Genetic Control of Branching Morphogenesis

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The genetic programs that direct formation of the treelike branching structures of two animal organs have begun to be elucidated. In both the developing *Drosophila* tracheal (respiratory) system and mammalian lung, a fibroblast growth factor (FGF) signaling pathway is reiteratively used to pattern successive rounds of branching. The initial pattern of signaling appears to be established by early, more global embryonic patterning systems. The FGF pathway is then modified at each stage of branching by genetic feedback controls and other signals to give distinct branching outcomes. The reiterative use of a signaling pathway by both insects and mammals suggests a general scheme for patterning branching morphogenesis.

Many essential organs—the lung, vascular system, kidneys, and most glands—are composed of ramifying networks of epithelial tubes that transport fluids. The exquisite branching patterns of these organs have fascinated biologists and mathematicians since Aristotle (*1*), but the mechanisms that generate these complex three-dimensional structures during embryonic development have remained a mystery. Even with the remarkable progress over the past two decades in other areas of developmental biology such as specification of the major body axes, the molecules and mechanisms that dictate complex organ structures have been elusive. Recently, however, key genes that direct the elaborate branching patterns of two organs, the airways of the fruit fly *Drosophila melanogaster* and the mouse lung, have been identified and the early steps in the genetic branching programs delineated. The two programs share several important organizational and molecular features, which suggests a general biological scheme for patterning branching morphogenesis.

Encoding Complex Branching Patterns

Essentially all branched tubular networks are constructed of an epithelial (sheetlike) monolayer of cells wrapped into a tubular structure. Most begin development as a simple epithelial sac or tube from which new branches successively bud, giving rise to a treelike structure of interconnected tubes (Fig. 1, A, C, and D) (*2*). In some organs, such as the lung, additional supporting cell layers develop around the epithelial tubes, but in other organs, like the *Drosophila* tracheal system, the tubes remain unadorned.

There are hundreds to millions of branches in most organs, and the patterns of branching, although exceedingly complex, are seldom random. At least for the early branch generations, the patterns are highly stereotyped, implying that they are under fixed developmental control. Furthermore, there are certain regularities in the structures of the branches. For example, in the lung there is a consistent relationship between branch generation and branch diameter (*3*), which facilitates flow through the network.

A tremendous amount of patterning information is required to configure such large numbers of branches. For each branch, the patterning information must specify (i) where the branch buds and the direction it grows, (ii) the size and shape of the branch, and (iii) when and where along the branch the next generation of branches will sprout. New branches typically arise as outpouchings of the epithelium, either by migration of a local region of the epithelium or by local, oriented cell divisions, in some cases accompanied by formation of a cleft in an existing branch (*4*). Thus, the patterning information must ultimately control fundamental cellular processes such as migration, proliferation, and changes in shape.

How is this vast amount of patterning information encoded in the genome? Mathematicians and theoretical biologists have formulated elegant algorithms that can generate branching patterns that rival the complexity of the natural forms and mimic certain structural features such as the regularities in branch diameter (Fig. 1B) (*3*, *5*). The appeal of these iterative or fractal models is that they are simple to encode genetically, because the same basic branching mechanism is used repeatedly. However, a major limitation is that they do not reproduce the natural branching patterns.

Iterative models assume that all branching events are alike and hence under the same genetic and molecular controls. Characterization of branching morphogenesis in the *Drosophila* tracheal system, and also the mouse lung, however, has revealed substantial differences in branching mechanisms: Different generations of branches form by different cellular mechanisms, express specific markers, and require different sets of genes for their formation. These contrasting views have begun to be reconciled by recent genetic studies that show how an iterative process can be repeatedly modified during development to give rise to different types and patterns of branches.

Genetic Dissection of *Drosophila* Tracheal Development

The larval tracheal system of *Drosophila* provides a paradigm of branching morphogenesis. The ramifying network of some 10,000 branches conducts oxygen from the spiracular openings to the internal tissues (Fig. 1C). The branch pattern is known in detail, and its development has been described at cellular resolution and analyzed genetically (*6*).

The tracheal system arises from segmentally repeated clusters of ectodermal cells that invaginate at mid-embryogenesis and form 20 epithelial sacs of about 80 cells. Each sac sprouts successively finer branches to generate a treelike structure (Fig. 1D). Remarkably, the entire branching process occurs exclusively by cell migration and changes in cell shape, without cell proliferation. The six primary branches form when one or two cells at six positions in each sac migrate out in specific directions. A small number of cells follow the lead cells, organizing into multicellular tubes as they migrate. Several hours later, secondary branches sprout from the ends of growing primary branches. Secondary branches are formed by individual tracheal cells that apparently roll up to form unicellular tubes. During larval life, secondary branches ramify into dozens of terminal branches, which arise as long cytoplasmic extensions that form fine (<1 μm in diameter) tubules that directly contact the internal tissues. As each sac generates an array of about 500 branches, specific branches fuse with branches from neighboring sacs to form an interconnected network (*7*).

Genetic screens have identified more than 50 genes required for tracheal development (*6*, *8*). Mutations in different genes disrupt the process at specific steps (Fig. 1D). However, some of the genes required for the first branching events are used again in the later stages of branching. Also, some of the genes required for early branching events trigger

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expression of genes needed in the later branching events. Thus, although each stage of branching is morphologically and genetically distinct, a core set of genes is used repeatedly while stage-specific genes are called into play at the appropriate times.

Establishing the Tracheal Sacs

Before branching begins, *trachealess* selects the tracheal primordia in the embryonic ectoderm and drives the conversion of these planar epithelial regions into sacs (*9*). The gene turns on in the tracheal primordia 1 to 2 hours before sac formation, and the basic helix-loop-helix (bHLH)–PAS domain transcription factor forms a complex with Tango (*10*), a broadly expressed bHLH-PAS protein homologous to mammalian ARNT. The Trachealess-Tango heterodimer presumably regulates target genes encoding cytoskeletal and cell surface proteins responsible for sac formation. It also readies the sacs for the branching events that follow by triggering expression of genes required for branching (*9*, *10*). These targets include *breathless*, a *Drosophila* homolog of mammalian fibroblast growth factor (FGF) receptors that is turned on throughout the sacs (*11*).

Patterning of Tracheal Branching by an FGF Pathway

A single gene, *branchless*, is the critical determinant of the tracheal branching pattern (*12*). Just before primary branching begins, *branchless* turns on in clusters of cells arrayed around the tracheal sacs, at positions where primary branches will bud (Fig. 2A). The secreted Branchless FGF binds the Breathless FGF receptor on nearby tracheal cells, stimulating the receptor's tyrosine kinase activity and downstream signal transduction cascades involving Ras, Raf, and a cytoplasmic protein encoded by *stumps* (*Dof*) (*11*, *13*). This signaling guides the migration of the tracheal cells as the primary branches bud (Fig. 2B).

Expression of *branchless* is highly dynamic. As each primary branch grows toward the nearby cluster of *branchless*-expressing cells, expression of the gene turns off and the branch stops growing. In some cases, another patch of *branchless* expression turns on at a more distant site and the branch continues to grow toward the new patch. Misexpressing the gene in novel positions causes ectopic branch outgrowth to the new sites. Thus, the pattern of *branchless* expression sets the pattern of primary branching.

Several hours after primary branches bud, secondary branches begin to sprout. The secondary budding pattern is also controlled by Branchless and Breathless, but by a different molecular mechanism. As primary branches extend toward the Branchless FGF signaling centers, cells at the growing end are exposed to high levels of the signal (Fig. 2B). This induces expression of secondary branch genes such as *pointed* (*6*, *12*), an ETS domain transcription factor (*14*), which drive formation of secondary branches. Paradoxically, Branchless also induces a potent inhibitor of branching called *sprouty* in the cells closest to the signaling center (*15*). Sprouty protein blocks Branchless signaling to more distant tracheal cells, thereby limiting secondary branch sprouting to positions closest to the FGF signaling sources.

Terminal branches bud several hours after the secondary branches and throughout larval life. The structure of these fine branches differs dramatically from that of previous generations of branches, and the pattern of branching is not rigidly fixed but variable and regulated by tissue oxygen need (*6*, *16*). Nevertheless, terminal branching is also controlled by the Branchless pathway (*17*). New genes come into play at this stage that change the expression pattern of the FGF ligand (rendering it oxygen-sensitive) and the tracheal cells' response to it. One of these genes is *blistered* (*pruned*), which encodes the *Drosophila* Serum Response Factor (*18*), a MADS domain protein proposed to function with a ternary complex factor as part of an FGF-activated transcription complex that regulates other terminal branch genes. The *blistered* gene turns on just before terminal branching begins, triggered by FGF signaling in the previous round of branching (*6*, *12*).

Thus, a core FGF pathway is used repeatedly to pattern each generation of tracheal branches. But at each stage, the mechanisms controlling expression or activity of the ligand are changed, and the signaling pathways downstream of the receptor are altered, resulting in different branching outcomes. Some of the changes are triggered by previous FGF signaling events: Thus, the different stages of the developmental program are coupled in a regulatory cascade that ensures that branching occurs in the proper sequence and generates distinct patterns at each stage.

Control of the Early Stages of Lung Branching by an FGF Pathway

Development of the mouse lung, although less well understood than the *Drosophila* tra-

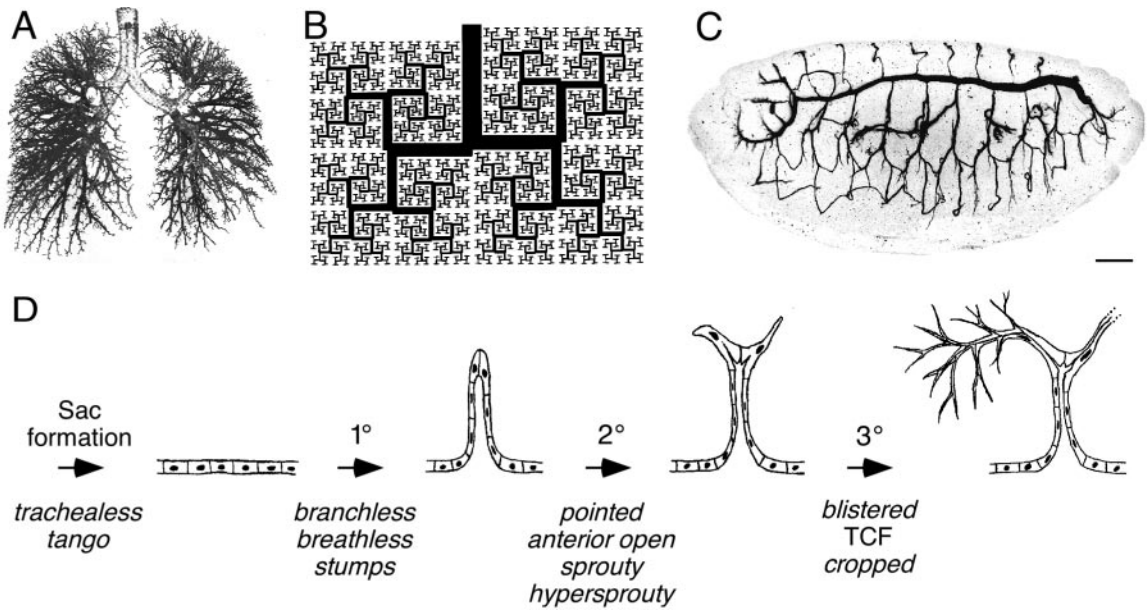

Fig. 1. Structure and formation of branching networks. (**A**) Latex cast of a human lung (*39*). There are 20 or more generations of branches. (**B**) Mandelbrot's model (*5*) of a branching network generated by 10 rounds of dichotomous branching. (**C**) Immunostain of the developing tracheal system in a 15-hour-old *Drosophila* embryo. In each segment, 6 primary and about 25 secondary branches have formed, and hundreds of terminal (3°) branches will sprout during larval life. (**D**) Schematic of *Drosophila* tracheal development. A portion of an epithelial sac is shown sprouting one primary, two secondary, and many terminal (3°) branches. Mutations in different genes block or cause misregulation of theprocess at the indicated steps. TCF, ternary complex factor. Bar is ~3 cm in (A) and 15 μm in (C).

cheal system, provides an instructive comparison. Like the *Drosophila* tracheal system, the mammalian lung develops by sequential rounds of branching. Beginning on embryonic day 9 in mouse (about day 25 in humans), one or two epithelial buds sprout from the gut into the surrounding mesenchyme to form the trachea and left and right primary bronchi. The primary bronchi grow and sprout secondary bronchi, which sprout tertiary bronchi, and so on. Branching continues for a total of 6 to 8 generations in the mouse and for about 20 generations in humans, forming the estimated 17 million branches of the human lung (*3*). Buds appear in a characteristic order and branch at characteristic locations (*19*), indicating that as in *Drosophila*, the early stages of branching are under fixed developmental control. Also, successive branches progressively diminish in size, and the histological structure of the epithelium and surrounding support layers change.

An isolated lung bud explant in culture develops into an extensively branched structure of apparently normal pattern (*19*). All of the patterning information is therefore contained in the several thousand cells of the pulmonary epithelium and surrounding mesenchyme that compose the explant. Experiments in which different portions of epithelium and mesenchyme were recombined in culture demonstrated that the mesenchyme is not only required for epithelial branching but also plays an important role in the patterning process (*19*, *20*). This suggested that the mesenchyme might contain spatially restricted cues that direct branching of the epithelium.

FGF10, one of 18 mammalian FGFs, was recently identified as a mesenchyme-derived factor that plays a critical role in patterning the early branching events. *Fgf10* knockout mice show a striking phenotype—the absence of lungs with just a blind-ended trachea remaining (*21*). A dramatic inhibition of bronchial branching is also seen in transgenic mice expressing a dominant negative form of an FGF10 receptor (Fgfr2-IIIb) in the pulmonary epithelium (*22*), indicating that the actions of FGF10 may be mediated through this receptor. As in the *Drosophila* tracheal system, the FGF receptor is initially expressed throughout the epithelium (*23*) while the ligand turns on in the surrounding tissue (*24*). *Fgf10* is expressed in a complex and dynamic pattern in the mesenchyme near the positions where primary, secondary, and tertiary bronchi bud. The buds grow toward areas of *Fgf10* expression (Fig. 2C), and when an FGF10-soaked bead is implanted, ectopic branches grow out and target the bead (*24*). Thus, FGF10 appears to direct early bronchial branching much as the Branchless FGF controls the initial branching events in *Drosophila*. Also like *Drosophila*, the FGF appears to be used repeatedly to pattern successive rounds of branching.

In addition to its chemoattractant function, FGF10 has another function in lung branching that parallels the situation in *Drosophila*: It induces later programs of gene expression in the growing branches (Fig. 2C). *Bmp4* expression is induced by FGF10 (*25*, *26)*, and the expression patterns of genes that encode Sonic hedgehog (Shh) (*27*) and mouse Sprouty homologs (*28*, *29*) suggest that they are induced in the same way. Thus, the tips of growing bronchial branches themselves become active signaling centers, with each secondary signal serving a different function. Bmp4 inhibits proliferation of the epithelium and hence may limit branch growth (*26*), and Sprouty2 functions like *Drosophila* Sprouty to limit branch formation (*28*).

Retrograde Signals from Epithelium to Mesenchyme

Another function of the secondary signals, one not required in the *Drosophila* tracheal system, is to pattern the surrounding mesenchyme. As the pulmonary epithelium branches, the mesenchyme grows and differentiates into support structures of the airway walls (cartilage, smooth muscle) and blood vessels. These processes must be coordinated with epithelial branching because the support structures ensheath the epithelial tubes, and pulmonary blood vessels follow their branch-

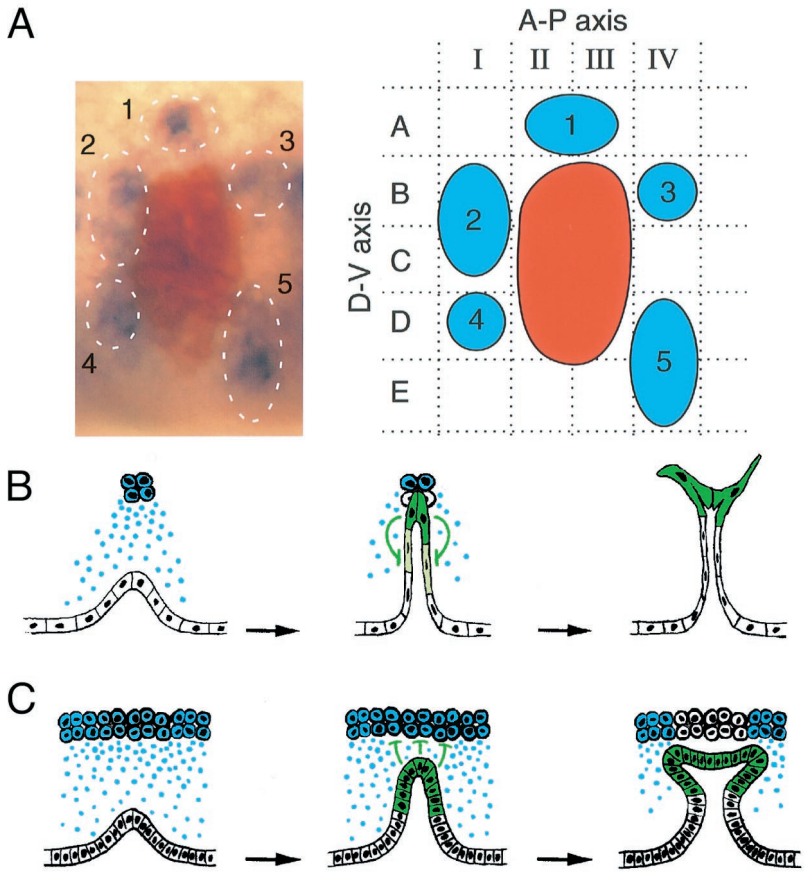

Fig. 2. FGF control of branching morphogenesis. (**A**) Five domains of *branchless* FGF mRNA expression (blue) surrounding a tracheal sac (*trachealess* expression, brown) at about 6 hours of development. Primary branches bud at these five positions and a sixth position of *branchless* expression deep to the focal plane. The schematic representation (right) shows the register of *branchless* and *trachealess* expression domains with the gridlike pattern of positional values set by the anterior-posterior (A-P) and dorsal-ventral (D-V) patterning hierarchies. Modified from (*12*, *32*). [Photomicrograph reproduced with permission from Cell Press] (**B**) Model of Branchless patterning of tracheal branching. Secreted Branchless FGF (blue) guides the migration of tracheal cells as they form primary branches. High levels of Branchless induce secondary branch genes such as *pointed* in the cells at the end of the primary branches (green), which reprogram these cells to form secondary branches. Another induced gene, *sprouty*, encodes an FGF pathway inhibitor that limits the range of FGF signaling (green inhibitory arrows) and restricts secondary branch formation to cells closest to the FGF signaling center. (**C**) Model of FGF10 patterning of mouse lung branching. FGF10 (blue) secreted by the mesenchyme guides bronchial branch outgrowth. It also induces new gene expression in the cells at the ends of the bronchial branches (green). Shh is proposed to function as a feedback inhibitor of *Fgf10* expression (green inhibitory arrows), which splits the *Fgf10* expression domain and promotes the next round of branching.

ing pattern. Signals from the epithelium back to the mesenchyme could promote mesenchymal growth and differentiation. Indeed, blood vessels and other mesenchymally derived elements fail to form in lungs of transgenic mice expressing a dominant negative FGF10 receptor in the epithelium, indicating that retrograde signals are induced by the FGF pathway (*30*). The expression pattern of *Shh* and the impairment of lung mesenchyme proliferation and differentiation in $Shh^{-/-}$ mutant mice suggest that Shh is such a retrograde signal (*27*, *31*).

Shh signaling to the mesenchyme may also play a role in branch patterning. Expression of *Fgf10* in the mesenchyme is highly dynamic and diminishes as each new branch approaches. Shh is proposed to function as a negative feedback signal that shuts off *Fgf10* expression in mesenchyme near growing tips, splitting the initial *Fgf10* expression domains into two smaller domains (Fig. 2C) (*24*). Two new buds then sprout, each targeting one of the remaining subdomains of *Fgf10* expression. Shh would thus arrest bronchial outgrowth and promote sprouting of the next generation of bronchi.

Feedback signals from the branching epithelium to the inducing tissue add an important element to branching models. One of the conceptual challenges in the field is understanding how the coarse-grained spatial information that patterns the major branches evolves into the fine-grained information that controls the later stages of branching. Signals from new branches that feed back and alter the expression domains of the inducing cues (or turn on new inducing cues) provide an appealing mechanism for recursively refining the patterning information.

Patterning the Branch Patterning Genes

Although the *Drosophila* and mouse studies demonstrate that FGFs play key roles in branch patterning and show how feedback signals like Shh and Sprouty can refine the patterning information during development, the question remains of how the complex initial expression patterns of the FGF genes themselves are established. What patterns the patterning genes?

The characteristic segmental positions of the *branchless* expression domains arrayed around each tracheal sac suggest that *branchless* expression is controlled by the earlier-acting gene regulatory hierarchies that specify positional values along the anterior-posterior (A-P) and dorsal-ventral (D-V) body axes (Fig. 2A) (*12*, *32*). There may be separate transcriptional enhancers for each spatial domain of *branchless* expression, each enhancer responsive to a different set of regulators differentially distributed along the A-P and D-V axes of each segment. Consistent with this idea, different domains of *branchless* expression are dependent on different genes in the A-P and D-V patterning hierarchies (*33*). The initial shape and position of each tracheal sac appear to be established in a similar way by other combinations of A-P and D-V patterning genes acting on *trachealess* (*9*). The initial function of *branchless* and *trachealess*, then, is to integrate the early, gridlike patterning information and transform it into more complex patterns that represent the initial form of the organ.

The upstream regulators that set the expression patterns of *Fgf10* and *Fgfr2* in the lung are less obvious, perhaps because the pathways specifying global positional values in mammals are not well defined. However, the global patterning pathway that dictates left-right asymmetry in the body regulates the lung branching pattern (*34*). Normally, the left and right lungs have distinct branching patterns. However, in $inv^{-/-}$ mutant mice, the patterns of the left and right lungs are reversed, and in other mutants where left-right asymmetry is lost, both lungs show the pattern of either the left or right lung. Thus, the lung patterning program lies downstream of *inv* and other genes in the left-right patterning pathway, implying that genes in this pathway, and presumably other global patterning pathways too, must ultimately control the expression pattern of lung branching regulators such as *Fgf10* and *Fgfr2*.

A Common Scheme for Patterning Branching Morphogenesis?

The genetic analyses of *Drosophila* and mouse respiratory system development suggest a general scheme for patterning branching morphogenesis (Fig. 3). The central element is that one basic signaling pathway, an FGF pathway in our examples, is used repeatedly to pattern successive rounds of branching. Global embryonic patterning pathways specify the initial positions of the inducing signal (FGF) as well as the position of the organ primordium and its ability to respond to the inducing signal. This sets the initial structure of the organ and patterns the first branching events. The inducing signal directs the cellular events of branch formation and outgrowth, and also triggers new gene expression in budding branches. Some of the induced genes encode downstream signaling components that change the response to the inducing signal in the next round of branching. Other induced genes encode signaling molecules themselves, which function as feedback signals that alter the expression or activity of the inducing signal. In this way, the pattern of branching and structure of the branches are modified at each stage of branching according to the specific genetic regulatory program. Although not as economical genetically as the strictly iterative schemes envisioned by theorists, these genetic programs generate reproducible patterns tailored to the organ's function.

Although an outline of the tracheal and lung patterning programs has emerged, many important questions remain. For example, in the lung, do other factors besides FGF10 contribute to patterning early branching events (*24*), and does FGF10 continue to play a central role after the first three branch generations? The *Fgf10* gene continues to be expressed, but other FGFs and a variety of other signaling pathways including epithelial growth factor (EGF) and transforming growth factor–β (TGF-β) are also active during these stages (*35*). In *Drosophila*, other signaling pathways, again notably EGF and TGF-β (Dpp) pathways, have been shown to function in tracheal branching, helping set the sizes of primary branches and boundaries between them (*33*, *36*). How are these other signaling pathways integrated with the FGF pathway? And how are the patterning signals transformed into the epithelial migrations and tube-assembly events they control? Once these and other genetic branching programs are thoroughly understood, it may be possible to design interventions that can reactivate the programs to restore vital organs like the lung that have been damaged by disease.

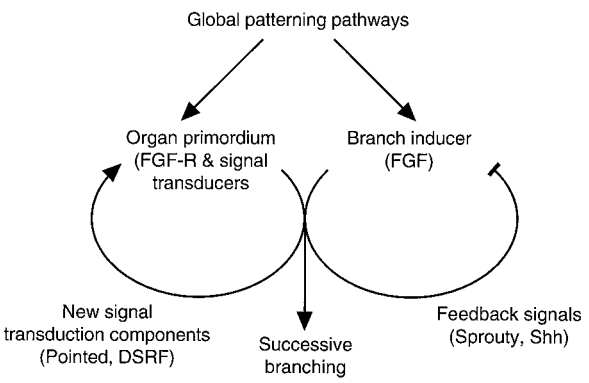

Fig. 3. A general scheme for patterning branching morphogenesis by reiterative FGF signaling. Global embryonic patterning pathways specify the initial positions of the organ primordium (and FGF reception and signal transduction ability) and a branching inducer (FGF) in localized regions near the organ primordium. This patterns the first round of branching. FGF signaling also triggers expression of feedback signals that alter the expression or activity of the branching inducer (or turns on new inducers), and expression of new FGF signal transduction components. The new modulators alter the pattern and structure of branches produced by the next round of signaling, and subsequent rounds of FGF signaling and branching are modified in a similar manner by genes induced in the previous round.

The central role of FGF pathways in development of the respiratory systems of both *Drosophila* and mouse is surprising because although insect tracheal systems and the mammalian lung share a common physiological function, they have always been believed to represent convergent evolutionary solutions to the general problem of oxygen transport. Perhaps they are after all homologous structures that evolved from a primitive airway present in our last common ancestor. A more plausible scenario is that an FGF pathway served to pattern some ancestral branched structure and this pathway was then coopted during evolution to pattern other branched organs (*37*). Indeed, FGF pathways are implicated in the development of many branched organs besides respiratory systems (*38*). If this view is correct, then the scheme outlined here may turn out to be a quite common means of patterning branching morphogenesis, with the basic genetic program embellished in different ways during evolution to generate the many exquisite biological patterns of branching.

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