CHAPTER FOUR

Cell Topology, Geometry, and Morphogenesis in Proliferating Epithelia

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Contents

Abstract

Epithelia are sheets of tightly adherent cells that line both internal and external surfaces in a vast array of metazoans. During development, an intrinsic consequence of coupling tight adhesion with cellular proliferation is the emergence of an epithelial form characterized by a stereotyped distribution of polygonal cell shapes. Despite the near universality of this constraint on cell shape and tissue

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organization, very little is known about the possible implications of cell pattern geometry for mechanical properties of tissues or key biological processes, such as planar polarization, tissue remodeling, and cell division. In this chapter, through an examination of increasingly complex models, we highlight what is known about the role of mitotic proliferation in the emergence of epithelial cell geometry, and examine some possible implications for tissue morphogenesis. Ideally, continued progress in this area will address a major conceptual challenge in biology, which is to understand aspects of morphogenesis that are not explicitly directed by genetic control, but instead emerge from the complex interactions between geometric and biomechanical properties of epithelial tissues.

1. Introduction

From the simplest to the most complex metazoans, epithelial morphogenesis is a fundamental component of development, organogenesis, and even disease progression. A staggering variety of organisms depend on epithelia and their derivatives for development and homeostasis. Across widely divergent evolutionary clades, epithelial architecture retains certain essential structural features. These include apical/basal cell polarization, formation of cell–cell junctions, and the constitution of a paracellular diffusion barrier, all of which enable epithelia to serve an even greater diversity of biological functions. Still, while the advantages of epithelial organization are clear, the corresponding constraints on morphogenesis are often poorly understood.

The paper-folding art of origami is a simple but powerful conceptual model for epithelial morphogenesis. Using a finite number of simple folding rules, an infinite number of scale-invariant morphologies can be achieved, which derive entirely from a simple, planar sheet (Huzita and Scimemi, 1989, as cited in Nagpal, 2001, 2002). This analogy is intended to emphasize the role of macroscopic architectural changes that occur seemingly independently of the microscopic and two-dimensional structure of the epithelium. In real epithelial sheets, however, an additional layer of constraint and complexity is introduced by the requirements for growth, proliferation, and the control of neighbor cell relationships. Consider a flat, monolayer sheet of proliferating columnar cells. Intuitively, cell division and cell rearrangement in the plane of the epithelium change the polygonal cell pattern geometry on the microscopic level. A critical and unresolved problem is thus to define the implications of the polygonal cell pattern geometry for macroscopic morphogenesis.

There is considerable evidence that morphological transformations at the cellular level are relevant for tissue-level morphogenesis. Three examples include coordinated apical constriction, oriented cell division, and

localized, differential control of cellular proliferation, among numerous others (Baena-Lopez *et al.*, 2005; Gong *et al.*, 2004; Lecuit and Lenne, 2007; O'Brochta and Bryant, 1985; Saburi *et al.*, 2008). However, these cases all involve some sort of global pattern control to orchestrate the cellular changes. An open question is whether uncoordinated aspects of the planar pattern geometry could also be of macroscopic relevance, for example, in setting the spatial or temporal noise in morphogenesis. Alternatively, do some microscale geometries make epithelial sheets more or less structurally stiff or strong? Could understanding the polygonal cell-packing pattern be relevant for understanding cell–cell signaling, for example, in planar cell polarity? These are speculative subjects, but they underscore the importance of understanding the cellular geometry of proliferating epithelia.

In this chapter, we highlight what is known about cell proliferation-dependent cell shape dynamics, with an emphasis on its broader relevance for higher-order aspects of morphogenesis. We first review epithelial structure, both molecular and cellular, to establish both the intrinsic and the emergent properties of epithelial architecture. Next, in order of increasing complexity, we consider models explaining the emergence of epithelial planar pattern geometry. We start with the simplest possible models, working up through complex geometric and biophysical simulators. Throughout, we consider the role of planar pattern geometry in epithelial morphogenesis, and conclude with an examination of the interactions among epithelial packing, biophysical processes, and tissue morphogenesis.

2. Conservation of Epithelial Architecture

The different types of epithelia are commonly classified by thickness, cellular morphology, and cellular connectivity. Simple epithelia are a single layer thick; stratified epithelia have two or more layers. Simple epithelia are typically classified as one of four types based on morphology of the component cells: squamous, cuboidal, columnar, or pseudostratified. Squamous cells, for example, are shaped like flattened, interlocking polygonal plates or scales, whereas cuboidal cells are isometric in vertical section (Gray, 1995). Columnar cells have height to width ratios significantly greater than one, and like cuboidal cells, are polygonal when sectioned horizontally (Gray, 1995). There is one additional epithelial category (considered to be a simple epithelium), the pseudostratified type, where elongate, spindle-shaped cells interdigitate their nuclei within the plane of the epithelium but nonetheless remain a monolayer (Wright and Alison, 1984). By analogy with the simple epithelia, the stratified epithelia also contain squamous, cuboidal, and columnar varieties. The critical difference between simple and stratified

epithelia is that at least one layer of the latter category has lost contact with the basal lamina, and differentiated (Wright and Alison, 1984). For simplicity, in this chapter we focus exclusively on simple columnar epithelia.

While the essential features of epithelial construction are conserved among metazoa, there are clear differences in the architecture among different evolutionary clades (Knust and Bossinger, 2002; Tepass *et al.*, 2001). The scope of animal epithelia and plant epidermis covered here is sufficiently expansive that a full enumeration of the comparative structural differences is not possible. For purposes of illustration in discussing epithelial architecture, we place emphasis on *Drosophila* simple epithelia, which are particularly well characterized, in terms of both macroscopic and molecular structure.

A primary feature of epithelia, in *Drosophila* and throughout the animal phyla, is cell polarization. Polarization, in turn, facilitates formation of a paracellular diffusion barrier, specialization of plasma membrane proteins, and directional transport in the form of secretion and absorption. The plasma membrane of each epithelial cell is divided into immiscible apical and basolateral domains (Tepass *et al.*, 2001). Importantly, both the apical and the basal domains of the neighboring cells align with each other, endowing the epithelium with a globally faithful, local polarity. Separating the apical and basolateral domains is the zonula adherens (ZA), an adhesive belt encircling the cell (Knust and Bossinger, 2002). The apical zone is subdivided into the apical surface and the marginal zone, where cell-cell contact occurs apical to the Zonula Adherens (Tepass *et al.*, 2001). In *Drosophila*, septate junctions (SJs) lie basal to the ZA and constitute a paracellular permeability barrier, functionally analogous to the vertebrate tight junction (Bilder, 2001; Genova and Fehon, 2003; Gibson and Perrimon, 2003).

The molecular architecture of epithelia has been studied extensively in animal tissues, partially owing to the prominent role they play in human cancers. ZA proteins in *Drosophila* include DE-cadherin, and the scaffold proteins Armadillo (the *Drosophila* orthologue of β-catenin), α-catenin, Canoe (homologue of mammalian Afadin). SJs include the transmembrane proteins Neurexin IV and Fasciclin III, and the scaffold proteins Scribble, Disks Large, Coracle, and Lethal giant larvae (Lgl) (Bilder, 2001; Gibson and Perrimon, 2003; Knust and Bossinger, 2002; Tepass *et al.*, 2001). Additional SJ proteins are encoded by such genes as *contactin*, *neuroglian*, *gliotactin*, *sinuous*, Na^+/K^+ *ATPase*, *lachesin*, and *megatrachea*, as well as *varicose* (Banerjee *et al.*, 2006; Wu *et al.*, 2007). Interestingly, several of the SJ proteins are critical for apical/basal polarity. Scribble, Disks Large, and Lethal giant larvae are also neoplastic tumor suppressors, thus linking epithelial morphology with control of epithelial proliferation (Hariharan and Bilder, 2006).

Currently, cellular geometry within simple epithelia is best understood in cases when it can be modeled as a planar network, such as at the apical

junctions, where the mechanics are constrained (Farhadifar *et al.*, 2007). When sectioned apically, monolayer epithelial cells form ordered polygonal arrays, resembling a froth of soap bubbles (Fig. 4.1). However, in the pseudostratified *Drosophila* wing imaginal disk epithelium, the three-dimensional cellular geometry is considerably more complex below the level of the SJs where cells no longer tightly adhere. This cellular disorder can be attributed to the fact that the relatively large cell nuclei cyclically migrate along the apical–basal axis in concert with the phase of the cell cycle. During cell division, the nuclei are just beneath the apical surface, and the morphology of the dividing cell is almost spherical. The dividing cell thus deforms the apical geometries of its neighbors. Nevertheless, the contacts between neighboring cells tightly adhere and do not rearrange, in spite of the stretching and compression induced by their mitotic neighbor (Gibson *et al.*, 2006). As a result, the ''interkinetic'' mode of cell division, reliant on cell cycle phase-coupled nuclear movements, has little effect on the polygonal geometry of the apical epithelial surface. By comparison with the deformable cell contacts of animal epithelia, the geometry of plant epidermis appears to be simple, stiff, and regular, and without the complication of nuclear migration along the apical/basal axis. Cucumber epidermal cells, for example, have a slight apical curvature, and are either flat or have a shallow pyramidal point at the basal level. Overall, they are close to being simple, stiff, polygonal prisms (Lewis, 1928). In light of these fundamental structural differences, one might expect animal epithelia and plant epidermis to have very different cellular geometries. In fact, their cellular geometries resemble one another to an unexpected degree, at least in apical cross section.

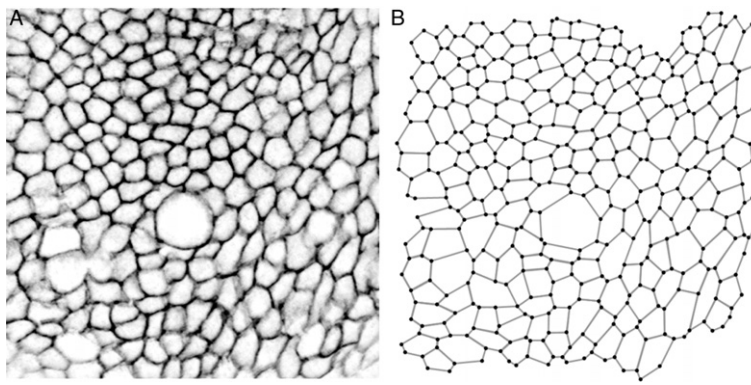

Figure 4.1 Epithelial topology at the level of cell–cell junctions. (A) An apical cross section through the pseudostratified *Drosophila* wing disk epithelium, stained with antibodies against the SJ component Disks Large to outline cell boundaries. (B) A polygonal approximation to the apical section geometry. Note the presence of non-hexagonal cells.

3. Introduction to Cellular Topology

In contrast to cellular geometry, which specifies cell shape, *cellular topology* refers to the connectivity among cells in a tissue. Intuitively, one can imagine stretching or deforming a sheet of cells in such a way that the cells' respective shapes change, but all neighbor relationships are preserved. For such deformations, the sheet's topology remains unchanged. By contrast, processes such as perforation or tearing, in which cell contacts are broken, or convergent extension, in which cell contacts are both made and broken, can change the sheet's topology significantly (Zallen and Zallen, 2004). In epithelia, various elementary processes, such as cell division, cell rearrangement, and cell disappearance, can be shown to modify cell sheet topology in stereotyped ways (Dubertret and Rivier, 1997). Moreover, in many biological systems, cell topology is expected to correlate with geometric variables, such as cell area (Rivier and Lissowski, 1982). Therefore, as a first approximation to geometrically complex morphogenetic processes, topological descriptions can provide fundamental insight into how tissue-level connectivity emerges from elementary cellular transformations.

4. Conservation of Topological Structure in Proliferating Epithelia

The columnar cells of both animal epithelia and plant epidermis, which differ in both cellular morphology and molecular architecture, nevertheless look quite similar when viewed in apical section. Quantitative analysis of the topological distributions in both types of monolayers reveals unexpected similarities that distinguish epithelial cell packings from other cellular structures such as soap bubble foams. This conservation of topological structure raises questions about why a given pattern geometry might be preferable to another, or whether the stereotypical polygonal pattern is simply an inevitable consequence of cell division.

The observation that apical sections of proliferating epidermal sheets have constant distributions of polygon types was first made by Lewis (1928) in the cucumber. The distributions of cellular polygons have since been measured in a wide range of divergent organisms, both animal and plant (Gibson *et al.*, 2006; Korn and Spalding, 1973; Zallen and Zallen, 2004). The polygon distributions are remarkably similar within select metazoan epithelia (differing by only a few percent), and are also similar between certain metazoans and some plant epidermis (Fig. 4.2). For example, the cucumber epidermis and the *Drosophila* larval wing disk epithelium have an almost identical distribution of polygon types, with a peak of approximately

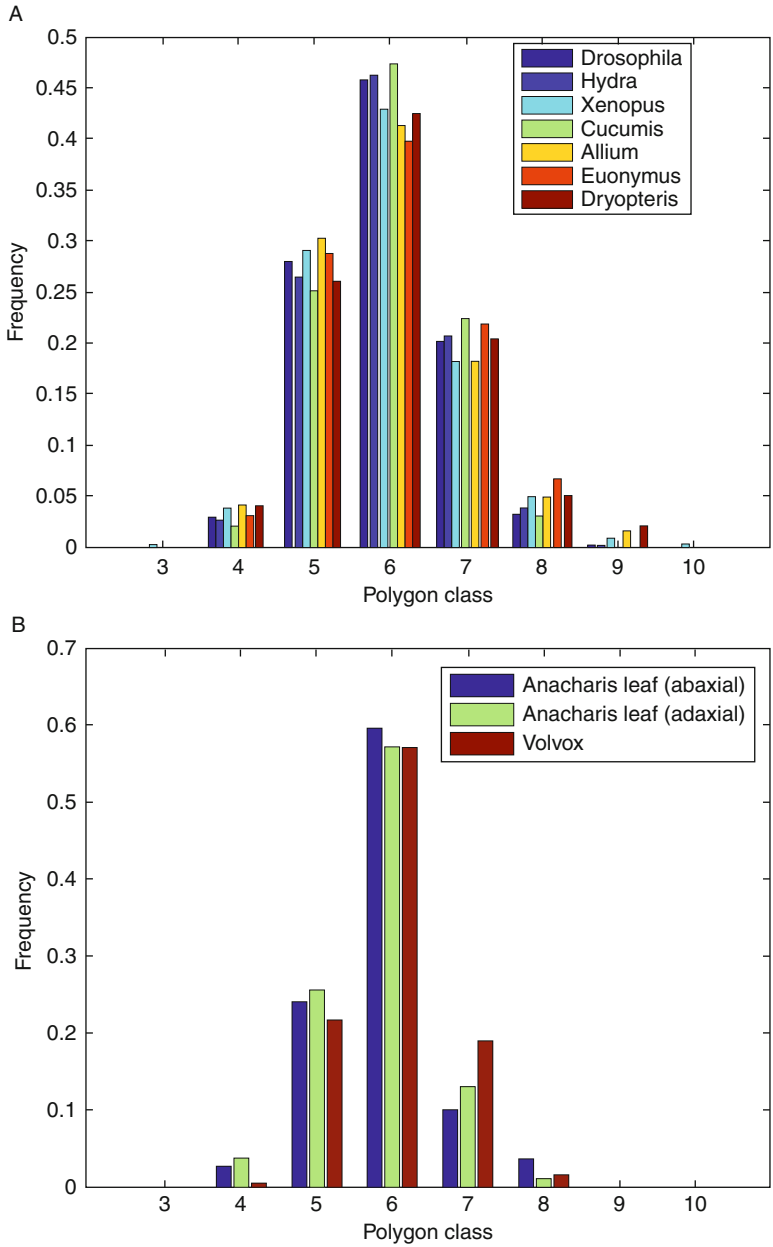

Figure 4.2 Distributions of cellular polygons in epithelia and plant epidermis. (A) The distribution of polygonal cell types in diverse animal epithelia and plant epidermis. Note the mode of hexagons, and the conservation of the general form in both plants and animals. (B) Two distributions of polygonal cell types that differ from the widely observed distribution seen in (A). Sources of data: *Drosophila*, *Hydra*, *Xenopus* (Gibson *et al.*, 2006); *Cucumis* (Lewis, 1928); and *Allium*, *Dryopteris*, *Euonymus*, *Anacharis*, *Volvox* (Korn and Spalding, 1973).

45% hexagons (Gibson *et al.*, 2006; Lewis, 1928). Moreover, a similar distribution of polygons is also observed in onion epidermis, fern epidermis, and the epithelium of the simple cnidarian, *Hydra* (Gibson *et al.*, 2006; Korn and Spalding, 1973). On the other hand, some species, such as the plant *Anacharis*, with a hexagonal frequency near 60%, have a significantly different distribution of polygon types (Fig. 4.2B; Gibson *et al.*, 2006; Korn and Spalding, 1973; Lewis, 1928). This indicates while there may be significant topological conservation in ''default state'' cell layers (which exhibit uniform proliferation and little cell rearrangement), biological mechanisms can clearly produce variant distributions under a range of circumstances in both animals and plants.

The distributions of cellular polygons observed in proliferating epithelia seem to share two prominent features. First, without exception, the mode of the distribution is at six-sided cells. Second, the form of the distribution is unimodal, with a rapidly decreasing right tail (Fig. 4.2B). Four-sided cells are rare (between 1% and 5% of the population), whereas three-sided cells are either ultra-rare ($<1/10^6$) or nonexistent (*Xenopus* epithelia present an exception to this rule). The reason for the absence of three-sided cells is unknown, but is probably due (at least in part) to highly symmetric mitoses in epithelial cells having very regular geometries. Cells most likely to give rise to three-sided daughters via division, such as four-sided cells, may also have extremely low division probabilities.

The widely observed similarities in epithelial cell topology naturally lead to the question of whether these similarities arise from conserved division mechanisms. While this question is unsolved theoretically, experimental evidence consistent with this hypothesis has been previously reported (Korn and Spalding, 1973). Quantitatively different planar pattern geometries are seen in plant tissues having qualitatively different division mechanisms (Fig. 4.2B). While the proper controls have not been done, the study suggests that quantitatively different division mechanisms could generate quantitatively different distributions of polygon types. For a first look at theoretical treatment of such questions, see (Cowan and Morris, 1988).

5. Topological Inference in Epithelia: Maximum Entropy Methods

The simplest models of epithelial geometry are actually direct statistical inferences about packed sheets of polygons known as *maximum entropy methods*. In epithelia, such methods algebraically compute the most likely configuration of polygons based on a small number of basic geometric assumptions (Rivier *et al.*, 1995). Maximum entropy calculations have yielded excellent local predictions about the neighbor relationships among

the different polygon classes (Dubertret and Rivier, 1997; Peshkin *et al.*, 1991). The basic prediction is that many-sided cells and few-sided cells are more likely to neighbor one another than would be expected by chance. Such correlations are not only relevant for modeling epithelial topology, they also provide fundamental insight into tissue architecture. Many-sided cells are expected to be larger than fewer-sided cells, both based on experimental observation and based on statistical inference (Lewis, 1928; Rivier and Lissowski, 1982). By anticorrelating the many-sided and the few-sided cells, the tissue reduces the frequency at which multiple large cells are crowded together, or at which multiple small cells are stretched to remain neighbors. Because these tissues are built using division mechanisms, the inference suggests an indirect link between proliferation and epithelial biophysics, and by extension, morphogenesis.

6. Topological Models: The Simplest Models of Epithelia

To complement statistical inference methods, simple topological models incorporate biologically plausible mechanisms to generate the empirically observed polygonal cell shape distributions. For a proliferating epithelial sheet, the three most likely candidate mechanisms include cell division, cell rearrangement, and cell disappearance. Empirical studies indicate that the polygon frequencies are in equilibrium or nearly so (Gibson *et al.*, 2006; Korn and Spalding, 1973; Lewis, 1928). Therefore, the simplest possible model to describe the polygon frequencies will specify the rates at which each polygon type is created and destroyed in an attempt to match the distributions observed empirically. During cell division, the mother polygon cell is destroyed, whereas two new daughter polygon cells are created. In addition, the two polygon cells that abut the division plane gain one side each (Fig. 4.3). The myriad ways in which cells can divide and gain sides, when combined with neighbor correlations between different polygon classes, makes predicting topological dynamics nontrivial. At least three groups have independently built mathematical models that closely approximate steady-state topological dynamics (Dubertret and Rivier, 1997; Gibson *et al.*, 2006; Korn and Spalding, 1973). Analytical models are essential for understanding the dynamics of dividing cell sheets. Nevertheless, such models have limitations. In particular, they predict global average dynamics in terms of local average dynamics, which in turn depend on neighbor correlations. Currently, such neighbor correlations are inferred based on equilibrium assumptions and maximum entropy (Dubertret and Rivier, 1997; Miri and Rivier, 2006; Peshkin *et al.*, 1991). Thus, only division rules having a stable equilibrium, and which are well captured

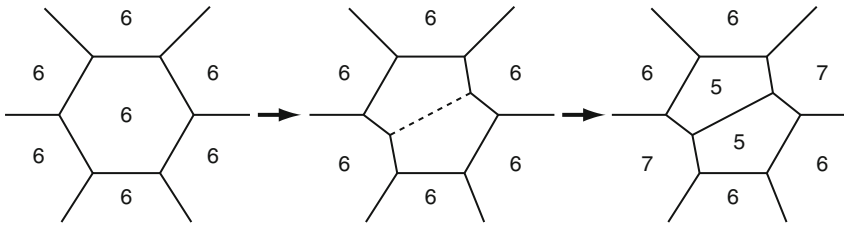

Figure 4.3 Mitosis in polygonal cells. Following division of the central cell, the two neighboring cells abutting the cleavage plane effectively gain one side each, transitioning from hexagons to heptagons. Daughter cells of the division tend to have a lesser number of sides than the mother polygon. These simple transformations drive the epithelial topology to a heterogeneous, yet predictable, equilibrium.

in terms of local mean-field behavior, can be represented by such analytical models. Topological simulators, which explicitly represent and store neighbor relationships between cells in computer memory, make no such approximations and permit a more general space of division mechanisms to be explored.

In the future, we anticipate that topological simulators will permit answering general questions about topological dynamics. For example, simulators may be useful for determining whether a particular cell division mechanism uniquely corresponds to a specific steady-state distribution of polygon types. When uniqueness holds, the division mechanism can automatically be inferred from the polygon distribution, which is useful for interpreting empirical polygon distributions. For example, the experiments of Korn and Spalding (1973) found several epithelia having hexagonal frequencies close to 60%, a significant deviation from the other studied epithelia, where the frequency is closer to 45%. Uniqueness would imply that these epithelia must also be using quantitatively different division mechanisms or cell rearrangements, which is consistent with the study's qualitative observations (Korn and Spalding, 1973).

A related question concerns the full range of possible polygon distributions that can be reached by a system using all possible division mechanisms. This second question has direct biological relevance for *Drosophila* wing development. For example, it is not currently known why cells undergo extensive neighbor exchanges to achieve hexagonal repacking during pupal wing morphogenesis. During this process, hexagonal frequency in the wing changes from about 45% to nearly 80% (Classen *et al.*, 2005). During larval development, the 45% hexagonal frequency appears to be achieved as a direct consequence of cell division (Gibson *et al.*, 2006). This raises the question of whether the tissue must employ cellular rearrangement to elevate the frequency of hexagons, perhaps because achieving an 80% frequency of hexagons is impossible by division alone. In this case,

rule-based modeling could be used to explore the full range of topologies achievable through cell division, and provide new hypotheses about when and why epithelial tissues utilize directed cell rearrangements during morphogenesis.

In spite of the promise that topological models hold for answering fundamental questions about epithelial proliferation and morphogenesis, they have important limitations. First, by definition, topological models do not incorporate space into their dynamics. Therefore, no matter how well epithelial dynamics are captured, topological models cannot predict large-scale macroscopic changes. Second, because any two polygons of the same class are assumed to have the same properties irrespective of their spatial location, processes that involve spatially correlated variables or directionality cannot be properly captured. For example, although topological relationships are a useful conceptual framework for understanding complex cell dynamics such as rosette formation and convergent extension during *Drosophila* germ-band elongation, meaningful simulations of these processes must be geometrical in nature (Bertet *et al.*, 2004; Blankenship *et al.*, 2006; Rauzi *et al.*, 2008). An even simpler example concerns oriented cell divisions, in which case the topological dynamics may look identical for oriented versus nonoriented divisions despite the fact that the geometrical dynamics are fundamentally different (Baena-Lopez *et al.*, 2005; Gong *et al.*, 2004). These limitations of topological models lead us to next consider methods for modeling geometrical aspects of epithelial organization.

7. The Smallest Geometrical Model: Cleavage Plane Orientation in a Single Cell

The geometric complexity within the plane of an epithelium emerges from interactions between cell shape, cell adhesion, and cell division. Very simple geometric models of proliferating epithelia can be built by assuming that cell division orientation is determined by a cell's local geometry, which emerges from mechanical interactions. Importantly, there is substantial evidence, in both plant and animal cells, that local cell geometry is the default mechanism for determining the cleavage plane.

As early as the nineteenth century, several geometrical rules for cell division were observed in plants. Hofmeister observed that cells tend to divide orthogonal to their long axes. Errera formulated a rule holding that cleavage planes tend to find the shortest path that will divide a cell into two equal halves, which has been confirmed in trichome cells of the Venus flytrap (Dumais, 2007; Smith, 2001). Sach's rule holds that dividing plant cells orient their newly formed cell walls perpendicular to previously formed walls (Lynch and Lintilhac, 1997). Still, the mechanistic puzzle of

how the geometry of a dividing cell influences its cleavage plane orientation is not fully understood. Using laser microsurgery in *Nautilocalyx* cells, Goodbody *et al.* (1991) demonstrated that the strands connecting the pre-mitotic nucleus to the cellular cortex are under tension. Arguing that the cortical connection points for the tensile strands are able to move along the cortex (as demonstrated in a simple analogue model), the work supports a minimal distance configuration of tensile strands (Flanders *et al.*, 1990; Goodbody *et al.*, 1991). Under this model, the spatial distribution of tensile strands, and therefore the spatial distribution of internal tension, depends on the geometry of the cell cortex (Flanders *et al.*, 1990).

In the context of plant cells, the distribution of internal tension is important because tension has been implicated as a regulator of cleavage plane orientation. Dividing tobacco cells imbedded in agarose gel blocks under compression have been reported to orient their cleavage planes either parallel to, or perpendicular to, the direction of the principle stress tensor. As a more direct link to cell geometry, the same study suggests that the short axis of the dividing cell is strongly correlated with the compressive stress tensor (Lynch and Lintilhac, 1997). Looking more globally at plant tissue, compression has been shown to induce coplanar cleavage orientations in otherwise disorganized tissue (Lintilhac and Vesecky, 1984). Therefore, it is tempting to speculate that, at least in some cases, dividing cells sense the direction of stress in a tissue based on their geometric strain, and then respond by orienting their cleavage planes to relieve the stress. Recently, Hamant *et al.* (2008) demonstrated a strong correlation between the direction of maximum stress and the orientation of microtubules in the *Arabidopsis* meristem. These experiments suggest that in *Arabidopsis*, cell-autonomous, stress-guided, microtubule alignment-based processes feed back on morphogenetic processes, including tissue folding and cell division. The mechanisms guiding such feedbacks are unknown, but may involve mechanotransduction (Ingber, 2006; Wang *et al.*, 1993).

The geometric biophysics of division plane orientation in animal cells is arguably less well understood than it is in plants. Orienting the division plane so as to divide the long axis is thought to be a default orientation mechanism, although sufficient data to make a general statement are lacking (Strauss *et al.*, 2006). Following a cell cycle-dependent time lag, dissociated *Xenopus* blastula cells with experimentally induced long axes divided perpendicular to the long axis up to 100% of the time (Strauss *et al.*, 2006). Similar division orientation preferences have been shown for the first cleavage of compressed *Xenopus* eggs and mouse zygotes, and also in the blastular wall of starfish embryos (Black and Vincent, 1988; Gray *et al.*, 2004; Honda, 1983). Mitotic spindle orientation is a valuable, if imperfect, predictor of eventual division plane orientation in some systems, and thus revealing about how division planes are determined in animal cells. Spindles in frog blastulas have been shown to orient according to the long axis the

majority of the time (Strauss *et al.*, 2006). Additionally, cultured normal rat kidney (NRK) cells reorient the spindle in a dynein-dependent manner so as to divide the long axis when the cellular cortex is deformed (O'Connell and Wang, 2000). Thus, without any additional information, a line perpendicular to a cell's long axis appears to be the best estimate of division plane orientation for an animal cell.

By analogy with plant cells, geometric correlates of division orientation in animal cells are likely due to biophysical mechanisms. The work of Thery *et al.* (2005) shows that placing HeLa cells onto micropatterns printed with fibronectin, which interacts with integrins, is able to strongly bias their spindle orientation. The work also provides evidence that internal actin-binding protein distributions are correlated with these external ECM patterns, thus suggesting a partial mechanism (Thery *et al.*, 2005). Proof of principle was provided in a simple, torque-based model with impressive predictive power (Thery *et al.*, 2007). Such mechanisms may help explain how cells may use extracellular matrix proteins to biophysically "read" their geometry.

8. Nongeometric Mechanisms of Division Orientation: A Larger Morphogenetic Space

Genetically directed mechanisms of cleavage plane orientation not solely driven by geometry or biophysics make possible a substantially larger space of morphogenetic transformations, and thus morphologies. An important class of nongeometric division orientation mechanisms includes the molecular control of mitotic spindle orientation, which is partially correlated with division plane orientation. In plants, molecular mechanisms are well known to be involved in orienting cleavage orientation (Jurgens, 2005). For example, the preprophase band (PPB), a ring of microtubules and F-actin, designates the future site of the cleavage plane on the cell cortex (Jurgens, 2005; Smith, 2001). It was recently shown that the *Arabidopsis* protein *tangled* colocalizes with the PPB and predicts the future cleavage sites throughout mitosis and cytokinesis (Walker *et al.*, 2007). Moreover, in *tangled* mutants, cleavage plane orientation is aberrant (Smith *et al.*, 1996). Thus, at least some plant cells appear to decide on their division orientation long before cytokinesis begins. Such fine control over division orientation might be expected to be essential for the control of organ shape. However, in *tangled* mutants, organ shape is normal, suggesting that division-plane independent mechanisms are operating (Smith *et al.*, 1996).

Molecular mechanisms guiding division plane and/or mitotic spindle orientation are well studied in animal cells, but the cellular "decision" concerning division orientation is less well understood. In *Drosophila*, a

number cellular junction components have been implicated in spindle orientation mechanisms, including the adherens junction components E-cadherin and Canoe (Le Borgne *et al.*, 2002; Speicher *et al.*, 2008). In mammalian cells, α-catenin is an additional example (Lechler and Fuchs, 2005). Planar cell polarity (PCP) is also implicated in spindle orientation. In developing zebrafish, the dorsal epiblast divides the short axes (not the long axes) of the cells in a PCP pathway-dependent manner (Gong *et al.*, 2004). Additional players include integrins in both *Drosophila* and mammals (Fernandez-Minan *et al.*, 2007; Lechler and Fuchs, 2005). Also, the microtubule plus-end tracking proteins APC and EB1 are implicated in *Drosophila* and also in human cell culture (Draviam *et al.*, 2006; Green *et al.*, 2005; Lu *et al.*, 2001; Rogers *et al.*, 2002; Yamashita *et al.*, 2003). Thus, both intrinsic and extrinsic mechanisms are involved in spindle orientation, and by extension, division plane orientation. These findings suggest that attempting to model morphogenesis using only geometric rules for guiding the division plane can be a vast oversimplification.

9. Scaling Up: Geometrical Models and Cellular Mechanics in Proliferating Epithelia

By comparison with geometrical division orientation mechanisms, nongeometric mechanisms make tissue growth relatively more complex. Without question, such mechanisms are an essential part of proliferation and morphogenesis. Nevertheless, for the most basic understanding of tissue growth, nongeometrical complications are negligible, because over a range of tissue types and species, we expect nongeometrical biases to average out. In other words, it is reasonable to consider geometric mechanisms driving cleavage plane orientation as a default system that can be overridden in instances of direct molecular control. To consider the emergence of epithelial structure in the default geometrical frame, here we consider four types of models: Dirichlet models, cellular Potts models, subcellular element models, and finite-element models, as well as their implications for proliferation and morphogenesis.

9.1. Dirichlet models

Dirichlet models are remarkable both for their simplicity and for their accuracy in predicting epithelial geometries. A Dirichlet domain is best visualized in the 2D plane. Suppose many different random dots lie in the plane. The Dirichlet domain for a particular dot is the region of space that is closer to that dot than to all other dots (Honda, 1978). When such a domain is computed for every dot in the plane, and the borders separating the

individual domains are drawn, one is left with a polygonal tiling that looks strikingly like an epithelial sheet (Fig. 4.4A). Starting with an image of an epithelium, the Dirichlet approximation to the epithelial geometry is constructed by placing a dot at the center of mass of each cell, and then constructing the Dirichlet domains. Not all cellular structures match the Dirichlet domains, and the degree to which a structure deviates from the approximation can be quantified (Honda, 1978). Nevertheless, for a first-order approximation, it looks quite realistic, and is an illustration of how strong the space constraints are in epithelial sheets.

To probe the underlying forces specifying the geometry of an actual cellular sheet, one might consider the regularity of the cells. Using a "boundary shortening" procedure, one can, in an iterative fashion, shorten the

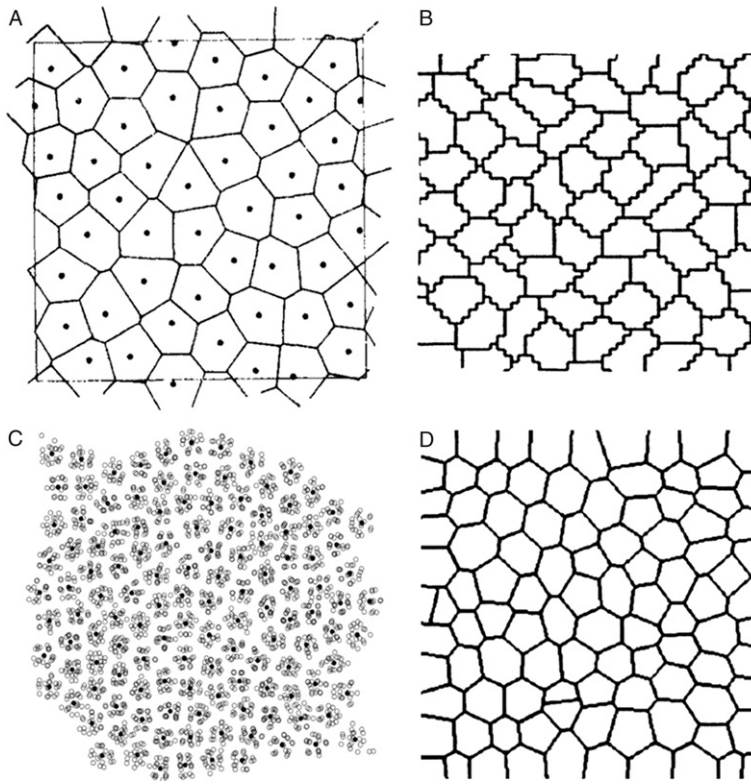

Figure 4.4 Visual output from models for epithelial geometry. (A) A Dirichlet model of space partitioning (Honda, 1978). Note the resemblance to natural epithelia. (B) Output from a cellular Potts model (Glazier and Graner, 1993). The cellular geometries are free to assume nonpolygonal shapes. (C) Visual output from a subcellular element model (Newman, 2005). Each cell is represented by a cloud of physically interacting points, visible in black. (D) Visual output from a finite-element model (Brodland and Veldhuis, 2002).

perimeters of the cells' boundaries without changing their areas (Honda and Eguchi, 1980). Interestingly, different cellular structures are able to shorten their boundaries to differing extents, depending on the regularity of the cellular shapes to begin with (Honda and Eguchi, 1980). Especially regular structures, such as soap bubbles, are already close to the minimum perimeter, whereas more irregularly packed cell sheets may be able to shorten their boundaries substantially. The differing abilities to shorten are thought to reflect a property of contractility in the cell boundaries, which was recently included in a model of epithelial geometry by Farhadifar *et al.* (Farhadifar *et al.*, 2007; Honda and Eguchi, 1980). Contractility of cells is a fundamental property that can influence morphogenesis in three dimensions, and may impact the dynamics of spindle orientation for planar representations.

The Dirichlet approximation has several applications to epithelia. First, it can be used to model how a sheet of cells responds to cellular disappearance (Honda, 1978). Second, in combination with an iterative, center-of-gravity-based relaxation procedure, it can be used to model cell division (Honda, 1983; Honda *et al.*, 1984). Thus, using only a few simple assumptions, a coarse-grained approximation of temporal froth evolution can be achieved for epithelial systems. These predictions demonstrate the degree to which epithelial proliferation, and morphogenesis by extension, is constrained by the geometry of the starting material.

9.2. Cellular Potts models

A second standard technique, the cellular Potts model (CPM) is a general method for simulating cellular dynamics and is useful for studying proliferating epithelia. Such models are based on Hamiltonians, or effective energy functions, in order to determine the probability that one mechanical configuration will transition to another. The art of designing these models is in specifying the Hamiltonian. Experimentally, the challenge is justifying the Hamiltonian being used. Practically, these models' utility is in simulating complex cellular shapes that could not be studied in closed form.

An extremely simple CPM for simulating proliferating epithelia can be found in the work of Mombach *et al.* (1993). Here, the energy function is based on interfacial energy. Mitosis (through the cell's approximate center of mass) occurs when the area to perimeter ratio exceeds a threshold. The significance of the model is that it demonstrates how the dynamics and geometry of epithelial sheets depend on fundamental mechanical quantities, such as interfacial energy. An ambitious and interesting model of morphogenesis can be found in the work of Graner and Glazier (1992, 1993), which simulates cell sorting for two classes of cells based on differential adhesion. Here, the energy function is based both on surface energy and on the difference between a cell's actual size and its ''preferred'' size (Graner and Glazier, 1992). More recently, Farhadifar *et al.* (2007) used the basic ideas of

the CPM to construct a more sophisticated incarnation incorporating line tensions to simulate proliferation and rearrangement in the *Drosophila* wing disk. Like the model of Graner and Glazier (1992), this model uses a preferred size in the energy function, but now the Hamiltonian also includes a contractility term and a preferred perimeter (Farhadifar *et al.*, 2007). The power of these models is that they are completely general—any cellular system can be simulated, with varying degrees of complexity, provided a proper energy function is specified. Because the energy functions are usually based on realistic physical principles, the framework allows large regions of developmental space to be explored by changing a few fundamental parameters. Developmentally, different parameter regimes may correspond to very different types of morphogenesis.

9.3. Subcellular element models

A new and exciting alternative to CPMs can be found in the subcellular element models developed by Newman (2005). These models simulate cellular interactions by representing each cell as a cloud of points. Each point belongs to exactly one cell, and has over damped, elastic interactions with the other points in the cell, which depend on absolute distances between particles. In addition to these interactions, there is also a random noise component. An additional term models interactions between points in neighboring cells when those points are in close proximity. Thus, cellular geometry and tissue morphology emerge as the collective interactions of these point masses (Fig. 4.4C). When division is introduced into such models, the cellular structures produced look qualitatively realistic (Newman, 2008). The true power of such models likely lies in the future, when three-dimensional biological complexities such as cell polarity and cell–cell signaling can be introduced. Nonplanar, dynamical rearrangements of epithelial sheets, such as folding or buckling, could be simulated in this manner. Such models also make it possible to compute the local stresses and strains that are operating in a cell at a particular instant, which might be useful for testing hypotheses about mechanotransduction (Ingber, 2006).

9.4. Finite-element models

A fourth class of models, termed finite-element models (Fig. 4.4D), simulates cellular mechanics in terms of deformable connections between point masses, with additional physical constraints. The most basic finite-element models essentially represent cells as idealized, polygonal rubber balloons. In such models, the tricellular junctions are small point masses, which are connected to the other tricellular junctions by idealized, straight springs. Keeping with the balloon analogy, the internal pressure is taken to be inversely proportional to the 2D cellular area (Prusinkiewicz and

Lindenmayer, 1990). On the other end of the complexity spectrum, Brodland and colleagues (Brodland and Wiebe, 2004; Chen and Brodland, 2000) have developed rigorous finite-element simulators of epithelial growth and morphogenesis based on mechanical properties including viscosities and interfacial tensions. Such models have been used to answer fundamental questions about the macroscopic mechanical effects of cellular anisotropy, and about whether lamellipodia might be sufficient to drive convergent extension (Brodland, 2006; Brodland and Veldhuis, 2002; Brodland and Wiebe, 2004). They have also been used as counterevidence against the differential adhesion hypothesis of cell sorting (Brodland, 2002). Thus, finite-element models are ideal for answering fundamentally mechanical questions. However, they are also among the most complex models to implement.

Ultimately, whatever the underlying model of the cellular mechanics, the goal of a geometrical model is to asymptotically capture the local geometry of cells. This is not just a modeling issue; dynamic control of cell geometry is a fundamental problem that a developing tissue must solve to grow and pattern itself in a robust, reproducible way. To consider just one of the challenges, when the new side is placed between the new daughter cells, how long should the side be? After many iterations of a division algorithm, if the lengths of the inserted sides are not realistically implemented, then the global statistics of cell geometry will be affected. Also, how much tension should a newly inserted side be under? This is a free parameter in most simulations, but could be estimated from the ablation methods of Farhadifar *et al.* (2007). Depending on the system, steady-state cell geometry may also feed back on tissue growth by influencing spindle orientation. In the future, we anticipate that robust geometrical models will serve as dynamic lattices upon which complex models of cell–cell signaling and morphogenesis can be layered. The intricate feedback between genetics, geometry, and biophysics will likely bring us one step closer to realistic simulations of development.

10. Putting It All Together: Genetics, Geometry, and Biophysics

Geometrical models of epithelial organization are alone insufficient to provide insight into how the simplest tissues grow and pattern themselves. Building on the geometric and biophysical machinery discussed above, it is necessary to integrate active cellular behavior into the picture, which will eventually be understood at the intersection of genetics, geometry, and biophysics. Below we discuss four systems in which all three components interact strongly, and consider the implications for morphogenesis.

One of the most basic aspects of organ development concerns how cells in a growing tissue know when to stop proliferating, or when they are proliferating too quickly. In the *Drosophila* wing disk, it has been observed that the proliferation rate is on average roughly uniform across the epithelium, with some heterogeneity (Dubatolova and Omelyanchuk, 2004; Milan *et al.*, 1996; Shraiman, 2005). In this system, cells that divide more slowly than their neighbors are eliminated by cell death, while those that divide too rapidly are able to target slower-dividing neighbors for elimination (Li and Baker, 2007). This phenomenon, termed cell competition, suggests that cells know how quickly they are proliferating relative to their neighbors (de la Cova *et al.*, 2004; Morata and Ripoll, 1975; Simpson, 1979; Simpson and Morata, 1981). How this information is computed is not known. Recently, Shraiman (2005) analyzed the continuous mechanical implications of differential rates of growth, and has proposed a mechanical basis for a negative feedback mechanism regulating cellular growth and apoptosis, which is implicated in cell competition. More recently, Hufnagel *et al.* (2007) demonstrated, using a geometrical, energy-based polygonal model not unlike the model of Farhadifar *et al.* (2007), that mechanical feedback is a potential mechanism for controlling organ size. This model achieves a uniform rate of cellular proliferation and uses a plausible mechanism for an organ to sense when it has reached a critical size (Hufnagel *et al.*, 2007). Such models demonstrate the sufficiency of mechanical stress as a negative regulator of growth to reproduce empirically observed growth trends. However, there are plausible alternatives. Recently, Senoo-Matsuda and Johnston (2007) demonstrated experimental *in vitro* evidence of bidirectional diffusible signaling molecules to explain how cells know their growth rates relative to their neighbors during cell competition. It is not clear whether such a mechanism could operate *in vivo*, or between wild-type cells. Perhaps a simple diffusion model based on these experiments could be extended in a geometrical framework as an alternative to the stress-based models of Hufnagel *et al.* (2007) and Shraiman (2005).

To consider a second system in which biophysics and cell geometry are both essential in the context of genetics, we look at the problem of hexagonal repacking that occurs during pupal wing morphogenesis, also in *Drosophila*. During this stage of wing development, the frequency of hexagons increases from approximately 45% to nearly 80%, and appears to result from iterative T1 transitions (Classen *et al.*, 2005). The work of Classen *et al.* (2005) suggests that cells recycle cadherin during junctional remodeling. Additionally, mutants for PCP proteins and dynamin are defective in hexagonal repacking (Classen *et al.*, 2005). Based on this evidence, it is tempting to speculate that the cells actively regulate the localization of cadherin so as to enable a T1 transition to a lower energy configuration at each junctional remodeling step, and thus bring their packing configuration closer to a hexagonal lattice. One might further

speculate that the PCP pathway biases each step in the sequence of iterations. Geometrical modeling would be a highly appropriate approach with which to study the repacking. An open question is why an iterative T1 procedure is not sufficient to make the lattice perfectly hexagonal. Is it simply a matter of structural noise, or is this the lowest energy state achievable?

A related question concerns why the pupal wing disk undergoes repacking in the first place. One possibility concerns the directional uniformity of the tiny hairs that point distally in wild-type wings, but can have different orientations in PCP mutants. Recently, Amonlirdviman *et al.* (2005) modeled PCP as a reaction–diffusion system utilizing directionally biased positive feedback on a perfectly regular hexagonal lattice. Importantly, however, the hexagonally packed pupal wing is not perfectly hexagonal and regular. A natural question is whether the proposed mechanism would function just as well on a more realistic irregular lattice. Recently, Ma *et al.* (2008) tested these assumptions both experimentally and computationally. Their analysis strongly suggests that planar packing geometry is a critical parameter for the proper functioning of the PCP-based distal hair alignment mechanism. The earlier work of Classen *et al.* (2005) did not uncover such a correlation, although their topological metric may not have been sensitive enough to detect such differences. A second possible reason for hexagonal repacking is simply structural. Would a more densely packed wing be stiffer or stronger, or result in structure with more homogenous mechanical properties? Might two hexagonally packed wings have better mirror-image symmetry than two irregularly packed wings? By simulating very regular versus very irregular packings, a finite-element model could yield insight into these questions. The *Drosophila rho-associated kinase* (*Drok*) gene has been shown to link PCP signaling to the cytoskeleton (Winter *et al.*, 2001). Therefore, it is also possible that PCP programs are able to change the packing geometry, which would bring the feedback full circle.

A third system in which biophysics strongly interacts with cellular geometry in a genetic context is in the *Drosophila* embryonic epithelium during germ-band extension. There are two very different analyses of this process, which have significantly different implications biophysically. The first analysis describes germ-band extension as an active process involving iterative, directional T1 transitions (Bertet *et al.*, 2004). The second describes complex rearrangements of cellular neighbor relationships, termed ''rosettes,'' which may involve local tissue-level coordination (Blankenship *et al.*, 2006). In terms of biophysical, geometric simulation, a very important issue concerns whether rosette-like structures are actively controlled tissue movements, or whether they are expected to emerge by chance from multiple, locally aligned T1 processes.

A recent study by Rauzi *et al.* (2008) provides evidence for the latter hypothesis based on cell and tissue-level biophysical simulations of

germ-band extension. The argument rests on two observations. First, using only T1 processes, and a physical model and parameter regime consistent with empirical measurements of germ-band elongation as a function of the number of T1 iterations, the authors observe similar frequencies of rosette like structures *in silico* and *in vivo*, which in both cases are rare. Second, it is shown experimentally that rosette structures can be decomposed into multiple T1-like processes using laser ablation. Moreover, such ablations can be used to infer similar levels of tension for the two processes (Rauzi *et al.*, 2008). Importantly, this analysis shows that chance alignments of three-way vertices, in combination with T1 processes, may be sufficient to produce rosettes at realistic frequencies. However, it does not preclude the existence of active biological programs which align and coordinate rosette formation. Analysis of the distribution of n-way vertices (where n is an integer), may be a useful way to distinguish between the two cases. Additional simulations, which consider different models of how elastic tension varies with junction orientation, are needed to ensure that the frequencies generated are not an artifact of the modeling assumptions (Rauzi *et al.*, 2008). Such methods might be complemented by geometric analysis of rosette formation, as well as genetic screens for rosette-formation defects. Thus, whether rosette formation is a process distinct from T1 transitions in the germ band is currently unresolved.

A fourth biophysically and genetically complex morphogenetic process is convergent extension in the notochord of the Ascidian *Ciona Savignyi*. Here, 40 cells in an initially rounded packing shape intercalate to produce a single column of flattened cells. The intercalation process involves penetration of cellular projections between neighboring cells (Miyamoto and Crowther, 1985). The system is especially well suited to address convergent extension in a biophysical context because cells (1) do not divide and (2) depend less strongly on neighboring tissues for their movement than they might in a vertebrate system (Veeman *et al.*, 2008). It was recently shown that normal convergent extension in *Ciona* requires the Prickled protein (encoded by *aim*), which biases the direction of lamellipodial extensions that are believed to drive convergent extension (Jiang *et al.*, 2005). A subsequent analysis of the mutant *chongmague* (*chm*) suggests that another reason that convergent extension fails in the *prickled* mutant is because PCP signaling is required for maintenance of the polarized distribution of a laminin protein encoded by *chm*. Moreover, even in the absence of PCP, *chm* mutants are able to partially complete convergent extension (Veeman *et al.*, 2008). Therefore, in this organism, there is a complex interaction between planar polarity signaling, laminin maintenance/polarization, and the biophysics of convergent extension. Mutation of the PCP player *disheveled* is also known to disrupt convergent extension in *Ciona intestinalis* (Keys *et al.*, 2002). In *Xenopus*, expressing a mutant form of *Disheveled* causes defects both in cellular polarization and in convergent extension (Wallingford *et al.*, 2000). The complexity of

convergent extension highlights why traditional genetic analysis alone is insufficient to describe these PCP and convergent extension phenotypes.

In terms of a biophysical understanding, the extended Potts model has been used to successfully capture the cellular rearrangements seen in convergent extension in *Xenopus* based on anisotropic differential adhesion. Interestingly, such models are also able to mimic Ascidian convergent extension (Zajac *et al.*, 2003). However, energy-based models are not mechanistic, and additional work remains to be done to determine how the separate forces indirectly associated with *chm* and *prickled* contribute to convergent extension. Previous models were also successful in reproducing convergent extension but require additional constraints (Weliky *et al.*, 1991). One possible next step might be to layer simulations of PCP components on top of the physical models to test how these rearrangements might be controlled by PCP.

11. Future Directions

Some of the primary challenges in understanding both cellular topology and cellular geometry are not theoretical, but instead empirical and technological. We suggest two key future directions. First, both statistical inference and mathematical modeling depend heavily on empirical constraints. Yet currently, there is no high-throughput means with which to gather empirical statistics on cellular geometry. Classen *et al.* (2005) employed an image processing software package to infer cellular topology, which we have independently tested (W. T. Gibson, unpublished data). Such programs represent a first step in the high-throughput transition, but are currently quite sensitive to experimental noise, scale, and imaging conditions. We therefore argue that the field is currently limited most by statistical power, and image analysis methods. For the same reason, most of the progress in the field has been made in studying static images. Solving problems in image processing will also give the field a substantial boost in studying dynamics. Currently, data are plentifully available, but the available image processing methods are the limiting factor.

One should not be left with the impression that the ''lower'' levels of the complexity hierarchy—topology and geometry—are in any way completely understood. Even at the most basic level, topology, there are areas of statistical inference that have not been attempted. For example, to our knowledge no study has yet attempted to predict the relative frequencies of the different classes of tricellular junctions (the number of such junctions bordering cells having i, j, and k sides, where i, j, and k are arbitrary), probably because such inferences would be difficult to verify empirically. As a consequence, there are limits to our ability to mathematically model the

processes of hexagonal repacking or convergent extension in terms of topology, because both depend on the relative frequencies of the different classes of tricellular junctions. It is also unclear whether dynamical topological models of epithelial proliferation will need to be revisited, because their topological kinetics have never been measured empirically.

Space partitioning plays a prominent role in setting up the geometric structure of epithelia, as can clearly be seen from Dirichlet constructions (Honda, 1983). Nevertheless, such constraints are not sufficient to fully specify geometry. A second major hurdle, as image processing improves, is to understand cell geometry as an emergent property of tissue mechanics and cellular rearrangements. The geometric parameters of rearrangement can be measured using live imaging. However, the underlying biophysical forces will have to be inferred, using a combination of statistical inference, modeling, and experimental methods. The work of Farhadifar *et al.* (2007) offers an example of how such properties might be tested experimentally. The final step is to construct geometrical simulators of cell and tissue mechanics, and then to test how closely such models are able to mimic the geometric parameters (including the statistical moments of angle measurements, side lengths, etc.) of actual tissues.

While this chapter has primarily focused on very simple animal tissues, the field has much to learn from the highly realistic models of tissue morphogenesis being developed by the plant community. In some respects, plant tissues are a more natural choice for a model system, due to their ease of culture, the viability of their genetic knockouts, their structural integrity, and their simple, elegant architecture. Recently, Grieneisen *et al.* (2007) considered the transport of Auxin in a growing root using both computational modeling and genetic perturbations. Importantly, the computational model incorporates realistic cellular geometry, and is therefore able to treat diffusion and permeability separately. The work considers the influence of cell geometry on Auxin distribution and transport, as well as the influence of Auxin transport on cell geometry and tissue patterning. Similarly complex models of plant phyllotaxis based on Auxin transport have been developed in the work of Smith *et al.* (2006). If even these complex plant tissues are amenable to computational modeling and experimental validation, then plant epidermis may provide an ideal model system for studying epithelial proliferation and morphogenesis in the future, as originally suggested by Lewis (1928).

12. Conclusion

Development is often considered in terms of gene networks and deterministic decisions, yet the emergent, biophysical properties of a developing tissue are essential for its morphogenesis. These properties emerge

stochastically and macroscopically, and cannot be explicitly encoded into the developmental-genetic program, even if the genetic program is tuned to exploit them. Consequently, these properties are difficult to understand through the traditional logic of molecular-genetic analysis, requiring the creative deployment of new modeling and simulation-based methodologies. Epithelial proliferation as it relates to morphogenesis is perhaps the simplest such relationship in development. The emergence of planar packing geometry is beginning to be understood, and it has certainly been shown to correlate with morphogenetic events. Still, much work remains to be done in order to understand the dynamic relationship between proliferation and epithelial cell packing, and to establish whether packing geometry plays an essential role in morphogenesis. Future work will likely expand the repertoire of quantitative models for tissue architecture and thereby extend our understanding of epithelial morphogenesis beyond the limits of traditional genetic analysis.

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