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Living Tissues Are More than Cell Clusters: The Extracellular Matrix as a Driving Force in Morphogenesis

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Abstract

In the study of morphogenesis, there is a general tendency to look at the extracellular matrix (ECM) as a mechanically passive agent that simply gives support to cells, and consequently, to place all the explanatory burden on cellular behaviors. Here we aimed to show that not only cells, but also the ECM may be an important force of morphogenesis. Understanding the mechanical role of the ECM broadens our view of morphogenesis and stresses the importance of considering embryonic tissues as a composite of cells and ECM.

Keywords

budding; evagination; invagination; mechanical forces; morphological motifs

1. Cell behaviors as drivers of morphogenesis

The tissue deformations that take place during the generation of body plans and organs are commonly understood in terms of collective cell behaviors (e.g., Firmino et al., 2016; Hopyan et al., 2011; Pearl et al., 2017). Cells can drive morphogenesis directly, by generating forces that deform the tissue, or indirectly, by changing the mechanical properties of the tissue, and thus, its response to an externally applied force (e.g., Davidson et al., 2009; Keller et al., 2008; Lecuit et al., 2011). Cells can actively generate *pulling forces* by contracting their cytoskeleton, a behavior that when coordinated in epithelial sheets can lead, for example, to the formation of tubes, closure of openings or invaginations (for a review see Sawyer et al., 2010). Epithelial cells can also generate pulling forces when undergoing apoptosis. Apoptotic cells remain strongly adhered to neighboring cells during their shrinkage, which produces a pulling force that can contribute to the closure of openings or the formation of epithelial invaginations (Ambrosini et al., 2017). In a similar way, epithelial cells also pull their neighbors when they become rounded during mitosis. Independently from their subsequent division, cells at the tracheal placode that round during mitosis accelerate its invagination (Kondo and Hayashi, 2013). Mesenchymal cells also generate

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pulling forces when they remain attached to their surrounding extracellular matrix. This tension can be transmitted to the epithelium and contribute to its remodeling (Ingber, 2006). In this manner, tissue invaginations would not only be driven by the activity of epithelial cells, but they could also originate when the epithelium is pulled by the underlying mesenchyme.

Cells do not only pull, but they also generate *pushing forces* when increasing in size, dividing, elongating, migrating or intercalating (e.g., Keller et al., 2008; Kinebrew and Hilfer, 2001). When randomly distributed throughout the tissue, these cell behaviors would mainly lead to changes in size, but when localized or oriented, they may extend the tissue along specific directions, thereby altering its shape. For several decades, it was commonly thought that the pushing forces generated by dividing cells at specific regions (i.e., localized cell proliferation) was the main driving force behind epithelial budding (e.g., Boehm et al., 2010; Ingber, 2006). This view has recently been challenged by studies showing, for example, that epithelial invaginations precedes rather than lags behind peaks of cells proliferation in branching morphogenesis (Nogawa et al., 1998), or that observed proliferation rates do not predict the morphogenetic changes undergone by the limb bud (Boehm et al., 2010).

Nowadays, pushing forces generated by directed cell behaviors in the mesenchyme are thought to be the main driving force of evaginating primordia (Hopyan et al., 2011). However, a recent study has shown that the limb epithelium is capable of contracting in response to the tension exerted upon it by the expanding mesenchyme, which suggests it may play an active role in limb bud morphogenesis (Lau et al., 2015). Pushing forces can also invaginate an epithelium. For example, the expansion of epithelial cells at their basal side, a mechanism called basal relaxation, is involved in the invagination of the optic vesicle in the chicken embryo, and accompanied by apical constriction, it contributes to the gastrulation of the *Drosophila* egg (for a review see Pearl et al., 2017).

During morphogenesis, some regions of the embryo – or the primordium – will actively generate forces, while others will be subjected to them. The deformation of a tissue in presence of an external force depends on its stiffness, which is determined by the mechanical properties and arrangement of its components (e.g., Davidson et al., 2009). Therefore, cells can indirectly alter the shape of a tissue by changing its stiffness. Cell stiffness is mainly regulated by the cytoskeleton and the cytosolic pressure: cells with either a high content of stress fibers or a high cytosolic pressure are stiffer, i.e., more resistant to shape deformation, than cells with low levels of stress fibers or a low cytosolic pressure (e.g., Chan and Ulfendahl, 1997; Gavara and Chadwick, 2016). These two cellular components can directly contribute to tissue stiffness. For example, the inhibition of either F-actin polymerization by latrunculin B, or myosin II activation by Y27632, reduces the stiffness of the embryonic tissues of the frog *Xenopus laevis* up to 70 % and 50 %, respectively (Zhou et al., 2009). Disruption of actin filaments by blebbistatin decrease the stiffness of embryonic tendons in the chicken embryo by almost 40% (Schiele et al., 2015). However, this direct contribution of the cytoskeleton to tissue stiffness would not be applicable to all its components. For example, microtubules can alter stiffness in some cell lineages, but they do not contribute to tissue stiffness in the frog embryo (Zhou et al., 2010).

Regarding intracellular pressure, it has been shown, for example, that cell swelling straightens and increases the stiffness of the notochord of *Xenopus laevis* embryos (Adams et al., 1990).

It is important to stress that cell behaviors can be either the driving forces of a morphogenetic process or the passive response of an externally applied force. For example, as commented previously, cell extrusion generates a pulling force that contracts the tissue. This force may be a driver of both the dorsal closure and the formation of leg invaginations in *Drosophila* (Ambrosini et al., 2017). However, cell extrusion can also be induced when the tissue is externally compressed: if compression becomes too high, the tissue may release the compressive load by extruding cells (Eisenhoffer et al., 2012). This dual role is applicable to other common cell behaviors, (e.g. cell intercalation, cell migration, cell polarity) (e.g., Bénazéraf et al., 2010; Keller et al., 2008). Therefore, one of the main aims in studies of morphogenesis will be to discern which of the observed cell behaviors represent driving forces and which side-effects.

In the absence of internally generated or externally applied forces, tissues can spontaneously generate forms due to their intrinsic material properties. Tissues behave to some extent like liquids, which adopt the configuration that minimizes their surface free energy. The surface free energy of a cell cluster is minimized when cells pack closely together, maximizing their contact area. In contrast to liquids, the free energy at the cell-cell interfaces or *interfacial tension* is not solely determined by cell adhesion, but also by cortical tension: cell adhesion decreases interfacial tension by increasing the contact area among cells, whereas cortical tension –mainly generated by the cytoskeleton – increases interfacial tension by decreasing the contact area among cells (Lecuit and Lenne, 2007; McMillen and Holley, 2015).

According to the Differential Interfacial Tension hypothesis, an aggregate of cells with different cohesiveness will self-assemble into layers where more cohesive cells are surrounded by less cohesive cells, which minimizes the surface free energy of the tissue (for a review see McMillen and Holley, 2015). Embryonic germ layers differ in their material properties, e.g., the surface ectoderm is less adhesive and stiffer than the deep mesoderm, suggesting that differences in the material properties among embryonic germ layers may contribute to the formation of body plans (Krieg et al., 2008; Maître et al., 2012). Differences in cell adhesion has also been proposed, for example, to explain the separation of the prospective limb mesenchyme from the flank mesenchyme during early limb bud morphogenesis (Damon et al., 2008).

Other morphological motifs that would self-assemble according to the principle of minimization of free energy are the formation of lumens, if the distribution of cell adhesion molecules is not uniform, but localized at a specific region of the cell membrane, and tissue elongation, if cell shape is anisotropic, i.e., cells are longer along one axis (Newman and Bhat, 2008). It is important to stress that the morphogenetic potential derived from the liquid-like behavior was not possible until the appearance of classical cadherins, which conferred fluidity to tissues by allowing the formation of transient cell-cell contacts, and Wnt morphogen, which is involved in cell polarity (Newman, 2016). Liquid tissues is a

distinctive feature of metazoans, which represents a major transition in the evolution of multicellularity (Newman, 2016).

2. A look at the extracellular matrix

Cells can both generate forces and change the mechanical properties of the tissue *indirectly*, by altering their surrounding ECM. This implies that the ECM has the potential of driving morphogenesis (for other roles of the ECM see Daley and Yamada, 2013; Rozario and DeSimone, 2010). The ECM can generate *pulling forces*. For example, collagen fibers contract in response to the traction forces exerted upon them by migrating mesenchymal cells (Harris et al., 1981). In some branching organs, collagen fibers align parallel to the epithelial surface, where a flux of migrating mesenchymal cells has been observed. The contraction of these polarized collagen fibers by mesenchymal cells could compress and fold the epithelial layer, contributing to the formation of clefts (i.e., the split of the tip of a primordium into two parts) (Hieda and Nakanishi, 1997; Wan et al., 2008) (Fig. 1a). This idea is supported by the observation that degradation of collagen inhibits the formation of clefts (for a review see Hieda and Nakanishi, 1997).

By increasing its volume through matrix deposition and/or swelling, the ECM can also generate *pushing forces*. This mechanism has been proposed to explain, for example, the formation of the primary invagination in the sea urchin embryo or the lumen expansion of the *Drosophila* hindgut (Lane et al., 1993; Syed et al., 2012). The sea urchin blastula is formed by a spherical mass of mesenchyme, the blastocoel, surrounded by a monolayer of epithelial cells (Fig. 1b). These epithelial cells are covered internally by the basement membrane, and externally, by the apical ECM. The latter is formed by an inner and an outer hyaline layer, which is in direct contact with the external environment (Fig. 1b).

Experimental data suggest that the secretion of ECM by invaginating epithelial cells at their apical side plays a fundamental role in this morphogenetic process. Based on this finding, it has been proposed that the swelling of the newly deposited matrix at the inner, but not at the outer hyaline layer, makes the apical ECM bend in a way analogous to a heated bimetallic strip (Fig. 1b) (Lane et al., 1993). The bending of the apical ECM would push the epithelial layer inwards, forming the primary invagination (Fig. 1b).

In the *Drosophila* embryo, the hindgut is formed anteriorly by a small curved intestine (Si), and posteriorly, by a large intestine (Li) that connects to the rectum. From stage 14 to 16, the lumen diameter of the hindgut changes from being uniform to being twofold wider at the Si. It has been suggested that these changes in lumen expansion are regulated by the secretion of a matrix protein, called tenectin (Tnc), which is expressed at a higher levels at the Si (Syed et al., 2012) (Fig. 1c). In support of this hypothesis, *tnc* mutants present a hindgut of a similar length but markedly narrower than wild type embryos. Ectopic expression of *tnc* increases the diameter of the lumen and stretches the surrounding cells, suggesting that the intraluminal accumulation of Tnc expands the hindgut by increasing its internal volume (i.e., producing a pushing force) (Syed et al., 2012). However, to what extent the swelling of the new deposited matrix is necessary for tenectin to expand the tube remains unknown.

By increasing fiber crosslinks, fiber density and/or fiber orientation, the ECM can alter tissue stiffness, and consequently, the shape changes the tissue will undergo in presence of an externally applied force. At early stages of eye morphogenesis, the optic vesicle contacts with the surface ectoderm, which induces the thickening (i.e., placode) and secretion of ECM at their contact area. Subsequently, both placodes invaginate to form the optic cup and the lens vesicle, respectively (Fig. 2a). The surface ectoderm is necessary for inducing both the placode and matrix secretion by the optic vesicle, but it does not directly contribute to its invagination. It has been shown that disrupting the ECM at early stages of invagination causes the optic vesicle to round again, suggesting that the ECM plays a relevant role during this process (Oltean et al., 2016). Based on the observation that the ECM is markedly stiffer than the optic vesicle, a differential growth mechanism analogous to the differential expansion of a heated bimetallic strip, has been proposed, in which the expansion of the fast growing retinal placode is constrained by a stiff ECM attached to it, forcing the placode to invaginate (Oltean et al., 2016) (Fig. 2a). (Note that the swelling of the ECM generates the pushing force that leads to invagination of the sea urchin blastula, whereas in the morphogenesis of the optic cup, the ECM contributes by resisting the force exerted upon it by the proliferating retinal placode).

Drosophila eggs transform from a sphere to an ellipsoid early in development. It has been shown that during this stage, Type IV collagen forms polarized fibrils perpendicular to the axis of body elongation, suggesting that the ECM may act as a “corset” which shapes the egg by constraining growth along the dorso-ventral axis (Haigo and Bilder, 2011) (Fig. 2b). In agreement with this hypothesis, elongated eggs with polarized Type IV collagen fibrils become spherical when they are exposed to collagenase (Haigo and Bilder, 2011). The ECM sheath that surrounds the notochord of *Xenopus* may play a similar role. The orientation of the fibers that form this sheath would determine in which direction the notochord would expand, once it is inflated by cell swelling (Adams et al., 1990).

It has been observed that the ECM at the epithelial-mesenchymal interface, i.e. the basement membrane, is not uniformly distributed during morphogenesis, but it is thinner at those regions where the epithelium is highly remodeled and thicker where it is more stable (Bernfield, 1977). Based on this observation, it has been proposed that the thickness of the basement membrane could drive tissue morphogenesis by regulating the mechanical pliability of the epithelium, as a thinner layer of matrix fibers would be easier to deform by external forces than a thicker layer (Harunaga et al., 2014; Ingber, 2006) (Fig. 2c).

In the absence of internally generated or externally applied forces, the configuration an aggregate of cells will take depends not only on cell-cell interactions, but also on cell-matrix interactions. When the affinity between the more cohesive cells and the ECM is greater than the homotypic affinity of the less cohesive cells, and only the more cohesive cells interact with the ECM (cell-ECM interaction is binary), the presence of ECM reverses the configuration predicted by the Differential Interfacial Tension hypothesis (Cerchiari et al., 2015) (Fig. 3). It has been demonstrated that this binary cell-ECM interaction dominates cell sorting, ensuring the tissue will adopt the appropriate configuration in spite of changes in cell-cell interactions, i.e., that the outer cells will occupy the tissue boundary – and

consequently, the luminal cells the inner layer – independently of their cell-cell interactions (Cerchiari et al., 2015).

3. Conclusions

This brief overview of the role of mechanical forces in morphogenesis highlights three relevant points. First, there is not a one-to-one relationship between morphogenetic mechanisms and morphological motifs, but many different mechanisms can generate the same morphological motif (i.e., a many-to one relationship), whereas the same mechanism can contribute to the formation of different morphological motifs (i.e., a one-to-many relationship). Considering epithelial cell behaviors only, there are at least seven mechanisms that can drive an invagination, as for example, cell shrinkage at the apical faces due to either actomyosin contraction (apical constriction) or the apicobasal displacement of the nucleus, which in a densely packed epithelium lead to cell wedging (basal wedging), cell expansion at the basal faces (basal relaxation) or the pulling of the epithelial surface by cell apoptosis (apical cable-driven buckling) (Pearl et al., 2017). As examples of a one-to-many relationship, matrix deposition and/or swelling may lead to the formation of invaginations by a mechanism analogous to the expansion of a heated bimetallic strip (Lane et al., 1993), but when secreted by luminal cells, it contributes to the formation of tubes (Syed et al., 2012). Depending upon their location, the pulling force generated by apoptotic cells can move the edges of two epithelial sheets closer, contributing to the closure of an opening, or buckle the epithelial surface, generating an invaginating primordium (Ambrosini et al., 2017).

Second, a major part of the morphogenetic mechanisms are not mutually exclusive, which implies that a morphological motif may be the result not of a single driving force, but of the coordinated contribution of several mechanisms. For example, during the dorsal closure of the *Drosophila* embryo, the leading edges of the lateral epidermis progressively approximate to each other, at the same time the extraembryonic epithelium that occupies the opening, called the amnioserosa, is being internalized and eventually degraded. There are, at least, two mechanisms that contribute to the closure: 1) the contraction of an actomyosin cable at the leading edge of the lateral epidermis, 2) the autonomous contraction of the amnioserosa, which may be due to cell apoptosis (Kiehart et al., 2000).

Third, all the components of an embryonic tissue can *potentially* contribute to its morphogenesis. The possibility that some of these components play a secondary role should not be assumed a priori, but experimentally tested. Importantly, the contribution of different components to morphogenesis may differ across taxa, which implies that their central role in one species should not be generalized to other species. For example, the ECM is a major component of the mechanical properties of the sea urchin blastula, but not of the frog blastula (Davidson et al., 1999; Zhou et al., 2009).

A look at the ECM can deeply transform our view of a morphogenetic process. For example, during early stages of gastrulation in amniotes, a remarkable, large-scale movement of cells gives place to a central furrow, called the primitive streak, which establishes the midline of the embryo. Although the embryonic tissue at these early stages already possesses a prospective basement membrane, the ECM is often thought to remain static, and that these

tissue movements are driven by directed cell migration. To test this hypothesis, the movement of both cells and ECM fibers was tracked using live imaging of chicken embryos with fluorescent labeled cells and fibronectin fibers. Contrary to the expectations, it has been shown that the ECM moves as much as cells do, and there is little cell autonomous movement relative to the ECM, rather both components move together (Zamir et al., 2008). At present, to what the extent the ECM moves by itself or as a consequence of an externally applied force is unknown (Loganathan et al., 2016), but in any case, these results suggest that directed cell migration may not be a main driving force of early amniote gastrulation. It is also important to mention that, like other cell behaviors, directed cell movement can be a driving force or the passive response of an externally applied force. For example, *in vitro* experiments have shown that both cells and inert particles can be transported in a directional way by forces generated by the assembly of ECM components, a mechanism called matrix-driven translocation (Newman et al., 1985).

Finally, we hope the present work can contribute to change our perspective on the role of the ECM in morphogenesis, as the formation of the primitive streak illustrates, it has the potential to challenge common views in the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Morphogenetic mechanisms of the extracellular matrix via force-generation

The contraction (green arrows) of collagen fibers aligned parallel to the epithelial surface (black fibers) can contribute to the generation of clefts (a). A heated bimetallic strip will curve towards the side with the higher expansion rate (b) (*top*). The swelling of new matrix (orange dots) in the inner layer (IL), but not the outer layer of the apical matrix (OL), will make the ECM to bend inwards in a manner analogous to a bimetallic strip (*bottom*). The bending of the apical matrix will generate a pushing force that could form the primary invagination of the sea urchin embryo (*middle*) (based on Lane et al. 1993). The deposition of the matrix protein Tenectin (Tnc) expands the *Drosophila* hindgut by increasing its luminal volume (i.e., generating a pushing force) in a dose-dependent manner: low (light green) and high (dark green) secretion of Tnc will lead to a thinner or a thicker hindgut region, respectively (c) (based on Syed et al. 2012).

Fig. 2. Morphogenetic mechanisms of the extracellular matrix *via* tissue stiffness

In the chicken embryo, the retinal placode (RP) is attached to the lens placode (LP) by a stiff ECM (orange fibers) (a). This stiff matrix would behave like the low expansion component of a bimetallic strip (*top*), forcing the optic vesicle (OV) to invaginate by constraining the expansion of a fast growing RP, thereby forming the optic cup (*bottom*) (SE: surface ectoderm; LV: lens vesicle) (based on Oltean et al. 2016). The polarization of the basement membrane (green fibers) in the *Drosophila* egg allows growth along the anterior-posterior axis, while constraining it along the dorso-ventral axis. This differential growth elongates the egg (redrawn from Harunaga, 2014) (b). The thinning of the basement membrane (blue fibers) may increase the mechanical pliability of the epithelium, which would consequently invaginate due to the tension exerted upon it by the mesenchymal cells (green arrows) (c).

Fig. 3. Morphogenetic mechanisms of the extracellular matrix *via* cohesiveness

According to the Differential Interfacial Tension hypothesis, an aggregate of cells (center) will self-assemble into layers where the more cohesive cells (pink) will be surrounded by the less cohesive cells (green), the configuration that minimizes the surface free energy of the tissue (left). This configuration can be reversed in presence of ECM (right) (see text for details).