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Mechanics of Tissue Morphogenesis

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Abbreviations

αSMA	α-Smooth muscle actin
AV	Atrioventricular
CREB	cAMP response element-binding protein
dpf	Days postfertilization
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FGF10	Fibroblast growth factor 10
HH	Hamburger–Hamilton
hpf	Hours post-fertilization
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor
MAPK	Mitogen-activated protein kinase
miRNA	microRNA

PDGF	Platelet-derived growth factor
PIV	Particle velocimetry analysis
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
SRF	Serum response factor
TGF	Transforming growth factor
VEGFR	Vascular endothelial growth factor receptor

12.1 Introduction

How are we made? How does the apparently (and perhaps, deceptively) simple fertilized egg transform from a sphere to the complex geometry of a mature organism? These questions, in one form or another, have fascinated scientists and laypeople for thousands of years. Some of the earliest ideas were that organisms develop from miniature versions of their adult selves, often referred to as homunculi or animalcules, hiding within the heads of sperm. This concept, of *preformationism*, was largely abandoned in the nineteenth century when the cell theory of life became the predominant viewpoint: all living things are made of cells and so development requires that the single fertilized egg divide successively to give rise to the differentiated cell types of the mature organism.

Recent scientific pursuits have been focused on uncovering the genetic *blueprints* of morphogenesis, those genes within the fertilized egg and its subsequent progeny that direct the formation of tissues, organs, and whole organisms. This decidedly reductionist approach has been fruitful, yielding information about thousands of gene products required for embryogenesis and tissue morphogenesis. Nonetheless, the genetic blueprint paradigm has oftentimes bordered on preformationist as well, simply shifting the requirement of a map of the mature organism from one constructed of miniaturized cells and tissues to one encoded by bits of DNA. The completed sequencing of the human genome (and that of other animals) suggests that the answer to the question "How are we made?" may be more complicated than the genes themselves, since there are only ~30,000 gene products and far more morphogenetic events required to turn an egg into a person. Is it possible that all morphogenetic movements, all changes in tissue geometry, can be reduced to a series of predetermined genetically encoded routines? Is it necessary that these details be encoded precisely in the genome?

Somewhat in parallel to investigations focused on the genetics of development have been a series of studies focused on the *mechanics* of development, the forces required to fold the progeny of that single fertilized egg into the tissues and organs that make each of us complete. These studies have viewed developing tissues as physical entities, subject to the laws of matter and physics and therefore responsive to mechanical manipulations. The emerging story is that forces are essential for tissue development and that cells both exert forces and respond to them as well. In some ways, the study of the mechanics of morphogenesis is a rejection of genetic preformationism. It is an acceptance of the laws of physics that all changes in tissue geometry must result from physical forces exerted by or on the tissue that is undergoing morphogenesis.

12.2 Passive Forces in Morphogenesis

The cells and tissues of the developing embryo are exposed to both active and passive mechanical forces. Active forces result from cells and tissues pulling and pushing on each other through ATP-dependent processes. Passive forces include those that result simply from the physical environment of the embryo: pressures and flows from interstitial fluids, buckling of adjacent tissues, and forces from surface tensions. Both active and passive forces can convey morphogenetic information to the tissues upon which they act, and both have been implicated recently in studies of tissue development (Nelson and Gleghorn, 2012; Heisenberg and Bellaiche, 2013; Mammoto et al., 2013). Here, we discuss work implicating fluid forces (flow and pressure) and elastic buckling in tissue morphogenesis.

12.2.1 Fluid Flow

The mature vertebrate animal can basically be considered as a bag of tubes—vessels and ducts that partition different fluids from each other and from the outside world (Figure 12.1). Flow of these fluids through the tubular organ systems of the body is driven by convective transport down pressure gradients. In the cardiovascular system, blood flows from the high-pressure arterial system (mean of 100 mm Hg in the adult human) to the low-pressure capillary and venous system (2–5 mm Hg). This pressure gradient is driven primarily by contraction of the heart, which is an active process, but the fluid flow itself conveys mechanical information to the cells within the vessel wall (Freund et al., 2012). Flow is typically sensed by the cells lining the wall of the tube containing the fluid and is transmitted to these cells via shear stresses exerted upon them. Wall shear stress (τ)

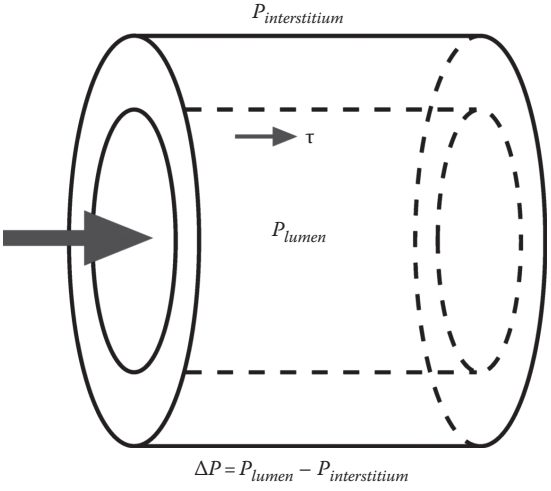

FIGURE 12.1
Passive fluid forces within developing tissues. Fluid is transported within embryonic tubes during morphogenesis. The pressure within the tube (P_{lumen}) minus that outside of the tube ($P_{interstitium}$) gives the transmural pressure across the tube. Mechanical forces from flow of fluid down the tube are transmitted to the layer of cells lining the tube in part through wall shear stress, τ.

is simply the product of the fluid viscosity (μ) and the derivative of the velocity of the fluid at the wall, $\tau = \mu \left.\frac{\partial u}{\partial r}\right|_{\text{wall}}$.

Although it is perhaps not obvious from its mature four-chambered structure, the heart initiates its development as a linear tube (Lopez-Sanchez and Garcia-Martinez, 2011; Miquerol and Kelly, 2013). The heart is the first functioning organ in the embryo and begins contracting (as an elastic impedance pump) early in its own development and while still in a tubular form, prior to the formation of any chambers or valves (Forouhar et al., 2006). Intriguingly, the fluid flow that results from heart function is also *required* for aspects of cardiac morphogenesis in a variety of species, an excellent example of positive reciprocal feedback during embryonic development.

High-speed confocal imaging of the embryonic zebrafish heart and vasculature has enabled quantification of the hemodynamics of blood flow at both early and late stages of development (Hove et al., 2003; Anton et al., 2013; Lee et al., 2013). At 37 h postfertilization (hpf), which is prior to the development of the heart valves, particle image velocimetry (PIV) analysis revealed that the average red blood cell travels through the heart tube at a velocity of approximately 0.5 mm/s. This velocity of flow, coupled with the high viscosity of the fluid and small radius of the developing vascular system, suggests a wall shear stress greater than 1 dyn/cm^2. At 40–50 hpf, the heart tube loops, corresponding to a significant increase in wall shear stress, and later forms the atrium, ventricle, and atrioventricular (AV) valve (Lee et al., 2013). At 4.5 days postfertilization (dpf), PIV analysis showed that the average red blood cell travels an order of magnitude faster than at earlier stages of development, at a rate of >0.5 cm/s through the nascent valves of the heart, which would suggest wall shear stresses of >75 dyn/cm^2 for this region of the tube (Hove et al., 2003). PIV analysis also revealed the presence of vortical flow patterns within the heart itself.

This flow profile appears to be critical for normal morphogenesis of the zebrafish heart tube. Disrupting flow by blocking the tube with an implanted bead leads to a 10-fold reduction in the wall shear stress and prevents formation of the valves and cardiac looping (Hove et al., 2003), the morphogenetic process that builds the chambers from the single heart tube (Taber et al., 2010). Similarly, disturbed flow patterns, such as those observed during vortical flow, are critical for formation of the valves in the zebrafish heart (Vermot et al., 2009). In particular, retrograde flow induces the expression of the Krüppel-like transcription factor *klf2a* in the endothelium of the AV canal; morpholino-mediated knockdown of *klf2a* leads to defects in valvulogenesis, suggesting that this transcription factor indirectly transduces signaling from retrograde flow to the morphogenetic program (Vermot et al., 2009). This signaling pathway may be conserved: reversing flows have been reported in the developing cardiovascular system of many species (Groenendijk et al., 2008), reversing flows induce the expression of *Klf2* by vascular endothelial cells in culture (Dekker et al., 2002), and mice lacking *Klf2* exhibit heart failure and reduced cardiac output (Lee et al., 2006). Normal morphogenesis of the heart valves abolishes the reversing flow patterns and permits the establishment of unidirectional flow. (Of course, other aspects of the mechanics of zebrafish heart development are not universal: cardiac looping is impervious to disrupted blood flow in the early chicken embryo, which is more similar to the human case than is the zebrafish (Aleksandrova et al., 2012).)

In culture, cardiac endothelial cells respond to the forces of fluid flow by altering their actomyosin cytoskeleton, changing their shapes, and modifying their gene expression profile (Hahn and Schwartz, 2008; Boon and Horrevoets, 2009). Shear stress increases

signaling through MAPK/ERK kinase (MEK5) (Surapisitchat et al., 2001), which is thought to enhance the stability of *Klf2* mRNA, thus leading to increased levels of Klf2 protein (Parmar et al., 2006; van Thienen et al., 2006). This signaling is mediated at least in part by microRNA (miRNA)-92a, a negative regulator of *Klf2* that is downregulated in response to flow (Bonauer et al., 2009; Wu et al., 2011). Klf2 enhances the expression of many flow-regulated genes in cultured endothelial cells, including endothelial nitric oxide synthase (eNOS) and thrombomodulin (Dekker et al., 2005; Atkins and Jain, 2007). The extent to which Klf2-mediated regulation of these genes controls mechanical regulation of cardiac morphogenesis remains unclear.

miRNAs are small noncoding RNA molecules that silence the translation of mRNAs (Chen and Rajewsky, 2007; Jackson and Standart, 2007). Several miRNAs are induced in cardiomyocytes and smooth muscle cells in response to mechanical stress (van Rooij et al., 2006; Mohamed et al., 2010). In addition to miR-92a, expression of miR-21 is induced within the endocardium of the embryonic zebrafish heart during the stages at which the valves are formed, in constricted regions of the bending tube in response to reversed blood flow (Banjo et al., 2013). This expression of miR-21 is required for valvulogenesis; miR-21 appears to act at least in part by suppressing the expression of *Sprouty-2* (*spry2*) (Banjo et al., 2013), an inhibitor of extracellular signal-regulated kinase (ERK) signaling in a variety of organs and contexts. Spry2 expression disrupts morphogenesis of the heart by altering cell proliferation.

The molecular structures within the endothelium that sense and respond to fluid flow have been under intense investigation. Several candidates have been proposed to act as mechanotransducers, including ion channels, G-protein-coupled or tyrosine kinase receptors, adhesive proteins, and the glycocalyx (Ando and Yamamoto, 2009). Shear stress has been shown to activate flow-responsive K^+ channels and Cl^- channels (Cooke et al., 1991). The outward flux of K^+ ions causes hyperpolarization of the plasma membrane and induces the inward flow of Ca^{2+} (Luckhoff and Busse, 1990), leading to Ca^{2+}-induced signaling pathways including Ca^{2+}-mediated release of eNOS (Isshiki et al., 1998). Shear stress can also directly activate vascular endothelial growth factor receptors (VEGFRs), independently of ligand binding (Shay-Salit et al., 2002; Jin et al., 2003; Lee and Koh, 2003), and induce downstream signaling through ERK and phosphoinositide 3-kinase (PI3K) (Tseng et al., 1995; Go et al., 1998). One promising candidate mechanotransducer is the primary cilium, a sensory organelle that projects from the apical surface of polarized cells, since defects in ciliogenesis within the endothelium disrupt heart development in mice (Slough et al., 2008). The primary cilia on the surfaces of endothelial cells are thought to bend in response to shear stress, and this bending increases the permeability of ion channels and thus permits the influx of Ca^{2+} (Nauli et al., 2003; Hierck et al., 2008; AbouAlaiwi et al., 2009). Flow can thus both directly and indirectly induce signaling within cells in intimate contact with fluid.

While endothelial cells lining the developing vascular system are the direct recipients of information from fluid flow, other cells not immediately adjacent to the fluid respond as well. Looping of the embryonic heart requires that the heart tube bend and twist around itself, while ballooning corresponds to the emergence of bulges within the tube that create the shape of the cardiac chambers. In the zebrafish heart tube, these morphogenetic events appear to be driven by changes in the shape of individual cardiomyocytes within the walls of the tube, with cells at the outer curvature of the nascent chambers becoming flattened and elongated (Auman et al., 2007). Importantly, blood flow is critical for these shape changes, as they are abolished when flow is disrupted. Although it remains unclear how the cardiomyocytes are sensing fluid flow, there are two obvious possibilities. First, the

endothelium may be responding to flow and communicating this response chemically to the myocardium. Second, the myocardium may be sensing pressure, rather than flow, and responding to this parameter. Future work is needed to define precisely how far the forces from fluid flow can be transmitted into the vessel wall during its morphogenesis.

12.2.2 Fluid Pressure

In any system of fluid-filled tubes, pressure differentials can develop along a given tube or across its wall (Figure 12.1). The latter is known as a transmural (*across wall*) pressure and is prevalent within both mature and developing organisms. The magnitude of this pressure difference (pressure in the lumen minus pressure surrounding the tube) appears to be necessary for normal morphogenesis of several organ systems.

In the developing lung, the fetal airways are lined by one or more layers of epithelial cells that surround the lumen. The developing pulmonary epithelium secretes fluid into the lumen of the airways (Alcorn et al., 1977; Fewell et al., 1983; Harding and Hooper, 1996), and this fluid is sufficient to generate a positive distending transmural pressure of approximately 200–400 Pa (1.5–3 mm Hg) in several animal models because the fetal larynx is closed at these stages of development (Vilos and Liggins, 1982; Hooper et al., 1993; Blewett et al., 1996; Schittny et al., 2000). In fetal lambs and rabbits, chronic drainage of the airways resulting from tracheostomy leads to pulmonary hypoplasia (underdeveloped lungs with a reduced number of airways and alveoli) (Alcorn et al., 1977; Fewell et al., 1983). In contrast, tracheal ligation or laryngeal atresia results in a buildup of fluid and leads to pulmonary hyperplasia (enlarged lungs with an increased number of airways) (Alcorn et al., 1977; Wigglesworth et al., 1987; Moessinger et al., 1990; Nardo et al., 1995; Keramidaris et al., 1996; Kitano et al., 1998; Nardo et al., 1998; Kitano et al., 1999). Fetal pulmonary hypoplasia is a common finding in neonatal autopsies and often copresents with anatomic changes to the fetal chest cavity that would disrupt transmural pressure (Finegold et al., 1971; Goldstein and Reid, 1980; Liggins and Kitterman, 1981; George et al., 1987; Greenough, 2000; Jesudason, 2007).

Although the underlying molecular mechanisms remain to be uncovered, changes in transmural pressure lead to significant alterations in gene expression in the developing lung. Tracheal occlusion leads to an increase in cell proliferation and expression of insulin-like growth factor (IGF)-II in fetal lambs (Hooper et al., 1993). Conversely, draining the airways of the lung leads to a reduction in proliferation and expression of IGF-II.

At least some of the effects of pressure on morphogenesis, gene expression, and cell differentiation in the example of the developing lung appear to result from mechanical stretch. In any thick-walled viscoelastic tube, changing the relative magnitude of the pressure within the tube will change the extent to which the wall is stretched. Applying intermittent mechanical stretch was found to result in an increase in the proliferation of fetal rat lung epithelial cells and fibroblasts in culture (Liu et al., 1992), consistent with the effects of increased transmural pressure on fetal lungs in vivo (Hooper et al., 1993). Mechanical stretch led to an increase in the expression of platelet-derived growth factor (PDGF), and antisense-mediated downregulation of PDGF or treatment with anti-PDGF function-blocking antibodies blocked the effects of stretch on the proliferation of fetal rat lung cells (Liu et al., 1995a). Mechanical stretch also led to an increase in calcium influx in fetal lung cells through stretch-activated ion channels (Liu et al., 1994) and activated signaling via protein kinase C (PKC) (Liu et al., 1995b). Since PKC has been shown to regulate the expression of PDGF in endothelial cells (Hsieh et al., 1992), it is possible that stretch enhances the proliferation of the cells of the fetal lung through a similar mechanism.

At the same time as the airway epithelium is developing, the surrounding pulmonary mesenchyme is differentiating into the cell types present in the mature lung. The most prominent mesenchymal cell type to form during this period is the pulmonary smooth muscle, which differentiates in a cranial to caudal direction and forms a sheath that envelops the developing airway epithelium (Collet and Des Biens, 1974; Sparrow et al., 1999). Transmural pressure appears to be critical for development of the airway smooth muscle and also acts at least in part through stretch. Mechanical stretch induces the expression of smooth muscle–specific genes, including α-smooth muscle actin (αSMA) and smooth muscle myosin, by undifferentiated pulmonary mesenchymal cells (Yang et al., 2000). This increase in myogenesis correlates with an increase in the expression of serum response factor (SRF), as well as a decrease in the expression of the splice variant SRFΔ5 (Yang et al., 2000). SRF is a transcription factor that stimulates the expression of a wide range of smooth muscle–specific genes (Pipes et al., 2006). Conversely, SRFΔ5 blocks myogenesis in culture (Belaguli et al., 1999; Kemp and Metcalfe, 2000) and is expressed aberrantly in hypoplastic lungs from human fetuses (Yang et al., 2000), suggesting that transmural pressure regulates lung development in part by regulating alternative splicing.

Transmural pressure represents the difference in fluid pressures between a fluid-filled tube (such as the airways of the developing lung or the arterial system) and the tissue surrounding the tube. Although normally atmospheric, under some conditions, interstitial fluid can build up and generate magnitudes of interstitial fluid pressure (IFP) (Figure 12.1) greater than atmospheric (Schmid-Schonbein, 1990). Interstitial fluid accumulates between the cells within tissues due to transvascular passage of plasma fluid and is normally drained in the mature organism by the lymphatic system (Aukland and Reed, 1993). Recent studies have revealed that the development of the lymphatic vasculature, a process known as lymphangiogenesis, is regulated in part by IFP (Planas-Paz and Lammert, 2013). In the mouse, increases in IFP correlate with stretch and proliferation of lymphatic endothelial cells, and decreasing IFP reduces the activity of the major lymphatic regulator, VEGFR3 by these cells (Planas-Paz et al., 2012). Fluid pressure, whether transmural or interstitial, can thus have significant impact on tissue morphogenesis in the developing embryo.

12.2.3 Buckling

Many vertebrate organs, including the lungs, esophagus, intestine, blood vessels, exocrine glands, and kidneys, are comprised of an epithelial compartment surrounded by one or more layers of mesenchyme. This topological arrangement separates the epithelial lumen from the surrounding tissue and permits the fluid within the lumen to undergo convective transport down the tube or diffusive exchange across the wall of the tube. For organs that specialize in nutrient exchange, such as the intestine, the surface area of the epithelium is a limiting factor in the rate of transport. The luminal epithelium in these organs thus increases its surface area by adding a third dimension in the form of wrinkles or folds, in a process known as mucosal folding.

Intriguingly, these epithelial surfaces do not start out wrinkled, as most are initially smooth layers of epithelium in the form of tubes or sheets. Several mechanisms have been proposed to explain the morphogenesis of epithelial wrinkling, including localized expression of wrinkle-inducing genes in the surrounding mesenchyme (Nelson, 2013). Physically, however, epithelial tubes can be considered as elastic thick-walled cylindrical shells. When subjected to external compression, elastic shells are unstable and will buckle inward in predictable patterns that depend on the geometry of the shell (thickness and

diameter) and its mechanical properties (elastic modulus and Poisson ratio) (Wang and Ertepinar, 1972; Papadaki, 2008). The compression that induces the buckling can be driven by active forces, such as something contracting around the shell, or passive forces, such as an increase in hydrostatic pressure resulting from a buildup of fluid surrounding the shell. Either way, however, the elastic shell itself undergoes a passive change in shape. Physical explanations of epithelial wrinkling thus suggest that the transformation of a smooth surface to a wrinkled one results from a mechanical instability of the inner mucosal cylinder (the epithelium) under compression from the surrounding cylinder of smooth muscle (Ben Amar and Jia, 2013).

The buckling epithelium hypothesis brings with it several predictions. First, the diameter of the epithelial ring is predicted to be larger when the mucosal epithelium is surgically separated from its surrounding mesenchyme. This is indeed the case for the porcine esophagus (Yang et al., 2007) and chicken small intestine (Shyer et al., 2013). Second, the number of folds is predicted to decrease as the stiffness of the epithelial wall relative to that of the surrounding mesenchyme increases. Put another way, to achieve any given arrangement of folds in the epithelium, the external forces applied by the surrounding smooth muscle would have to increase as the stiffness of the mucosal layer increases. This appears to be true for some disease states, including the esophagus of patients with systemic scleroderma (Villadsen et al., 2001), the bronchial airways of patients with asthma (Wiggs et al., 1997; Hogg, 2004), and the pharynx of patients with obstructive sleep apnea (Kairaitis, 2012). These conditions have been approximated computationally using continuum models of growing cylindrical surfaces (Hrousis et al., 2002; Papastavrou et al., 2013).

In the duodenal portion of the small intestine, the epithelium is surrounded by three layers of smooth muscle that differentiate from the mesenchyme during the same time period as the epithelium transforms from a smooth surface to a wrinkled sheet (Coulombre and Coulombre, 1958). In chickens and humans, the smooth epithelial sheet sequentially folds into parallel ridges, then zigzag-shaped folds, and then into pillars known as villi. The sequential genesis of these folds matches temporally the sequential differentiation of the three layers of smooth muscle that surround the epithelial wall. Formation of the intestinal villus was first proposed to result from mucosal buckling in the 1950s and was thought to depend on external compression provided by contraction of the three layers of smooth muscle (Coulombre and Coulombre, 1958). However, disrupting smooth muscle contraction by surgically removing portions of the smooth muscle wall does not disrupt epithelial wrinkling (Burgess, 1975). Nonetheless, the presence of the three layers of smooth muscle is critical for folding of the intestinal epithelium. As the surrounding mesenchyme differentiates into smooth muscle, the mesenchyme becomes stiffer; this increase in stiffness appears to be sufficient to compress the epithelium as it grows, thus causing it to buckle inward (Shyer et al., 2013). Replacing the smooth muscle with a sheath of silk of similar mechanical properties is sufficient to induce buckling of the intestinal mucosal epithelium (Shyer et al., 2013), in surprisingly the same sequence of geometric patterns as are observed in the embryo.

Similarly, the cortex (outer surface layer) of the vertebrate brain is initially smooth. In several mammals, including humans, the cortex folds during development to produce the fissures, sulci, and gyri of the mature brain (Molnar and Clowry, 2012). Cortical folding is essential for brain function, as defects are associated with severe mental disorders including autism and schizophrenia (Pavone et al., 1993; Sallet et al., 2003; Hardan et al., 2004; Nordahl et al., 2007; Cachia et al., 2008). Several hypotheses have been proposed to explain the physical mechanisms that underlie cortical folding, including buckling. In contrast to

epithelial tubes, which appear to buckle along their inner surfaces, the developing brain would buckle along its outer surface (Richman et al., 1975; Raghavan et al., 1997). Such surface buckling could arise, as in the intestine, from a mechanical instability between the outer cortical layer and the deeper layers of the brain. In this case, differences in the rates of growth of the cortical layer and its *foundation* tissues would produce compressive stresses that lead to buckling. As in mucosal buckling, cortical buckling would also require that the cortical and subcortical regions of the developing brain be of different stiffness, with one model suggesting a 10-fold difference (Richman et al., 1975). However, a recent study using microindentation approaches found similar mechanical properties for both cortical and subcortical regions of the neonatal ferret brain at any given developmental stage, with a shear modulus ~40 Pa (Xu et al., 2010). A clear answer to this problem will require advanced strategies to measure in situ the mechanical properties of the different regions of the developing brain.

In lieu of purely elastic buckling, an alternate but related mechanism for cortical folding was reported recently by Taber and colleagues (Xu et al., 2010; Bayly et al., 2013). Here, the mechanical stresses between the two layers feed back to induce patterns of growth within the brain, and these patterns of differential growth in the subcortical regions are sufficient to induce folding of the cortex. The pattern of folds (wavelength and depth) depends on the relative rates of growth in the cortex, which expands as a sheet, and growth within the subcortical regions. Nonetheless, the growth rates of different regions of the cortex are presumed to be similar (Van Essen and Maunsell, 1980). Regardless of the underlying biological mechanism, tissue buckling appears to be a common mechanism to fold sheets of cells in the embryo.

12.3 Active Forces in Morphogenesis

Passive forces from fluid pressures and flows act on tissues in much the same way as they act on nonliving elastic materials, bending or buckling the material to result in a change in tissue form. Active forces, however, arise from active contractions by the cells within the tissues (Mammoto et al., 2013). These behaviors can change the shape of the tissues by changing the shapes of the individual cells relative to their neighbors, such that they lead to local or global displacements of the tissue.

12.3.1 Cell Contractions

Cells can generate forces by polymerizing and contracting their cytoskeletal machinery. Most cell-generated contractions result from the sliding of motor proteins, such as myosin, along actin filaments, which results in a local rearrangement and/or shortening of the cytoskeleton (Figure 12.2). Cytoskeletal contractions are transmitted to neighboring cells by spot welds present in the plasma membrane. The most common force-supporting intercellular junction is the adherens junction, which is comprised mainly of cadherin molecules that form homophilic interactions between neighboring cells and connect to the actin cytoskeleton via association with α- and β-catenin (Abe and Takeichi, 2008, Cavey et al., 2008). The forces that result from actomyosin contractions in one cell can thus be transmitted to neighboring cells directly via adherens junctions. When cells are connected together in tissues, actomyosin contractions can generate forces that

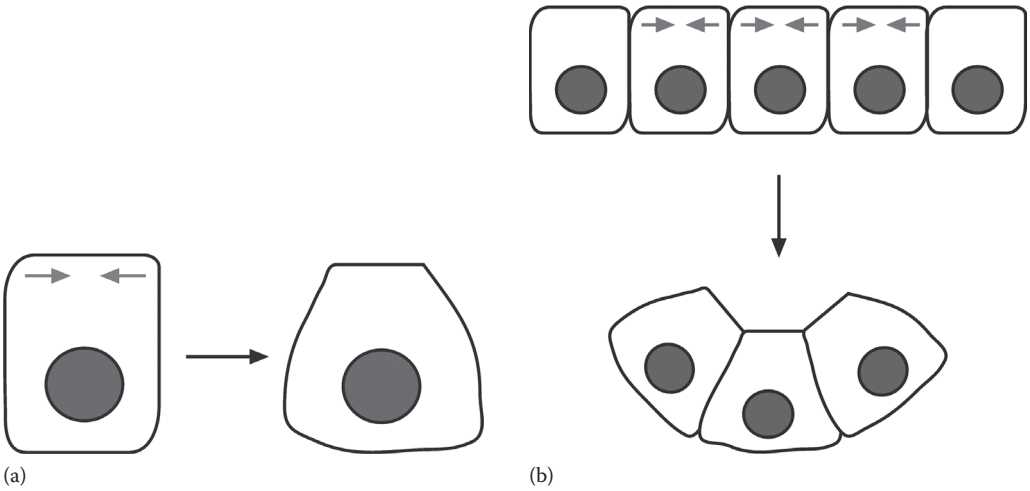

FIGURE 12.2
Active contractions induce morphogenesis. (a) Apical constriction changes the geometry of cells from cubic to trapezoid. (b) When one or more cells within a sheet undergo apical constriction, this generates sufficient force to bend the sheet.

are transmitted several hundred micrometers (several cell diameters) across the tissue (Gjorevski and Nelson, 2012). Such forces can also be transmitted indirectly through the surrounding extracellular matrix (ECM), which acts as a mesh-like substratum supporting the cells. Cells form adhesive junctions with the ECM via transmembrane complexes such as focal adhesions or hemidesmosomes. The former are comprised mainly of integrins, which also link to the actin cytoskeleton via interactions with cytoplasmic plaque proteins including vinculin and talin (Schiller and Fassler, 2013). Actomyosin contractions can thus generate forces on the underlying ECM substratum by pulling on integrins, and these forces can be transmitted to neighboring cells via deformation of the elastic ECM meshwork (Sen et al., 2009).

Coordinated and directed cellular contractions can lead to changes in tissue shape that drive early embryonic development and tissue morphogenesis (Gjorevski and Nelson, 2011). One such commonly observed contractile event is apical constriction, when the actomyosin cytoskeleton localized along the apical cortex of a cell contracts more than that along the basal or lateral surfaces (Sawyer et al., 2010; Rauzi and Lenne, 2011). Apical constriction causes the apical surface area to shrink relative to that of the basal surface and typically transforms cells from a cuboidal or rectangular geometry to a trapezoidal shape (Figure 12.2). Apical constriction drives invagination of the mesoderm during gastrulation in *Drosophila* (Leptin and Grunewald, 1990; Leptin, 1995) and ingression of the endoderm during gastrulation in *Caenorhabditis elegans* (Roh-Johnson et al., 2012). In both cases, pulsatile myosin contractions cause the apical surface to contract periodically, leading to a coordinated inward movement of the cell–cell junctions (Martin et al., 2009; Martin et al., 2010; Roh-Johnson et al., 2012). This change in cell shape causes the tissue to fold.

A similar role for apical constriction has been observed for branching morphogenesis of the airways of the avian lung (Kim et al., 2013). In the embryonic chicken, new buds form sequentially in a cranial-to-caudal direction along the dorsal surface of each primary bronchus via a process known as monopodial (or lateral) budding (Gleghorn et al., 2012).

As each new bud forms, the cross-sectional area of the primary bronchus transforms from circular shape to lemniscate (figure-eight shaped) (Kim et al., 2013). Quantitative morphometric analysis of time-lapse imaging revealed that the airway epithelium of the primary bronchus undergoes apical constriction along both the dorsal and ventral surfaces as new buds form, and both experimental and computational studies showed that this apical constriction was necessary and sufficient to induce the cylindrical tube to fold locally into a bud (Kim et al., 2013). Active mechanical forces thus cause a change in the shape of a subpopulation of cells, inducing morphogenetic folding.

Actomyosin contractions have also been found to be important for driving the early morphogenetic events in the development of the vertebrate brain. As with the heart, the early embryonic brain is initially a relatively straight tube comprised of neuroepithelium. The brain tube then bends and swells to form three primary vesicles (corresponding to the forebrain, midbrain, and hindbrain), and the hindbrain bulges into rhombomeres (Goodrum and Jacobson, 1981; Lowery and Sive, 2009). Regulated contractions of the actomyosin cytoskeleton occur on the basal surface of the neuroepithelium to form the boundary between the midbrain and hindbrain in zebrafish (Gutzman et al., 2008). In contrast, contraction of the apical surface of the neuroepithelium appears to regulate formation of the boundaries and rhombomeres in the brain tube of chicken embryos (Filas et al., 2012). How these local contractions are regulated in the development of any tissue remains unclear, but it will be interesting to determine any similarities between organs as disparate as the brain and the lung.

12.4 Long-Range Morphogenetic Signals: Mechanics, Morphogens, and Electrical Signaling

Tissue morphogenesis is a physical process that results from the changes in shapes of multicellular structures as a function of time. The regulation of this process is undoubtedly complex, but it is clear that the mechanical deformations are both a consequence and a cause of morphogenesis (Ingber, 2005; Nelson et al., 2005). Mechanical forces provide information to individual cells, but are also transmitted over long distances within tissues and whole embryos. This long-range transmission of information occurs rapidly, at the rate of elastic deformation waves (Beloussov et al., 1994), providing an information transfer–biological response system that can operate at the quick rates of embryonic development. Long-range signaling is not limited to mechanical forces, however. Morphogen gradients and electrical signaling have both been found to provide long-range signals that instruct positional cues in developing tissues. It is likely that these integrate with long-range signals from mechanical stresses in the final morphogenesis of tissues.

12.4.1 Morphogen Gradients

Morphogens are biomolecules that are produced or stored in specific locations within an embryo or tissue (Turing, 1952). Transport from their source location causes the formation of concentration gradients in space, such that some populations of cells are exposed to higher concentrations than others. In the strictest definition of the term, the local concentration of the morphogen determines the response of the cells (Wolpert, 1969; Crick, 1970), which depends on intracellular signaling networks and dynamic changes in the activity of

transcription factors (Kicheva et al., 2012). Morphogen-mediated patterning has been well described for the morphogenesis of the early embryo, mammary gland, and lung.

Patterning of the early *Drosophila* embryo is driven in part by diffusion of morphogens including bicoid and dorsal (Grimm et al., 2010). In the earliest stages, this embryo is a syncytium—a single cytoplasm containing several to hundreds of nuclei, depending on the stage of development. Because of the absence of plasma membranes separating the nuclei, any molecule in principle can act as a morphogen in the early *Drosophila* embryo, including those that are normally cytoplasmic or nuclear. (For all other situations, morphogens are limited to extracellular molecules.) The well-studied morphogen, bicoid, is a transcription factor deposited as mRNA at one end of the fertilized egg by cells from its mother (Driever and Nusslein-Volhard, 1988; St Johnston et al., 1989). The protein translated from this mRNA diffuses across the length of the embryo, forming a concentration gradient; the concentration of bicoid in the nucleus of each cell along the length of the embryo defines the gene expression pattern of that cell and the resulting cell fate (Driever and Nusslein-Volhard, 1989). Investigations into the generation of the bicoid concentration gradient have revealed that this morphogen forms its long-range signaling pattern via diffusion, but that the concentration profile is modified by local degradation of the bicoid protein (Gregor et al., 2005, 2007a,b).

Branching morphogenesis of the vertebrate lung is also thought to be driven by concentration gradients of morphogens. In this case, the master regulator of epithelial branching is considered to be fibroblast growth factor 10 (FGF10). FGF10 is expressed in a focal pattern in the submesothelial mesenchyme of the lung, such that new branches in the epithelium emerge at positions adjacent to those with high expression of FGF10 (Bellusci et al., 1997; Park et al., 1998; De Moerlooze et al., 2000; Weaver et al., 2000). The extent to which diffusion might play a role in establishing the concentration profile of FGF10 is unclear. Branching occurs recursively in the lung, and focal expression of FGF10 within the mesenchyme is presumed to as well, suggesting that this protein may have a rather short half-life within the mesenchymal environment of the developing lung. Also unclear is precisely how FGF10 exerts its effects on the epithelial cells to induce branching, since some models presume a chemotactic role (Weaver et al., 2000), whereas others presume a role in proliferation (Menshykau et al., 2012) or differentiation (Volckaert et al., 2013). Computational models have shown that if the concentration of FGF10 directs morphogenesis solely by altering cell proliferation, then this could also lead to the generation of a space-filling epithelial tree (Clement et al., 2010). The critical assumption here is that patterns of proliferation are equal to patterns of morphogenesis, and which has been found to be false for most tissues and organs (Beloussov and Dorfman, 1974).

In the two examples described above, the source of the morphogen is distinct from the cells upon which it acts: bicoid is deposited maternally, whereas FGF10 is secreted by mesenchymal cells to act on adjacent epithelial cells. However, morphogens can also act on their source cells. Branching morphogenesis of the mammary epithelium is a stochastic process involving bifurcations of the terminal ends and lateral branching, depending on the species (or strain of mouse). As it develops, the epithelium synthesizes and secretes transforming growth factor β (TGFβ), which acts as an inhibitor of branching (Silberstein and Daniel, 1987; Daniel et al., 1989; Robinson et al., 1991; Daniel and Robinson, 1992; Pierce et al., 1993; Soriano et al., 1995; Bergstraesser et al., 1996; Joseph et al., 1999; Ewan et al., 2002; Crowley et al., 2005; Serra and Crowley, 2005). Since TGFβ is secreted by the epithelium itself, its diffusion forms a concentration gradient emanating along and away from the epithelium. This results in a lower concentration of TGFβ at the ends of the developing ducts and adjacent to areas of convex curvature (Silberstein et al., 1992, 1990; Ewan et al., 2002),

which are precisely the regions that induce new branches (Nelson et al., 2006; Pavlovich et al., 2011; Zhu and Nelson, 2013). Diffusion of morphogens thus plays a role in instructing the morphogenetic events that build the final architectures of tissues.

12.4.2 Electrical Gradients

While chemical gradients resulting from nonuniform distributions of chemical species have a demonstrated importance in long-range morphogen signaling, electrical gradients (voltages) arising from nonuniform distributions of charge are important in biological patterning as well. A role for bioelectricity in animal physiology was first observed in the eighteenth and nineteenth centuries with the study of nerve stimulation and injury potentials, but it was not until the early twentieth century that bioelectricity was first investigated in morphogenesis (Vanable, 1991). This early work would provide evidence for qualitative correlations between measured voltages and developmental characteristics, such as the degree of growth of embryonic salamanders (Burr and Hovland, 1937) and the final shape of developing gourds (Burr and Sinnott, 1944). Bioelectric patterning has since been investigated in many morphogenetic processes (Adams and Levin, 2013), including limb generation (Altizer et al., 2001), craniofacial development (Vandenberg et al., 2011), and asymmetric left–right patterning (Levin et al., 2002; Fukumoto et al., 2005).

As in classical physics and engineering systems, electrical potential differences within biological systems result from separation of charge. Membrane proteins, predominantly ion channels and transporters, drive this charge segregation across cellular domains and membranes by generating differential ion flow (Adams, 2006). In fact, K^+ flux across the plasma membrane is thought to be a primary contributor to the membrane potential of a cell. Here, Na^+–K^+ pumps import K^+ in exchange for Na^+ to establish intracellular potassium levels that are two orders of magnitude greater than extracellular levels. In a cell without a potential difference across the membrane, this concentration difference will promote the diffusion of K^+ through the membrane via K^+ leak channels. This results in a net movement of positive charge into the extracellular space. It can thus be imagined how a resting membrane potential on the order of −60 mV arises as a by-product of balancing the transport of charged species in an electric field with transport driven by concentration differences across the membrane.

On a larger scale, transepithelial potentials can form across layered epithelia (Adams, 2006). In the skin of the adult frog, a transmembrane potential of ~100 mV is sustained due to net inward flow of Na^+ into the body through Na^+–K^+ ATPases (McCaig et al., 2005). Here, tight junctions prevent leakage so as to maintain separately charged domains. If the epithelium is damaged, this transepithelial potential is short-circuited at the wound site, creating additional electric fields along the apical and basolateral sides of the epithelium, which are thought to drive epithelial cell migration toward the wound site (Zhao, 2009). This may occur through activation of the PI3K signaling pathway at the cathodic side of the cell, where leading edge protrusions form. Other signaling pathways, such as those involving epidermal growth factor receptors (EGFRs) and mitogen-activated protein kinase (MAPK), have also been implicated in mediating a cellular response to an electric field. Furthermore, cells can also sense changes in membrane voltage through voltage-sensitive transport channels (Stock et al., 2013), which alter activity through voltage-dependent conformational changes, in addition to voltage-mediated changes in gene expression, for example, through the cAMP response element-binding protein (CREB) pathway (Deisseroth et al., 2003). In this way, cells and tissues contain the machinery necessary to create and sense voltages.

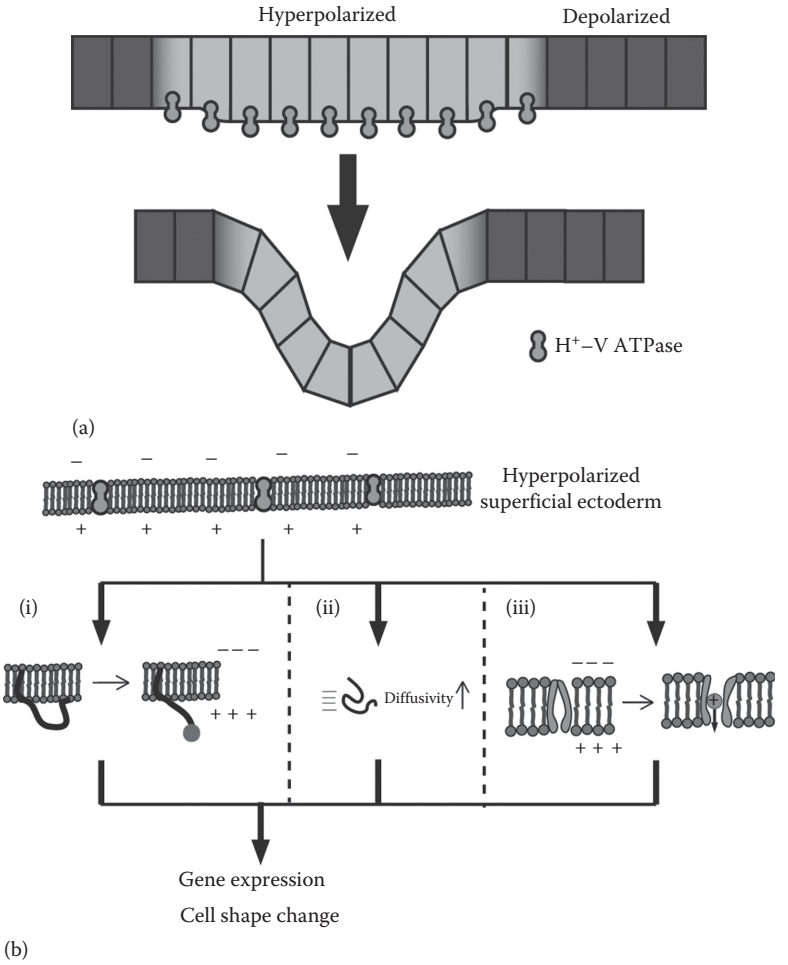

FIGURE 12.3
Membrane voltage patterns invagination and signaling during *Xenopus* neural tube closure. (a) Regions of hyperpolarization, specified by increased H^+–V ATPase pumping activity, pattern locations that will subsequently invaginate. (b) A mechanism proposed by Vandenberg et al. (2011) suggests that the electrophysiological state of the superficial ectoderm cell could (i) (de)activate voltage-gated surface receptors, (ii) alter the diffusivity of molecular signaling components in the extracellular space, and/or (iii) de(activate) voltage-gated ion channels. As a result, this could influence gene expression and cell shape changes of that cell and, through long-range signaling, of neighboring deep ectoderm cells.

The cellular membrane potential is maintained over a long time frame (seconds to days), as opposed to an action potential operating on a millisecond timescale, and has gained attention as a key patterning mechanism in morphogenesis. In developing *Xenopus* embryos, waves of membrane hyperpolarization are observed beginning in stage 13 (Vandenberg et al., 2011). One particular wave occurs during neural tube closure, with streaks of hyperpolarized cells defining regions that will subsequently invaginate (Vandenberg et al., 2011) (Figure 12.3). The H^+–V ATPase, which harnesses the energy of ATP hydrolysis to pump H^+ against its electrochemical gradient, plays a central role in regulating membrane voltage and intracellular pH. Here, this transporter is required just prior to and during neural tube closure. In addition to neurulation, bioelectric patterning is also important in early

asymmetric left–right patterning of vertebrate embryos. After formation of the primitive streak in Hamburger–Hamilton (HH) stage 3 chicken embryos, cells to the left of the streak become depolarized relative to those to the right of the streak (Levin et al., 2002). A voltage as large as 20 mV can be measured across the streak, although it diminishes through HH stage 4. In conjunction with this voltage, gap junctions and H^+–K^+ ATPase expression are required for correct localization of serotonin signaling and proper left–right asymmetry (Fukumoto et al., 2005). These findings suggest a possible mechanism by which differential influx of K^+, followed by K^+ loss via leakage channels in cells to the right of the streak (in chick) or ventral midline (in *Xenopus*), creates an electrical field across a cellular domain connected by gap junctions. According to this model, a low-molecular-weight determinant (perhaps serotonin) could then be transported asymmetrically via electrophoresis to initiate preferential gene expression on a particular side. The interested reader should be directed to Adams and Levin (2013) for additional examples of bioelectric patterning in morphogenesis.

12.4.3 Coupling Mechanical Signals with Morphogen Gradients and Electrical Gradients

Given the similarities in length scales upon which they act, perhaps it is not surprising that information from mechanical forces is increasingly recognized to be coupled with information from gradients in morphogens and electrical signaling. In the case of mammary epithelial branching morphogenesis, new branch sites emerge at positions that are dictated by both high mechanical stresses resulting from cellular contraction and low concentrations of TGFβ resulting from diffusion (Nelson et al., 2006; Gjorevski and Nelson, 2010). The situation is likely to be more complex than a simple Boolean logic AND gate, however, since TGFβ itself can be activated from its latent form by mechanical stress transmitted through integrins by actomyosin contraction (Annes et al., 2004; Jenkins et al., 2006). It remains unclear precisely how these two signals—mechanical and morphogen—are integrated by the tissue during morphogenesis.

There appears to be a similar coupling between mechanical forces and electrical signaling in a number of systems. During establishment of left–right asymmetry, myosin-driven leftward migration generates an asymmetric distribution of cells that secrete sonic hedgehog (SHH) and FGF8 around the node, which is induced by changes in bioelectrical activity regulated by a membrane H^+/K^+ ATPase. Mechanical loading of bone produces gradients in mechanical strains important for its morphogenesis and remodeling; these gradients in strain have been revealed to induce electrical potentials across the morphogenetic tissue (Beck et al., 2002). It will be interesting to determine the extent to which mechanical, chemical, and electrical gradients separately and synergistically regulate tissue morphogenesis.

12.5 Conclusions

One of the greatest mysteries of science is how multicellular organisms achieve their final forms. Although genes, and gene regulatory networks, clearly play a fundamental role in specifying the phenotypes of cells during morphogenesis, the products of these genes must necessarily be translated into mechanical and physical changes within and between

cells to induce tissues to change their shapes. Also clear, however, is that cells and tissues respond to physical forces, which play major roles in the morphogenesis of vertebrate organs in particular. How much of this information is truly passive? To what extent can mechanically induced deformations be explained by the effects of forces acting on viscoelastic materials? How much of this information is active, requiring activation or repression of gene expression? The answers to these questions will shape our understanding of the living world and define whether tissue morphogenesis is the result of emergence or preformationism (Levin, 2012).

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