An archetypal mechanism for branching organogenesis

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October 3, 2013

Keywords : morphogenesis, organogenesis, branching, lung, kidney

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 3D 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 obviously prevents from fitting the development of a specific organ, it suggests that few ingredients are actually required to achieve branching organogenesis.

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, but also the development of mammalian lungs, kidneys, or salivary glands, as well as insects tracheal system (Figure 1). While the vascular tubes are composed of endothelial cells, branched organs have their lumen lined by epithelial cells, and 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 such a process requires understanding the elementary branching mechanism: how two (or more) tubes can sprout from a pre-existing one? It also raises the question of the organization process: how can branching events be temporally and spatially regulated at the organ

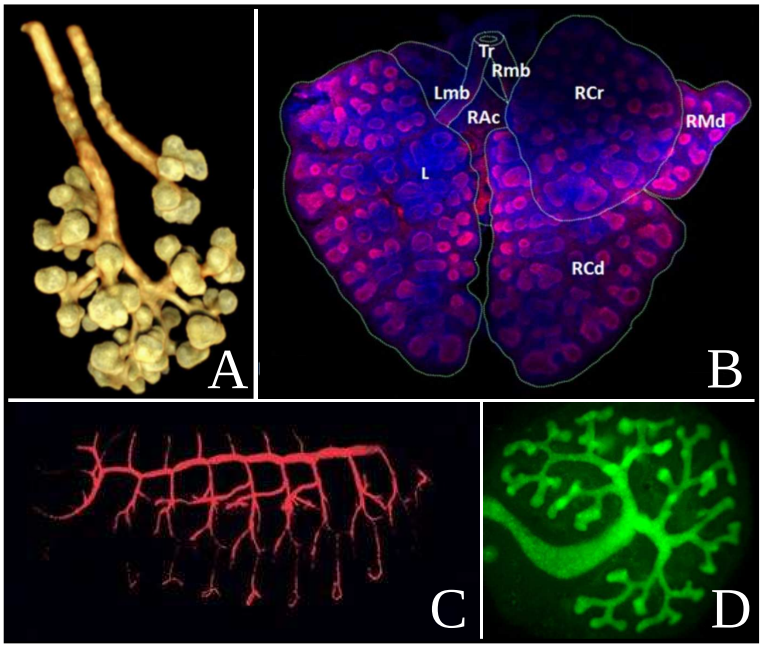

Figure 1: **Examples of branching organogenesis. A.** Mouse salivary glands at day 13.5 stained for E-cadherin (courtesy of Dr I. Smyth, Monash University). **B.** Mouse lungs at day 13.5 double stained for E-cadherin and DAPI. Legend shows the trachea (Tr), the right (Rmb) and left (Lmb) main bronchi, the right cranial (RCr), right middle (RMd), right accessory (RAc), right caudal (RCd) and left (L) lobes (adapted from [3]). **C.** Drosophila tracheal system imaged with a lumen antibody (courtesy of Dr S. Araujo [4]). **D.** Mouse kidney at E11.5 (courtesy of Dr C. Bates, adapted from [5]).

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 may be the elementary branching mechanism, branching events are somehow encoded by genetic routines and subroutines, establishing a complete developmental program. The branching points, branching angles and branches 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]. A consequence is that the resulting organ should be stereotyped among the individuals of a given species. The second scenario would rather propose that self-avoiding branching morphogenesis requires real-time response and adaptation to the spatial configuration of the neighbouring 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 known a strong regain of interest [8, 9, 10, 11]. Several arguments support this hypothesis: First, it has been recently demonstrated in lung [3] that the early branching process is less stereotyped than previously reported in mouse [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 pioneer 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, 15, 16] - the two latter 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, 18, 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, results were restricted to lung in a two-dimensional geometry. In this paper, we extend the model to three dimen-

sions 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. 3D 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, but 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, combined to the growth of involved tissues. The dynamics of ligands through diffusion, degradation, binding, and regulation cues, is organ specific; and simplified models of core signaling networks have therefore been proposed for specific organs [8, 22, 9, 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 ore 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 contribute 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. It is likely that their respective weight vary from one organ to the other, and therefore the precise shape of the gradient might vary as well.

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):

$$\nabla^2 c_L(x, y, z, t) = 0 \tag{1}$$

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 $\overrightarrow{J_L}$, with:

$$\overrightarrow{J_L}(\overrightarrow{x}, t) = -D_L \overrightarrow{\nabla} c_L, \tag{2}$$

and

$$u_e = f(J_L), \tag{3}$$

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 is 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 details in the supplementary material.

Description of the mesenchymal tissue

As we consider large time scale (developmental periods, i.e hours to days), we make the hypothesis that the mesenchyme behaves as an incompressible viscous fluid. The 3D motion of the mesenchyme and of its distal boundary is the consequence of two phenomena: first, the motion of the epithelial sheet, 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:

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

where p stands for the pressure and η for the viscosity of the mesenchyme. The second equation is the mass conservation: 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 equation Eq.2 and Eq.3, the velocity of the epithelial sheet. Finally, we assume the stress to be homogeneous (reference pressure) on the mesenchyme distal boundary.

3D Simulations

A gradient of ligand is sufficient to generate 3D 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 (Eq.1) in the current geometry and determine the velocity of the epithelial sheet (Eq.2 and Eq.3); 2/ we compute the velocity field u of the mesenchyme (Eq.4); 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 the width σ

(please see supplementary information for details). The simulations show that the self-avoiding tree-like structure is robustly found in this 3D model (Figure 2 - also see movie online). We 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, as 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 of 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, 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], the rigidity is implicit in the numeric model and corresponds to the cut-off of the surface mesh. This cut-off is chosen 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 towards 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, the epithelium invades the mesenchyme and reaches the external boundary (Figure 3A, left); on the other hand, if g is too large, the external boundary grows too quickly and the epithelium is unable to fill the mesenchyme (Figure 3A, 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 towards smaller values in the distal mesenchyme or between buds. Again, the choice

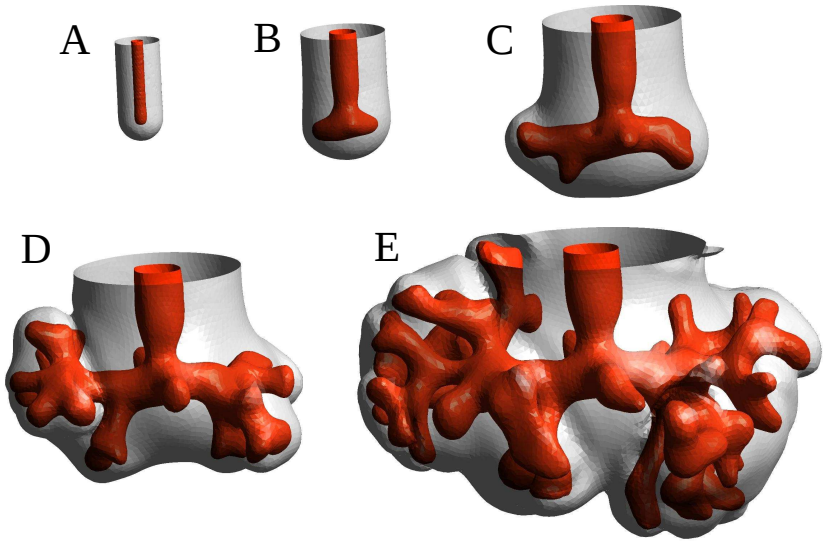

Figure 2: **A-E.** Time-lapse sequence of a growth simulation. The structure obtained is similar in many aspects to structures emerging during branching organogenesis: it is self-avoiding and space-filling, while a typical distance is set between epithelial tips and mesothelium. A movie is also available online.

of the function is driven by simplicity arguments, therefore we tested both linear functions of ∇c_L (Figure 3B) and sigmoid functions (Figure 3C). 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 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 3D).

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 as the role of epithelium-mesenchyme crosstalk in branching morphogenesis is very debated. 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, which 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 3D branching, but still qualitatively different from the original [35]. Qiao et al. 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 [36, 19, 37, 38, 39]. However 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 lung than in kidney, 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.

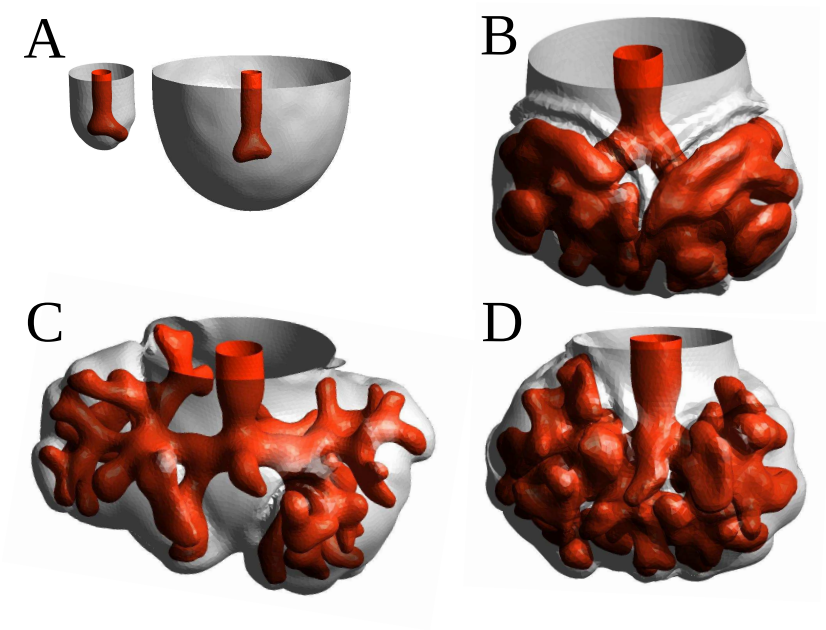

Figure 3: **A.** Simulation with homogeneous proliferation in the mesenchyme. If g is too small, the epithelium reaches the mesothelium (left). When g increases, the mesothelium moves too quickly, preventing the epithelium from receiving enough signal to grow normally (right). **B.** Mesenchyme proliferation 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).

Influence of the growth response

We tested the influence of the growth response f to the growth-promoting ligand. Similarly to what we described in the 2D lung model [11], we found that the morphology of the tree was finely tuned by the growth response. A good example 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. Resulting shapes suggest that the higher the threshold is (Figure 4A), the more tubular the branches are. This is in fact consistent with the instability mechanism that we described. When the threshold is high, the sensitivity to low ligand income is very poor. Branches sides thus undergo very little growth, as the gradient essentially concentrates on distal tips. When the threshold is decreased (Figure 4B), sensitivity to weak ligand incomes is increased and the spatial distribution of growth spreads on branches sides. Branches diameters are consequently 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 not 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 in lung or kidney impact the branching pattern and regularity but not the global tree-like structure.

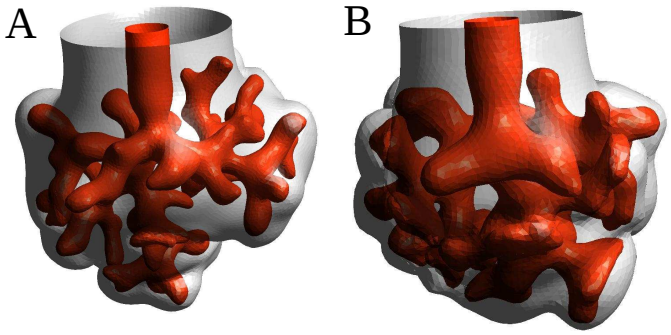

Figure 4: **A.** Growth response with a high threshold. Branches rather elongate than thicken. **B.** Growth response with a low threshold. Branches rather thicken than elongate and branches consequently have a greater diameter than in (A).

Initial geometry

A qualitative discrepancy between the model and branching organogenesis resides in the initial geometry: we made the choice of simplicity and chose an ideal tubular geometry, which is not the case in vivo, as 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 - viscous fingering - that the repetition of the same experiment in an ideal geometry results in random branched structures sharing the same statistical properties [41]. But 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 exterted 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. 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 towards $v/(d_0(k-1))$, 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 - 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 built an organ-scale model based on a gradient of growth-promoting ligand between proximal and distal mesenchyme. Implementing the model numerically in 3D, we found that this sole ingredient allows, through ligand diffusion, the emergence of a self-avoiding, space-filling branching epithelium. It suggests that specific regulation might not be required to to achieve branching, to organize branching events into a self-avoiding structure and to set an equilibrium distance between bud tips and external boundary. As 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 2D representation, we provide here the demonstration that they robustly hold in a general 3D 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 be the integration of realistic organ-specific parameters such as experimental growth responses, realistic external constraints, qantitative 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 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, as 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 Stéphane Leduc or D'Arcy W. Thompson [45, 46]: at the beginning of the nineteenth century, they hypothesized 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, as the evolution of the shape is mostly determined by shape itself (Figure 5).

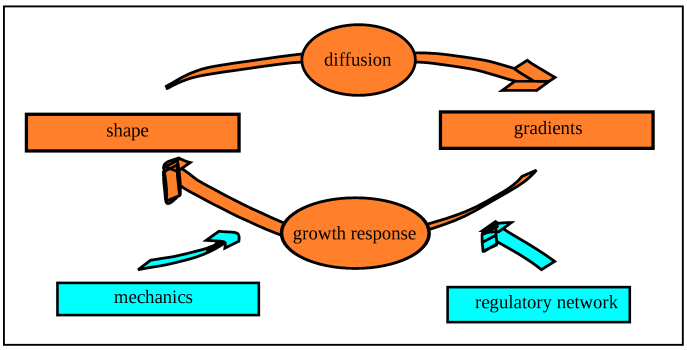

Figure 5: **Feedback loop between shape and gradients.** Shape boundaries limit diffusion domains and determine gradients. In return, gradients influence the shape through the growth response to gradients of signaling molecules. The growth response is underscored both by the regulatory network and by 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.

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

Acknowledgments

Authors warmly thank Stephane Douady for his contributions to this project since its very beginning; Pierre Blanc and Vincent Sapin for early discussions concerning lung development; and Erwan Poindron for making nice movies out of the simulations.

Part of this work has been funded by the program "Aide aux jeunes chercheurs" from the city of Nice, France; and by the CNRS program PEPS (Projet exploratoire premier soutien, Physique Théorique et Interfaces).

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Short title

An archetypal mechanism for branching organogenesis