

Symmetry Breaking during Morphogenesis in the Embryo and in Engineered Tissues

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During embryonic development, cells divide and migrate and tissues change shape. These changes in tissue shape constitute the highly choreographed dynamic process of morphogenesis that collectively turns simple forms into the complex architectures of mature organs. Although the key signaling pathways are determined genetically, the geometries of these simple tissue forms are instructive as well, and help to elaborate symmetry-breaking events required for morphogenesis. Understanding the role of tissue geometry during morphogenesis may reveal novel strategies for constructing engineered tissues *ex vivo*.

Tissue development is an intrinsically dynamic process. Changes in chemical reactions and physical forms (which specify the geometry of the reaction volume as well as its boundary conditions) as a function of time work to turn a fertilized egg into a full living being, comprised of multiple tissues and an exquisite variety of differentiated cell types. This dynamic change in form is known as morphogenesis, a process which is encoded in part within the genome, but which is also responsive to external stimuli.

How do we study this problem of morphogenesis? When viewed as a series of chemical reactions, it is natural to examine morphogenesis using computational approaches, and such techniques have indeed revealed novel control mechanisms.^{1,2} These studies are elegant, but can run the risk of lacking relevance if taken to an extreme. When viewed as part of the whole organism, morphogenesis can be examined *in vivo* using either classical embryology techniques or more recently developed genetic approaches. These studies have been particularly fruitful when completed using small and simple organisms. When viewed as an independent unit of function, morphogenesis can be examined using engineered tissues, thus combining experimental control with computational accessibility. These studies may be directly

applicable to regenerative medicine, but, in general, the conclusions derived from engineered systems need to be tempered and framed with respect to what is already known about the embryo. In this Perspective, the author will describe the current understanding of how symmetry is broken during development *in vivo*, and discuss how those lessons might inform the design of engineered tissues.

Symmetry Breaking Events in the Embryo: How to Establish a Coordinate System

The animal kingdom is incredibly diverse. From the tiny fruit fly with its elegant wings, to the camouflaged squid with its eight strong arms and piercing eyes, to the hammerhead shark, to the common house mouse, a seemingly endless variety of structures has populated our planet. Remarkably, all of these different forms begin as essentially the same shape—a spherical oocyte, that when fertilized undergoes hundreds of divisions to yield all of the cells that make up the final adult organism. This transition, from a sphere to a hammerhead, so to speak, is the essence of developmental biology and the major challenge for engineers hoping to recapitulate organ formation in the design of engineered tissues.

To turn a sphere into a functional organism requires a breaking of symmetry. Where is the top of the embryo? Where is the bottom? Where will the head form? The wing? The heart? Is this specification stochastic, or does that initial sphere already contain a coordinate system? In the fruit fly *Drosophila melanogaster*, the main coordinate systems of the embryo, those that specify the positions of the head and the tail (the anterior-posterior axis) and the back vs. the belly (the dorsal-ventral axis), are defined long before fertilization.³ These axes are encoded by the localization of messenger RNA within the oocyte: *bicoid* localizes to what will become the anterior end of the animal, *oskar* to the future posterior, and *gurken* to the anterior-dorsal quadrant.⁴ The deposition of these mRNA molecules depends on the geometry of the egg chamber in which the oocyte is developing, which is already asymmetric. In short, in the fruit fly the

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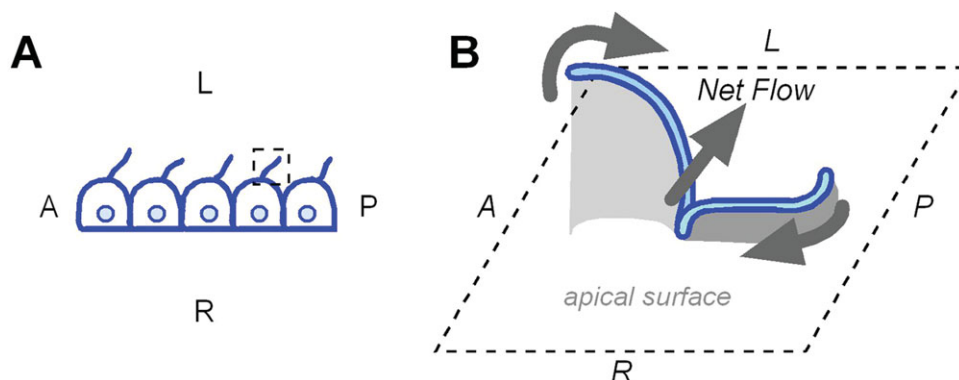


Figure 1. Left-right symmetry breaking is driven by leftward fluid flow.

(A) The apical surfaces of cells within the mouse embryo are studded with cilia. The cilia tilt in the posterior direction, rather than standing perpendicularly, and (B) clockwise rotation of cilia that have a posterior tilt drives the flow of fluid to the left. When in the leftward phase of rotation, a tilted cilium drags more fluid than when it is in the rightward phase of rotation. A, anterior; P, posterior; L, left; R, right. Schematic adapted from¹⁰.

symmetry is broken even before the oocyte has become an oocyte.³

In the roundworm *Caenorhabditis elegans*, the coordinate system is also present at a very early stage, and similarly defined by the localization of specific proteins at either the anterior or posterior end.⁵ Here, however, the symmetry breaking occurs upon fertilization, with the posterior end of the embryo being specified by the site at which the sperm enters the oocyte.⁶ Although the precise mechanisms by which the body axes are established have been challenging to examine in many vertebrate systems, it is clear that sperm entry plays a role in some species, such as the frog *Xenopus laevis*.⁷ What are essentially physical disturbances from the environment around the oocyte can thus pattern the future embryo.

In addition to the anterior-posterior and dorsal-ventral axes, vertebrates also specify their left vs. right sides. On the exterior, vertebrates are apparently bilaterally symmetric. On the interior, however, asymmetries are present in both the position and shape of the organs, and these asymmetries are conserved among individuals and between species. For example, fish, frogs, mice, and people all have their hearts on the left sides of their bodies. The mysteries underlying this left-right specification are beginning to be unraveled, and the mechanisms are apparently physical in nature. As with the other two axes of the embryo, left-right symmetry breaking involves the differential localization of key signaling proteins, including nodal.⁸ This differential localization does not arise from the entry of the sperm or from the geometry of the egg chamber, however, but rather from directional fluid flow resulting from the chiral nature of motor proteins. The surfaces of cells are studded with slender, finger-like protrusions known as cilia, which contain within them microtubules and motor proteins. The dynamics of these proteins cause the cilia to wave on the surface of the cell. In the embryo, the cilia are tilted sidewise to the posterior and have been found to rotate in a clockwise manner (Figure 1), which causes extra-embryonic fluid to flow from the right to the left side.^{9,10} Disrupting this low Reynolds number flow destroys the left-right asymmetry in mouse embryos, and causes defects in heart position and development.¹¹ Although quantitative details of the fluid flow velocity and cilia rotation speed differ across species, the overall

hydrodynamics, as represented by the Péclet number, appears to be conserved.¹²

Symmetry breaking in the early embryo thus emerges spontaneously from instabilities caused by physical perturbations: in the geometry (*Drosophila* egg chamber), in the membrane (*C. elegans* sperm entry point), and in the convection of fluid (vertebrate cilia). These alterations in the mechanical properties and boundary conditions lead to alterations in chemical signaling, which amplify into differences in gene expression and cellular behavior. Importantly, we have found that these basic principles are not limited to the early embryo, but are also used in the symmetry breaking required for organ development, as discussed below.

How to Break Symmetry in Developing Organs—Branched Systems

To find an extreme example of repeated symmetry breaking during development, one need look no further than to the ramified, tree-like architectures of organs such as the lung. In mammals, the airways of the mature lungs are magnificent to behold—up to 23 generations of branches make up the conduits through which air flows to its final destination, the alveoli, which are the extremely thin-walled gas-exchange surfaces present at the terminal ends. The lung airways themselves begin as very simple outpouchings of the ventral foregut endoderm (Figure 2A). This primitive structure then undergoes reiterative bifurcating and lateral branching morphogenesis to form a space-filling architecture (Figure 2B). A similar developmental process is responsible for building organs as diverse as the collecting ducts of the kidney, the milk ducts of the mammary gland, and the intercalated ducts of the salivary gland.¹³

The intrinsic beauty and self-similar nature of branched organs has been recognized for centuries. The earliest known descriptions of branched architectures within the body were by Aristotle, who suggested that the branching of the blood vessels within the vascular system was reminiscent of that of rivers dividing into ever-smaller tributaries.¹⁴ This analogy persisted into the 20th century, as pulmonary physiologists adopted the mathematical tools and terminologies invented by hydrologists to characterize the branching patterns of

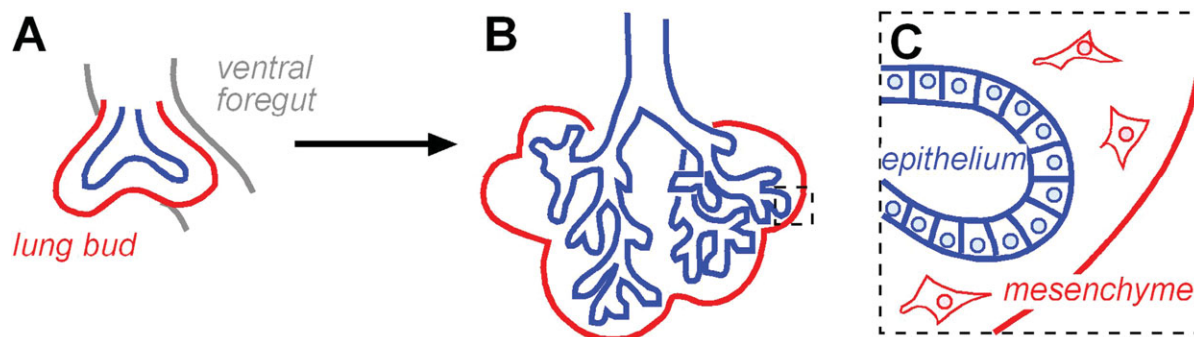


Figure 2. Branching morphogenesis builds the airways of the lung.

(A) Initial lung buds emerge as an omega-shaped structure from the ventral foregut epithelium, (B) successive rounds of branching transform the simple organ primordium into the tree-like airways, and (C) as with all branched organs, the tree itself is comprised of a highly organized sheet of epithelial cells (blue) surrounded by a more loosely organized mesenchyme (red).

drainage basins.^{15,16} We still use this terminology today: in “dichotomous” or bifurcating branching, the parent branch or “stem” divides into two “daughter” branches, which may themselves go on to branch again. When developing the theories underlying fractal mathematics, Benoît Mandelbrot noted that the branching airways show signs of fractal geometry, and used the rules of fractals to build two-dimensional (2-D) analogs of branching architectures that were reminiscent of the lungs.¹⁷ Since that initial description, more realistic space-filling algorithms have been implemented to simulate the morphogenesis of the mammalian airways,¹⁸ and fractal analysis has been used to describe the morphology of the mature bronchial tree.¹⁹

Whereas they are beautiful and intuitively satisfying, mathematical models of the development of branched organs that use fractals as their source cannot explain the physical mechanisms underlying morphogenesis. All branching organs are comprised of multiple cell types, including an epithelium that forms the branched structure and a surrounding mesenchyme that provides signals and support (Figure 2C). These two types of tissues are fundamentally distinct from each other. The epithelium is a continuous tissue in which individual cells are connected to their neighbors through specialized adhesions; the locations of these adhesive structures give the cells and their composite tissue a polarity. The mesenchyme is a looser tissue in which individual cells move freely within a polymeric network of extracellular matrix proteins. It is the behaviors of these populations of cells that drive branching. New branches extend either by epithelial migration or by bending and folding the epithelial tissue into the surrounding mesenchyme.²⁰ These physical behaviors have been modeled mathematically as arising from viscous fingering effects that occur when one liquid is immersed in another liquid of different viscosity.²¹ These purely physical models come closer to approximating the properties of the continuous tissues, but simultaneously neglect the behaviors of the constituent cells.

One often overlooked aspect of morphogenetic tissues is that they rarely begin as spheres—most start out as a population of cells, either an outpouching of epithelium or a placode, that is already somehow asymmetric.²² The earliest outpouching of foregut epithelium that eventually undergoes branching morphogenesis to build itself into the airways of the lung is an ω -shaped tissue²³ (Figure 2A). Each half of

the ω develops into a primary bronchus, the largest of the conductive airways that supply the left or right lobes of the lung. Similarly, the mammary epithelium that undergoes branching morphogenesis during puberty already exists as a small simply branched structure prior to the induction of the morphogenetic process by ovarian hormones.²⁴ Thus, unlike the earliest stages of the embryo, the geometry of developing organs has already broken symmetry.

The author and her collaborators have shown that this initial geometry by itself can play a major role in patterning the subsequent symmetry-breaking events that characterize branching morphogenesis.^{25,26} First, morphogenesis of most epithelial trees is directed by diffusible signals, both proteins and other small molecules, that are secreted by the epithelial cells themselves (so called “autocrine” signals) or by the surrounding mesenchyme (“paracrine” signals). In the case of autocrine morphogens, the epithelial tissue acts as a localized source of molecular flux; simple diffusion away from this source enables the formation of a concentration gradient around the tissue. When the epithelial tissue is nonspherical in shape, a concentration gradient will also form along the surface of the tissue, such that cells will be exposed to different concentrations of the morphogen depending on their location (Figure 3). In the case of the inhibitory morphogen transforming growth factor-beta ($TGF\beta$), the development of

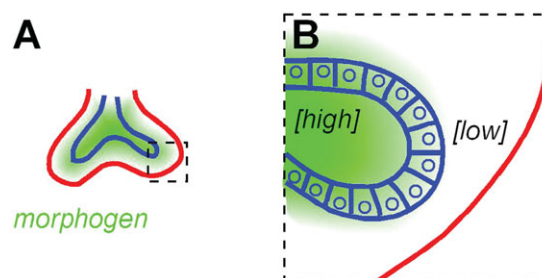


Figure 3. Tissue geometry specifies concentration profile of secreted diffusible morphogens.

(A) A morphogen (green) secreted from the epithelium (blue) diffuses away from its source, and (B) epithelial cells located far from the centroid of the morphogen-secreting tissue will be surrounded by a lower concentration of morphogen than those near the centroid.

this concentration profile was sufficient to pattern branch initiation in engineered mammary epithelial tissues.²⁵ Cells were able to form branches when located in regions of low concentration of TGF β , but were prevented from doing so when located in regions of high concentration,²⁵ and the magnitude of this concentration, rather than any local gradient, was responsible for the divergent cellular behaviors.²⁷ Computational models suggest that a similar mechanism may be used in the patterning of the branching architecture of angiogenic vascular endothelium.²⁸ Indeed, this “self-loathing” behavior, where branches repel each other via diffusible inhibitory signals, may be ubiquitous across organ systems.^{29–31}

Whereas inhibitory morphogens are commonly observed to play a role in the development of branching epithelial tissues, branch initiation itself requires a stimulatory signal, which often also takes the form of a diffusible morphogen.¹³ In mammalian lungs, such as those of the mouse, fibroblast growth factor-10 (FGF10) is expressed by cells located in the distal mesenchyme, from whence it diffuses to bind to receptors and induce proliferation and branching at the tips of nascent epithelial buds.^{32–35} FGF10 induces the expression of sonic hedgehog (SHH) and bone morphogenetic protein-4 (BMP4), a member of the TGF β family, by the epithelium, which act as diffusible inhibitors to limit FGF signaling.^{34,36} The FGF10-SHH signaling axis was recently shown computationally to result in the development of Turing-like patterns that could lead to the spatial arrangement of daughter branches on a parent branch¹; importantly, branching in this model was encoded to result purely from localized FGF10-induced epithelial proliferation, which has not been definitively proven to drive branching *in vivo*.^{37–39}

In some ways, it is remarkable that simple diffusion can pattern structures as complex as branching organs, and it is important to note that all of the results discussed in the preceding paragraphs were obtained using systems in which the diffusible signal was presumed to have a constant diffusivity within the surrounding mesenchyme. Other more complicated diffusion scenarios have been proposed to play a role in the patterning of different branched organs *in vivo*. For example, most of the development of the embryonic chicken lung is characterized by branching morphogenesis, but instead of branches a unique structure called the air sac forms on the ventral surface of the avian lung.^{40,41} This cyst-branch difference in morphogenesis by the epithelium depends on the mesenchyme,⁴² and in particular on regional differences in the diffusivity of FGF10.⁴³ FGF10 has a higher diffusivity through the ventral mesenchyme than it does through the dorsal mesenchyme, which results from the lower concentration of the FGF10-binding heparan sulfate proteoglycan present in the latter.⁴³ It will be interesting to determine how regional differences in diffusion coefficients are interpreted by cells to instruct morphogenesis.

In addition to concentration gradients of diffusible morphogens, the initial geometry of a tissue can pattern the behaviors of its constituent cells via gradients in mechanical stresses.^{26,44–46} Cells are inherently contractile, and this contractile behavior results directly from the dynamics of myosin motors moving along the actin cytoskeleton. Epithelial cells connect to their neighbors and to the surrounding insoluble extracellular matrix network through spot weld-like

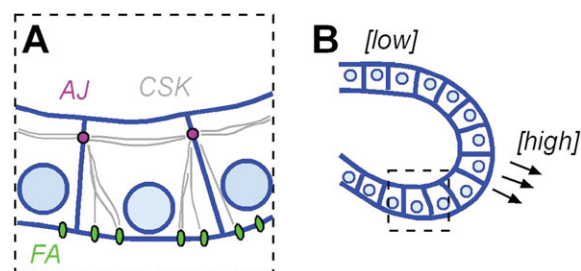


Figure 4. Tissue geometry specifies profile of endogenous mechanical stresses.

(A) Epithelial cells connect to neighboring cells through adherens junctions (AJ; purple) and to the surrounding extracellular matrix through focal adhesions (FA; green). Both structures also connect to the actin cytoskeleton (CSK; gray), and (B) contraction of the actin cytoskeleton within the epithelium leads to regions characterized by high and low mechanical stresses.

structures within their membranes.²⁰ These connections to neighboring cells and to the matrix, most commonly in the form of adherens junctions and focal adhesions, respectively, link not only to the world outside of the cell, but also connect to the actin cytoskeleton within (Figure 4A). Therefore, when a cell contracts it pulls on the adjacent cells and extracellular matrix, and can thus transmit forces over large distances.^{20,22} When a population of epithelial cells contracts, mechanical stresses become concentrated within specific regions of the epithelium if the tissue itself has a nonspherical geometry^{26,44,46} (Figure 4B). The author and her collaborators have found that cells located within regions of high mechanical stress activate mechanotransductive signaling,^{26,45} express a unique gene signature,⁴⁷ and are able to form nascent branches in response to inductive stimuli.²⁶ In contrast, cells located in regions of low mechanical stress exhibit none of these changes. Importantly, it is the initial geometry of the epithelium that sculpts the mechanical stress profile. In this way, tissue geometry is both a cause of symmetry breaking events as well as a consequence.

Engineering Tissues: Lessons from the Embryo?

It is clear and perhaps not surprising that the geometrical features of a tissue at a given point in time play a role in its later morphogenesis and help to define the final geometry attained. Morphogenesis, both in the early embryo and in the construction of organs, emerges from feed-forward of asymmetries, with symmetry-breaking events instructing later ones; as such, form begets form. As with nonbiological systems, the initial and boundary conditions of a morphogenetic tissue are critical.

The past 50 years of research in cell and developmental biology have defined several key but redundant and overlapping signaling pathways that are encoded genetically, revealed by experimental analysis of cultured tissues and organs and transgenic and knockout animals. Perhaps we can take advantage of this already encoded redundancy, and design strategies to mimic embryonic developmental signaling pathways in the creation of engineered tissues. This

might include both the gradients in morphogens as well as those in mechanical stresses that permit distant regions of tissues to affect each other and instruct development. Such a strategy may not require much in the way of genetic manipulation, if we can find a way to ride this domino effect of symmetry breaking and determine how to trigger tissues to build themselves *ex vivo*.

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