails to form [46–48]. Thus, FGF 10 may serve as a chemoattractant to guide migration and/or a mitogen during branching of lung epithelium [30]. In the kidney, both Fgf7 and Fgf10 are expressed in mesenchymal cells, and the loss of their receptor Fgfr2 in epithelium causes a significant reduction in branching [49]. Thus, FGF signaling also plays a role in renal branching.

In the mammary gland, FGF signaling has long been associated with normal development and with breast cancers [50]. However, its role in branching morphogenesis has not been defined due, in a large part, to an early requirement for FGF signaling in embryonic mammary placode development [51]. Using Cre-mediated gene disruption and mosaic analysis, we have found that FGFR2 function is required in TEBs for proper ductal elongation and bifurcation (P. Lu and Z. Werb, unpublished results). Likewise, down-regulation of FGF signaling by a function-blocking antibody against FGFR2 reduces branching in the submandibular gland [52]. Interestingly, the dosage of FGF 10 appears to be critical for branching morphogenesis in the lacrimal gland, since loss of only one wild-type allele is sufficient to cause defective branching in heterozygous mice [53].

In addition to FGFs, other RTK ligands are required for vertebrate branching. For example, EGF signaling regulates lung development, as a 50% reduction in branching is observed in Egfr mutant mice [54]. Similarly, mesenchymal GDNF is important both for kidney induction and for renal epithelial branching [25]. In the mammary gland, insulin growth factor (IGF) signaling appears to function downstream of female hormones to promote epithelial branching [55,56]. Hepatocyte growth factor (HGF) has also been proposed to play a role in mammary branching based on gain-of-function studies [57], although this idea has yet to be tested through loss-of-function analysis.

Given their similar downstream signaling events, in particular their overlapping activation of the Ras–Raf–MAPK pathway, it is reasonable to assume that several mesenchymal RTK ligands may cooperate to induce epithelial migration and branching. This may indeed be the case for FGF and GDNF in the kidney. As mentioned earlier, GDNF can stimulate renal epithelial cell migration *in vitro*, and ectopic GDNF is sufficient to induce supernumerary ureteric buds [25,58]. Likewise, FGF beads can cause formation of ectopic ureteric buds [143]. It is thus tempting to suggest that FGF and GDNF signaling act concordantly to promote renal epithelial branching. Alternatively, they may act sequentially in the same signaling pathway. Future studies will help distinguish between these models.

Another interesting, but challenging, question is how mesenchymal RTK ligands serve distinct functions, as they appear to do in the fly, if their downstream signaling events are similar. In air sacs, Bnl/Fgf and EGF, both of which depend on Ras function, act as guidance cues and survival factors, respectively [35]. However, at least two strategies are implemented to ensure that these two RTK ligands function independently. First, they seem to differentially impinge upon MAPK activities such that it is more strongly activated by Bnl/Fgf in cells of the leading edge. Second, they recruit distinct co-factors to initiate separate downstream events [35]. Aside from differentiating whether the mesenchymal factors act as survival or guidance cues, another major challenge will be to determine how different downstream events are elicited to control specific aspects of branching morphogenesis in the vertebrate.

Epithelial Responsiveness to Mesenchymal/Stromal Cues

Given the importance of mesenchymal/stromal factors in regulating epithelial cell behavior, it comes as no surprise that mesenchymal cell functions or signals need to be controlled. Indeed, there are at least two ways to accomplish this. First, epithelial cells can modulate their own responsiveness to mesenchymal factors. Second, the epithelium can signal the mesenchyme and proactively influence the production of cues from the latter tissue.

The *Drosophila* Sprouty (*Spry*) gene was first identified in a screen for mutations that cause defective branching in the tracheal system. In *Spry* mutant flies, more secondary branches are produced from the lead cell of primary branches [59]. It was discovered subsequently that *Spry* functions in a cell-autonomous manner to inhibit Btl/Fgfr signaling in tracheal epithelium. In its absence, the lead cells are over-sensitized to Bnl/Fgf stimulation and, as a result, excessive secondary branches form. In addition, consistent with the aforementioned role of Bnl-Btl signaling in specification of the lead cell, *Spry* mutant cells are more likely to adopt the lead cell fate than neighboring non-mutant cells in a mosaic environment [44]. Importantly, it is now known that *Spry* is not a specific antagonist to FGF signaling; rather, it also inhibits EGFR and presumably other RTKs in the fly [60–63].

Four vertebrate homologues of the fly *Spry* gene, *Spry* 1–4, have been identified in the mouse and some of them have been shown to play a role in branching morphogenesis in the mouse [60,64,65]. Mice lacking *Spry*1, for example, show polycystic kidneys due to formation of extra ureteric buds, similar to having excessive GDNF/Ret signaling during kidney induction [66]. Indeed, reducing GDNF/Ret signaling activity by removing one wild-type *Gdnf* allele, and thus reducing the dosage of GDNF, rescues the phenotype of *Spry*1 mutant mice. These results suggest that *Spry*1 normally functions to inhibit GDNF/Ret signaling in ureteric bud formation. Importantly, *Spry*1 also functions at later stages during renal branching as ectopic branching has been observed in the mutant kidney [142]. Similarly, data suggest that *Spry*2, which is expressed in the distal lung epithelium, negatively regulates branching morphogenesis of the lung. For example, down-regulation of *Spry*2 function using an antisense RNA strategy can stimulate lung branching, whereas gain of *Spry*2 function causes the opposite phenotype [67,68]. However, it remains unclear which RTK signaling pathway, and which cellular behaviors are regulated by *Spry*2.

Sprouty-related protein (*Spred*) and *Similar* expression to *Fgf* (*Sef*) genes, have also been shown to antagonize RTK signaling [69–74]. Interestingly, *Spreds* are expressed in the developing lung and may play a role in vertebrate branching [75]. Given the importance of RTKs in both invertebrate and vertebrate systems, it will be important to determine how RTK signaling is regulated by these antagonists during branching morphogenesis in mammals.

Role of Epithelial Cues in Mesenchyme/Stroma

In addition to adjusting its responsiveness to mesenchymal/stromal cues, the epithelium also directly signals the mesenchyme and modulates its activities. Such mesenchymal modulation by epithelium appears to be a unique feature of vertebrate branching morphogenesis. In the fly tracheal system, for example, although the epithelium can fine-tune Bnl/Fgf signaling activities by expressing *Spry*, so far there has been no evidence to suggest that the epithelium can alter Bnl/Fgf expression in return. In fact, Bnl/Fgf expression is believed to be pre-determined by global patterning genes irrespective of the epithelium [4,5]. Interestingly, in the lung, where branch pattern is also stereotyped and thought to be hard-wired, mesenchymal Fgf10 expression appears to be modulated by the epithelium [5]. In the following sections, we discuss several signaling pathways that have been proposed to modulate mesenchymal functions in vertebrate branching.

Hedgehog (HH) Signaling—The first line of evidence that lung mesenchyme is modulated by the epithelium derived from the observation that Fgf10 expression is up-regulated in mice lacking Sonic HH (Shh) function [76]. Since Patched-1 (Ptc-1), which encodes the receptor for Shh protein, is expressed in lung mesenchyme, it was suggested that Shh signaling from the epithelium restricts Fgf10 expression to discrete locations in this tissue [21,76]. Importantly, despite up-regulation of Fgf10 expression, Shh mutant lungs fail to branch beyond primary budding [76,77], suggesting that Shh may play additional roles in lung branching morphogenesis (see below).

Shh is also expressed in epithelium of the submandibular gland. Moreover, inhibition of Shh signaling by cyclopamine, a chemical inhibitor of this pathway, reduces branching in salivary gland organ cultures. These results suggest that HH signaling is required for salivary gland branching [78]. Surprisingly, Smoothened, a positive transducer of HH signaling, Gli3, an HH-responsive transcription activator, and Ptc-1 are expressed in the epithelium. In this context, therefore, Shh may function in an autocrine manner within the salivary epithelium compartment [78].

HH signaling may play a role in branching morphogenesis of the mammary gland. Loss of one wild-type allele of Ptc-1, which causes excess HH signaling, leads to hyperplasia of ductal epithelium [79]. Conversely, loss of stromal Gli2 causes defective ductal epithelium in pregnant animals [80]. Since both Gli2 and Ptc-1 are expressed in the stroma, HH signaling must act upon the epithelium via an unidentified stromal factor(s). However, mammary glands lacking either Shh or Indian HH (Ihh) show no defects in postnatal branching morphogenesis [81,82]. Thus, it remains unclear at present whether Shh and Ihh play a redundant role in mammary branching. If so, removal of both genes may be required to cause discernible defects in this developmental process.

Interestingly, hedgehog-interacting protein (Hip), an inhibitor of Shh function, is expressed in the lung mesenchyme to counteract HH signaling activities. In mice lacking Hip function, Fgf10 expression is down-regulated due to excess HH signaling and, as a result, lung branching is significantly reduced [83]. Moreover, the reduction of lung branching in Hip mutants is exacerbated by additional increase of HH signaling, for example in mutants lacking one wild-type allele of Ptc-1, which is another inhibitor of HH function [83].

TGF- β Super-family—The transforming growth factor β (TGF- β) super-family contains a large number of members, including at least eight bone morphogenetic proteins (BMPs), three TGF- β s, five activins and other related proteins that function as ligands for serine-threonine kinase receptors at the cell surface [84,85]. Binding of these ligands to their type I and type II heterodimeric receptors causes phosphorylation and kinase activation, which in a Smad-dependent or -independent manner lead to transcription of target genes [84,85]. Available data show that TGF- β super-family ligands play an important role in a variety of developmental processes, including branching morphogenesis.

BMP—During fly tracheal development, Dpp/BMP in dorsal domains regulates Bnl/Fgf expression. In the absence of Dpp/BMP signaling in this context, Bnl/Fgf is not expressed and dorsal tracheal branches do not form [86]. The function of BMP signaling in vertebrate branching morphogenesis has been difficult to analyze. This is in part due to the fact that multiple BMPs are often expressed in rather broad and overlapping domains, where they may play redundant roles, and in part due to their dynamic nature, whose functions are spatiotemporally dependent. The complexity of BMP function is best illustrated in vertebrate limb development. In this setting, several BMPs, including Bmp2, Bmp4 and Bmp7, are expressed in a dynamic fashion in both ectoderm and mesenchyme of the developing limb [87,88]. Using loss-of-function studies, it has been shown that BMP signaling in the ectoderm

is required initially for the formation of apical ectodermal ridge (AER) [89,90], a crucial signaling center in limb development, and, counter-intuitively, for its subsequent demise when AER function is no longer required [90].

Several BMPs are expressed in the developing lung [30,91,92], including Bmp4, which is expressed in distal epithelium [30]. Since removal of BMP receptor 1a in the epithelium causes cell death and reduced branching, it has been suggested that Bmp4 functions as an autocrine factor in the lung epithelium to promote cell survival [93]. In contrast, BMP signaling appears to inhibit induction, and possibly branching morphogenesis, of the kidney. For example, ectopic ureteric buds form in Bmp4 heterozygous null mice, suggesting that BMP4 normally functions to inhibit GDNF/Ret signaling during kidney induction [94]. Consistent with this notion, the opposite phenotype, a failure of ureteric bud formation, has been observed in mice lacking the BMP antagonist Gremlin. This is because loss of Gremlin causes excessive BMP signaling, which in turn prevents GDNF/Ret from inducing formation of the kidney [95]. At present it is not known whether BMP signaling plays an analogous role in branching morphogenesis of the mammary gland.

TGF- β —Generally speaking, TGF- β signaling has been found to inhibit vertebrate branching morphogenesis [2,7,19,45,91,96]. Indeed, TGF- β family members can inhibit branching in organ cultures of lung [97,98] and kidney [96,99]. In addition, over-expression of TGF- β 1 in epithelium causes reduced branching in the mammary gland [100]. Based on results from these gain-of-function studies, one would predict that ectopic branching should be observed in the absence of TGF- β (s). However, loss-of-function studies have not been straightforward. For example, despite the expression of TGF- β 1, TGF- β 2 and TGF- β 3 in the developing lung [91], loss of any one of these three genes is not sufficient to cause defective branching, although TGF- β 3 mutant mice show hypoplastic alveoli during late stage lung development [91,101,102]. From these results, one might speculate that different TGF- β s play redundant roles, and thus, simultaneous removal of two or all three may be required to cause a discernible phenotype in lung branching. It turns out, however, that this hypothesis is difficult to test, given that maternal TGF- β 1 can traverse the placenta and rescue immune-deficiency and possibly other phenotypes in mutant embryos [101]. Therefore, the issue of maternal supply has so far hindered analysis of TGF- β function in the development of most branched organs in the mouse, which develop primarily before birth.

In retrospect, it comes as no surprise that the function of TGF- β signaling in branching morphogenesis is best understood in the mammary gland. This is because the mammary gland develops primarily after birth, and therefore maternal TGF- β 1 does not play a major role. Indeed, in TGF- β 1 heterozygous females, in which TGF- β 1 protein is expressed at ~10% of normal levels, epithelial invasion is accelerated [103]. This phenotype can in part be explained by a much higher rate of cell proliferation in the epithelium. Tissue-recombination experiments and expression analysis have revealed that mammary epithelium is the source of active TGF- β 1, as opposed to the stroma [103–105]. However, it appears that mammary stroma is the target for TGF- β 1 since loss of TGF- β receptor II (T β RII) function in the stroma accelerates epithelial branching [106–108]. Together, these results suggest that TGF- β 1 inhibits epithelial branching by targeting stromal production of a yet unidentified factor(s), which normally functions to regulate epithelial branching. Several lines of evidence suggest that stromal FGF may be regulated by TGF- β . Indeed, TGF- β 1 has been shown to inhibit Fgf10 expression in fibroblasts [109], as well as in lung [110] and prostate [111] explant cultures. Future studies should address which FGF factor functions in mammary stroma and, importantly, whether its expression is indeed negatively regulated by TGF- β signaling *in vivo*.

Additionally, TGF- β signaling may regulate branching morphogenesis by affecting ECM remodeling, which, as discussed below, plays an essential role in vertebrate branching. For

example, TGF- β 1 expression has long been associated with ECM deposition. TGF- β 1 regulated ECM components include collagen I, collagen III and fibronectin in developing lungs and mammary gland [112–114]. Moreover, pellets soaked with TGF- β 1 alter ECM production in the mammary gland [113]. Taken together, it appears that mammary epithelial branching is highly regulated by TGF- β , likely through control of expression of stromal factors and ECM deposition in stromal cells.

Epithelium as the Source of Mesenchymal Survival Factors—Aside from modulating the production of mesenchymal cues, epithelial signals regulate cell proliferation and survival in the mesenchyme. As mentioned earlier, Shh plays dual roles in the developing lung; one by restricting sites of Fgf10 expression, the other by promoting survival of mesenchymal cells [115]. Thus, in the absence of Shh function, despite an up-regulation of Fgf10 expression, the lung fails to develop [76,77]. Another survival factor for lung mesenchyme is Fgf9, which, curiously, is derived from both mesothelium and epithelium [115]. Similarly, in addition to its aforementioned role in epithelial branching [49], renal FGF signaling has recently been shown to play a survival function for metanephric mesenchyme [116].

In the mammary gland, some of instructive or permissive epithelial signals are provided by members of the EGF family. The first indication that EGFR signaling regulates mammary branching derives from the observation that EGF-related ligands can rescue branching defects in mice lacking ovarian hormonal stimulation or estrogen-receptor (ER) function [117–119]. Loss-of-function analysis showed that amphiregulin (AREG), but not EGF or TGF- α in the mammary epithelium is required for normal branching [120]. Interestingly, although EGFR is expressed in both epithelial and stromal compartments, its function is required only in the stroma [117]. Our laboratory has shown that ADAM17, a metalloproteinase, is required for cell surface release of AREG and other EGFR ligands. Indeed, ADAM17 mutant mice have reduced mammary [121] and lung branching [122], as do EGFR deficient mice [123]. Thus, ADAM17 plays a key role in the development of these and probably other branched tissues by releasing critical cell surface EGFR ligands, thus enabling EGFR activation and its downstream consequences to occur.

Role of ECM in Branching Morphogenesis

The extracellular matrix (ECM) is a complex, highly dynamic and critical component of all tissues in a multicellular organism [124]. Research has shown that ECM regulates cell proliferation, survival, migration, cell fate specification and other fundamental aspects of cell biology. It is now well known that regulation of ECM is required for many essential developmental processes during tissue morphogenesis, injury and repair, and that its deregulation plays a critical role in the pathogenesis of cancer and many other diseases [124, 125]. Nevertheless, our understanding of the role of ECM production and remodeling in vertebrate and invertebrate branching systems remains rudimentary. In general, functions of the ECM during branching morphogenesis can be divided into two broad categories: structural and biological.

Structural Role of ECM

The structural role of ECM in branching morphogenesis refers to its relatively inelastic and stiff properties, which act as a physical barrier to branching epithelium. For example, the site of fibronectin deposition has been correlated with branching pattern in the lung and kidney such that it is more abundant in clefts and around stalks, than in front of budding tips [126]. Similarly, sulfated glycosaminoglycans (GAGs) and type I collagen are more strongly expressed in the cleft and around ducts than in areas in front of mammary TEBs [113,114]. Indeed, in submandibular gland organ cultures, down-regulation of fibronectin blocks

branching, whereas adding fibronectin to the growth medium facilitates it [126]. It is therefore likely that regional deposition of fibronectin and other ECM proteins is a common strategy to execute branch-point decisions and guide growing epithelium in vertebrate organs.

In addition to localized synthesis, ECM is constantly being removed and/or remodeled to keep up with the dynamic nature of branching morphogenesis. For example, cells of the TEB face an ever-changing environment, where they need to remove interstitial ECM and lay down new matrix components during epithelial invasion. Moreover, the physical barrier posed by extracellular basement membrane (BM) needs to be removed for cells in the ductal epithelium to undergo side-branching. Remodeling of ECM is largely accomplished by matrix-modifying enzymes, including matrix metalloproteinases (MMPs), GAG-degrading enzymes (e.g., β -glucuronidase) and polysaccharide synthetases.

The importance of these enzymes for branching morphogenesis was first shown in cultured organs, including the kidney, lung, salivary gland and pancreas, where collagenase or hyaluronidase treatment can change branch patterns [127,128]. In the salivary gland, collagenase appears to block epithelial bifurcation by preventing deposition of type I collagen fibrils, whereas inhibitors of collagenase cause the opposite phenotype [129]. In the kidney, selective inhibition of MMP-9 with function-perturbing antibodies causes abnormal branching, suggesting that MMP9 plays a role in renal branching morphogenesis [130]. Moreover, the kidneys of MMP9-deficient mice have 12% fewer nephrons than normal, probably reflecting a mild *in vivo* defect in branching morphogenesis [131]. MMP9-null mice have no overt mammary branching phenotype. However, MMP2- and MMP3-deficient mice show defective mammary ductal elongation and side-branching, respectively [132]. In addition, regulation of lung branching morphogenesis by EGFR is partly accomplished through EGFR-mediated induction of MMP14/MT1-MMP expression, and activation of MMP2 by the former enzyme [123]. Likewise, MMP14-mediated MMP2 activation and clearance of type I collagen by both enzymes appears to be required for normal mammary branching morphogenesis. This pathway probably also lies downstream of EGFR (M. Egeblad, M.D.S. and Z.W., unpublished results).

Biological Role of ECM

The outdated notion that ECM is a mere scaffold or barrier for cells has been revised to reflect the fact that it conveys important contextual information and actively regulates cell behavior [133]. Indeed, ECM components can influence cell shape, polarity, motility, growth, differentiation and survival by acting as fixed ligands for adhesion molecules that transduce ECM-derived signals to the cell interior and by sequestering other signaling molecules, such as growth factors and their binding proteins. By extension, enzymes that modify or degrade the ECM can affect cell behavior by altering the content and organization of ECM-associated information. For instance, proteolytic ECM remodeling can (1) alter or abolish existing ECM signals, (2) remove physical barriers to ductal branching and elongation, (3) unmask hidden structural cues, such as cryptic integrin binding sites on fibrillar collagen, a bio-active laminin-5 fragment that promotes epithelial motility, and anti-angiogenic fragments of collagens IV (tumstatin) and XVIII (endostatin), and (4) release ECM-sequestered signaling molecules, such as amphiregulin, FGFs, Wnts and TGF β [124].

Thus it is not surprising that the ECM, its receptors and its proteolytic remodeling influence branching. Indeed, multiple ECM receptors may participate, such that genetic ablation of any one receptor may show only subtle defects in branching. For example, deletion of the discoidin domain receptor-1 (*DDR1*) gene, which codes for a receptor tyrosine kinase that transmits signals upon binding fibrillar collagens, results in delayed and abnormal mammary development [134]. Thus, fibrillar collagens provide more than structural support. This fact may partly explain the delayed mammary branching phenotypes that occur in mice lacking collagenolytic enzymes MMP2 or MMP14, or that express cleavage-resistant or mutant type

I collagen (M. Egeblad, M.D.S. and Z.W., unpublished results). In addition, mice that lack integrin $\alpha_2\beta_1$ collagen/laminin receptors due to deletion of the α_2 integrin gene show diminished but otherwise normal mammary branching [135]. Likewise, antibodies against β_1 integrin cause diminished ductal elongation, as do antibodies against the laminin γ_1 chain (i.e., part of a major ligand for β_1 integrins) [136]. In contrast, targeted deletion of β_1 integrin using MMTV-Cre is reported to have no effect on mammary development [137]. Over-expression of another laminin receptor, (β -1,4-galactosyltransferase, elicits abnormal mammary development [138], and yet another laminin receptor, dystroglycan, has been shown to regulate normal mammary epithelial cell polarity [139]. In addition, transitions between different modes of adhesion due to spatial differences in ECM deposition may play a role in ductal branch-point selection. For instance, localized fibronectin deposition is required for cleft formation in submandibular gland cultures and is associated with a switch from E-Cadherin to $\alpha_5\beta_1$ integrin-mediated adhesion, i.e., from cell-cell to cell-ECM adhesion, a transition that may stabilize bifurcation sites [126].

Concluding Remarks

It is amazing how nature uses sometimes similar, if not identical, molecular gadgets to build what clearly are non-homologous structures from the fly trachea to the human lung, kidney and mammary gland. Although a great deal has yet to be learned, several signaling pathways and common themes have emerged as conserved regulators of branching morphogenesis in diverse organs (Fig. 3 and Table 1).

An interesting question, then, is what such molecular conservation really means. Perhaps it means that there are “branching modules” consisting of a defined set of molecules and pathways that are activated whenever epithelial branching is required [140]. Alternatively, it may indicate that branching morphogenesis, irrespective of what species or organs it occurs in, is the sum of several cellular events, each of which is controlled by specific signaling pathways. Lastly, the molecular conservation may merely reflect regulatory relationships between signaling pathways that often work together in many different times and places during development. For example, directional FGF signaling, its regulation by Spry family proteins and its participation in epithelial-mesenchymal interactions are recurring themes in organogenesis, from tracheal development in flies to limb and tooth development in vertebrate organisms. So it should, after all, not be surprising that a small number of genes and pathways are used repeatedly to generate branched organs. While continuing our quest for molecules that play conserved roles in branching morphogenesis in diverse organs, we should also take note of mechanisms that determine specific differences in development of various branched structures.

One obstacle that hinders our progress in future studies on branching in vertebrate organ systems, in general, and in the mammary gland, in particular, is the lack of detailed information on cell behavior in this process. Technical improvements in organ culture and live imaging, for example, may help facilitate a better understanding of cellular events involved in mammary branching. Ultimately, such knowledge will be essential to our dissection of the effects different mesenchymal factors have on the epithelial cells and whether these factors act as guidance cues, mitogens, survival factors, or a combination thereof.

Another important area of future research will be to identify upstream regulators and downstream effectors of the signaling cascades known to influence branching morphogenesis, as well as those that have not yet been discovered, and to determine how these various pathways interact with one another in a holistic sense to direct and carry out the overall process of branching morphogenesis. What, for instance, are the critical downstream targets of FGF signaling? How do these events lead to production of epithelial signals that can affect mesenchymal activities, and vice versa? What are the downstream targets of EGF signaling in

the mammary stroma? Does EGFR signaling alter production of FGFs or other stromal factors that in turn effect the epithelium; and if so, how? Lastly, we have primarily considered cellular and molecular events that occur at the tips of epithelial branches, which is where many of the branch-point decisions are made, yet side-branching also occurs [3,7,19,21–23]. Although the main cellular and molecular events that regulate branching may be similar in primary branching and side-branching, deciphering their differences may be crucial to our eventual understanding of mechanisms that define mammary-specific branching and function.

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Abbreviations

ADAM	a disintegrin and metalloproteinase
AEL	after egg laying
AER	apical ectodermal ridge
BMP	bone morphogenetic protein
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial–mesenchymal transition
EGFR	EGF receptor
FGF	fibroblast growth factor
FGFR	FGF receptor
GDNF	glial-cell-line derived neurotrophic factor
GFP	green fluorescent protein
HGF	hepatic growth factor
IGF	insulin growth factor
MMP	

	matrix metalloprotease
RTK	receptor tyrosine kinase
SHH	sonic hedgehog
TEB	terminal end bud

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Figure 1.

Fate map of the tip and duct of branching renal epithelium. **a.** Relative contribution of the tip and duct to ductal elongation. The overall increase of ductal length during renal epithelial invasion is the sum of new ductal epithelium produced by extension of the existing duct (*light gray*) and by derivation from the tip (*dark gray*). **b.** Ductal participation in the remodeling of a branch-point. As the tip bifurcates and gives rise to two new tips. The original ductal epithelium is remodeled so that it contributes to the proximal side of each new trunk, whereas the rest of the trunk epithelium derives from each new tip. E_d , ductal contribution of elongation; E_t , tip contribution of elongation; E_n , net increase of ductal length. Adapted from Shakya et al. [25].

Figure 2.

Differential RTK signaling pathways control cell proliferation and migration in *Drosophila* tracheal air sac. Coordination of cell behavior in the outgrowth of a fly larval tracheal air sac. FGF signaling is required for migration of cells at the leading edge. Bnl/Fgf ligands are expressed in the cells adjacent (*dark gray cloud*) to the tracheal epithelium. Activation of Btl/Fgfr function in cells at the leading edge (*gray*) triggers changes in the cytoskeleton and cell shapes, and thus causes cells to migrate toward the source of signal. EGF signaling is required for all epithelial cells to proliferate and/or to survive and thus plays a permissive role in air sac outgrowth. Although sharing major downstream effectors, these two RTKs use different molecules to ensure distinct cellular effects. Adapted from Cabernard and Affolter [35].

Figure 3.

Common themes controlling branching morphogenesis in diverse organs. The mesenchyme produces guidance cues (*Gm*) and survival factors (*Sm*) for the epithelium during branching morphogenesis. The epithelium responds to these mesenchymal factors, via an unknown mechanism (dotted arrow and *question mark*), by making survival factors (*Se*) for the mesenchyme. In addition, the epithelium produces factors (*Me*) that can modulate the activities of factors produced by the mesenchyme (via an unknown mechanism, dotted arrow and *question mark*). The epithelium adjusts its responsiveness by expressing *Re* and fine-tuning its sensitivity to mesenchymal inputs. Conversely, the mesenchyme adjusts its responsiveness (*Rm*) to epithelial inputs. *Epi*, epithelium; *Gm*, mesenchymal guidance cues; *Me*, epithelial modulators; *Mes*, mesenchyme; *Re*, epithelial responsiveness; *Rm*, mesenchymal responsiveness; *Se*, epithelial survival factors; *Sm*, mesenchymal survival factors.

Table 1
Molecular basis of the common themes that govern branching morphogenesis in diverse organs.

	Fly trachea	Fly air sacs	Lung	Kidney	Mammary gland
Gm mesenchymal guidance cues	Bnl/Fgf	Bnl/Fgf	Fgf7, Fgf10	Fgf7, Fgf10, GDNF	IGF, FGF, HGF?
Sm mesenchymal survival factors	-	Egf	Spry2	Spry1	Spry2
Re epithelial responsiveness	Spry	N/A	Shh, TGF- β ?	TGF- β	TGF- β
Me epithelial modulators	-	-	Shh, Fgf9	Fgf	AREG
Se epithelial survival factors	-	-	Ptc-1, Hip, Spry4?	N/A	Ptc-1
Rm mesenchymal responsiveness	-	-			