

Patterning Mechanisms of Branched Organs

Pengfei Lu and Zena Werb*

Branching morphogenesis is one of the earliest events essential for the success of metazoans. By branching out and forming cellular or tissue extensions, cells can maximize their surface area and overcome space constraints posed by organ size. Over the past decade, tremendous progress has been made toward understanding the branching mechanisms of various invertebrate and vertebrate organ systems. Despite their distinct origins, morphologies and functions, different cell and tissue types use a remarkably conserved set of tools to undergo branching morphogenesis. Recent studies have shed important light on the basis of molecular conservation in the formation of branched structures in diverse organs.

Branching morphogenesis is the process whereby a cell or a group of cells expands its surface area by forming cellular or tissue extensions during development. It was one of the most common processes in the emergence of organ systems as metazoans explored and adapted to previously untapped niches in nature. Various invertebrate and vertebrate organs (for example, fly trachea and mammalian salivary gland, lung, kidney, and mammary gland) (Fig. 1) undergo branching morphogenesis as an essential part of their ontogeny (1–3). Branching can occur in a single cell, such as a neuron as it forms short branches; or dendrites, to communicate with a myriad of other neurons and long branches; or axons, to relay nerve impulses to target tissues at a distance. Alternatively, branching can occur with a group of cells in the vasculature, where a network of blood vessels is formed to deliver oxygen and nutrients and remove metabolic wastes.

Historically, the mechanisms of branching and guidance of nerves and blood vessels have been well studied. Due to their structural simplicity and genetic accessibility, the *Drosophila* tracheal and air sac systems have given insight into understanding how epithelial branching occurs in the more complex organ systems of vertebrates (1, 4). With recent technical advances, including modern mouse genetics, cell fate-mapping, mosaic analysis, and live imaging of organ cultures, our understanding of vertebrate branching mechanisms has dramatically improved. One of the great surprises from

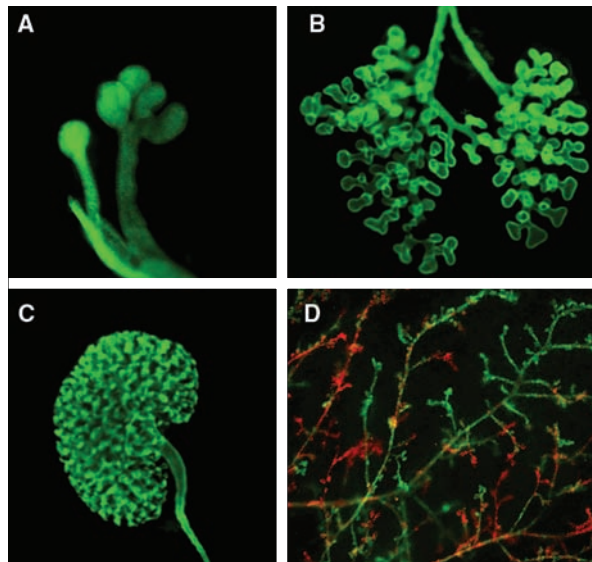


Fig. 1. Branching morphogenesis in mouse organs. (A to C) Immunofluorescent staining of E-cadherin in the branching epithelium of whole-mount salivary gland (A), lung (B) and kidney (C) from embryos at E13.5 to E15. (D) Branches of mammary epithelium derived from progenitors that were marked by expression of green and red fluorescent proteins as described previously (32).

these studies has been the realization that molecules essential for neuronal branching also play important roles in branching of invertebrate and vertebrate organs and the vasculature (3). Here, we review current understanding of the cellular and molecular mechanisms of branching morphogenesis. Comparison of the events underlying branching of different cell types has yielded insight into both emerging common themes and organ-specific mechanisms that render each of these cell and tissue types unique.

Mechanisms of Branching Morphogenesis

Different cells and tissues form branches with distinct branch patterns, defined by length, diameter, shape, and spacing. Yet, there are general themes in how branches are generated and main-

tained. It seems that there exists a branching “engine,” a special structure composed of either a single cell, a part of a cell, or a group of cells at the leading front of branch tips. This branching engine should be able to respond to an inductive signal that initiates, directs, and maintains branch outgrowth. The inductive signal must be subject to local and global regulation to achieve cell- or organ-specific branch patterns during development and remodeling processes.

Structure of the branching engine. More than a century ago, Ramón y Cajal described the growth cone, a highly specialized structure at the tip of chick neuronal axons (3). By forming actin-rich finger-like projections, or filopodia and sheet-like projections, or lamellopodia, the growth cone can respond to various guidance cues (Fig. 2A). Similarly, during vertebrate angiogenesis, migration of endothelial cells depends on tip cells, which lead trailing stalk cells as they colonize the avascular area of the embryo and form the vascular network (Fig. 2B). Likewise, in the embryonic *Drosophila* trachea, tip cells lead stalk cells as they invaginate from an epithelial sac and form the initial or primary branch (Fig. 2C). The tip cells of the primary branch are further elaborated by cellular extensions called secondary and terminal branches (5). At the late instar larval stage, the tracheal branches associated with the wing disc undergo further morphogenesis and give rise to the thoracic air sacs (1) (Fig. 2D). Again, tip cells at the migrating front send out filopodia and lamellopodia and play an important role in guiding stalk cells in a stereotypical direction (1).

In vertebrates, epithelial branching appears, at first glance, to be a relatively simple process, involving reiterative events of branch-point formation and duct elongation (also called the trunk or stalk). Branch points form through bifurcation [on rare occasions, trifurcation (6)] of the tip (also called the end bud or ampulla) or side-branching, whereby a group of cells buds out from duct epithelium. How these two modes of branch-point formation are deployed is organ-specific (6, 7) and presumably is genetically programmed. At the cellular level, vertebrate epithelial branching shows important differences from other branched systems. Unlike branch tips in the vasculature or fly trachea, those of vertebrate epithelium contain a heterogeneous and sometimes a multilayered cell population, as in the mammary gland (Fig. 2E) (8). Extensive cell proliferation is necessary for branching, especially in the tips (2, 8).

Inductive signals for branch initiation. A central event in branching morphogenesis is determining where to initiate a new branch. Remarkably, nerves, blood vessels, and epithelium all use growth factor ligands of the receptor

Department of Anatomy and Program in Developmental Biology, University of California at San Francisco, San Francisco, CA 94143–0452, USA.

*To whom correspondence should be addressed. E-mail: zena.werb@ucsf.edu

tyrosine kinase (RTK) family as inductive signals to form new branches. Neurons, for example, respond to the RTK ligand nerve growth factor (NGF), which is secreted by target tissues, for example, muscles, devoid of neuronal innervation (3) (Fig. 3). Likewise, endothelial cells are beckoned toward hypoxic tissues, which secrete the RTK ligand vascular endothelial growth factor (VEGF) during angiogenesis (3).

In the epithelium of both invertebrate and vertebrate branched organs, members of the fibroblast growth factor (FGF) family play a dominant role in branch initiation (2). In fly trachea and air sacs, for example, mesodermal cells express *Branchless* (*Bnl/Fgf*), which causes migration and branch initiation of adjacent epithelial

cells expressing the receptor *Breathless* (*Btl/Fgfr*) (4, 5). In addition to its role as a chemoattractant, *Bnl/Fgf* and its downstream events determine whether a cell becomes a tip cell or a stalk cell. Although all tracheal epithelial cells express *Btl/Fgfr* and respond to *Bnl/Fgf*, they compete for the ligand, and the cells with the highest *Bnl/Fgf* signaling activities become tip cells (9). Once tip cells have been determined, they are the only cells of the primary branch that depend on *Bnl/Fgf* signaling; the remaining stalk cells follow tip cells in a *Bnl/Fgf*-independent manner (9). Likewise, in both fly air sacs and the mammary gland, FGF signaling activity is necessary for cells to remain in the tip but not in the stalk or the duct (1, 10).

A major challenge for understanding vertebrate epithelial branching is the presence of a plethora of RTK ligands from the same or different families. These different RTK ligands can elicit collaborative, independent, or even opposing cellular behaviors, presumably depending on their specific downstream events during epithelial branching. In renal epithelium, beads soaked in either FGF (11) or glia-derived neural factor (GDNF) can induce ectopic branches (12). In addition, GDNF signaling is required for epithelial cells to remain in the tips of renal epithelium (12). These results suggest that FGF and GDNF signaling pathways may collaborate by acting in parallel or sequentially to regulate branch initiation and/or outgrowth in the kidney. On the other hand, in fly air sacs, FGF and epidermal growth factor (EGF) act independently. Thus, while FGF facilitates cell migration, EGF promotes cell proliferation of air sac cells (1). Finally, FGF7 and transforming growth factor α (TGF- α), a member of the EGF family, oppose one another during mammary epithelial branching (13).

Local and global regulation of branching patterns. The formation, maintenance, and subsequent outgrowth of a new branch are under extensive local and global regulation. Different tissues have the intrinsic ability to regulate the number of branches that form. In the fly trachea, the number of branches is regulated by mutual inhibition, whereby epithelial cells inhibit each other to take the leading position as they compete for branch-inducing factors. In both fly trachea and the vasculature, such mutual inhibition depends on Notch signaling (1, 9, 14) (Fig. 2, B and C).

Mutual inhibition also appears to be at work in the branching epithelium of vertebrates. It has long been recognized, for example, that mammary epithelial cells have self-avoidance properties. Thus, a new branch often starts off at a sharp angle and turns away or stops growing upon approaching another branch (15). In addition, when exogenous mammary epithelial cells are introduced into the mammary stroma, they can grow out and repopulate the whole gland, except in the presence of endogenous epithelium, which in-

hibits their growth. Indeed, in the mammary gland, epithelial geometry determines the potential branching sites due to self-inhibition (16). In this case, though, self-inhibition depends, at least in part, on TGF- β signaling activities (Fig. 2E). Finally, other extrinsic factors, including WNTs, hedgehogs (HHs) and bone morphogenetic proteins (BMPs), are expressed in the mesenchyme and play an important role in regulating branch initiation (2).

Because the embryonic mesenchyme and postnatal stroma are heterogeneous, it is not surprising that specific cell types participate in organ development. For example, in the mammary gland, macrophages, eosinophils, and mast cells all play a role in normal branching (17). It remains unclear, however, whether different cell populations control unique aspects of epithelial branching in vertebrate organs, for example, by secreting one or more extrinsic factors.

Finally, in many systems, including the fly trachea, the mammalian lung, large nerves, and blood vessels, branching patterns are highly conserved and stereotyped, suggesting that they are genetically programmed. Indeed, in mutant mice with reversed left-right asymmetry, the branching pattern of lung epithelium becomes the mirror image of that in the normal lung (7). For other branched organs, for example, the mammary gland and prostate, where branching is not stereotypical, branch patterns are still influenced by global physiological and/or hormonal status of the organisms. In the mammary gland, epithelial branching is regulated by growth hormone and estrogen (17), whereas in the prostate it is regulated by androgen (18).

Coordination of Branching Morphogenesis of Nerves, Blood Vessels, and the Epithelium

Branching morphogenesis of nerves, blood vessels, and the epithelium needs to be integrated, as manifested by their alignment along each other (3, 7, 19). But how is branching of different tissues coordinated during organ formation? Two effective ways of accomplishing this goal are communication between tissues and the use of common branch-regulating genes and pathways.

Communication between different tissues. It is well established that epithelial tissues attract endothelial cells and neuronal axons by secreting VEGF and NGF, respectively (Fig. 3). When *Vegf* is removed from lung epithelium, the vasculature develops abnormally (3). Likewise, disruption of NGF function or its gradient prevents neurons from innervating their target tissues (3). Conversely, endothelial cells are essential for specification and differentiation of the epithelium in the lung, pancreas, and liver (20); thus, they may play a role in epithelial branching as well. For example, lung endothelium secretes hepatic growth factor (HGF), which is essential for distal lung epithelial morphogenesis (3). Blood vessels and nerves also take advantage of one another to

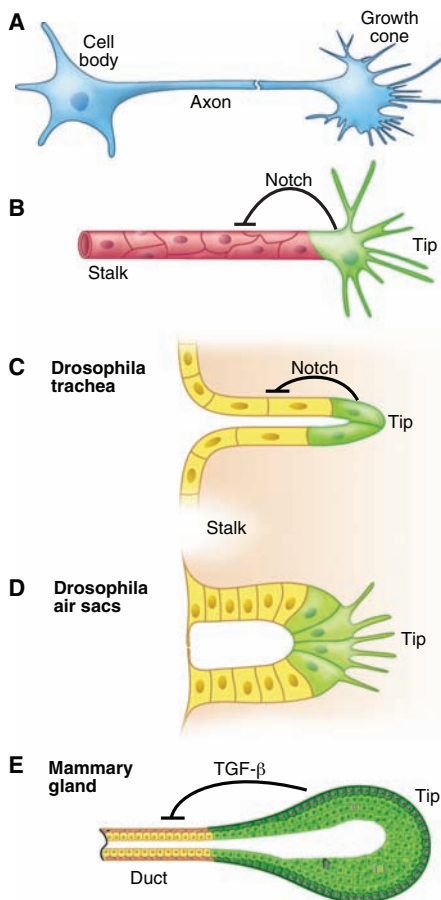


Fig. 2. Anatomy of the branching “engine.” A schematic presentation of various branched tissues: neuron (A), vasculature (B), *Drosophila* trachea (C) and air sacs (D), and mammary gland (E). Note the finger-like projections of the neuronal growth cone (A) and those of tip cells (green) of endothelium during angiogenesis (B) and *Drosophila* air sacs during larval development (D). To become tip cells, cells inhibit each other to take the leading position. In endothelium (B) and *Drosophila* trachea (C), mutual inhibition depends on Notch signaling. In the mammary gland, mutual inhibition depends on TGF- β signaling (E).

Organ Development

follow the same path. Endothelial cells produce molecules such as artemin and neurotrophin-3 (21) to attract axons to travel alongside the pioneer blood vessels, much like early axons guiding later axons. Likewise, nerves can produce various forms of VEGF and attract blood vessels to their side (Fig. 3).

Common genes and signaling pathways in branching tissues. Neurons rely on a small, well-conserved set of molecules to guide them toward their target tissues. Most of these guidance cues, belonging to four families, Slits, Netrins, Ephrins, and Semaphorins, can attract or repel axons (3). A major surprise is that members of all four families of axon guidance cues play roles in branching and guidance of the vasculature and invertebrate and vertebrate epithelium.

Robo4, a receptor for Slits, is expressed in the vasculature, and its activation causes repulsion of endothelial cells *in vitro* (21). Likewise, in the fly trachea, Slit and its receptors, Robo and Robo2, are expressed in the mesoderm and the adjacent epithelium, respectively. Interestingly, binding of Slit by Robo and Robo2, expressed in different branches, cause opposite reactions; whereas Slit attracts *Robo*-expressing epithelial cells, it repels *Robo2*-expressing ones (22). In the kidney, *Slit2* is expressed in the Wolffian duct from which the initial branch (i.e., ureteric bud) of renal epithelial network is induced. *Robo2* is expressed in the mesenchyme. Rather than directly guiding cell migration as in nerves, vessels, and fly trachea, *Slit2* regulates *Gdnf* expression and limits the number of ureteric buds that form. In the absence of *Slit2* or *Robo2*, the *Gdnf* expression domain expands and, as a result, ectopic ureteric buds form (12).

Other axon guidance cues also function in branched organs in a manner similar to or distinct from that in nerves. Like Robo4, the Netrin receptor *Unc5b* is expressed in endothelial tip cells. Its loss in mice causes aberrant extension of tip cell filopodia and excessive branching, which suggests that *Unc5b* normally represses endothelial branching *in vivo* (3). Consistent with these results, Netrin1 causes filopodial retraction when added to cultured endothelial cells (3). In contrast, in mammary epithelium, Netrin1 is required for cell adhesion. Thus, loss of either *Netrin1*, which is expressed in body cells of the tip, or its receptor *Neol1*, which is expressed in basal cap cells of the tip, the bilayered tip epithelium is not held together, so the tip collapses (23). Netrins and their receptors are also expressed in lung epithelium and may restrict side-branching (23).

Semaphorins signal through multimeric receptor complexes; whereas membrane-bound

semaphorins bind plexins, secreted forms bind the coreceptors neuropilins. In the vasculature, semaphorin-3a (Sema3a) represses branching by inhibiting lamellopodia formation of endothelial cells, which express various plexins and neuropilins (21). Likewise, in both the lung and kidney, *Sema3a* inhibits epithelial branching during organ formation (23, 24). However, in the salivary gland, *Sema3a* promotes branching by regulating cleft formation (25). Thus, the exact mode of *Sema3a* function in vertebrate organs remains unclear.

Ephrins and their Eph receptors are unusual in that ligand-receptor binding leads to bidirectional signaling: forward signaling in cells expressing Eph receptors and reverse signaling in those expressing the ligands. As in the nervous systems, EphrinB2 and its receptor EphB4 function as repellants in arteries and veins, respectively, and are essential for vessel maintenance by preventing intermixing of arterial and venous

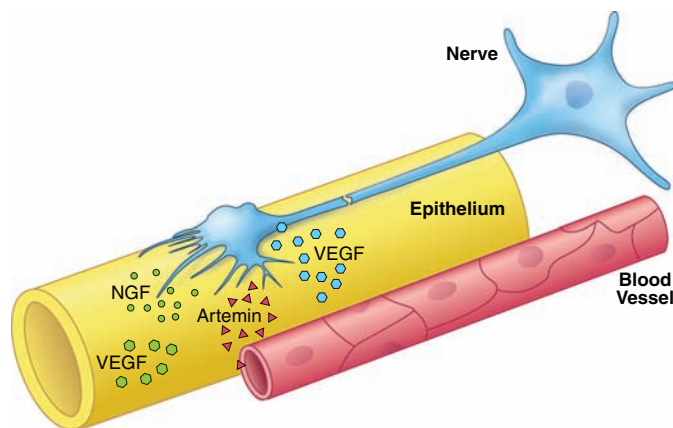


Fig. 3. Communication between epithelium, nerves, and blood vessels. Epithelium secretes NGF and VEGF (green) to attract nerves and vessels, respectively. Nerves also secrete VEGF (blue) to attract small arteries to track alongside nerve fibers. Blood vessels produce artemin and neurotrophin-3 (not shown), neurotrophic guidance signals, to guide neuronal axons.

endothelial cells (21). In the mammary gland, *EphrinB2* is expressed in luminal cells, whereas *EphB4* is expressed in basal cells (23); however, their function remains unclear. Finally, many factors, especially WNTs, BMPs, and HHs, which are known to regulate epithelial morphogenesis, also play a role in branching and guidance of nerves and blood vessels (26). By sharing these morphogens and guidance cues, branching morphogenesis of different tissues can be coordinated efficiently during organ formation.

Stromal Role in Development and Regeneration of Branched Organs

During formation of branched organs, several essential events, including organ specification, control of branch size, and cell differentiation and homeostasis, occur concomitantly or sequentially with epithelial branching. To understand development of vertebrate branched organs fully, we

must determine how these events are incorporated into branching morphogenesis. As a major source of branching regulators, mesenchyme and stroma are also essential for coordinating various aspects of vertebrate organogenesis (Fig. 4).

The instructive function of the mesenchyme during formation of several branched organs was first established by classic transplantation experiments. When combined with embryonic mammary mesenchyme, skin epithelium from mouse, or even chick or duck, embryos can be respecified to form mammary branches and milk-producing alveoli (17). The age and the differentiation state of the epithelium determine its plasticity to instructions from the mesenchyme. Thus, whereas cells of Rathke's pouch (future pituitary) epithelium from an embryonic day 8.5 (E8.5) mouse embryo can be respecified to form the salivary gland, those from an E12 embryo cannot (17). Moreover, although salivary gland mesenchyme can instruct embryonic mammary epithelium to form branches with patterns specific to the salivary gland, the grafts still retain the ability to form milk-producing alveoli (17). These results suggest that cell differentiation and branch pattern are differentially controlled by both intrinsic and extrinsic factors.

Stroma, which regulates epithelial differentiation and branching, plays an essential role in the stem cell biology of adult organs and is indispensable for organ homeostasis and regeneration in vertebrates (27, 28). The mammary gland is a nonvital organ that is readily accessible to experimental manipulations and thus is a valuable tool for understanding adult stem cell biology in vertebrates. When endogenous epithelium is surgically removed before puberty, the mammary gland

fatty stroma, which remains largely unoccupied by invading epithelium, can readily be used as a host to exogenous cells. This method has shown that a single mammary stem cell can regenerate the entire organ (29, 30). Remarkably, when testicular or neural stem cells are introduced along with mammary epithelial cells into mammary stroma, they form an epithelial branching network and differentiate as milk-producing cells (31). These results suggest the presence of a stem cell "niche" in the postnatal mammary gland, which contains essential signals that not only maintain mammary stem cells but can reprogram stem cells from other origins (Fig. 4).

Without definitive markers, it has been difficult to determine where stem cells are located in branched organs and how they are maintained throughout organ development. In the mouse mammary gland, stem cells are distributed throughout the organ and are not greatly affected

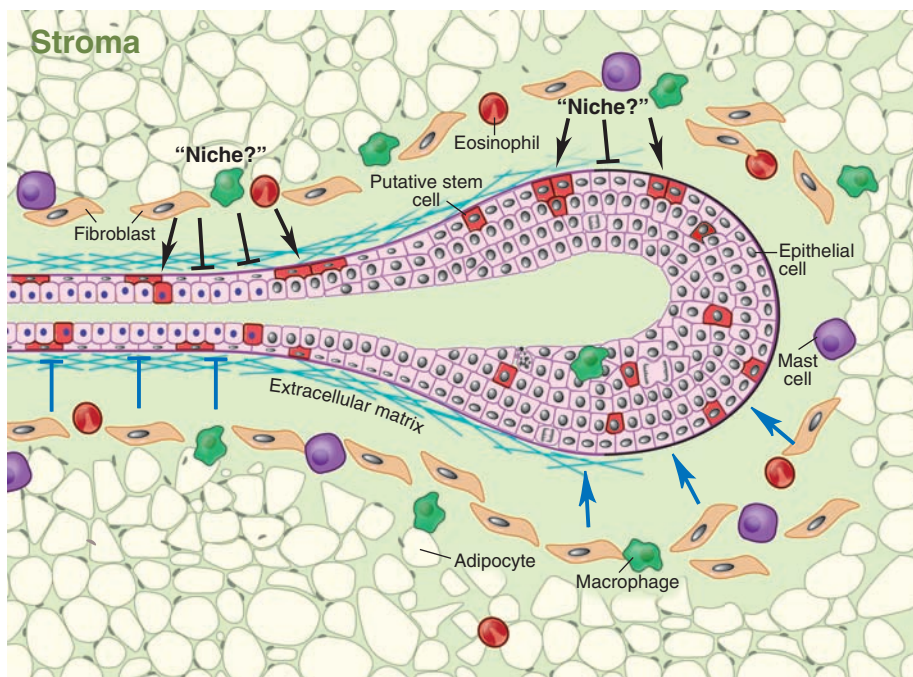


Fig. 4. Stroma role in development of vertebrate branched organs. A schematic presentation of the epithelium and stroma during mammary gland development. Stroma, which has a heterogeneous cell population, plays an important role in determining branching patterns (blue arrows and bars indicate stimulatory and inhibitory cues acting on the epithelium). Stroma also regulates biology of stem cells by contributing to a presumptive stem cell “niche.”

by the mouse’s age or reproductive history (31). This distribution may have important implications for where new branches are initiated, since multiple stem cells can contribute to a single branch (32). It may also allow formation of the maximum number of alveoli, which undergo recurring cycles of cell proliferation and death during each pregnancy. In addition, “terminally” differentiated cells, for example, from the mammary gland (31) or fly trachea (33), can regain developmental potency and form various cell types under appropriate conditions. These results underscore the importance of understanding the molecular basis of stem cell differentiation and stromal role in regulating stem cell potency.

Conclusions and Future Directions

Despite their distinct origins and functions, different tissues and organs use common genes and signaling pathways for regulating branching during organ formation. One possible explanation for such molecular conservation is the existence of “branching modules,” consisting of a defined set of genes that can be activated whenever branching is needed (34). Alternatively, and perhaps more likely, branching morphogenesis, irrespective of cell and tissue types, requires a common set of cell behaviors, for example, regulation of cytoskeletal machinery that is controlled by evolutionarily conserved genes and pathways. It is thus formally possible that the genes and signaling pathways involved in neural

branching and guidance, which evolved first, were later “co-opted” by blood vessels and epithelia of various branched organs.

While continuing the quest for molecules that play conserved roles in various branched organs, we should also focus on molecules that promote organ-specific morphogenesis and functions to understand how different organs evolved. For instance, in the embryonic lung or kidney, branching of the epithelium accompanies enormous growth of the overall organ. In contrast, in the mammary gland, the epithelium primarily invades and forms branches in the stroma that have already been laid down and barely changes in size during pubertal development. Thus, in the mammary gland, unlike in most other branched organs, there is constant remodeling of the stromal compartment, extracellular matrix, and vasculature as mammary epithelium populates the fat pad. Indeed, the mammary gland may use different genes than do other organs for epithelial branching. *Shh*, which is indispensable for lung and kidney epithelial branching, is not required for mammary development (2).

Identifying stromal factors will be important for understanding essential aspects of development of vertebrate branched organs. To understand how organs are formed and maintained, and whether they can be regenerated, it will be important to determine whether a stem cell “niche” exists in vertebrates and, if so, how it regulates stem cell self-renewal and differentia-

tion. In addition, what is the nature of adult stem cells? At least in the mammary gland, the frequency and property of adult stem cells remain puzzling and do not fit current models (31, 35). Thus, it will also be important to determine the intrinsic factors that respond to the “niche” and maintain stem cells and how they can be reprogrammed upon experimental manipulations. With the emergence of the mammary gland as an amenable platform for functional testing, we can be optimistic about a better understanding of the intrinsic and extrinsic factors that regulate stem cell development in branched organs.

References and Notes

- M. Affolter, E. Caussinus, *Development* **135**, 2055 (2008).
- P. Lu, M. D. Sternlicht, Z. Werb, *J. Mammary Gland Biol. Neoplasia* **11**, 213 (2006).
- P. Carmeliet, M. Tessier-Lavigne, *Nature* **436**, 193 (2005).
- R. J. Metzger, M. A. Krasnow, *Science* **284**, 1635 (1999).
- A. Ghabrial, S. Luschnig, M. M. Metzstein, M. A. Krasnow, *Annu. Rev. Cell Dev. Biol.* **19**, 623 (2003).
- T. Watanabe, F. Costantini, *Dev. Biol.* **271**, 98 (2004).
- R. J. Metzger, O. D. Klein, G. R. Martin, M. A. Krasnow, *Nature* **453**, 745 (2008).
- A. J. Ewald, A. Brenot, M. Duong, B. S. Chan, Z. Werb, *Dev. Cell* **14**, 570 (2008).
- A. Ghabrial, M. A. Krasnow, *Nature* **441**, 746 (2006).
- P. Lu, A. J. Ewald, G. R. Martin, Z. Werb, *Dev. Biol.* **321**, 77 (2008).
- L. Chi *et al.*, *Development* **131**, 3345 (2004).
- F. Costantini, R. Shakya, *Bioessays* **28**, 117 (2006).
- J. E. Fata *et al.*, *Dev. Biol.* **306**, 193 (2007).
- C. A. Jones, D. Y. Li, *Curr. Opin. Genet. Dev.* **17**, 332 (2007).
- G. B. Silberstein, *Microsc. Res. Tech.* **52**, 155 (2001).
- C. M. Nelson, M. M. Vanduijn, J. L. Inman, D. A. Fletcher, M. J. Bissell, *Science* **314**, 298 (2006).
- M. D. Sternlicht, H. Kourou-Mehr, P. Lu, Z. Werb, *Differentiation* **74**, 365 (2006).
- G. S. Prins, O. Putz, *Differentiation* **76**, 641 (2008).
- V. N. Patel, I. T. Rebutini, M. P. Hoffman, *Differentiation* **74**, 349 (2006).
- N. Bahary, L. I. Zon, *Science* **294**, 530 (2001).
- P. Carmeliet, *Nat. Rev. Genet.* **4**, 710 (2003).
- C. Englund, P. Steneger, L. Falileva, N. Xylourgidis, C. Samakovlis, *Development* **129**, 4941 (2002).
- L. Hinck, *Dev. Cell* **7**, 783 (2004).
- A. Tufro, J. Teichman, C. Woda, G. Villegas, *Mech. Dev.* **125**, 558 (2008).
- L. Chung *et al.*, *Development* **134**, 2935 (2007).
- F. Charron, M. Tessier-Lavigne, *Development* **132**, 2251 (2005).
- J. M. W. Slack, *Science* **322**, 1498 (2008).
- K. S. Zaret, M. Grompe, *Science* **322**, 1490 (2008).
- M. Shackleton *et al.*, *Nature* **439**, 84 (2006).
- J. Stingl *et al.*, *Nature* **439**, 993 (2006).
- G. H. Smith, D. Medina, *Breast Cancer Res.* **10**, 203 (2008).
- B. E. Welm, G. J. Dijkgraaf, A. S. Bledau, A. L. Welm, Z. Werb, *Cell Stem Cell* **2**, 90 (2008).
- M. Weaver, M. A. Krasnow, *Science* **321**, 1496 (2008).
- J. A. Davies, *Bioessays* **24**, 937 (2002).
- K. U. Wagner, G. H. Smith, *J. Mammary Gland Biol. Neoplasia* **10**, 25 (2005).
- We thank G. Dijkgraaf for taking the photograph of the fluorescent mammary gland and M. Chan, O. Klein, J. Phillips, and M. Zeiger for helpful discussions and insightful comments. We regret that many authors’ work could not be cited because of space limitations. This work was supported by grants ES012801 and CA057621 from NIH.

10.1126/science.1162783