HIGHLIGHTED TOPIC | Lung Growth and Repair

Cellular and molecular mechanisms involved in branching morphogenesis of the Drosophila tracheal system

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Cabernard, Clemens, Marc Neumann, and Markus Affolter. Cellular and molecular mechanisms involved in branching morphogenesis of the Drosophila tracheal system. J Appl Physiol 97: 2347–2353, 2004; doi:10.1152/japplphysiol.00435.2004.—Recent comparative studies have shown that, in many instances, the genetic network underlying the development of distinct organ systems is similar in invertebrate and vertebrate organisms. Genetically well-characterized, simple invertebrate model systems, such as Caenorhabditis elegans and Drosophila melanogaster, can thus provide useful insight for understanding more complex organ systems in vertebrates. Here, we summarize recent progress in the genetic analysis of tracheal development in Drosophila and compare the results to studies aimed at a better understanding of lung development in mouse and man. Clearly, both striking similarities and important differences are apparent, but it might still be too early to conclude whether the former or the latter prevail.

THE QUESTION OF HOW PARTICULAR ORGANS form during development and how these organs ultimately function during adult life has moved to center stage in developmental genetics. The conservation of genetic regulatory cascades and networks during evolution argues that simple model organisms might contribute important information concerning organ formation, although the architecture of these organs is simpler and their function more limited compared with higher organisms, such as mice and humans.

The fruit fly Drosophila melanogaster is a particularly well-characterized model organism. Genetic analysis over the past century have led to the characterization of approximately one-fourth of the estimated 13,500 genes present in the genome (2). All major signaling pathways involved in cell-cell interactions during development of higher animals are conserved in the fruit fly, and many of the organ-specific transcriptional regulators play similar roles in the fly compared with mice or humans (37, 57). Several organ systems have been dissected in Drosophila using genetic and molecular approaches (1, 5, 15, 17, 21, 47). Here, we briefly summarize the current state of knowledge concerning the development of the respiratory organ of the fly (the trachea) and mention parallels between tracheal development and the development of the lung in mammals.

CELL MIGRATION CONTROLS BRANCH FORMATION IN THE EMBRYO

The larval tracheal system consists of hundreds of interconnected tubes that transport oxygen and other gases throughout the body (3, 18, 51). Tracheal branches are simple tubes consisting of an epithelial monolayer wrapped into a tube around a central lumen. The development of the tracheal system is initiated in the early embryo on the determination of 10 bilaterally symmetrical clusters of ~20 tracheal precursor cells; on invagination and two additional cell divisions, each of the tracheal sacs undergoes a similar sequence of developmental events to generate one segment of the network in the absence of further cell divisions (Fig. 1, A–H). The transformation, in a few hours time, of a simple epithelial sac into a highly ramified, yet stereotyped, tubular network is a fascinating phenomenon and has attracted the attention of a number of research groups sharing the aim of better understanding the cellular and molecular principles underlying the control and regulation of branching morphogenesis. As it turns out, three key cellular processes are involved in branch formation: directional cell migration, cell rearrangements, and cell shape changes. To interconnect the individual metameres, certain tubes ultimately fuse with corresponding tubes in neighbor segments (38, 48).

The most insightful finding with regard to the spatial control of tracheal branch formation in the Drosophila embryo came from the identification and characterization of two genes, breathless/FGFR (btl/FGFR) and branchless/FGF (bnl/FGF). The absence of either of these two gene products leads to a complete failure of branch outgrowth despite proper determination of tracheal cells (29, 45). bnl/FGF encodes a fibroblast growth factor (FGF) receptor expressed in tracheal cells but not
Invited Review

DROSOPHILA TRACHEAL SYSTEM MORPHOGENESIS

Fig. 1. Tracheal development in the Drosophila embryo. A–D: development of the embryonic tracheal system. The tracheal system arises from 10 placodes on each side of the embryo that bud in from the epidermis. On invagination of the tracheal cells and after 2 rounds of cell division, the individual tracheal metameres consist of ~80 cells (A). From this placode, 6 branches grow out into different directions (B–D). Subsequently, some of these branches fuse to form a continuous network that will be used for oxygen supply in the hatching larva. E–H: schematic drawing of the development of an individual (boxed) tracheal metamere. Lateral views show the stereotyped branching pattern of the tracheal epithelium (TR; red) as well as the expression of the fibroblast growth factor (FGF) ligand bnl (blue). Light blue indicates fading bnl/FGF expression. Top views (e–h) indicate the connection to the epidermis (EP) and the flattening of the tracheal sac on progression of development. Note that embryonic branching morphogenesis does not rely on cell division but on cell migration, cell intercalation, and cell shape changes. I: nomenclature of the tracheal branches. DB, dorsal branch; DTa/p, dorsal trunk anterior/posterior; VB, visceral branch; SB, spiracular branch; LTa/p, lateral trunk anterior/posterior. K–N: live images of a dorsal branch expressing actin tagged with green fluorescent protein. Branch outgrowth is driven by Bnl/FGF, which induces filopodia in tracheal tip cells (arrows). During branch outgrowth, the cells are rearranged from a side-by-side (K) to an end-to-end (N) arrangement, visualized here by the rearrangement of the nuclei (*).

in surrounding epithelial cells (29), whereas bnl/FGF encodes the FGF ligand of Btl/FGFR and is expressed in individual cells or cell clusters surrounding the invaginating tracheal placode (50). Gain-of-function and loss-of-function studies (50) combined with live imaging (46) revealed that the Bnl/FGF ligand induces a migratory behavior by promoting cytoskeletal dynamics in a few cells at the tip of the tracheal branches (see Fig. 1, K–N). The stalk cells appear to follow these tip cells passively; during branch formation, all tracheal cells maintain their epithelial character and remain attached to each other via their subsapical adherens junctions (AJ).

It is not clear yet whether Bnl/FGF acts as a true chemotactant or as a motogen. A function as a chemotactant is supported by the finding that bnl/FGF expression in ectopic positions induces branch outgrowth toward such sites (49, 50). However, it was shown that the direction of migration is influenced by other signaling systems, such as Dpp/BMP (46, 52). In the absence of Dpp/BMP signaling, prospective dorsal branch cells start to migrate dorsally but ultimately reintegrate into the dorsal trunk and thus never form a definitive dorsal branch (for nomenclature, see Fig. 1I) (46). Conversely, ectopic Dpp/BMP signaling is able to direct cells from migration along the anterior-posterior axis toward migration along the dorsal-ventral axis (52). How Dpp/BMP influences the direction of migration is not known and remains to be investigated.

What are the molecular consequences of FGF signaling in tracheal cells, or, in other words, how are cells instructed to become motile and follow directions under the control of Bnl/FGF? Genetic studies have led to the identification of a mutation in a gene called downstream-of-FGFR (dof), which displays a tracheal phenotype identical to bnl/FGF and btl/FGFR (25, 40, 53). Dof turns out to be a cytoplasmic signaling adaptor protein, present in only those cells of the embryo that express FGF receptors. Dof interacts constitutively with the Drosophila FGF receptors and becomes phosphorylated by the latter on activation, leading to the recruitment of the Corkscrew phosphatase (44, 59). Corkscrew recruitment represents an essential step in Bnl/FGF-induced cell migration and in the activation of the Ras/MAPK pathway. However, it appears that Ras activation is not sufficient to activate the migration machinery in tracheal cells (44). Additional proteins binding either to the FGFRs, to Dof, or to Corkscrew appear to be crucial for a migratory response, and it will be important to identify these missing links. Somewhat surprisingly, tracheal cells also migrate properly when the spatial information of Bnl/FGF ligand expression is transmitted to the cell interior by the kinase domain of a different receptor tyrosine kinase; this was shown via the expression of chimeric receptor molecules carrying the extracellular domain of Btl/FGFR and the intracellular kinase domain of unrelated receptor tyrosine kinases, such as epidermal growth factor receptor or Torso (16). These results demonstrate that tracheal cells respond with directed migration to receptor tyrosine kinase signaling (whereas other cells in the developing embryos respond differently). Therefore, it will be important to decipher the regulatory program that leads to the determination of epithelial cells toward the tracheal fate; identification and characterization of genes that are specifically activated or repressed in tracheal cells might shed light on why and how tracheal cells respond to FGF signaling with migration.

These studies on the formation of the tracheal tree provide the first insights into molecular events of guided cell migration controlled by FGF signaling. However, the developing embryo of Drosophila is not an ideal model system to investigate these
molecular events at a much more detailed level using a genetic approach. This is due to the fact that many proteins essential for either cell signaling (i.e., Ras) and/or cytoskeletal rearrangements (i.e., Rac) are maternally provided (large amounts of RNA and/or protein products of numerous genes are transferred from the polyplloid nurse cells of the mother to the oocyte and are sufficient for the early development of the embryo) and thus often fail to be detected in standard genetic screens for zygotic effects. In addition, the lack of proteins involved in important, basic cellular events might lead to dramatic defects in embryonic stages before tracheal branching, making analysis of their contribution to branching morphogenesis more difficult. However, and as we will outline below, FGF signaling via Bnl/FGF and Btl/FGFR is again used during larval and pupal stages to induce branching morphogenesis in a growing tracheal epithelium, the developing air sac (49). This system allows the identification of genes involved in branching morphogenesis in a genetically more amenable context, and major progress toward a better molecular understanding of FGF signaling in branching morphogenesis is expected to be obtained by studying this system.

CELL REARRANGEMENTS AND CELL SHAPE CHANGES LEAD TO BRANCH ELONGATION IN THE EMBRYO

Quite obviously, the active migration of a small number of tracheal tip cells away from the saclike structure of the invaginated tracheal placode has to be accompanied by cell rearrangements and cell shape changes to generate elongated tracheal branches. Because tracheal cells are at all time part of a tightly sealed epithelium, epithelial AJ must be remodeled to a significant extent during branch formation. Indeed, extensive AJ remodeling is required for the generation of most tracheal branches of a late *Drosophila* embryo, and individual steps in the remodeling process have been described in dorsal branch formation (27, 48). During the early stages of branch formation, tracheal cells are initially arranged in a side-by-side-side fashion (see Fig. 1K). Through cell intercalation (Fig. 1, L–M), this conformation is transformed into an end-to-end arrangement (Fig. 1N). The dorsal branch (as well as most other branches) finally consists of single cells wrapped around the lumen and closed up by autocellular AJs (27, 48).

The molecular events underlying AJ remodeling have not yet been elucidated, but it appears that the coordination of cell rearrangements with cell migration requires additional developmental signaling systems. For example, the outgrowth of a particular branch, the dorsal branch, required Dpp/BMP signaling in addition to Bnl/FGF signaling (46). The lack of Dpp/BMP leads to the absence of dorsal branches, despite the presence of filopodia formation and initial dorsal branch outgrowth under the control of Bnl/FGF signaling; in the absence of Dpp/BMP signaling, cells in the initial dorsal bud reinteregrate into the major tracheal branch, the dorsal trunk (46). Thus Dpp/BMP signaling appears to be required for proper cell rearrangements to take place, and in the absence of concomitant cell rearrangements cell migration is not sufficient to bring about the formation of distinct tracheal branches. It also appears that the activity level of the small GTPase Rac has a strong impact on cell rearrangements; reduced Rac activity inhibits tracheal cell rearrangements, whereas hyperactivation leads to the loss of tracheal cell adhesion (12). These effects might be due to a regulation of the levels of E-cadherin in tracheal cells, but how exactly Rac activity levels and E-cadherin protein levels are coordinated remains to be determined.

Interestingly, a recent study has made a link between tracheal cell rearrangements and the presence of proteins secreted apically into the luminal space (27). Piopio and Dumpy, two proteins containing a zona pellucida domain (28), have been shown to contribute to the apical extracellular matrix and to play an important role during the intercalation process (see Fig. 1, K–N) and autocellular AJ formation in tracheal development (27). In the absence of these two proteins, all fine tracheal branches form cysts instead of tubes, leading to a disruption of the tracheal network. Although the precise role of the two proteins remains to be firmly established, this study demonstrates for the first time an important requirement for luminal matrix proteins in tubulogenesis.

DEVELOPMENTAL CONTROL OF TUBE SIZE, TUBE DIAMETER, AND LIQUID CLEARANCE

The size and shape of the branches making up a tubular organ are fundamental parameters in the control of transport demands. Like in other tubular organs, tracheal tubes increase in size and diameter as the animal grows during development. At the end of larval life, tracheal tubes have expanded up to 40 times their initial size. This increase in tube size does not depend on cell division or on the number of cells in the branches but is controlled mostly at the apical surface of tracheal cells; although there is a dramatic increase in the inner tube diameter, little change is observed in the outer tube diameter (7).

Tube size is controlled genetically and is not modified under high- or low-oxygen conditions. A number of mutants, in which the length as well as the diameter of tracheal branches is altered, have been described (7). The two *Drosophila Claudin* family members *Megaschemea* (6, 7) and *Sinuous* (60) as well as the cell surface protein Lachesin (36) are components of septate junctions, and mutations in these genes show overgrown tubes. Other components of septate junctions, such as Coracle and Neurexin, also show similar phenotypes in the tracheal system when absent (6, 43). However, whether the developmental control of tube shape, e.g., the increase in tube diameter in larval stages, is exerted via these genes remains to be investigated.

Apart from septate junction components, the control of the growth of the apical membrane seems to be equally important for maintaining a balanced tube size and length. The transcription factor Grainy head (Grh) limits apical membrane growth; in *grh* mutants, several branches are convoluted, and *grh* tracheal cells show an expansion of the apical membrane in late embryonic stages, resulting in an enlarged and anomalous luminal surface (22). Neither Grh target genes nor other effector proteins involved in the control of the apical surface of tracheal cells have been identified so far.

Liquid clearance of the tracheal system provides a developmentally controlled physiological aspect of this tubular organ. At the end of embryogenesis, 2 h before larvae emerge, the liquid in the tracheal system is cleared and replaced by air (38). Clearance occurs by active epithelial absorption, and epithelial Na⁺ channels appear to be involved in this process. Several
Degenerin/epithelial Na\(^+\) channel family members are expressed quite specifically in the tracheal system. Inhibitor as well as RNAi studies targeting the products of two of these genes (PPK4, PPK11) inhibited liquid clearance (34), suggesting that the regulated activity of these channels contributes to the proper differentiation of tracheal cells.

**PHYSIOLOGICAL ASPECTS OF TRACHEAL DEVELOPMENT AND FUNCTION**

Quite in contrast to the stereotyped, developmentally controlled organization of the major branches of the larval tracheal system, the organization and ramification of the so-called terminal branches are under physiological control. Terminal branch formation is regulated by the gene *DSRF/blistered*, which encodes the *Drosophila* homolog of serum response factor (4, 20, 42). *DSRF/blistered* is specifically expressed in terminal tracheal cells and required for the formation of tens to hundreds of long cytoplasmic processes by each of these cells during the larval stages. These cytoplasmic processes form an intracellular lumen, which connects up to the lumen of the main tracheal network. Larvae grown under nonphysiological, low-oxygen conditions show a dramatic increase in terminal branching; conversely, larvae grown under high-oxygen tension show the opposite phenotype, having relatively few terminal branches (26).

A link between terminal branching and oxygen conditions has been established through the analysis of *bnl/FGF*, which is expressed broadly in the larva and in all tissues that become heavily tracheated. Strikingly, *bnl/FGF* transcription is enhanced in many cells under low hypoxic conditions. Oxygen deprivation stimulates expression of *bnl/FGF*, and the secreted growth factor functions as a chemoattractant that guides new terminal branches to the expressing cells. This implies that, in *Drosophila*, *Bnl/FGF* is either a or possibly the key mediator of the hypoxic response (26).

These experiments suggested that an oxygen-sensing pathway exists in *Drosophila*. Indeed, it has been shown more recently that the hypoxia-inducing factor-α, encoded by the gene *similar* (*sima*), as well as the hypoxia-inducing factor-β subunit encoded by *tango*, are required for the hypoxic response. *Sima* and *tango* code for basic helix-loop-helix PAS transcription factors that form heterodimers. It was also proposed that Sima protein stability is controlled by oxygen through the Prolyl 4-hydroxylase homolog encoded by *CG1114*; a mutant in which *CG1114* protein expression is reduced shows an upregulation of Sima under normal physiological oxygen conditions (31). Thus fine terminal branches are formed from terminal tracheal cells as a result of oxygen needs in target tissues, but how the outgrowth of terminal branches is coordinated and controlled by FGF signaling remains to be established.

**COORDINATION BETWEEN CELL MIGRATION AND CELL DIVISION DURING AIR SAC FORMATION IN THE LARVA**

A striking difference between the development of branched organs in higher vertebrates and the formation of the tracheal system in the *Drosophila* embryo lies in the fact that cells do not divide during the branching process in the embryonic tracheal system of the fly; the increase in the three-dimensional complexity of the network is due to cell migration, cell rearrangements, and cell shape changes. During larval development, however, the tracheal system is remodelled tremendously to satisfy the needs in the adult fly, and the formation of new structures includes, and presumably relies on, controlled cell proliferation (38).

Recently, the formation of the thoracic air sacs of the adult fly has been investigated (49). The thoracic air sac is an offshoot of an existing tracheal branch, the transverse connective, which attaches to the wing imaginal disc (see Fig. 2). In the early-third instar larva, some 50 h before pupation, a few tracheal cells of the transverse connective branch start to proliferate and migrate out in a stereotypical manner. Both *btl/FGFR* and *dof* are expressed in the tracheal cells of this growing air sac, suggesting that the FGF signaling system,
which shapes the embryonic branches, might also be involved in late branching. Indeed, both the proliferation and migration of tracheal cells in the developing air sac are dependent on a functional FGF signaling pathway (49). The ligand Bnl/FGF itself is produced and secreted from a small number of columnar epithelial cells in close proximity to the tracheal cells of the transverse connective (Fig. 2, A and B). As air sac tracheoblasts undergo intensive proliferation and migrate toward the Bnl/FGF-expressing columnar epithelial cells (Fig. 2, A and B), cells at the tip of the growing sac extend filopodia, probably as a result of Bnl/FGF signal reception. Bnl/FGF appears to guide the migration of air sac tracheal cells, an interpretation that is supported by the finding that filopodia extend toward ectopic Bnl/FGF sources in genetic mosaic experiments. During pupal stages, the air sac does not cease to grow and eventually forms three distinct branches, the mediocular, the laterocular, and the scutellar sac, in the thorax of the adult fly (49). Intriguingly, Bnl/FGF fulfils a dual role during air sac formation, serving both as a chemoattractant/motogen as well as a mitogen. The molecular basis for this dual role has not been addressed so far, and many questions remain open concerning the development of air sacs.

As mentioned above, there are a number of serious limitations to the genetic analysis of cell migration in the developing embryo. The proliferative capacity of the air sac tracheoblasts in late larval stages opens the door for the application of genetic mosaic analysis, namely the generation of genetically mosaic animals. Generation of mosaic air sacs, including small clusters of mutated cells, will allow an assessment of the involvement of the FGF signaling pathway as the molecular pathways that link FGF signaling to cytoskeletal remodeling, in particular the formation of tubes with autocrine AJ.s, is important for the formation of fine terminal branches in the lung. Thus it remains to be seen to what extent similarities between Drosophila and mice or humans exist with regard to cell rearrangements.

Recent studies have demonstrated a requirement of luminal zona pellucida-domain proteins during the branching process in Drosophila (27). Zona pellucida-domain proteins are also major components of lumen-containing epithelial organs in vertebrates (10, 24, 30, 32, 33, 39, 56). It remains to be seen whether they contribute to the proper formation of these organs during development or whether they play physiological roles. Without doubt, the number of molecules linked to the process of branching morphogenesis of specific organs will dramatically increase in the future, and it is hoped that this will lead not only to an increase in the molecular complexity underlying the branching process but also to an enhancement of clarity with regard to developmental logic.

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REFERENCES


