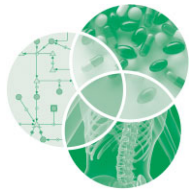
Focus Article



Branch formation during organ development

Nikolce Gjorevski[1] and Celeste M. Nelson[2]*

Invertebrates and vertebrates use branching morphogenesis to build epithelial trees to maximize the surface area of organs within a given volume. Several molecular regulators of branching have recently been discovered, a number of which are conserved across different organs and species. Signals that control branching at the cellular and tissue levels are also starting to emerge, and are rapidly unveiling the physical nature of branch development. Here we discuss the molecular, cellular, and physical processes that govern branch formation, and highlight the major outstanding questions in the field. © 2010 John Wiley & Sons, Inc.
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INTRODUCTION

Branch formation is a morphogenetic process used extensively across the animal kingdom to construct organs comprised of elaborate epithelial networks, including the *Drosophila* trachea and salivary gland, and the vertebrate lung, kidney, salivary gland (Figure 1), and mammary gland. Branched systems maximize surface area, used for the exchange and transport of gases and fluids, within a constrained volume. The spatial patterns of branched organs are highly complex but surprisingly organized. How subpopulations of cells are instructed to form branches and how the process is reiterated numerous times to form epithelial trees have fascinated scientists across disciplines for many years. Advanced genetic tools, culture models, and imaging techniques have recently unveiled many signals that regulate branch formation.

The morphology and organization of branches vary between organs. The branching patterns in the *Drosophila* trachea and mammalian lung are stereotyped and nearly identical between individuals,[3,4] suggesting that branch formation in these organs is under 'hardwired' genetic control. On the other hand, branching appears to be nonstereotyped in the mammary gland and prostate,[3] and in these organs cell fates and behaviors depend on the context. Despite the great variation of branching morphologies, a number of biological principles are conserved. Here, we cover some of the mechanisms that govern branch formation, list major unanswered questions, and discuss potential systems biology strategies to address them effectively.

THE KNOWNS: CONSERVED AND UNIQUE ASPECTS OF BRANCH FORMATION

Molecular basis of branch formation

Perhaps the most conserved aspect of branch formation is that growth factors of mesenchymal origin act as instructive cues. Members of the fibroblast growth factor (FGF) family direct branching of the *Drosophila* trachea and mammalian lung, mammary and salivary glands (reviewed in Refs 3,5). Multiple growth factors often regulate a single branching system. For instance, branching of the kidney is driven by glial cell line-derived neutrophic factor (GDNF), epidermal growth factor (EGF), and FGFs,[6] whereas EGF and hepatocyte growth factor (HGF) regulate branching of the mammary gland and lung (reviewed in Refs 3,7). It is unclear what role these shared instructive cues play in different organs, or how they interact within a single organ to supply local information to subpopulations of cells.

How can mesenchymally derived growth factors induce branching? A relatively well documented mechanism is chemotaxis. During the development of the *Drosophila* trachea, clusters of mesenchymal cells produce the FGF molecule Branchless (Bnl) at stereotyped locations.[8] Epithelial cells extend filopodia toward the sources of Bnl, suggesting that Bnl

*Correspondence to: celesten@princeton.edu

[1]Department of Chemical Engineering, Princeton University, Princeton, NJ 08544, USA

[2]Departments of Chemical Engineering and Molecular Biology, Princeton University, Princeton, NJ 08544, USA

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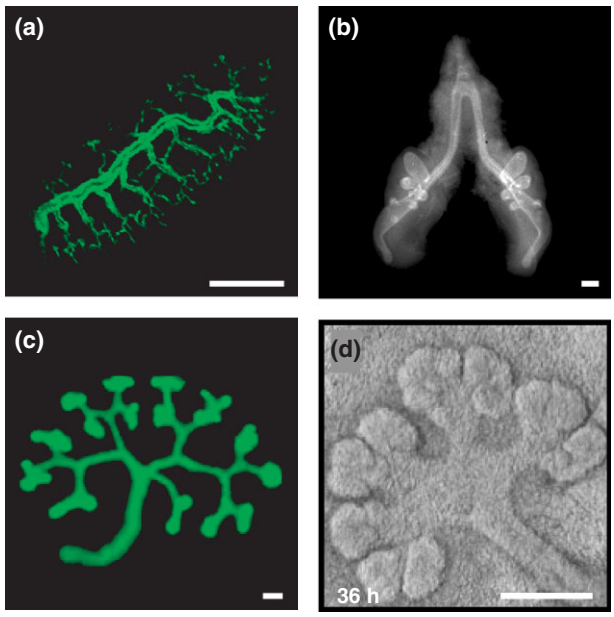

FIGURE 1 | Organs constructed by branch formation. (a) *Drosophila* trachea, (b) chicken lung and (c) mouse kidney (Reprinted with permission from Ref 1. Copyright 2009 Elsevier), and (d) mouse salivary gland (Reprinted with permission from Ref 2. Copyright 2006 Macmillan Publishers Ltd.). Scale bars, 100 μm.

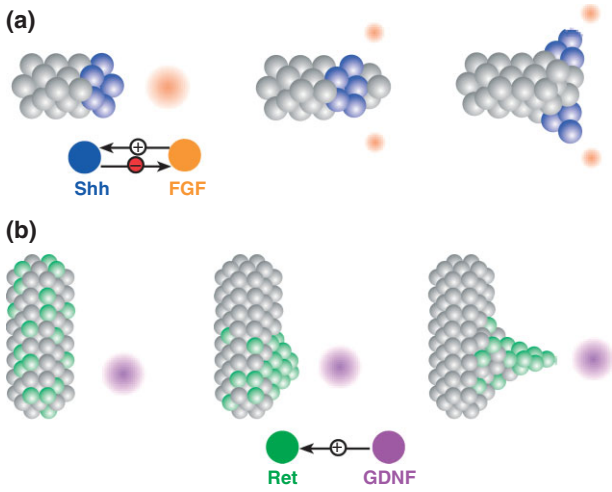

FIGURE 2 | Branch formation by chemoattraction. (a) Fibroblast growth factor (FGF) source (orange) guides branch extension by enhancing motility of tip cells. FGF induces expression of Sonic hedgehog (Shh) (blue), which in turn suppresses and splits the FGF source. The split source of FGF gives rise to branch bifurcation. (b) Cells expressing high levels of Ret (green), persistently migrate toward the source of glial cell line-derived neutrophic factor (GDNF) (purple), forming a patch of high Ret activity, which ultimately forms the ureteric bud.

induces branching by enhancing cell motility via cytoskeletal rearrangements.[9] A similar chemotactic mechanism is hypothesized to drive branch formation in the mammalian lung (Figure 2(a)).[5] Here, the levels and spatial profile of FGF10 are thought to be refined by Sonic hedgehog (Shh), which is expressed at the branch tips (reviewed in Ref 10). It has been postulated that FGF10 is locally downmodulated by Shh as the branch tip approaches. The FGF10 profile is thus split into two sources, leading to branch bifurcation. This interplay between Shh and FGF10 suggests a possible mechanism to space branches during morphogenesis of the lung airways. During kidney development, chemotaxis appears to be restricted to ureteric bud formation rather than branching. In this context, cells expressing the highest levels of the Ret receptor, initially randomly dispersed throughout the epithelium, migrate persistently toward the mesenchymal source of GDNF[11,12] (Figure 2(b)). Once there, the cells form a cluster of high Ret activity, which swells outward, forming the ureteric bud. It is unclear, however, what role GDNF plays in branch initiation and guidance by chemoattraction in the subsequent stages of kidney development.[12]

Conversely, there is no evidence for chemoattraction in specifying and guiding branches in the mammary gland. Nonetheless, as in all other branched organs, epithelial–stromal interactions are critical, and matrix metalloproteinases (MMPs) have emerged as key mediators. Elevated levels of both MMP2 and MMP14 have been observed at the tips of mammary and ureteric branches *in vivo*,[13,14] and MMP activity is required for mammary branch formation.[15] MMPs could act in a path-clearing or signaling capacity: they could promote branching by degrading the extracellular matrix (ECM) adjacent to branch sites and facilitating cell invasion, or alter signaling by generating bioactive ECM fragments, cleaving cell–cell or cell–matrix adhesions, activating latent transforming growth factor (TGF)-β or releasing ECM-bound growth factors.[7,13,16] Indeed, a recent study demonstrated that MT2-MMP-mediated proteolysis of collagen IV releases NC1 domains, which regulate branching in the salivary gland by increasing epithelial proliferation.[17] Accordingly, in these organs MMPs might supply the patterning instructions, whereas FGF and other growth factors might serve a more global role.

In addition to positive instructive cues, branching organs are sculpted by pervasive inhibitory cues. TGF-β is a potent inhibitor of branching in the lung, kidney, and mammary gland (reviewed in Ref 6). TGF-β is secreted by mammary epithelium and acts as an autocrine inhibitor in part by stimulating the synthesis and deposition of ECM along ducts.[18] Moreover, the concentration profile of TGF-β, dictated by

the geometry of the existing epithelium, acts locally to supply patterning information by defining new sites of branching.[15] Bone morphogenetic proteins (BMPs) play a similar role in the lung and kidney: BMP4 directs proximal–distal differentiation of the lung tree,[19,20] whereas BMP2 and BMP7 inhibit ureteric branching.[21]

Considering the downstream signaling cascades activated by the positive and negative cues discussed above reveals further overlap of the regulatory mechanisms employed by different organs. FGFs, EGF, HGF, and GDNF all signal through the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase cascades, whereas TGF-β and BMP mainly signal through Smads. However, even molecules that belong to the same family or signal through a common downstream pathway appear to contribute to branching in distinct ways. In the salivary and lacrimal glands, FGF7 induces formation of new branches whereas FGF10, which also signals through the FGFR2b receptor, leads to branch elongation.[22] In mouse mammary organoids, FGF7 induces growth and TGF-α induces branching, even though both growth factors elicit their effects through MAPK.[23]

Cellular basis of branch formation

Even when the underlying signaling pathways are conserved, strikingly different cellular behaviors are used to drive branch formation. For instance, *Drosophila* tracheal branching is accomplished exclusively by cellular migration, as cell division is fully completed prior to branch outgrowth.[24] In contrast, cell division is critical for branch initiation and growth in vertebrates.[23,25] The end buds of the pubertal mammary gland are highly proliferative,[26] and inhibiting proliferation prevents mammary branching.[23] Branching of different organs is accompanied and possibly driven by distinct cellular morphologies. Full epithelial polarization is maintained during ductal elongation in the zebrafish kidney and *Drosophila* salivary gland,[27,28] whereas mammary end buds are multilayered and partially depolarized.[26] Reports of neoexpression of mesenchymal markers at the tips of mammary ducts *in vivo* and in culture[15,29] hint that mammary branching might occur through partial loss or alteration of epithelial character.

Whereas the organs discussed thus far use cell migration, division, and shape change to drive branching via budding, the mammalian salivary gland initiates branches through clefting. Here, the epithelial cells undergo vigorous rearrangements, but the lack of choreographed motions has led to the conclusion that epithelial motility alone is insufficient for pattern formation.[30] Instead, it is the directional assembly of fibronectin that patterns branching, although cell motility may aid the process by allowing remodeling.[2,30] Nonetheless, proliferation also plays a role in salivary gland branching. Although proliferation is not required for cleft initiation, it is increased in the distal regions of the epithelium and is critical for cleft progression and branch outgrowth.[31] Future studies will need to determine whether these distinct cellular behaviors (division, migration, clefting) are the final events in branching, or if they actively feed back to control the process.

Branching as a physical process

In addition to biochemical stimuli, developing organs receive and interpret biophysical and mechanical signals from the microenvironment. Many of the processes that drive branching—cell migration, shape change, rearrangement, budding, clefting and bifurcation—are fundamentally mechanical. In contrast to the different molecular regulators that are used by different organs, the putative physical mechanisms are universal. The process of branch formation can be thought of physically as a viscous fluid (epithelium) elongating and bifurcating against a second fluid (stroma or mesenchyme) of different rheological properties. This situation is modeled in physics with the free-boundary problem, a simple case of which is the phenomenon of viscous fingering.[32] Here, a given pressure gradient exists at the interface between the two fluids (Figure 3). The formation of a small bulge in the inner fluid (the epithelium) redistributes the gradient, such that the bulge region experiences a sharper pressure drop, which propels it forward. As the bud grows, it displaces the mesenchyme laterally, thus reducing the pressure gradient and consequently the driving force for growth at what have now become the stalk regions. Instabilities at the tip can lead to bifurcation and generation of a complex self-similar structure reminiscent of the lung and kidney trees.[32]

Cytoskeletal contraction has also been implicated in branch formation. Inhibiting actomyosin contractility prevents branching in the kidney, lung, and salivary gland.[31,33,34] Conversely, activating contractility promotes branching in the lung.[35] Given reports that the basement membrane thins at sites of branch formation,[15,34] Ingber has proposed a 'run-in-a-stocking' mechanism, based on the postulate that contractility renders branching tissues in a state of isometric prestress. When the ECM is locally degraded, the adjacent prestressed epithelium is no longer physically hindered and invades in the form of a bud.[34] This prestress itself could lead to basement membrane thinning

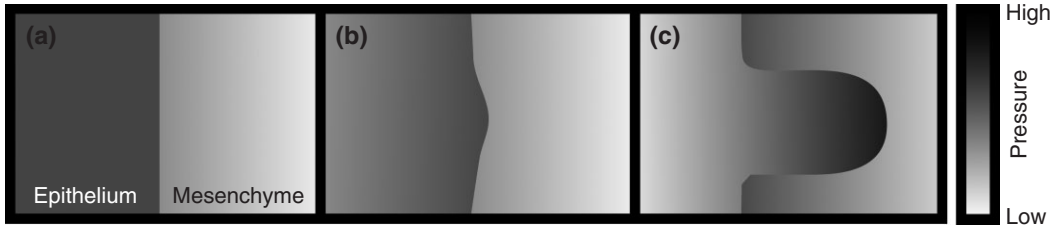

FIGURE 3 | Branch formation by viscous fingering. (a) The pressure in the epithelium is initially uniform, whereas that of the mesenchyme decreases away from the epithelium. (b) A small bulge in the epithelium protrudes and encounters a sharper pressure drop, which drives further protrusion. (c) As the bud grows, it displaces the mesenchyme toward the stalk regions, reducing the pressure drop there and lowering the driving force for motion.

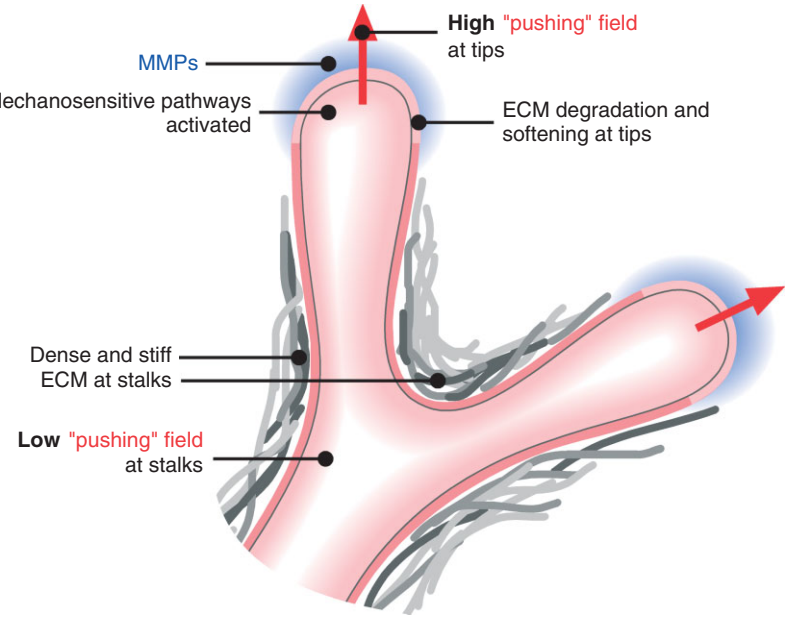

FIGURE 4 | Mechanical control of branching. The high mechanical fields at the tips enhance branching by physically propelling the epithelium forward and by activating proteases and signaling pathways that enhance cellular motility and invasiveness.

through local stimulation of MMP production,[36,37] consistent with reports of elevated MMP2 and MMP14 at the leading edge of mammary and ureteric buds[13,14] and the tips of engineered mammary tubules.[38] High mechanical stress could also activate cell motility and invasion. Thus, physical fields could not only assist branch formation by mechanically propelling the epithelium forward, but also by controlling the biochemical regulators (summarized in Figure 4).

SYSTEMS APPROACHES TO 'CONNECT THE DOTS'

From the mechanisms discussed here, and those omitted because of space limitations, it is clear that branching requires various growth factors, morphogens, ECM molecules, proteases, and mechanical signals from the microenvironment. It is unclear how these signals (identified separately by different investigators) interact to provide patterning information that makes a lung airway distinct from a kidney tubule. Perhaps the time is ripe to dissect the precise roles and interactions of the regulatory cues. Are the different signals coupled or independent? Is the regulation of branch formation hierarchical, such that removing a signal would induce a catastrophic collapse of the process, or is it structured redundantly? Are there a minimum number of cues that can orchestrate formation of branches with the correct histology and function? Many of these questions would likely be answered by taking a systems biology approach to the problem and organizing the critical signals into a 'branching regulatory network'.

In order to describe branch formation using a systems perspective, it is imperative that we first rigorously delineate the sequence of events and morphologies during branching. The most comprehensive description of a branching program to date is the study by Metzger et al.[4] In this formidable effort, the authors mapped out the complete three-dimensional branching pattern of the mouse bronchial tree. In

doing so, they uncovered a conserved set of three basic subroutines (domain branching, planar, and orthogonal bifurcation). Each of the subroutines could in turn be described as a combination of four basic spatial operators (periodicity generator, domain specifier, bifurcator, and rotator). To fully define the branching regulatory network, we must investigate the molecular control mechanisms that execute each level of the branching program.

Large-scale approaches to analyze genetic changes that accompany branching have been reported for several organs. DNA microarrays have been used to describe the temporal gene expression profiles during kidney development *in vivo* and in culture.[39,40] A genome-wide transcript analysis identified genes with altered expression levels between the terminal end bud, duct, and stromal microenvironments of the mammary gland.[29] A separate study distinguished the gene expression levels in the cleft and bud regions of the salivary gland.[41] These efforts should be extended to define the full spatiotemporal gene expression profiles for each organ. The resulting information would instruct better-targeted perturbation of gene expression to examine both the effect of molecular signals on the branching phenotype and their interactions. As these large amounts of data accumulate, the challenge will lie in interpretation and synthesis into plausible multiscale models and regulatory networks of branch formation. To that end, sophisticated *in silico* approaches will likely prove helpful, if not necessary.

Surprisingly, there have been few efforts to computationally model the process of branch formation. Hartmann and Miura modeled *in vitro* lung branching using the free-boundary problem described above.[42] A different model described the salivary epithelium and the surrounding mesenchyme as fluids of different viscosities, and demonstrated that a mesenchymally localized force field, which could arise from fibroblast contraction, is sufficient to induce clefts in the epithelium.[43] The authors improved this two-dimensional model, in which the force was located at a single point and in a fixed direction, by devising a three-dimensional model which tracked the concentration of the contractile fibroblasts in the mesenchyme.[44] Both models suggest that, whereas salivary epithelium does branch when cultured in the absence of mesenchyme, the latter significantly increases the rate of branching. Nevertheless, these models do not describe the budding mechanism employed by other organs, and the presence of an advecting mesenchymal force field has yet to be confirmed *in vivo*.

Perhaps, the most complex model of branching thus far is that developed by Hirashima et al.[45] Instead of modeling the epithelium as a continuous medium, the authors used the cellular Potts model to describe individual cells and cellular behaviors such as growth, division, and chemotaxis, all under the control of a split GDNF source. The model suggests that the balance of cell proliferation and chemotaxis is the major determinant of branch shape. While this model is commendable for incorporating biochemical and cellular complexity, it does not provide a mechanism for the localization of GDNF and the transduction of the chemical signal into motile and invasive behaviors.

The shortcomings of past approaches motivate the need for models that reflect the *a priori* conviction of systems biology that no process can be understood predictively until a majority of its components are included in the analysis.[46] In that sense, a comprehensive understanding of the topology of the branching network is unlikely until the models incorporate the full molecular and physical complexity seen *in vivo*. In devising such models, connections must be drawn between spatiotemporal profiles of gene expression and the cellular processes that perform branching (proliferation, migration, invasion, shape change). Although the field is in its infancy and data acquisition should certainly continue, the present body of experimental knowledge is probably sufficient to begin connecting the dots. Tentative maps have already been composed for organs such as the salivary gland.[47] As the downstream-most processes of clefting, budding, and shape change are ultimately physical, tentative molecular networks should be coupled with a physical component to fully simulate branching *in silico*.

First, such models could provide tractable platforms for testing the plausibility of proposed network topologies. For instance, the regulatory network for branching in the lung should yield structures that recapitulate the four branching modes described by Metzger et al.[4] in the correct sequences and in the correct spatial and temporal registration. Nigam and colleagues have recently postulated that branching regulatory networks resemble autocatalytic networks, in which complex emergent properties arise from the interaction of several members within the pathway.[48–52] Given that gene expression changes between the T-shaped ureteric bud and the multiple rounds of branching are modest,[40] the authors consider the possibility that the set of proteins interact in the context of an autocatalytic network thus giving rise to the iterative tip and stalk generation. Could the existence of such autocatalytic networks also explain how a set of conserved molecular regulators interact to yield emergent organ-specific patterns of branching? Comprehensive computational models can potentially shed light on this question and others.

Further, sophisticated models could play a predictive role by identifying connections not readily apparent from previous experimental work and thereby direct future experiments. Finally, detailed simulations of branch formation could help elucidate where and how normal developmental mechanisms are disrupted in disease, and even unveil strategies for reconstructing branched organs *ex vivo*.

CONCLUSION

The past decade has seen great progress in identifying the molecular signals that globally regulate branch formation. Surprisingly, many of these signals are used even by evolutionarily distant organs such as the fly tracheal system and the mammalian lung, kidney, and mammary gland. Nevertheless, the conserved set of regulatory molecules effect branch formation via distinct cellular behaviors and there are enough unique molecular aspects across different organs to still keep us far from a unified theory of branching. Further, major questions about how cell behaviors are patterned remain unanswered. We still do not fully understand how branch sites are selected and what determines the correct spacing between individual branches. Reinforcing the classic genetic, pharmacologic, and tissue recombination techniques used to study branch formation with detailed maps of the branching program in each system, large-scale gene expression screens and computational models that recapitulate the molecular complexity and the physical phenomena observed *in vivo* will likely prove helpful, if not necessary, for addressing the outstanding questions in this important process.

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