Patterned cell and matrix dynamics in branching morphogenesis

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Many embryonic organs undergo branching morphogenesis to maximize their functional epithelial surface area. Branching morphogenesis requires the coordinated interplay of multiple types of cells with the extracellular matrix (ECM). During branching morphogenesis, new branches form by "budding" or "clefting." Cell migration, proliferation, rearrangement, deformation, and ECM dynamics have varied roles in driving budding versus clefting in different organs. Elongation of the newly formed branch and final maturation of the tip involve cellular mechanisms that include cell elongation, intercalation, convergent extension, proliferation, and differentiation. New methodologies such as high-resolution live imaging, tension sensors, and force-mapping techniques are providing exciting new opportunities for future research into branching morphogenesis.

Introduction: Overview of branching morphogenesis

During embryonic development, simple bud-like organ precursors undergo dramatic branching to generate the complex branched architectures of many organs. This process expands an organ's epithelial surface area by orders of magnitude to maximize secretion or absorption by epithelia located in thousands of acini or alveoli and their ducts (Davies, 2005).

Branched structures are common in both plants and animals. For example, plants have tree branches and leaf veins. In animals, branching morphogenesis has been studied extensively in *Drosophila melanogaster* trachea (Ghabrial et al., 2003; Affolter and Caussinus, 2008), mammalian lung (Morrisey and Hogan, 2010; Warburton et al., 2010; Varner and Nelson, 2017), blood vessels (Meadows and Cleaver, 2015), kidney (Costantini and Kopan, 2010), pancreas (Shih et al., 2013), mammary gland (Gray et al., 2010; Inman et al., 2015), and salivary gland (Patel et al., 2006; Larsen et al., 2010). The morphology of these organs can differ dramatically, ranging from predominantly "lobular" in salivary glands to completely "tubular" in blood vessels (Fig. 1, A and B). Their appearance largely depends on the aspect ratio (length-to-diameter) of branches between branch points, as well as the relative size of branch tips compared with stalks.

During development, the lobular versus tubular appearance of some organs can change. For example, the ureteric bud of the mouse kidney is relatively lobular at early stages of branching morphogenesis, with bulging tips connected by relatively short branches (Fig. 1 C, left). However, later in development, the branches (future collecting ducts) elongate dramatically to become very tubular (Costantini and Kopan, 2010), resembling a collection of lines radiating from the ureter (Fig. 1 C, right). In contrast, the mouse mammary gland begins as a tubular network (Fig. 1 D, top) that is later remodeled to become lobular during pregnancy and lactation (Hennighausen and Robinson, 2005; Fig. 1 D, bottom).

Branching morphogenesis can be stereotypic or stochastic. The branching patterns of both mammalian lung and *Drosophila* tracheal system are highly stereotyped (Ghabrial et al., 2003; Metzger et al., 2008), suggesting their branching programs are genetically hardwired. The topology of kidney branches appear stereotyped, though individual kidneys differ in branching patterns (Short et al., 2014; Sampogna et al., 2015). In contrast, branching of blood vessels and mammary gland appears stochastic without defined patterns, presumably because of the absence of space constraints (Andrew and Ewald, 2010). Other organs, such as the salivary gland, also exhibit stochastic branching patterns, at least in organ culture.

Morphogenesis of a branched organ requires both the formation of new branches and remodeling of existing branches. Forming new branches in mammalian organs occurs through two geometrically distinct processes, "budding" or "clefting" (Fig. 2 A and Table 1). In this paper, we define budding as de novo branching from the surface of a primordial epithelium or from the lateral side of a preexisting branch, whereas clefting splits a preexisting branch tip into several tips (usually two or three). Topologically, each budding event generates a new branch tip, whereas each clefting event simultaneously eliminates an old branch tip and generates at least two new tips (Fig. 2 A).

At the cellular level, new branch formation can be driven by collective cell migration, patterned cell proliferation, coordinated cell deformation, and/or cell rearrangement. Plant tissues can lack cell migration and cell shape plasticity, so they rely on patterned cell proliferation and growth for branching. In contrast, animal organs often use multiple cell and tissue remodeling strategies.

In this review, we examine the complex cell biology underlying budding and clefting in various branched organs.

A Mouse submandibular gland (lobular)

Epithelium
Mesenchyme

E13.5   E16

B Blood vessels (tubular)

C Mouse kidney (transition to tubular)

Epithelium
Mesenchyme

E13.5   E18.5

D Mammary gland (transition to lobular)

Epithelium   Fat pad   Lymph node (LN)
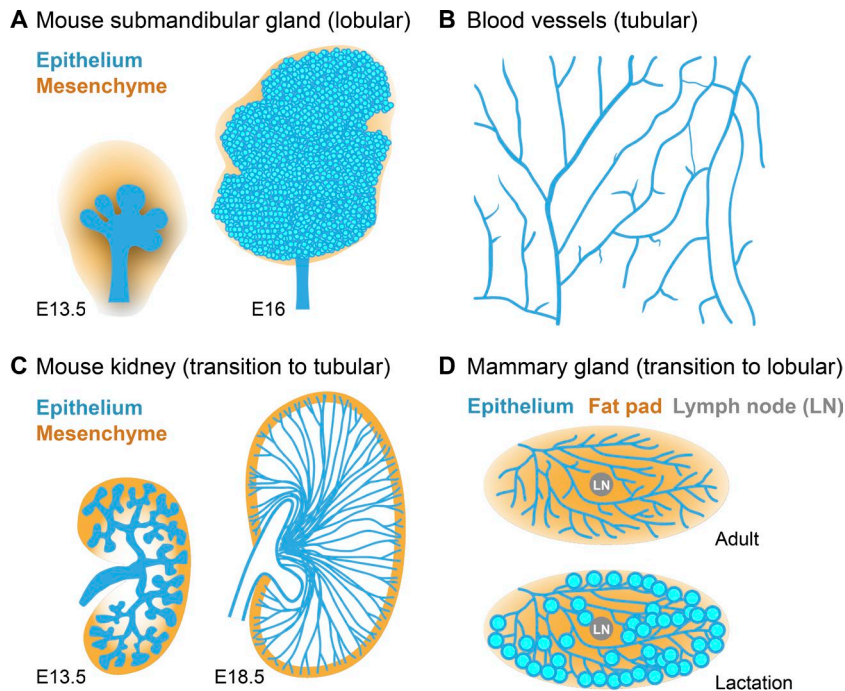
LN

Adult

LN

Lactation

Figure 1. **Varying morphology of branched organs.** (A) Schematics of mouse submandibular glands from embryonic day (E) 13.5 and E16 stage embryos. Submandibular gland morphology is predominantly lobular. (B) Schematic of blood vessels from a mouse retina. The branched vascular network is completely tubular. (C) Schematics of the ureteric bud of mouse kidney from E13.5 and E18.5 stage embryos. At E13.5, mouse kidney is relatively lobular. At E18.5, the elongated collecting ducts convert kidney morphology to predominantly tubular. (D) Schematics of mouse mammary gland at young adult stage and lactation stage. At the young adult stage, the mammary gland is a tubular network. During pregnancy and lactation, dramatic remodeling occurs in the mammary gland so that lactating alveoli form at branch tips, which transforms the structure to primarily lobular.

Budding in blood vessels and *Drosophila* trachea occurs by an invasive form of collective cell migration, by which a leader cell forms extensive protrusions and migrates outward, followed by other cells (Fig. 2, B and C; Scarpa and Mayor, 2016). Budding in mammalian epithelial organs, however, appears to be powered by a noninvasive form of collective cell migration along with cell proliferation (Fig. 2, D and E; Ewald et al., 2008).

In contrast, clefting at the branch tip in lung and kidney requires proliferation to enlarge the tip, which deforms and splits (Fig. 3, A and B; Watanabe and Costantini, 2004; Schnatwinkel and Niswander, 2013). In salivary gland, clefting can occur without cell proliferation, and cytoskeletal and ECM remodeling mediate cell rearrangement and epithelial bud deformation (Fig. 3 C; Daley and Yamada, 2013; Harunaga et al., 2014).

Other processes, such as branch elongation, lumen formation, and tip maturation, help to shape branched organs by adjusting the aspect ratio of branch stalks and tips. At the cellular level, branch elongation can occur through cell rearrangement by intercalation or convergent extension, cell elongation, and/ or cell proliferation (Fig. 4, A–C; Andrew and Ewald, 2010). Lumen formation occurs concomitantly with branching in many organs, though is substantially delayed in salivary glands (Andrew and Ewald, 2010). Tip maturation in lung and glandular organs is driven by cell proliferation and deformation to form alveoli or acini (Fig. 4, D and E), whereas the kidney represents a special case in which tips of ureteric bud branches or collecting ducts need to fuse with nephrons developed from the surrounding metanephric mesenchyme (Costantini and Kopan, 2010).

Patterning of cell behavior in branching morphogenesis

Branched organs contain multiple cell types plus their ECM. The core structure of all branched organs consists of tightly associated epithelial cells. The epithelium is surrounded by the basement membrane, a dense network of ECM glycoproteins and proteoglycans. Beyond the basement membrane is usually a loosely organized mixture of mesenchymal cells and their ECM. In addition to these common structures, the presence and morphogenetic roles of other cell types including smooth muscle cells, neurons, and blood vessels have been described in multiple epithelial organs. During branching morphogenesis, the epithelial cells actively interact with the ECM and these other cell types, both biochemically and biophysically.

Patterning of cell behavior is thought to involve patterned signaling. To date, numerous growth factors and regulatory signaling pathways have been implicated in branching morphogenesis. We have cataloged the loss-of-function phenotypes of major signaling pathway genes in Table S1 and transcription factors in Table S2. Despite this extensive knowledge, how these different regulators are integrated into patterns governing the various branching mechanisms remains poorly understood. A fundamental requirement for one or more receptor tyrosine kinase (RTK) signaling pathways has been established for most branched organs (Schuchardt et al., 1994; Luettke et al., 1999; De Moerlooze et al., 2000; Olsson et al., 2006). The ligands of these core RTK signaling modules are usually produced by the mesenchyme, whereas the corresponding RTK receptors are expressed in the epithelium. Modulators of RTK signaling have been identified in the mesenchyme, ECM, and epithelium itself. The regulation and cross talk of these pathways has been reviewed extensively (Hennighausen and Robinson, 2005; Costantini and Shakya, 2006; Olsson et al., 2006; Patel et al., 2006; Jørgensen et al., 2007; Lu and Werb, 2008; Affolter et al., 2009; Costantini and Kopan, 2010; Blake and Rosenblum, 2014). In this review, we will limit our discussion of signaling pathways to their roles in pattern formation.

New branch formation by invasive collective cell migration

An invasive form of collective cell migration can drive budding of new branches in *Drosophila* trachea (Samakovlis et al., 1996) and mouse retina blood vessels (Gerhardt et al., 2003). In both cases, a protrusive leader cell migrates outward at the vanguard of a group of follower cells that rearrange to form a new branch
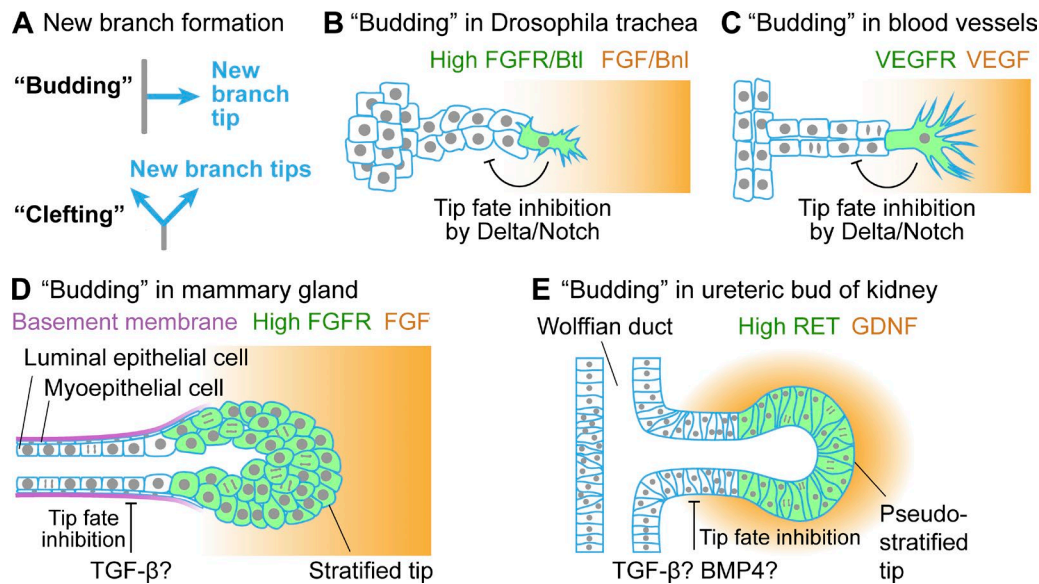
Figure 2. **New branch formation by budding.** (A) New branches can form through two geometrically distinct processes, budding or clefting. Schematics of budding in *Drosophila* trachea (B) and mouse retina blood vessels (C), where budding occurs by an invasive form of collective migration. The identity of the protrusive tip cell is specified by high RTK signaling. A Delta/Notch-mediated lateral inhibition mechanism prevents follower cells from becoming leader cells. Schematics of budding in mouse mammary gland (D) and the ureteric bud of mouse kidney (E), where budding occurs by a noninvasive form of collective migration and regionalized cell proliferation. The stratified tip of the mammary gland, or TEB, has high FGF receptor (FGFR) activity and higher proliferation rate than the stalk, which contains two cell layers. The tip of the ureteric bud at this early stage is pseudostratified, and its identity is specified by high RET signaling activity. EGFR, EGF receptor; VEGFR, VEGF receptor.

(Fig. 2, B and C; Scarpa and Mayor, 2016). The identity of the leader cell is specified by RTK signaling in both systems: FGF (Branchless/Bnl) in *Drosophila* trachea and VEGF in blood vessels (Gerhardt et al., 2003; Ghabrial and Krasnow, 2006). In addition, a Delta-Notch–mediated lateral inhibition mechanism prevents the follower cells from becoming leader cells in both systems (Ghabrial and Krasnow, 2006; Hellström et al., 2007; Suchting et al., 2007; Lebreton and Casanova, 2014).

Differences exist in the responses of stalk cells to RTK signaling. In *Drosophila* trachea, FGF/Bnl activation is only important in the leader cell at the tip, whereas the follower stalk cells respond passively to pulling forces generated by leader cells (Caussinus et al., 2008; Lebreton and Casanova, 2014). During blood vessel branching, both tip and stalk cells respond to VEGF, which regulates tip cell migration and stalk cell proliferation (Gerhardt et al., 2003).

New branch formation by noninvasive collective cell migration

A noninvasive form of collective migration has been described in budding of the mammary gland, where multilayered terminal end buds (TEBs) grow out from an epithelial cyst ex vivo (Ewald et al., 2008). However, unlike blood vessels and *Drosophila* trachea, cells at the leading edge of the TEB lack invasive protrusions penetrating the basement membrane (Fig. 2 D; Ewald et al., 2008; Huebner et al., 2016). Moreover, cells within the TEB can dynamically switch positions at the migration front. Whether outward migration of the TEB is driven by pushing forces from the stalk (Friedl and Gilmour, 2009) could be tested by analyzing TEB behavior after laser ablation of the stalk versus the tip.

Cell behavior similar to mammary gland TEB budding occurs in budding of mouse lung (Schnatwinkel and Niswander, 2013) and ureteric bud of kidney (Fig. 3 B; Chi et al., 2009;

Riccio et al., 2016), although the term "collective migration" was not used. Live imaging of sparsely labeled lung and ureteric bud cells reveal a group of cells that remain at the tip and are apparently pushed outward by cell division and rearrangement of the forming stalk. Unlike mammary gland TEB, branch tips in both lung and ureteric bud (kidney) are both single-layered, although the newly formed ureteric bud tip is pseudostratified (Chi et al., 2009; Schnatwinkel and Niswander, 2013).

Tip identity establishment and maintenance by cell competition

A recurring theme in branching morphogenesis by budding is that RTK signaling-dependent cell competition appears to maintain the identity of the tip cells (Fig. 2, B–E). As mentioned in the New branch formation by invasive collective cell migration section, leader cell identity for invasive branching in *Drosophila* trachea and blood vessels is mediated by FGF and VEGF signaling, respectively, whereas the leader cell inhibits the followers through Notch signaling in both systems. Strikingly, chimeric analysis in *Drosophila* trachea reveals that mutant cells defective for FGF signaling never take the leader cell position (Ghabrial and Krasnow, 2006). Similarly, genetic mosaic analysis in mammary glands shows that *Fgfr2*-null cells are progressively outcompeted by *Fgfr2*-positive cells in the TEBs (Lu et al., 2008), whereas *Ret*-null cells (*Ret* encodes the RTK receptor important for kidney branching; Schuchardt et al., 1994) are excluded from the tips of chimeric ureteric buds (Chi et al., 2009). Furthermore, this RTK-dependent cell competition is dose dependent, because ureteric bud cells with up-regulated RTK signaling (*Sprouty1* null cells) outcompete wild-type cells to occupy the branch tips (Chi et al., 2009). How tip cells inhibit the stalk cells in mammalian epithelial organs is not entirely clear, though TGF-β is a candidate for mammary gland (Nelson et al., 2006; Lu and Werb, 2008) and kidney (Rivitos et al., 1995). Taken together, a

conserved role for RTK signaling appears to establish, as well as to maintain, the identity of cells at the branch tip.

New branch formation by patterned cell proliferation

Patterned cell proliferation operates at two levels: regionalized proliferation and oriented cell division. Regionalized proliferation at the branch tip appears important for formation of new branches by budding in most cases, except for *Drosophila* trachea and blood vessels (Fig. 2, B–E). Studies using BrdU or radioactive thymidine labeling reveal a consistent pattern of higher proliferation in epithelial buds than in the stalks of many vertebrate organs (Bernfield et al., 1972; Goldin and Wessells, 1979; Goldin and Opperman, 1980; Michael and Davies, 2004; Lu et al., 2008; Schnatwinkel and Niswander, 2013). This pattern of high proliferative activity often mirrors the RTK activity (Costantini and Shakya, 2006; Patel et al., 2006) and could drive budding; in fact, elevated proliferation often precedes or coincides with budding (Nogawa et al., 1998; Michael and Davies, 2004; Schnatwinkel and Niswander, 2013). In contrast, localized proliferation activity has never been observed to precede clefting at branch tips. Consistent with this difference between budding and clefting, blocking cell proliferation abolishes budding in cultured mouse lung (Goldin et al., 1984) and mammary gland (Ewald et al., 2008), whereas clefting in salivary gland still proceeds (Nakanishi et al., 1987; Spooner et al., 1989).

Another type of patterned cell proliferation—oriented cell division—appears to contribute to clefting of lung branch tips. In mouse embryonic lung, live imaging of GFP–Histone 2B revealed no regional differences in rates of cell division inside the tip before clefting. However, cells toward the center divide preferentially along the axis of flattening (Fig. 3 A, black arrow in the center panel), which helps expand the bud in the same direction in preparation for clefting (Schnatwinkel and Niswan-

der, 2013). It is not clear how the orientation of cell divisions in different regions of the branching lung tip is regulated; however, planar cell polarity (PCP) regulators and external compression by smooth muscle cells are possible candidates (see Physical constraint by basement membrane or smooth muscle cells in clefting; Yates and Dean, 2011; Kim et al., 2015).

A similar homogeneous distribution of mitotic cells within branch tips before clefting has also been described in mouse kidney (Michael and Davies, 2004), which undergoes clefting by a similar sequence of enlarging, flattening, and clefting (Fig. 3, A and B; Watanabe and Costantini, 2004; Schnatwinkel and Niswander, 2013). Interestingly, cell division in kidney during ureteric bud branching occurs in a manner similar to lung, where premitotic cells first delaminate from the single-layered epithelium, complete cell division in the lumen, and reinsert back to the epithelium (Fig. 3, A and B; Packard et al., 2013; Schnatwinkel and Niswander, 2013). However, whether preferential orientation of cell division occurs in the clefting tips of kidney requires further examination. In sum, both regionalized proliferation and orientation of cell divisions can contribute to new branch formation, depending on the organ.

New branch formation by cell rearrangement and coordinated deformation

Besides proliferation, recent studies have highlighted the importance in branch tip clefting of actomyosin contractility, differential cell motility, ECM remodeling, and external physical restraints. These studies reveal unifying themes and organ-specific differences.

Contributions of actomyosin contractility to budding and clefting

One emerging theme is the contrasting roles of actomyosin contractility in the budding versus clefting mechanisms of new

Table 1. **New branch formation by budding versus clefting**

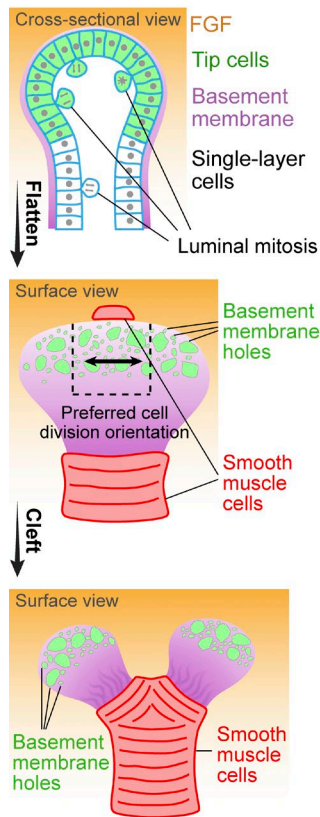| Cellular mechanisms	Budding	Clefting
Initiation of new branches	Protrusion of a group of cells outward from an epithelium to generate a new branch tip (in blood vessels, *Drosophila* trachea, mammary gland, lung, kidney,[a] and pancreas[b])	Separation of cells in a preexisting branch tip to generate two or more new branch tips (in salivary gland,[c] lung, kidney, and pancreas[b])
Collective cell migration		
Invasive	*Drosophila* trachea	—
	Blood vessels	
Noninvasive	Mammary gland	—
	Lung (?)	
	Kidney (?)	
Patterned cell proliferation		
Regionalized proliferation	Lung	—
	Kidney	
Oriented cell division	—	Lung
		Kidney (?)
Actomyosin contractility	Not required in blood vessels and lung	Salivary gland, kidney,
	Required in mammary gland (?) and pancreas[b]	lung, pancreas[b]
Differential cell motilities	Mammary gland	Salivary gland
	Pancreas[b]	Pancreas[b]
Physical constraint		
By basement membrane	—	Salivary gland
By smooth muscle cells	—	Lung

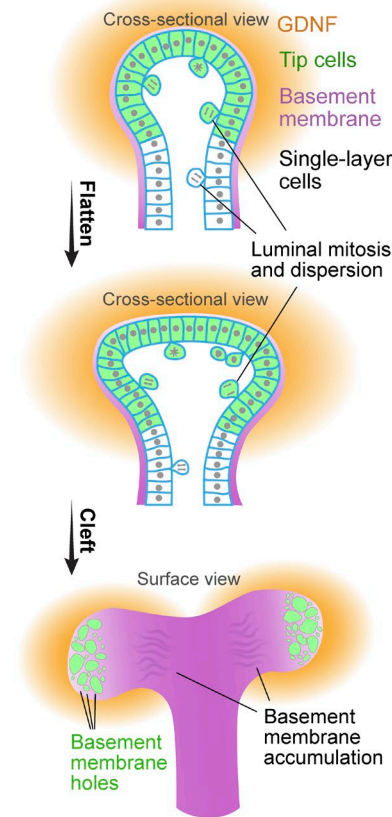—, not yet described or established.
[a]Ureteric bud in kidney.
[b]Mechanisms of pancreas branching may be complex, including a unique process involving the formation of microlumens.
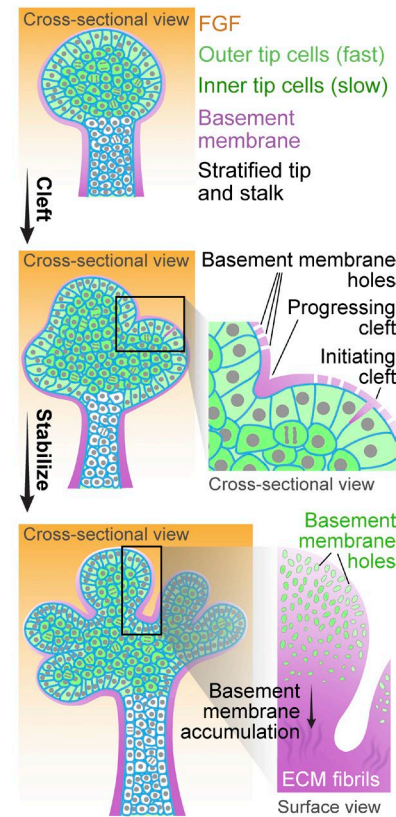[c]Submandibular salivary gland.

Figure 3. New branch formation by clefting. (A) Schematics of clefting (or terminal bifurcation) in mouse lung. The clefting tip of developing lung contains a single layer of cells that flattens before clefting. Within the tip, cells toward the center (dashed box in the center panel) divide preferentially parallel to the axis of flattening (black arrow in the center panel). Before clefting, smooth muscle cells differentiate at the future clefting site, which helps to deform the flattened tip to complete clefting. (B) Schematics of clefting in the ureteric bud of mouse kidney. The clefting tip of kidney contains a single layer of cells that also flattens before clefting. For cell proliferation, premitotic cells delaminate from the single-layered epithelium, complete cell division in the lumen, and then reinsert into the epithelium. (C) Schematics of clefting in mouse salivary gland. In these images, the clefting tip (bud) is stratified. The outer tip cells are more columnar and more regularly arranged than the inner tip cells. The outer tip cells also move much faster than the inner tip cells. Shallow clefts form stochastically with ECM invasion into the outer layer of epithelium, and they widen and stabilize to complete clefting. Clefting in all three systems is accompanied by microscopic perforations in the basement membrane toward the tip and accumulation of basement membrane components away from the tip. For each organ, a critically essential growth factor regulator is listed, although others contribute. GDNF, glial cell–derived neurotrophic factor.

branch formation: budding usually does not rely on actomyosin contractility, whereas clefting always does. A caveat for this process is that complex, sometimes contradictory, results exist in the earlier literature, which can often be explained by specific choices of inhibitors or organ culture conditions. We have omitted reviewing results using problematic inhibitors (e.g., the myosin inhibitor 2,3-butanedione monoxime; Ostap, 2002) and will discuss results that appear contradictory superficially, but not fundamentally.

Inhibition of actomyosin contractility in clefting-dominant branching organs (Fig. 3, B and C) consistently inhibits branch formation. In mouse salivary gland, inhibiting myosin II activity by blebbistatin, or Rho kinase (ROCK) activity by Y-27632 or ROCK I siRNA (but not ROCK II siRNA; Daley et al., 2009; Hsu et al., 2013), all inhibit branching, even though shallow initial clefts still form (Daley et al., 2009). Similarly, mouse ureteric bud branching is strongly suppressed by inhibiting ROCK by Y-27632 or actin polymerization by cytochalasin D (Michael et al., 2005). A conflicting study found Y-27632 stimulates rat kidney branching, but in mesenchyme-free Matrigel culture (Meyer et al., 2006).

Importantly, studies inhibiting actomyosin activity in the mouse lung, an organ using both budding (domain branching) and clefting (Fig. 3 A; terminal bifurcation), demonstrate differential requirements for actomyosin activity in budding versus clefting (Moore et al., 2005; Schnatwinkel and Niswander, 2013). Although Moore et al. (2005) initially reported overall inhibition of lung branching by inhibiting ROCK or actin polymerization, deeper analyses by Schnatwinkel and Niswander (2013) revealed that either blebbistatin inhibition of myosin activity or ML7 inhibition of myosin light chain kinase (MLCK) disrupt bifurcation (clefting) without blocking domain branch formation (budding). Inhibition of myosin, but not MLCK, further altered domain branch position and the shape of bifurcating buds.

Inhibiting actomyosin activity in budding-dominant branching systems often fails to prevent new branch formation. In a 3D culture model of blood vessels, where budding is driven by the invasive form of collective migration (Fig. 2 C), Fischer et al. (2009) elegantly demonstrated that a local loss of cortical myosin preceded filopodia protrusion and tip cell outgrowth. In fact, global myosin inhibition by blebbistatin greatly enhanced

blood vessel branching, and local application of blebbistatin triggered filopodial extension of tip cells.

However, the mechanisms appear more complicated in mouse mammary gland organoid culture (Fig. 2 D). Although inhibiting ROCK by Y-27632 did not prevent branching, inhibiting either Rac (NSC23766) or MLCK (ML7) did block branching of mammary gland organoids (Ewald et al., 2008). Because these ex vivo organoids undergo a characteristic luminal clearance and filling process to become a "branching-competent" complex cyst (Ewald et al., 2008), treating the organoids at the complex cyst stage might help distinguish roles of Rac and MLCK in branching versus organoid remodeling. A study of budding in embryonic chick lung described prominent enrichment of actin filaments at the apical surface of forming buds, hinting at an apical constriction–mediated contractile budding mechanism (Kim et al., 2013). However, inhibition of new bud formation by the myosin II inhibitor blebbistatin was marginal and did not rule out a role for regionalized proliferation (Figs. 2 I and 4 D with different y axes in Kim et al., 2013). We suggest that live imaging could distinguish the different possibilities.

To summarize, the published literature appears to support the concept that new branch formation by clefting requires actomyosin contractility, whereas budding may not. As discussed in New branch formation by patterned cell proliferation, regionalized proliferation instead seems to be more crucial for budding (Fig. 2, D and E). Interestingly, recent work in mouse pancreas and salivary gland suggests that patterned actomyosin contractility, rather than global contractility, is more important for pancreas branching. Either globally activating or inactivating contractility blocked pancreatic branching (Shih et al., 2016), suggesting that either a certain level of contractility is critical or a pattern of contractility is important for branching. In both pancreas and salivary gland, outer (peripheral) cells at the branch tip have a characteristic columnar shape (Fig. 3 C); importantly, this columnar shape becomes either cuboidal or misshapen upon actomyosin perturbation to resemble inner cell shape (Hsu et al., 2013; Shih et al., 2016), suggesting possible differential contractility of outer versus inner cells. Whether these specific differences between outer and inner cells and locally tuned differences in contractility are crucial for branching remains to be determined.

Differential cell motilities in branching morphogenesis

In addition to differences in shape, differential motilities of outer and inner cells at branch tips (or epithelial "buds") that could contribute to branching have been quantified in both mouse salivary gland and pancreas, but there are several differences. Outer cells touching the basement membrane move more than twice as fast as inner cells in tips (buds) of the salivary gland and show greater displacement (Fig. 3 C; Larsen et al., 2006; Hsu et al., 2013). Cells in these two regions move at similar speeds in the pancreas, and the major regional difference in pancreas involves directionality, with outer cells undergoing twofold greater displacement than inner cells (Shih et al., 2016). Although both systems require integrins to branch, integrin inhibition has different effects. Although inhibiting β1 integrin to disrupt cell–ECM interactions slows down outer cells in the salivary gland to rates comparable to inner cells (Hsu et al., 2013), it reduces the directionality of pancreatic outer cells without altering their velocity (Shih et al., 2016).

The most dramatic difference between salivary gland and pancreas involves opposite effects of perturbing the cell–cell adhesion molecule E-cadherin. In salivary gland, blocking E-cadherin slightly slows the outer cells and significantly accelerates the inner cells, which eliminates the differential velocity and inhibits branching (Walker et al., 2008; Hsu et al., 2013). In contrast, pancreatic deletion of E-cadherin almost doubles the branching index and partially rescues the nonbranching phenotype of pancreatic β1-integrin deletion (Shih et al., 2016). Lastly, although both pancreas and salivary gland seem to down-regulate E-cadherin in the outer cells, they seem to do so through distinct pathways. Although a fibronectin–Btbd7 axis is important for this regulation in the salivary gland (Sakai et al., 2003; Onodera et al., 2010), an integrin–Src axis appears to operate in the pancreas (Martinez-Rico et al., 2010; Shih et al., 2016). Taken together, these studies highlight ECM-associated differential cell behavior involved in branching, but the specific mechanisms can differ significantly in different organs.

Physical constraint by basement membrane or smooth muscle cells in clefting

In addition to its functions in cell motility, the basement membrane plays other biochemical and biophysical roles in branching morphogenesis. For example, modulation of growth factor signaling by heparan sulfate proteoglycans and cleaved collagen IV fragments has been described in many cases (Patel et al., 2006, 2014; Rebustini et al., 2009; Crawford et al., 2010; Shah et al., 2011). A fundamental requirement for the ECM protein fibronectin in branching morphogenesis has been demonstrated in several branched organs (Sakai et al., 2003). Whether their mechanisms of action differ in clefting versus budding deserves further examination.
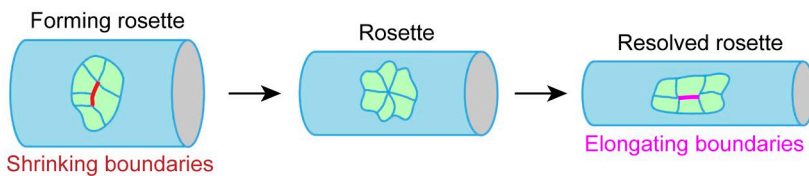
Classical studies established the importance of basement membrane in organ branching, with a general pattern of thinner basement membrane at branch tips during remodeling (Grobstein and Cohen, 1965; Bernfield and Banerjee, 1982; Moore et al., 2005). Recently, a deeper examination by live imaging reveals that the basement membrane at branch tips is remarkably dynamic (Harunaga et al., 2014). Surprisingly, the basement membrane ECM at branch tips of salivary gland, lung, and kidney becomes perforated by numerous well-defined microscopic holes that facilitate pliability (Fig. 3). Additionally, the basement membrane as a whole constantly moves rearward away from the tip. Both the microscopic holes and the rearward movement require protease activity and actomyosin contractility (Harunaga et al., 2014). The rearward movement of basement membrane hints at a potentially important mechanism for cleft formation: a stochastically formed nascent cleft (Sakai et al., 2003) could initiate, progress deeper, and become stabilized by basement membrane components (Kadoya and Yamashina, 2010). This combination of pliability at the tip/bud with basement membrane translocation and thickening at the cleft bottom to stabilize morphology may permit both rapid tip outgrowth and stabilization of clefts (Fig. 3 C). Laser ablation studies of the basement membrane should test these predictions.

Besides the ECM-based basement membrane, physical constraint can also be provided by cells. Kim et al. (2015) reported recently that tip clefting in lung is always preceded by the local appearance of mesenchyme-derived smooth muscle cells at the future cleft. The differentiated smooth muscle cells locally deform and cleft the tip (Fig. 3 A). Globally inhibiting or
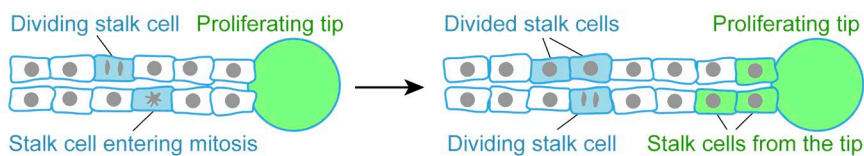
A Stalk elongation by cell elongation and cell intercalation (Drosophila trachea)
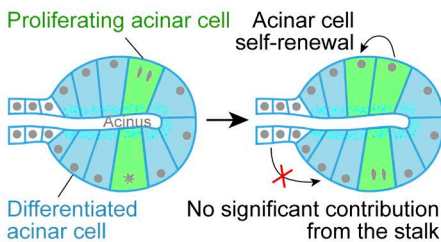
Stalk cell intercalation (SCI)
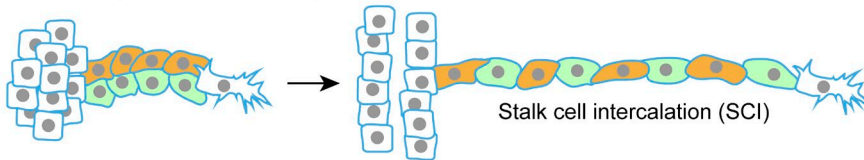
B Stalk elongation by rosette-based convergent extension (kidney collecting duct)

Forming rosette
Rosette
Resolved rosette

Shrinking boundaries
Elongating boundaries

C Stalk elongation by cell proliferation (most mammalian epithelial organs)

Dividing stalk cell   Proliferating tip
Stalk cell entering mitosis

Divided stalk cells   Proliferating tip
Dividing stalk cell   Stalk cells from the tip

D Acinar branch tip (adult salivary gland)

Proliferating acinar cell   Acinar cell self-renewal
Acinus
Differentiated acinar cell   No significant contribution from the stalk

E Alveolar branch tip (adult lung)

Squamous alveolar type 1 (AT1) cells
Alveolus
AT2-mediated renewal
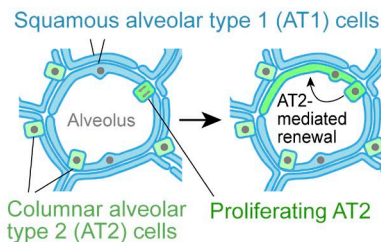Columnar alveolar type 2 (AT2) cells   Proliferating AT2

Figure 4. **Refining organ architecture by branch elongation and maturation.** (A–C) Schematics of stalk elongation by cell elongation and intercalation in *Drosophila* trachea. (A) In response to the pulling force of the migrating tip cell, stalk cells elongate and intercalate, resulting in >2-fold increased stalk length. (B) Schematics of stalk elongation by rosette-based convergent extension in vertebrate kidney collecting duct. (C) Schematics of branch stalk elongation by cell proliferation in most mammalian epithelial organs. Some cells divide within the stalk, whereas some cells are deposited by the proliferating tip to elongate the stalk. (D) Schematics of the acinar branch tip in adult mouse salivary gland. During maturation, cells at the branch tip differentiate to become columnar cells that surround a relatively small cavity to form an acinus for saliva secretion. Homeostasis of the acinar cells is maintained by self-renewal with little contribution from the stalk. (E) Schematics of the alveolar branch tip in adult mouse lung. During maturation, lung cells at the branch tip differentiate into flat, squamous AT1 cells and columnar AT2 cells. AT2 cell–mediated self-renewal can be triggered by AT1 injury.

promoting smooth muscle differentiation disrupted lung bifurcation or clefting (Kim et al., 2015). Collectively, local physical constraints seem to be particularly important for clefting-mediated branch formation, whether by ECM or cells.

What remains mysterious in salivary gland and lung is the origin of pattern formation. A key characteristic of a pattern generator is that either global activation or inhibition should disrupt the pattern. The inhibitor studies by Kim et al. (2015) point to a key role for sonic hedgehog (SHH), because both activating SHH signaling by smoothened agonist (SAG) or inhibiting SHH by cyclopamine disrupt the stereotyped differentiation of smooth muscle and bifurcation. Interestingly, reaction-diffusion modeling using only three components—FGF10, SHH, and the SHH receptor—is sufficient to generate bifurcation patterns (Menshykau et al., 2012). Further efforts are clearly required to generate quantitative models with sufficient predictive power to explain cleft patterning.

Beyond epithelium: Contributions of other cell types

As we have seen, the epithelium of branching organs is crucial for branching morphogenesis. However, establishing the architecture of branched organs requires contributions from many other cell types, including the surrounding mesenchyme, as well as blood vessels and neurons.

The encapsulating mesenchyme adjacent to the basement membrane contributes to epithelial branching both biochemically and biophysically. A crucial function of the mesenchyme is to produce the ligands for key RTK receptors, including VEGF, FGFs, and glial cell–derived neurotrophic factor (Fig. 2, B–E; and Fig. 3; Lu and Werb, 2008). If certain growth factors are provided, mesenchyme-free cultures of many embryonic organs can generate branched structures (Shamir and Ewald, 2014), demonstrating that the epithelia themselves are able to branch. However, these branched structures often appear morphologically different from in vivo organs or whole-organ explant cultures—for the most part, they appear to recapitulate budding and stalk elongation much more effectively than clefting (Morita and Nogawa, 1999; Qiao et al., 1999; Meyer et al., 2004; Steinberg et al., 2005; Ewald et al., 2008; Ghosh et al., 2011; Greggio et al., 2013; Huch et al., 2013). Thus, the epithelium may require interactions with the mesenchyme to prepattern local clefting signals. Importantly, studies using heterotypic recombinations of epithelium and mesenchyme from different organs have found the pattern of branching is often dictated by the tissue origin of the mesenchyme rather than the epithelium (Lubkin, 2008). For example, recombination of mammary gland epithelium with salivary gland mesenchyme yields an epithelial structure morphologically similar to salivary gland even though it produces milk (Kratochwil, 1969; Sakakura et al., 1976). Lubkin (2008) suggests that viscosity of the mesenchyme largely determines epithelial morphology, which will be interesting to test experimentally. To summarize, the epithelium appears competent to generate branches autonomously by budding, likely through RTK signaling–based cell competition, but it requires epithelial–mesenchymal cross talk to generate local cleft patterning.

Branched epithelial organs are also often "copatterned" with blood vessels and neurons, consistent with requirements for nutrition and neuronal regulation (Nelson and Larsen, 2015). Interestingly, diametrically opposed perfusion-independent roles for blood vessels in branching morphogenesis of lung and pancreas have been described. Although expression of a decoy VEGF receptor to reduce blood vessels inhibits branching and alters branching stereotypy of the lung (Lazarus et al., 2011), blood vessels in the pancreas instead seem to restrict its growth and branching (Magenheim et al., 2011). It is not clear, though, whether vascularization itself requires signals from the epithelial cells. Consistently positive roles for parasympathetic innervation have been described in branching of both salivary gland and lung, though through different pathways. In the salivary gland, parasympathetic innervation maintains epithelial progenitor cells through acetylcholine/muscarinic M1 and EGF receptor signaling (Knox et al., 2010). Removing the parasympathetic ganglion reduced but did not abolish branching (Knox et al., 2010). In contrast, laser ablation of parasympathetic neurons on one side of the mouse lung completely halted branching on that side, and acetylcholine signaling does not appear to be required (Bower et al., 2014).

Interestingly, parasympathetic innervation in both salivary gland and lung initiates after branching has started. In fact, salivary gland epithelial cells first secrete Wnt ligands to guide parasympathetic innervation, which in turn promotes epithelial growth and branching at later stages (Knosp et al., 2015). For other branching organs, the temporal sequence and interdependence of epithelial branching compared with recruitment of neurons and blood vessels need clarification.

Refining organ architecture by branch elongation and maturation

The overall morphology of a branched organ depends not only on the pattern of new branch formation, but also on the aspect ratio of branch stalks and tips. Diverse cellular mechanisms control branch stalk elongation and tip maturation, including cell rearrangement, elongation, proliferation, and differentiation.

Branch stalk elongation can occur by several mechanisms: cell rearrangement by intercalation or convergent extension, cell elongation, and cell proliferation (Fig. 4, A–C; Andrew and Ewald, 2010). Elongation of *Drosophila* tracheal branches occurs without cell division; instead, the stalk cells elongate along the outgrowth axis and intercalate to elongate the branch (Fig. 4 A; Caussinus et al., 2008). Another type of cell rearrangement, rosette-based convergent extension, plays an important role in elongation of both mouse and *Xenopus laevis* kidney tubules (Fig. 4 B; Lienkamp et al., 2012). This convergent extension depends on both Wnt and PCP signaling (Karner et al., 2009; Lienkamp et al., 2012). Additionally, PCP signaling directs the orientation of cell divisions to further promote kidney tubule elongation (Fischer et al., 2006; Saburi et al., 2008). Oriented cell division is also important for regulating branch shape in mouse lung, where cell division is thought to orient longitudinally by default or to be modulated by the activity of extracellular signal–regulated kinase (Tang et al., 2011). In mammary gland and branching ureteric bud, mitosis at branch tips increases stalk length (Ewald et al., 2008; Chi et al., 2009), though it is not yet known how proliferation preferentially increases stalk length rather than diameter. Overall, different organs clearly employ different mechanisms to elongate branch stalks.

Maturation of branched organs often involves cell differentiation at branch tips. At this stage, epithelial cells at the branch tips of salivary gland are slightly wedge-shaped cuboidal cells surrounding a small lumen to form an acinus (Fig. 4 D; Aure et al., 2015). However, tip epithelial cells in lung have differentiated into two types of cells: flat, squamous alveolar type (AT) 1 cells and cuboidal, surfactant-secreting AT2 cells, which together surround a comparatively large lumen to form an alveolus (Fig. 4 E; Morrisey and Hogan, 2010). Branch tips of the mammary gland also form alveoli for lactation, which can regress and reform between pregnancies (Hennighausen and Robinson, 2005; Akhtar et al., 2009, 2016; Akhtar and Streuli, 2013). For maturation of other branched organs, there are several excellent recent reviews for lung, kidney, and pancreas (Costantini and Kopan, 2010; Morrisey and Hogan, 2010; Shih et al., 2013).

Future directions

Branching morphogenesis has been studied intensively, not only because it is an intrinsically beautiful, dynamic process, but also because it holds promise for regenerative medicine. Two important and related future goals for studying branching morphogenesis include achieving a quantitative understanding of this process and bringing regenerative medicine to fruition by replacing or regenerating impaired branched organs.

To achieve a full understanding of branching morphogenesis, we predict that recursive applications of quantitative observation, mathematical modeling, and precisely controlled experimental perturbations will be crucial. For quantitative observation, live organ imaging at high spatiotemporal resolution as well as local force measurements will be valuable. Recently, several high-resolution 3D imaging methods with low phototoxicity have been developed, including dual-view inverted selective plane illumination microscope (Wu et al., 2013; Kumar et al., 2014) and lattice light-sheet microscopy (Chen et al., 2014).

For tracking the behavior of individual cells in the crowded cellular environment of developing organs, valuable tools will include chimeric labeling strategies such as Brainbow (Livet et al., 2007), photo-convertible dyes such as KikGR (Tsutsui et al., 2005; Hsu et al., 2013), and analysis software such as RACE (real-time accurate cell-shape extractor; Stegmaier et al., 2016). Application of advanced microscopy methods to chimerically labeled organs should help characterize cell dynamics and shape changes in more detail to catalyze new hypotheses. For quantitative modeling of branching morphogenesis, recently developed intracellular tension sensors will be useful (Kumar et al., 2016). For measuring tension and compression forces in live tissues, approaches such as oil microdroplet force-mapping will also be valuable (Campàs et al., 2014). For example, mapping actomyosin contractile forces during branch formation will help elucidate the different roles of actomyosin contractility in clefting versus budding. Complementary descriptive gene expression data can be obtained using single-cell or laser microdissection transcriptomic sequencing (RNA sequencing).

Once a speculative model is derived from descriptive observations, mathematical modeling can simulate the process, followed by tests of its experimental predictions to guide further model refinement. Modeling signaling pattern formation by reaction-diffusion (Turing, 1952; Kondo and Miura, 2010) and mechanical interactions between different cells (Lubkin, 2008; Varner and Nelson, 2017) may be particularly illuminating. For example, a simplified model focusing on FGF and SHH

has provided initial limited success in explaining lung branching (Menshykau et al., 2012; Iber and Menshykau, 2013), and a cell-based model simulates cleft progression in salivary gland (Ray et al., 2013).

Besides further application of gene ablation and RNAi approaches, precise perturbations of specific proteins and local forces will be valuable. Recent advances in the temporal control of protein depletion using an auxin-inducible protein degradation method (Nishimura et al., 2009) could be added to the Cre-LoxP gene ablation toolkit. For spatial control, laser-based inactivation and activation will be useful. For example, laser ablation can be used to measure tensile forces at specific locations or to ablate specific cells or ECM structures. Laser micropatterned activation or inactivation based on optogenetic switches (Zhang and Cui, 2015) and chromophore-assisted laser inactivation (Sano et al., 2014) will help test hypotheses and models. Photo-caged inhibitors and activators may also be useful (Lee et al., 2009). The rapid evolution of CRISPR-Cas9–mediated genome-editing methods (Doudna and Charpentier, 2014; Wright et al., 2016) will accelerate development of novel perturbation methods.

Regenerative therapies could be achieved by transplantation of tissue-specific stem cells/progenitors or gene therapy to restore impaired organs or by implanting a functional bioengineered organ to replace damaged or lost organs. Both approaches are active areas of research. For stem cell therapy to be developed as a regenerative therapy, the locations of potentially useful progenitor cells need to be identified. Although progenitor cells can reside in duct regions, recent studies reveal that such cells involved in homeostasis of mature lung and salivary gland are located in terminal alveoli or acini (Fig. 4, D and E; Desai et al., 2014; Aure et al., 2015; Hauser and Hoffman, 2015). Alternatively, successful restoration of both salivary gland and lacrimal gland function by transplantation of bioengineered embryonic organ germs has been described in mice (Hirayama et al., 2013; Ogawa et al., 2013). Potential future embryonic stem cell or induced pluripotent stem cell redifferentiation to organ-specific progenitors, organoids, or even functional organs can be envisioned (Shamir and Ewald, 2014). All of these approaches will benefit from applying new knowledge about the mechanisms of normal branching morphogenesis to guide successful regenerative therapy.

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