

An archetypal mechanism for branching organogenesis

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Abstract

Branched structures are ubiquitous in nature, both in living and non-living systems. While the functional benefits of branching organogenesis are straightforward, the developmental mechanisms leading to the repeated branching of epithelia in surrounding mesoderm remain unclear. Both molecular and physical aspects of growth control seem to play a critical role in shape emergence and maintenance. On the molecular side, the existence of a gradient of growth-promoting ligand between epithelial tips and distal mesenchyme seems to be common to branched organs. On the physical side, the branching process seems to require a mechanism of real-time adaptation to local geometry, as suggested by the self-avoiding nature of branching events. In this paper, we investigate the outcomes of a general three-dimensional growth model, in which epithelial growth is implemented as a function of ligand income, while the mesenchyme is considered as a proliferating viscous medium. Our results suggest that the existence of a gradient of growth-promoting ligand between distal and proximal mesenchyme implies a growth instability of the epithelial sheet, resulting in spontaneous self-avoiding branching morphogenesis. While the general nature of the model prevents one from fitting the development of specific organs, it suggests that few ingredients are actually required to achieve branching organogenesis.

Keywords: morphogenesis, organogenesis, branching, lung, kidney

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Introduction

The emergence of ramified structures is a fundamental and recurring feature of living systems [1]. In animals, branching patterns are ubiquitous and underscore the morphogenesis of the nervous and vascular systems, they are also responsible for the development of mammalian lungs, kidneys, and salivary glands, as well as an insect's tracheal system (figure 1). While the vascular tubes are composed of endothelial cells, branched organs have their lumen lined by epithelial cells. The tree-like structure is systematically achieved by the repeated self-avoiding branching of the epithelial sheet into the surrounding mesoderm [2].

The understanding of this process requires an understanding of the elementary branching mechanism. Specifically, how can two (or more) tubes sprout from a pre-

existing tube? It also raises the question of the organization process. Specifically, how can branching events be temporally and spatially regulated at the organ scale in such a way that branches homogeneously fill the mesenchyme in a self-avoiding manner?

The latter question has generated two main scenarios. The first supposes that, whatever the elementary branching mechanism may be, branching events are somehow encoded by genetic routines and subroutines, establishing a complete developmental program. The branching points, branching angles and branch diameters should be exhaustively specified in order to systematically achieve a self-avoiding structure. Genetic models have been proposed for several branched organs [6, 7]. One consequence of this first scenario is that the resulting organ should be stereotyped among the individuals of a given species. The second scenario proposes that

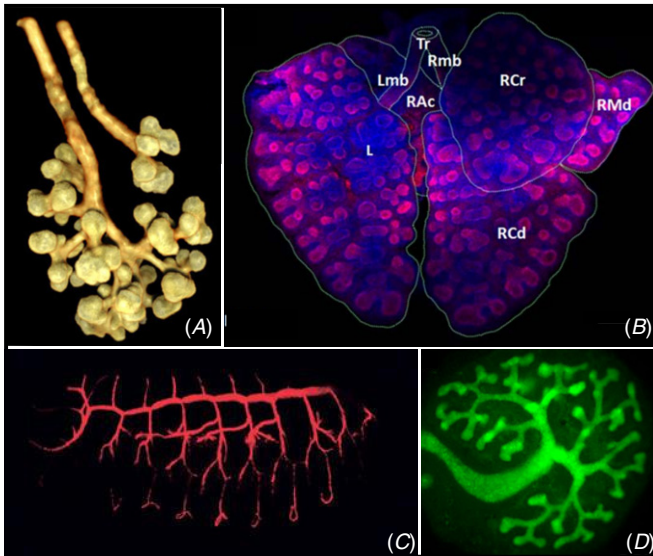


Figure 1. Examples of branching organogenesis. (A) Three-dimensional reconstruction of mouse salivary glands at day 13.5 stained for E-cadherin (courtesy of Dr I Smyth, Monash University). (B) Mouse lungs at day 13.5 double stained for E-cadherin and DAPI (adapted from [3]). (C) *Drosophila* tracheal system imaged with a lumen antibody (courtesy of Dr Araujo [4]). (D) Mouse kidney explant at E11.5 (courtesy of Dr Bates, adapted from [5]).

self-avoiding branching morphogenesis requires real-time response and adaptation to the spatial configuration of the neighboring buds to achieve the self-avoiding tree. In this scenario, the exact structure of the tree is not genetically predetermined, and the ability to fill available space to overcome spatial and temporal variations in the branching process should be intrinsic to the branching mechanism itself.

While the first scenario, underscored by genetic pre-determination, is as of today the prevailing hypothesis, the second scenario has recently received more interest [8–11]. Several arguments support this hypothesis. First, it has been recently demonstrated in lungs [3] that the early branching process is less stereotyped than that previously reported in mice [6]. Although spatial, temporal and morphological variations are frequent, the new buds continue to fill the mesenchyme in a self-avoiding manner so that their distribution remains statistically homogeneous. These observations are consistent with the pioneering morphometric human data [12, 13]. Second, both developmental disorders and mutant phenotypes show that self-avoiding branching organogenesis is very robust when geometry changes occur [14–16]. The last two points suggest that there may actually be real-time adaptation of branching to geometry. Third, exhaustive genetic programming, even hierarchized in subroutines and master routines, requires such a huge quantity of information that its selection by evolution would probably be jeopardized.

In the vein of previous experiments and models proposing diffusion-limited growth as a potential mechanism for branching morphogenesis [17–19], we have developed [11, 20] a model of lung morphogenesis suggesting that self-organization might indeed play a major role in the emergence and maintenance of branching. However, our results were restricted to lungs in a two-dimensional geometry. In this

paper, we extend the model to three dimensions and introduce a more general description of mesenchyme's and mesothelium's motions based on Stokes equations of fluids, taking into account the viscous nature of the mesenchyme. We only assume the existence of a gradient of growth-promoting ligand, which makes the model general enough to be relevant to the development of several branched organs [21], although it prevents quantitative fitting of specific organs. Three-dimensional numerical simulations show that the spontaneous self-avoiding branching morphogenesis of the epithelial sheet robustly holds in this description. The variety of tree-like morphologies obtained by changing the parameters suggests that the morphological diversity of branched organs might arise from organ-specific regulation networks and physical parameters, while the branching process is general. These results also suggest that specific encoding might not be required to organize branching events at the organ scale and that organization might rather result from the constant interplay between boundaries and diffusing gradients. They finally assemble into a comprehensive scenario for branching organogenesis, in which patterning, diffusion and mechanics allow the real-time self-regulation of the developing shape.

Model

The concentration of growth-promoting ligand

An exhaustive and quantitative model of organogenesis should describe the full dynamics of growth promoters and inhibitors in the mesenchyme, which should be combined to the growth of involved tissues. The dynamics of ligands through diffusion, degradation, binding, and regulation cues, is organ specific. Consequently, simplified models of core signaling networks have been proposed for specific organs [8, 9, 22, 23]. Such a quantitative description is not the purpose of this paper; thus, we will only hypothesize the maintenance of a gradient of growth-promoting ligand between proximal and distal mesenchyme.

It has been pointed out that a gradient of ligand concentration emerges from at least two mechanisms shared by branched organs [21]. While the signaling pathways involved vary according to the organ considered, branched organs are submitted to epithelial growth promotion by one or several ligands diffusing from the mesenchyme (FGF10 in lung [24], GDNF/FGF10 in kidney [25, 26], BNL in *drosophila* trachea [27], etc). First, this signal is eventually subject to down-regulation by one or more inhibitors expressed by epithelial cells (SHH/SPRY2 in lung [28, 29], SPRY1 in kidney [25], SPRY in *drosophila* trachea [27]—note that SPRY proteins act at the intracellular level). Second, reception of the signal by epithelial receptors (FGFR2 [30] in lung, FGFR2/RET in kidney, BTL in *drosophila* trachea), induces the partial internalization and degradation of the ligand. Reception combined to proximal down-regulation contributes to form a gradient of growth-promoting ligand concentration between distal mesenchyme and proximal mesenchyme [21, 31, 32]. The reader should note that concentration gradients and transcriptional gradients are two different

things and that the formation of ligand gradients do not require gradients of transcriptional activity, although they can definitely contribute to their formation.

It is unclear which mechanism prevails for gradient formation. In addition, it is likely that their respective weight varies from one organ to the other and, therefore, the precise shape of the gradient might also vary.

The steady-state concentration field resulting from diffusion is given by Laplace's equation. Thus, a Laplacian field is a good qualitative model for a smooth variation of the ligand concentration c_L from $c_{\min}$ (proximal mesenchyme) to $c_{\max}$ (distal mesenchyme):

$$\nabla^2 c_L(x, y, z, t) = 0. \quad (1)$$

The epithelial response to signal

Epithelial proliferation relies on the reception of growth-promoting ligand. We will simply write the normal velocity u_e of the epithelial sheet as a function of the incoming flux of signaling ligand $\vec{J}_L$, with:

$$\vec{J}_L(\vec{x}, t) = -D_L \vec{\nabla} c_L, \quad (2)$$

and

$$u_e = f(J_L), \quad (3)$$

where D is the diffusion coefficient of the ligand in the mesenchyme. Nothing is *a priori* known about the epithelial growth response f to the reception of ligand, except that it should be increasing with the flux J_L . The fact that the growth response is a local function of the gradient of concentration is discussed in detail in the supplementary material (available from stacks.iop.org/PhysBio/11/016003/mmedia).

Description of the mesenchymal tissue

Since we consider large time scale (developmental periods; that is, hours to days), we make the hypothesis that the mesenchyme behaves as an incompressible viscous fluid. The three-dimensional motion of the mesenchyme and of its distal boundary is the consequence of two phenomena: first, the motion of the epithelial sheet, which is induced by the reception of the growth-promoting ligand; and, second, the proliferation of mesenchymal cells in the tissue. Mesenchyme dynamics can, thus, be described by Stokes equation with non-zero divergence:

$$\begin{cases} \vec{\nabla} p = \eta \nabla^2 \vec{u} \\ \text{div}(\vec{u}) = g, \end{cases} \quad (4)$$

where p stands for the pressure and η for the viscosity of the mesenchyme. The second equation is the mass conservation equation: g stands for the proliferation rate of the mesenchyme, and can be either a constant or a scalar field in the case of inhomogeneous proliferation. $g = 0$ corresponds to an absence of proliferation. Last, boundary conditions are required for Stokes equation, both on epithelium and mesothelium. The ligand concentration c_L provides, thanks to equations (2) and (3), the velocity of the epithelial sheet. Finally, we assume the stress to be homogeneous (reference pressure) on the mesenchyme distal boundary.

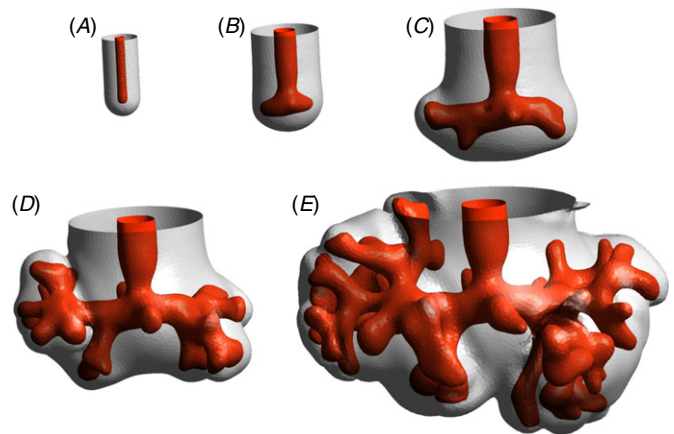


Figure 2. (A)–(E) Time-lapse sequence of a growth simulation. The structure obtained is similar in many aspects to structures emerging during branching organogenesis: it is self-avoiding and space-filling, while a typical distance is set between epithelial tips and mesothelium. A movie is also available online (available from stacks.iop.org/PhysBio/11/016003/mmedia).

Three-dimensional simulations

A gradient of ligand is sufficient to generate three-dimensional self-avoiding branching

Starting from an initial tubular geometry, the numerical model basically consists in repeating three successive steps: (1) we compute the Laplacian field c_L (equation (1)) in the current geometry and determine the velocity of the epithelial sheet (equations (2) and (3)); (2) we compute the velocity field u of the mesenchyme (equation (4)); and, (3) we apply the displacement field $u \times dt$ to the current geometry (where dt is a time step constant throughout the simulations). The geometry thus computed becomes the current geometry.

We used a smooth threshold function (i.e. a sigmoid) as the epithelial growth response to signal f . Sigmoids are the most physiologically relevant types of response to a signal. The threshold is noted G_0 , and so is the width σ (please see supplementary information for details). The simulations show that the self-avoiding tree-like structure is robustly found in this three-dimensional model (figure 2—also see movie 1 online (available from stacks.iop.org/PhysBio/11/016003/mmedia)). We have also found that an equilibrium distance to mesothelium is spontaneously reached by epithelial tips, preventing tips from any collision with the external boundary, which is an highly non-trivial feature of branching organogenesis since tips constitute the main sites of proliferation. Branching of the epithelium is spontaneous—no branching instructions of any kind are present in the model—and relies on the spontaneous focus of ligand diffusive flux on spatial perturbations of the epithelium, which eventually leads to bud outgrowth [11]. This ‘tip-effect’ on the flux is a well-known phenomenon and is formally similar to the lightning rod effect. The typical size of branches results from the competition between this instability and the mechanical rigidity of the epithelial sheet, which is stabilizing. As discussed in our previous works [11], this rigidity is implicit in the numeric model and corresponds

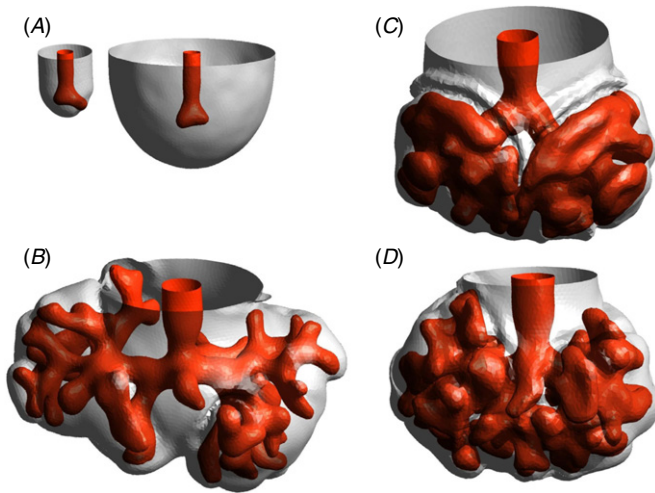


Figure 3. (A) Simulation with homogeneous proliferation in the mesenchyme. If g is too small, the epithelium reaches the mesothelium (left). When g increases, the mesothelium moves too quickly, preventing the epithelium from receiving enough signal to grow normally (right). (B) Mesenchyme proliferation rate g is proportional to ∇c_L . (C) Mesenchyme proliferation rate g is a sigmoid function of ∇c_L . (D) Mesenchyme proliferation rate multiplied by 0.8 compared to (C).

to the cut-off of the surface mesh. This cut-off is chosen to be a constant throughout the paper. Finally, self-avoiding growth is also spontaneous: when the space between two growing branches decreases, the local flux of signaling molecules tends toward zero, which prevents branches from any collision.

Influence of the mesenchyme proliferation rate

We first tested the influence of the growth rate g . An homogeneous growth rate g in the mesenchyme is physiologically unlikely. Mesenchymal proliferation is downstream of various pathways (FGF9 and SHH in the lung [33], SHH in the kidney [34],...), suggesting that the proliferation rate spatially varies within the mesenchyme. Indeed, setting a constant rate in the simulation, we found that if g is too small, then the epithelium invades the mesenchyme and reaches the external boundary (figure 3(A), left); on the other hand, if g is too large, then the external boundary grows too quickly and the epithelium is unable to fill the mesenchyme (figure 3(A), right). No equilibrium between tips and external boundary could be observed with a spatially constant rate g . It has been shown, for instance in the lung [33], that the proliferation rate is more important near sites of major epithelial proliferation. A convenient way to implement this behavior in the growth term g is to write it as a function of the field ∇c_L , which drives epithelial proliferation. This sets high values of g near epithelial sites of proliferation, and smooth g toward smaller values in the distal mesenchyme or between buds. Again, the choice of the function is driven by simplicity arguments; therefore, we have tested both linear functions of ∇c_L (figure 3(B)) and sigmoid functions (figure 3(C)). In both cases, a self-avoiding unstable epithelium is obtained but with very different morphologies. Finally, taking g as a given function of ∇c_L , we have tested the influence of the

amplitude of the function (i.e. weak proliferation versus strong proliferation). Our results tend to demonstrate the intuitive fact that interstitial space between buds increases with the mesenchymal proliferation rate. On the contrary, for low proliferation rates, the epithelial tree is very tightly packed in the mesenchyme (figure 3(D)).

These findings suggest that mesenchymal proliferation impacts the geometry of the branching pattern. This is not surprising, as the proliferation of the mesenchyme necessarily impacts the relative occupation of space by branches and mesenchyme. However this point should be carefully discussed because the role of epithelium–mesenchyme crosstalk in branching morphogenesis is point of much debate. In particular, *in vitro* cultured epithelia have been shown to display branching morphogenesis. Since there is obviously no control of gene expression exerted by epithelial or mechanical cues in a gel, this suggests that the main mechanism of gradient formation is the binding and degradation of the signal at the epithelial level—which does not require mesenchymal contact if adequate soluble factors are added, as is always the case for successful mesenchyme-free branching morphogenesis. However, the branching patterns observed are different from the ones observed *in vivo*. In kidney, ureteric buds cultured in gels with adequate soluble factors display three-dimensional branching but are still qualitatively different from the original [35]. Qiao *et al* have therefore suggested that, although epithelial branching did not require mesenchymal contact, such contact may play a key role in regulating branching elongation and regularity. Our results support this hypothesis and, moreover, suggest that proliferation in particular may contribute to details of the branching pattern. However, our interpretation differs since we do not conclude that an epithelial program of branching exists. Lung epithelia cultured in matrigel display differential growth and cusps that lead to the formation of buds [19, 36–39]. However, the buds collide, the self-avoidance is lost and the pattern is very different from the original. This might suggest that proximal inhibition plays a more important role in gradient formation in lungs than in kidneys, which is supported by the poorly branching *Shh*^{−/−} phenotype. Finally, it is worth noticing that elastic instabilities have been shown to induce bud formation in circular geometries [40], which might partly contribute to the initiation of branching in gel.

Influence of the growth response

We tested the influence of the growth response f to the growth-promoting ligand. Similarly to the results that we have described in the two-dimensional lung model [11], we have found that the morphology of the tree was finely tuned by the growth response. A good example of this is the sensitivity of the shape to the value of the threshold, G_0 . Figure 4 displays the outcome of two simulations with different values of G_0 . The resulting shapes suggest that higher thresholds (figure 4(A)) have more tubular branches. This is in fact consistent with the instability mechanism that we have described. When the threshold is high, the sensitivity to low ligand income is very

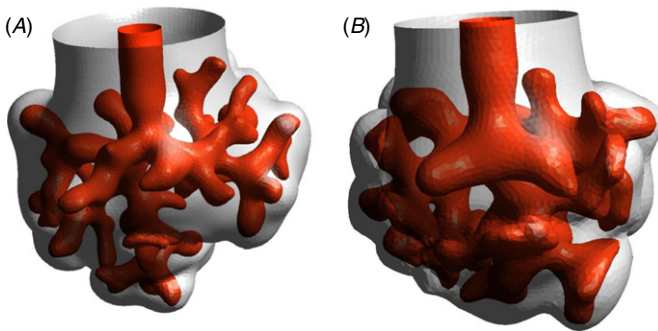


Figure 4. (A) Growth response with a high threshold. Branches rather elongate than thicken. (B) Growth response with a low threshold. Branches rather thicken than elongate and branches consequently have a greater diameter than in (A).

poor. Branch sides thus undergo very little growth because the gradient essentially concentrates on distal tips. When the threshold is decreased (figure 4(B)), sensitivity to weak ligand incomes is increased and the spatial distribution of growth spreads on the branch sides. Consequently, the branch diameters are increased, while the mesenchymal volume remains similarly filled by the epithelial tree. Again, this suggests that modifications in the growth response impact the fine geometry of the tree but do not impact the core mechanism. During organogenesis, the role of the complete regulation network, which is not described in our model, is partly to shape the growth response to the signal. Our results are thus consistent with the fact that mutations impact the branching pattern and regularity but not the global tree-like structure.

Initial geometry

A qualitative discrepancy between the model and branching organogenesis resides in the initial geometry. We have made the choice of simplicity and have chosen an ideal tubular geometry, which is not the case *in vivo* since the boundaries of the mesenchyme are constrained by surrounding tissues and organs. We think that this issue deserves specific investigation in future works. Indeed, it has been shown in other Laplacian branching systems—such as viscous fingering—that the repetition of the same experiment in an ideal geometry results in random branched structures sharing the same statistical properties [41]. However, the patterns obtained when a tiny constraint is imposed on the initial geometry can be very stereotypic, especially for the first generations of branching [42]. During organogenesis, stereotypic constraints on the boundaries are obviously exerted by surrounding organs, which we believe might lead to the observed branching stereotypy that mostly concerns the first rounds of branching [3]. In the model, such constraints could be implemented as an inhomogeneous stress on the distal boundary.

Homothety ratio

Branched organs share another striking feature: new branches are smaller than old ones. A simple hypothesis could be that, in the model, growth is only ligand-reception-dependent, although it is most likely that the whole organ undergoes cell

proliferation in addition to the inhomogeneous proliferation due to the ligand. By adding uniform growth (dilatation rate k) to ligand-induced growth (roughly described with branches elongation rate v), we find that the ratio L/D (length/diameter) tends toward $v/(d_0(k-1))$, where d_0 is the typical size of branch formation. The unstable length d_0 should be constant and determined by the parameters of the growth instability described in the model. If we call T the mean period between two bifurcations—also determined by the parameters of the instability—then the mean diameter of generation N asymptotically writes $d_{N+1} = d_N e^{-(k-1)T} < d_N$ (see supplementary information for details). Interestingly, this relates measurable parameters of adult organs to parameters of the instability, such as the homothety ratio $h = d_N/d_{N-1}$ or the length/diameter ratio L/D [43, 44].

Discussion

An integrative mechanism for branching organogenesis

Previous studies have pointed out that branching organogenesis seems to require the maintenance of ligand gradients. In this paper, we have built an organ-scale model based on a gradient of growth-promoting ligand between proximal and distal mesenchyme. By implementing the model numerically in three dimensions we have found that this sole ingredient allows, through ligand diffusion, the emergence of a self-avoiding, space-filling branching epithelium. This suggests that specific regulation might not be required to achieve branching, to organize branching events into a self-avoiding structure or to set an equilibrium distance between bud tips and external boundary. Since these striking features emerge spontaneously in such a basic representation, it seems likely that they are rather adjusted than designed by the rest of the regulatory network. Although these mechanisms were already intuited for lung in a two-dimensional representation, we provide here the demonstration that they robustly hold in a general three-dimensional model. Whereas shape details are organ specific and depend on the growth response of epithelial cells to ligand reception, or on the proliferation rate in the mesenchyme, the self-avoiding branching morphogenesis is very robust. This suggests that morphological diversity observed among branched organs might arise from organ-specific regulation networks and physical parameters, while the branching process is general. An evolution of the model should include the integration of realistic organ-specific parameters, such as experimental growth responses, realistic external constraints, quantitative modelling of the gradient formation, additional regulation cues, etc.

The feedback between shape and gradients: an efficient mechanism of self-regulation

The epistemological approach to morphogenesis varies greatly among scientific disciplines. On one hand, morphogenesis in physical systems is seen as the result of an interaction problem dynamically solved by the shape. On the other hand, developmental biology has benefited from the emergence of

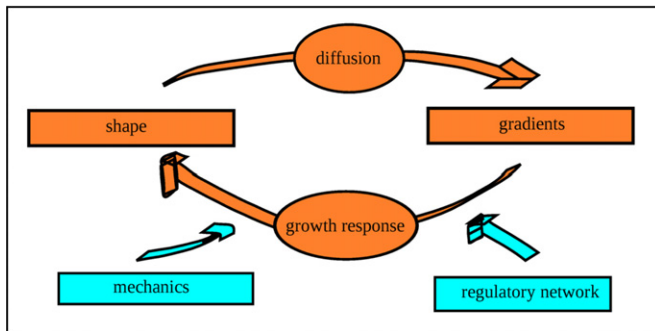


Figure 5. Feedback between shape and gradients. Shape boundaries limit diffusion domains and determine gradients. In return, gradients influence the shape through the growth response to gradients of signaling molecules. The growth response is underscored both by the regulatory network and by the mechanics of growth. In branching organogenesis, the maintenance of a gradient between the boundaries (epithelium and mesothelium) underlies the emergence of the branching pattern.

molecular tools and the study of morphogenesis has become oriented by the technological possibility to control gene expression and to generate mutants, providing a direct access to the genetic contribution to shape development. A huge achievement of modern biology has been the discovery of developmental disorders resulting from unique knock-outs. Nevertheless, it seems possible that such a gene-oriented construction fails to identify morphogenesis mechanisms in some cases since they sometimes result from the contributions of developmental actors (genes and proteins, cells, tissues, fluids, etc) *taken in their physical and geometrical context of interaction*. A link should be made with the seminal works of Leduc or Thompson [45, 46], who hypothesized at the beginning of the 19th century that chemical and physical laws of interaction may have a major role in the organization of living systems. Such approaches were mostly forsaken later in the century. This model suggests that such a self-organization process may be at play during the development of branched organs: the growth factor expression domain, the concentration and gradient of ligand are mostly determined by the geometry of the boundaries. In turn, the gradients have a direct influence on the growth of these boundaries. This is, in a way, a self-regulation of the shape since the evolution of the shape is mostly determined by shape itself (figure 5).

From an evolutionary perspective, the feedback loop between shape and gradients through the laws of physics, notably diffusion, turns out to be a very simple and robust way to achieve morphogenesis. In this case, it constitutes a very economic way to initiate and maintain branching regularity throughout development. The required amount of information encoded in the genome is tremendously reduced compared to a system in which each branching event is encoded individually and in which developmental errors would be inherited by next generations of branching, thus leading to organ-scale failure. Also, this feedback provides a simple framework to understand shape evolution toward more efficient geometries through natural selection: the self-avoiding branching pattern is robust, while the details of the geometry (diameter, length, aspect ratio, etc) vary with the growth response, which is

underscored by the regulatory networks. More generally, analyzing shape change processes through the interactions between shape and gradients might be a fruitful approach in developmental biology since it allows take the spatial dimension into account, which is sometimes absent from the regulatory network approaches.

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