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Expanding the boundaries of synthetic development

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Abstract

Embryonic tissue boundaries are critical to not only cement newly patterned structures during development, but also to serve as organizing centers for subsequent rounds of morphogenesis. Although this latter role is especially difficult to study *in vivo*, synthetic embryology offers a new vantage point and fresh opportunities. In this review, we cover recent progress towards understanding and controlling *in vitro* boundaries and how they impact synthetic model systems. A key point this survey highlights is that the outcome of self-organization is strongly dependent on the boundary imposed, and new insight into the complex functions of embryonic boundaries will be necessary to create better self-organizing tissues for basic science, drug development, and regenerative medicine.

Keywords

Synthetic embryology; Self-organization; Boundaries; Bioengineering

1. Introduction

Tissue boundaries are a defining feature of complex multi-cellular life. From the fibrous connective layers that encapsulate organs and muscles, to the basement membranes that divide epithelia from mesenchyme, to the myelin sheaths that electrically insulate nerve cells, these boundaries are ubiquitous throughout animal bodies. Tissue boundaries may be cellular or acellular, and most generally can be defined as discontinuities in tissue structure or in the transmission of chemical, mechanical, or other intercellular information. Their purpose is not only to divide different groups of cells from one another and so allow for specialization and co-existence within the same organism, but also to shape and organize the signals that different cells or different regions send and receive, thus permitting the emergence of higher-order functions.

The progressive creation and elaboration of boundaries during embryogenesis is of considerable interest to developmental biologists, and the field has learned a great deal about mechanisms of boundary formation as well as boundary fusion, such as occurs during neural

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tube closure (Ray and Niswander, 2012). The study of processes such as somitogenesis and hindbrain segmentation in mouse, as well as blastoderm and imaginal disc compartmentalization in fly, have helped identify many of the key molecules and mechanisms required for boundary formation (Fagotto, 2015, 2020; Dahmann et al., 2011). Consistent with their importance in animal evolution, developmental biologists have also found that these processes and the molecules that regulate them are largely conserved across diverse species.

Today it has become increasingly clear that embryonic boundaries fulfill additional and vital functions in development distinct from their role in the adult. For instance, many developmental boundaries are transient and dynamic, and either completely disappear or dramatically transform beyond recognition by the time the animal has reached adult form (see Fig. 1 for example). If only considering the function of boundaries from the perspective of their function in the adult organism, this presents a conundrum: what is the point of creating a boundary only to remove it or drastically change it later? The answer is that boundaries also guide development by breaking symmetries and constraining the possible outcomes of developmental events by limiting where cells can move or by limiting the transmission of signals to within specific regions of the embryo. Embryonic boundaries also create new chemical, mechanical, and electrical anisotropies that are necessary to catalyse and guide the next round of morphogenesis. Thus embryonic boundaries are endpoints that "lock in" newly patterned structure, but they are also foundations upon which subsequent morphogenetic events are assembled.

How boundaries constrain and guide developmental events is an exciting question that is challenging to address *in vivo* despite the emergence of powerful new imaging tools for tracking cells deep in living tissues, as well as genetic tools for perturbing cells like CRISPR. Boundaries can be nearly invisible, exist on multiple spatial scales, and are not encoded by individual genes. This often makes imaging whole boundaries *in vivo* impossible, genetic knockouts largely unviable or ambiguous, and surgical perturbations tricky and requiring high levels of expertise and practice.

Enter synthetic embryology. Synthetic embryology offers a fresh vantage point and unique opportunities for studying the role of boundaries during development for two reasons: (1) in trying to reconstitute or redesign a particular embryonic process *in vitro* the researcher must consciously introduce boundaries that would have been provided by default in the embryo, and (2), in this bottom-up approach the researcher gains a level of control over boundaries beyond what is possible *in vivo*. Together, the requirement to introduce boundaries and the freedom to choose how to do so presents an unrivalled opportunity to reveal new and unanticipated functions. Here we review recent progress from the emerging field of synthetic embryology to reveal the role of embryonic boundaries during development, and summarize the current state-of-the-art tools for creating and shaping boundaries *in vitro*. A central point we hope to illuminate is that the outcome of self-organization *in vitro* is strongly influenced by the boundary imposed, and new insight into the complex functions of embryonic boundaries has direct and immediate relevance to creating better self-organizing tissues for basic science, drug development, and regenerative medicine. Finally, we consider challenges

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to recreating boundaries *in vitro* and imagine what sort of boundaries we will need in the future if we are to create fully synthetic embryos and organs.

2. Boundaries in classical embryology

2.1. Tissue affinities and compartment boundaries

As outlined in several recent excellent reviews (Fagotto, 2015, 2020; Dahmann et al., 2011) and briefly summarized here, systematic studies of embryonic boundaries were founded on two striking observations: the ability of cells to organize and sort into distinct domains, and the ability of tissues to form nearly invisible sharp structures between domains across which cells do not mix. The first was originally observed in sponges (Wilson, 1907) and subsequently analyzed in greater depth using frog embryos (Holtfreter, 1939; Townes and Holtfreter, 1955) (Fig. 2a). Based on these analyses, researchers hypothesized that cells possessed “tissue affinities” that allowed them to recognize self and non-self tissues (Holtfreter, 1939). Uncovering the mechanistic basis for tissue affinities and sorting ability thus became a central goal for the field. Advances came with the discovery of cellular adhesion genes such as cadherins (Edelman, 1986), the “differential adhesion hypothesis” of Steinberg (Steinberg, 1970; Foty and Steinberg, 2005; Steinberg and Takeichit, 1994), and the importance of cell contractility in cell sorting (Fig. 2c). The synthesis of these ideas into the “differential interfacial tension hypothesis” ultimately explained these phenomena as arising from differences in interfacial tensions—the ability of cells to dynamically set the mechanical tension of its cellular interfaces and couple these tensions with neighboring cells through the activity of cell adhesion molecules—that drive the cell shape changes and cell rearrangements that underly sorting (Brodland, 2002; Krens et al., 2011; Harris, 1976).

The second striking observation was that the expansion of clones in *Drosophila* larval epithelia became restricted by anatomically imperceptible yet sharp and restrictive “compartments” (Garcia-Bellido et al., 1973; Crick and Lawrence, 1975; Dahmann and Basler, 1999) (Fig. 2b). Similar compartments were subsequently found and studied in the vertebrate embryo, such as the division of the pre-somitic mesoderm along the anterior-posterior axis into somites and the subdivision of the brain into forebrain, midbrain, and hindbrain, and the further segmentation of the hindbrain into rhombomeres (Dahmann et al., 2011). Together with the fly imaginal wing disc and blastoderm, these models form the basis of many classic studies of compartmentalization and boundary formation, and many genes, such as those involved in Eph-Ephrin signalling (Fagotto et al., 2014; Xu et al., 1999), have been discovered from their study and shown to be universally required for proper compartmentalization (Fig. 2c). Subsequent research programs today are also focused on identifying mechanisms of boundary fusion and elimination (Ray and Niswander, 2012), and how boundaries are maintained once formed, for example, by the deposition of extracellular matrix, by the onset of different types of in plane or out of plane cell polarity, or by the differentiation of cells on or immediately adjacent to the boundary (Cayuso et al., 2019).

2.2. Boundaries as organizers

An emerging area of interest, and the one we focus on here, concerns the role of boundaries as organizers of subsequent rounds of patterning and morphogenesis. This question arose

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during the earliest studies of boundary formation but was rarely the central focus of investigation. A notable exception is the initiation and patterning of the fly leg, which was first proposed to be a boundary dependent process by Hans Meinhardt (1983) (Fig. 2d). The fly leg arises precisely at the intersection of the anterior-posterior (AP) and dorsal-ventral (DV) boundaries, and Meinhardt reasoned that if cells in the three bounded adjacent zones produced different diffusible signals then in principle any cell in this neighbourhood would be able to deduce its relative circumferential location from this unique intersection point. Although the molecular details of how this might happen were unclear and the article was considered highly controversial at the time, this did not stop Meinhardt from grasping the importance of his insight for development in general:

> "It is stipulated that the boundaries resulting from the primary embryonic organization of a developing organism, act as organizing regions for secondary embryonic fields, e.g. imaginal discs in insects. This boundary mechanism would allow very reliable pattern formation in the course of development: Primary positional information leads to cells of different determination, separated by sharp borders. At these borders, in turn, positional information would be generated for the next finer subdivision, and so on … Thus, our model suggests a chain of relatively simple molecular interactions which could provide a basis for the reliable generation of structures during embryonic development."

As Meinhardt also noted, this scheme essentially inverts the roles of cause and consequence in the classical conception of pattern and boundary formation. Whereas classically boundaries are for the most part imagined as consequences of development that lock in a morphogen patterning, here boundaries are also causes of subsequent patterning. Meinhardt's model was validated decades later when the mechanism was revealed (Vincent and Lawrence, 1994). Briefly, the posterior region of the fly embryo secretes Hh, which then induces dpp in the bordering anterior dorsal domain and wg in the bordering anterior ventral domain. Wg and dpp are both needed to cooperate to turn on distal-less and commence the distal fly leg patterning program. Cells that fall just outside the meeting place of these three factors activate genes necessary for proximal identities, and this new proximal-distal interface leads to cells in between to activate genes for medial identities. In this manner, three major proximal to distal domains of the adult leg are established in the larval imaginal discs due to the previously determined AP and DV boundaries (Estella et al., 2012).

Today there is a renewed interest in this type of biophysical and geometric reasoning (Abzhanov, 2017; Briscoe and Kicheva, 2017) and this has spilled over to new attention on the foundational role of embryonic boundaries on subsequent morphogenesis and patterning. For example, in a seminal review Keller et al. established the importance of the mechanical boundaries and extra-cellular matrix (ECM) in directing morphogenetic movements (Keller et al., 2003), and this has been followed up and explored in many new contexts ranging from *C. elegans* and *Drosophila* to *Tribolium* (Münster et al., 2019; Kelley et al., 2015; Chen et al., 2019), as summarized in the recent review by Walma and Yamada (2020). In another example, the groups of Tabin and Mahadevan described the many ways boundaries function in the developing avian gut. For instance, the smooth muscle bounding the mesenchyme and endoderm causes buckling and stereotyped villi formation in chick (Shyer et al., 2013), the

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dorsal mesentery directs intestinal looping (Savin et al., 2011), and villi deformation patterns the underlying mesenchyme (Shyer et al., 2015).

Further exploration of the organizing functions of embryonic boundaries is likely to remain challenging and limited due to the difficulty of imaging and perturbing boundaries systematically. Further, the complexity of the developing embryo, with many different processes taking place at the same time, will remain a confounding limitation to *in vivo* studies no matter the level of technological sophistication. However, synthetic *in vitro* reconstruction efforts provide an alternative path by allowing specific developmental processes to be recreated and controlled separately from each other. Reconstituted tissues also require explicit boundaries, and, as we will showcase in the next section, the presence or absence of boundaries can have dramatic and unforeseen effects on subsequent patterning and morphogenesis.

3. Boundaries in synthetic embryology

Prior to modern efforts to study aspects of embryonic patterning and morphogenesis *in vitro*, the only boundary traditionally considered in cell culture was the cell-surface boundary. This was primarily viewed through the lens of improving the growth, viability, and differentiation state of various cell types. Most efforts build upon tissue-culture treated plastics and add numerous purified ECM proteins, gels, and defined chemicals (Ryan, 2008). However, these efforts largely conceptualize the cell-surface boundary as homogenous and fixed in time and acting on single cells individually, much as we treat most of the ingredients added to the culture media. They typically ignored structures present at the tissue-scale, as well as other boundaries such as the cell-media and cell-cell interfaces. It has taken a generation of pioneering bioengineers to correct this picture and explore how minute differences in substrate surface structure or composition can have large effects on emergent tissue properties, such as intercellular tensions, reciprocal gene expression programs, or overall morphology (Mui et al., 2016; Eyckmans and Chen, 2017; Bissell et al., 1982; Simian, Bissell; Nelson and Bissell, 2006). In this section, we show how this picture continues to evolve by highlighting two sets of recent examples from synthetic embryology. These examples showcase how small differences in the cell-media boundary or the cell-cell boundaries can result in large-scale and surprising tissue level reorganizations and patterning that dramatically improves the *in vitro* tissues similarity to that *in vivo*.

3.1. ECM boundary composition determines cell polarity and positioning

In the first set of examples the researchers modeled two different tissue architectures that were both discovered to have their structure dominated by the composition of the boundary on the cell-surface interface. Bedzhov and Zernicka-Goetz examined the pre-to post implantation transition of the mouse blastocyst, a point where the initially amorphous epiblast transforms into a polarized columnar epithelium within which the proamniotic cavity emerges (Bedzhov and Zernicka-Goetz, 2014) (Fig. 3a). Seeking to understand the basis of this transformation, the authors discovered that the extra-embryonic cells synthesized laminins while the epiblast cells upregulated β1-integrin receptors. They hypothesized that the basal lamina which surrounds the epiblast to separate it from the extra-

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embryonic trophectoderm and primitive endoderm might serve as an instructive cue to promote its apical-basal polarity. To test this hypothesis, they adopted an approach similar to efforts used to understand apicobasal polarity in the mammary gland and kidney (Barcellos-Hoff et al., 1989). They first extracted the inner cell mass of the blastocyst and cultured it in a 3D laminin-rich basement membrane (Matrigel) suspension and showed that epiblast cells were able to successfully polarize and lumenize, presumably due to laminin in the Matrigel. To prove this point, they cultured mouse embryonic stem cells (ES cells) (which resemble epiblast cells of the inner cell mass) in either Matrigel or agarose. The ES cells in Matrigel successfully polarized and created a central lumen, while those seeded in agarose remained as unpolarized clumps (Fig. 3b). Going further, they showed that β1-integrin was necessary for lumenization even when surrounded by Matrigel. Thus, this relatively simple synthetic assay demonstrated that the basal lamina functions as a morphogenetic cue to polarize the epiblast downstream of integrins, a role that extends beyond its function in dividing the epiblast from the extra-embryonic lineages.

Beyond cell polarity, the role of ECM in regulating the relative positioning of different cell types has been unclear. To understand the role of ECM on the positioning of different cell lineages, Cerchiari and colleagues examined the two-component and bilaminar cell system comprising the main lineages of the human mammary epithelium: the basally-positioned myoepithelial cells (MEPs) and the luminal epithelial cells (LEPs) that line the ducts (Cerchiari et al., 2015) (Fig. 3c). While technically an adult system, the mammary gland is unique among human organs because much of its structure arises and is elaborated postnatally during puberty, menstrual cycles, pregnancy, involution, menopause, and the early stages of malignant disease. The relative positioning of LEPs and MEPs are maintained during these dynamic processes and the authors aimed to understand how this was possible. The prevailing view was that cell positioning is often maintained by adhesion molecules such as the cadherins, yet surprisingly, previous work had found that deletion of E– and P-cadherin (the primary cell adhesion molecules expressed in the mammary gland) had no gross effect on MEP/LEP cell positioning. Cerchiari and colleagues found that rather than cadherin-based interactions among the cell lineages, the dominant interaction in this system was between MEPs and a self-generated tissue boundary provided by the basement membrane (Fig. 3d). Furthermore, this interaction was binary in that LEPs only weakly interacted with the boundary. Strikingly, an inverted tissue structure arose when basement membrane was removed, revealing the secondary role of cadherin-dependent sorting in the absence of the primary directing signal provided by ECM. Computational modelling demonstrated that exceptionally robust self-organization emerges from binary adhesion to a stationary ECM—effectively negating the effect of altering cell-cell adhesion across a wide range of parameters that would otherwise disrupt cell-sorting. Thus, both the chemical composition and the fixed position of the boundary play important roles in determining the outcome of self-organization.

3.2. Cell-cell boundaries determine patterning by sculpting morphogen gradients and mechanical stresses

In a second set of examples, experiments with 2D human embryonic stem cell (hESC) gastruloid systems showed how cell-cell boundaries can also create “pre-patterns” that have

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the potential to direct future morphogen-based patterning events. Seeking to understand the patterning of the human epiblast in the very first stage of gastrulation, Warmflash and colleagues designed a synthetic 2D micropatterned system that mimicked the epiblast at this stage (Warmflash et al., 2014) (Fig. 3e). In this system, hESCs are confined within micropatterns where they form tightly packed 2D monolayer disks 500 μm–1000 μm in diameter. Confinement and the outer boundary imposed on the hESC colony did not alter cell fates, as they remained pluripotent in the absence of cues triggering their differentiation. However, the addition of BMP triggered a remarkable transformation. Instead of exiting the pluripotent state and differentiating stochastically into different germ layer or extra-embryonic fates as would occur in colonies lacking clearly defined boundaries, cells in each micropatterned colony self-organized into highly regular concentric rings of distinct cell fates, with extra-embryonic fates on the outside, followed by endoderm, then mesoderm, and finally ectoderm in the center. Taking advantage of the control over micropattern size offered by their system, the authors used smaller diameter micropatterns to show that patterning was directed from the outer edge of the colony, as the width of the outer ring was fixed when varying the micropattern diameter, and the innermost fate was observed to vanish as micropattern diameter was decreased.

In follow-up work, Etoc and colleagues uncovered how the micropattern boundary specifically influenced cell patterning following BMP administration (Etoc et al., 2016) (Fig. 3f). First, BMP induced the transcription and secretion of its inhibitor Noggin. Due to the kinetics of its diffusion in two dimensions, Noggin accumulated in the center of each colony and was lost at the outer edge. This in effect created a BMP activity gradient, with cells receiving a more persistent and intense BMP signal on the outer regions of the colonies than in the center. Thus the boundary in this case functioned as a filter, sculpting a signalling gradient out of a uniformly administered morphogen. This mechanism was not quite enough to explain the patterning at all densities, and the authors also identified a second interesting function of the boundary in this system. As hESCs grew denser they apically-basally polarized, sequestering receptors under apical tight junctions. As a consequence, these cells were unable to receive any BMP signal that was restricted to the apical compartment of the tissue. In contrast, the discontinuity of cell-cell contacts at the outer boundary of each colony allowed BMP to access the exposed basolateral surfaces to activate BMP signalling proximal to the boundary. Importantly, *in vivo* studies in the mouse embryo later validated these *in vitro* observations (Zhang et al., 2019). Careful quantitative analysis and mathematical modelling showed how this strategy can buffer morphogen gradients to variations in ligand concentration, and additional work from Chhabra et al. showed how this first BMP asymmetry can lead to subsequent symmetry breaking and signal propagation in the downstream WNT and NODAL pathways as well (Chhabra et al., 2019).

Extending this work, Martyn and colleagues demonstrated that boundaries also affect Wnt signalling in hESC micropatterns by at least two mechanisms (Martyn et al., 2019) (Fig. 3f). First, uniform Wnt stimulation resulted in hESC patterning through a mechanism involving induction and shaping of gradients of its own secreted inhibitor DKK1. Here again, the boundary functioned as a sink for the secreted inhibitor and so created an effective morphogen gradient across the colony. As a consequence, mesoderm and endoderm emerge in the Wnt high region on the outermost ring of the colony, and pluripotent cells remain in

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the Wnt low region at the interior of the colony. Second, the boundary functioned independent of DKK1 by focusing mechanical strain on cells residing at the outermost edges of high density micropatterns. Thick actin-myosin cables were observed at the extreme edge of each colony, and asymmetry in E-cadherin pairing between cells at the edge resulted in the accumulation of non-membrane bound β-catenin, and consequently, increased sensitivity to Wnt signalling. Eliminating E-cadherin or treatment with small molecules that disrupted these mechanical forces eliminated Wnt asymmetry downstream of the boundary. Independent work by Muncie et al. also identified this boundary effect in micropatterned hESC colonies (Muncie et al., 2020). They proposed a mechanism whereby the local mechanical forces modulate the conformation of β-catenin within cadherin-catenin complexes, permitting Src-mediated phosphorylation and release of β-catenin from cell junctions which allows it to translocate to the nucleus and induce signalling and differentiation. Consistent with this model, β-catenin phosphorylation in response to local tissue mechanics was also observed *in vivo* in the mesoderm invagination in *Drosophila* embryos (Röper et al., 2018). Additionally, large-scale supracellular actomyosin rings are observed at the margin between the embryonic and extraembryonic territories in chick, and computational modelling suggests that the graded contraction of this bounding ring generates forces which drive and shape the stereotyped movements of the developing chick primitive streak and may even influence signalling pathways as well (Saadaoui et al., 2020). More generally, we note that it has also been recently shown in chick that β-catenin in the cells of the developing epidermis is mechanosensitive and responds to the aggregation of neighboring dermal cells by dissociating from cadherins junctions to transit to the nucleus to trigger the follicle gene expression program (Shyer et al., 2017). Thus, the role of boundaries in shaping mechanical stresses and consequent signalling appear to be common themes throughout development and across different species.

4. Getting the right boundaries: control and synthetic boundaries

These examples from synthetic embryology and reconstituted systems highlight the important and at times unexpected roles that boundaries serve in guiding self-organization. Moving from observation to engineering, the natural question is how can we leverage this insight to improve the fidelity or systematically alter the outcome of *in vitro* self-organization? Fortunately, in recent years there has been an explosion of innovation, spanning synthetic ECMs and microfluidics to bioprinting and engineered gene networks. Together, these new tools provide synthetic embryologists with powerful means to program self-organization and to specify custom boundary conditions.

4.1. Programmable ECM boundaries

Beginning with ECMs, there has been remarkable progress in controlling their chemical composition and emergent mechanical properties, such as elasticity, viscosity, density, porosity, and relative orientation (Chaudhuri et al., 2020). For example, working to reconstitute features of mammalian neurulation and cognizant of the possible importance of the ECM microenvironment, Ranga and colleagues took a systematic approach that avoided the commonly used Matrigel, a material they noted that is “poorly defined [and] whose properties cannot be readily modulated” (Ranga et al., 2016). To identify the biophysical and

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molecular components of the microenvironment that control neurulation, they investigated large libraries of defined synthetic matrices—including a variety of hydrogel scaffolds functionalized with the different ECM proteins that are the major components of Matrigel (such as collagen IV, entactin, laminin-111, perlecan, fibronectin, etc.)—using a high-throughput morphogenetic screen. While controlling for substrate mechanics, they identified optimal parameters for inducing more homogeneous neural tube-like structures having a higher proportion of apical-basal polarization and lumenization compared to Matrigel. Impressively, these conditions also allowed neural structures to self-organize and reproducibly break dorsal-ventral symmetry, with a Shh expression appearing on one side and Pax3 expression on the other. Beyond this achievement, the team was able to deduce three important principles governing the impact of the ECM boundary on patterning. First, adhesion ligands in the matrix (especially laminin) were required for proliferation, differentiation, and establishment of lumens. Second, non-degradable matrices favoured apicobasal polarization. Third, the parameters they explored were not completely independent of one another. For example, increasing the laminin concentration could compensate for otherwise excessively compliant substrata to drive polarization.

Chemically defined 3D hydrogels have also been leveraged to engineer more controlled human and mouse organoid morphogenesis (Cruz-Acuña et al., 2017; Holloway et al., 2019). In mouse intestinal organoids, for example, a stiff matrix is optimal for stem cell expansion, while a softer matrix is required for epithelial differentiation (Gjorevski et al., 2016). Optimal conditions for stem cell expansion followed by differentiation required engineering a dynamic ECM boundary: the team created PEG hydrogels that retained an intermediate stability to hydrolysis and so were able to soften over time. This illustrated an important and emerging concept in embryonic boundaries, that is their living character that changes dynamically through time.

In another example, Trushko et al. sought to understand the role of ECM stiffness and compressive forces in the epithelial folding process that occurs later in development and is common to the formation of many organ systems (Trushko et al., 2020). Buckling through compressive stresses imposed by an external boundary has been proposed to explain many instances of folding, but proving this mechanism *in vivo* is challenging since it requires measuring stress fields and material properties. Trushko and colleagues used a synthetic approach to encase epithelial cells in elastic spherical shells of defined size and material properties. They showed that within these shells cells created a monolayer epithelium and were able to spontaneously fold. By measuring the deformation of the shell, they were able to infer the relevant forces and show quantitatively that these forces were sufficient to account for buckling.

4.2. Programming spatial heterogeneities with micropatterning and microfluidics

Although defined matrices and purified ECM molecules offer improved control over *in vitro* systems, used by themselves they only offer control over bulk properties. When used in combination with increasingly accessible tools such as bioprinting, microfabrication, and programmed cellular assembly, they allow researchers to program spatial heterogeneities and anisotropies which can be used to create more complex and novel boundaries.

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In one such approach, Wang and colleagues used 3D microfabrication to sculpt collagen gels into crypt/villus domains (Wang et al., 2017). They then seeded human intestinal crypt fragments onto these gels, allowed them to form an epithelia, and placed them on transwell inserts between two different media reservoirs. When the one reservoir was filled with differentiation media and the other was filled with stem cell media, this generated a signalling gradient across the gel, leading to the formation of proliferative crypt-like zones and differentiated villus domains. In follow-up work, the same group used a similar approach to create molded crypts for human colonic epithelium (Wang et al., 2018). While not explored in this work, the technology has the potential to investigate how the shape of the micropattern and the length of the signalling gradient affects the pattern of differentiation in this tissue.

Recent work has integrated microfluidics into this general model of the intestinal epithelium. Nikolaev and colleagues used a microdissection laser to sculpt crypt/villi-like regions out of a hydrogel sandwiched between two inlet and outlet ports oriented along the long intestinal axis. They additionally included two larger media reservoirs along the apicobasal axis (Nikolaev et al., 2020). Like Wang et al. (2017), they seeded their scaffold with intestinal stem cells in stem cell media with and allowed them to form an epithelium before changing the media in the longitudinal chamber to differentiation media, followed by changing to differentiation media in the reservoirs along the apicobasal axis as well after an additional 3 days. This led to the cells in the crypts retaining their stemness while cells in the villi region differentiated. Although the team did not test to what degree the curvature of the sculpted crypt regions versus the delay in changing media along the apicobasal axis was responsible for the properly localized crypts, their design allows for such exploration together with the impact of rheological properties such as matrix stiffness and fluid shear forces in the interior.

In a different system based on mES and hESC spheroids, Zheng and colleagues investigated engineering approaches to break symmetry by exposing each hemisphere to unique morphogens, mimicking early developmental events (Zheng et al., 2019). They settled on a microfluidic approach with a parallel 3-channel construction in which the middle channel was separated from the other two by regular trapezoid-shaped supporting posts. These posts created regular gaps whose sizes were tailored to match the diameter of the spheroids. Filling the inner channel with Geltrex (similar to Matrigel), and then seeding single pluripotent hESCs in one of the parallel outer channels results in cells adhering to Geltrex exposed by these gaps and eventually forming luminal cysts that plugged the gaps. In this position the spheroids could be exposed to a gradient of morphogens by flowing different media through the two outer channels. Exploring different combinations of factors, the team found that a BMP gradient was sufficient to break symmetry and differentiate the BMP high half into amnion-like cells while the other half differentiated into primitive streak-like cells. Strengthening this gradient further by adding Noggin and IWP2 (a Wnt inhibitor) to the primitive streak side resulted in those cells remaining pluripotent and epiblast-like instead of differentiating. Intriguingly, they also observed the appearance of rare primordial germ cell-like cells from the amniotic region that are exceedingly difficult to study *in vivo*.

To break symmetry across even large length scales, Rifes and colleagues developed the microfluidic-controlled stem cell regionalization (MiSTR) system (Rifes et al., 2020). The

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backbone of this system is a microfluidic gradient generator that relies on sequential diffusive mixing of two inlet media (for example 0 and 100% of a morphogen) that ultimately flows over a cell growth area as a stable linear gradient. The authors loaded this region with ectodermally directed hESCs and created a Wnt gradient across a 20 mm region to mimic the gradient of Wnt *in vivo* that is thought to regionalize the developing neural tube. This strategy was successful, as the Wnt gradient resulted in distinct anterior and posterior regions separated by a sharp boundary. By modifying the concentration of Wnt in the inlet and outlet ports they were able to shift the gradient and observe a corresponding shift in the location of the new AP boundary as well. A potential limitation of these microfluidic-based systems is the need for constant flow, which will disperse slowly accumulating autocrine and paracrine factors generated by the tissue which could play an important role in subsequent morphogenesis.

4.3. Programming spatial heterogeneities with bioprinting and directed cell assembly

Bioprinting offers another tool to create spatial heterogeneities and custom boundaries, but users face the challenge of optimizing many different parameters in parallel, such as nozzle diameter, extrusion pressure, bioink composition, and cellular concentration. To overcome many of these hurdles, Brassard and colleagues chose to leverage the self-organizing properties of stem cell-based cellular inks combined with defined boundaries provided by ECM hydrogels (Brassard et al., 2020). They developed an easy-to-build proof-of-principle printing chassis comprising a syringe-based extruder coupled to a microscope with a manually controlled stage. This simple set-up facilitates experimental optimization and provides users with direct visual feedback and control. They demonstrated the approach by printing a line of mouse intestinal stem cells that self-organized into a single millimeter scale tube with a connected lumen. In a subsequent experiment, they combined intestinal stem cells with gastric stem cells to print a tube with a gradient of features spanning those of the stomach to small intestine. It will be exciting to see how this powerful strategy might be combined with state-of-the-art 3D printing platforms. Others have achieved sharper boundaries between neighboring tissue regions by squeezing together organoids of different identities or by embedding cells engineered to produce key morphogens inside organoids in a manner analogous to classically used morphogen releasing beads—observing emergent phenomena that depends on the identity and shape of the new boundary (Bagley et al., 2017; Birey et al., 2017; Koike et al., 2019; Xiang et al., 2017; Cederquist et al., 2019).

What is the maximum spatial resolution with which a synthetic embryologist might specify a boundary? Printed droplet microfluidics is an emerging microfluidic-based technology that can provide deterministic control over the number and types of cells in small tissues, but it has not yet been additionally directed to applying flexible spatial control (Cole et al., 2017). Another method investigators can turn to is “DNA Programmed Assembly of Cells” (Todhunter et al., 2015; Chen et al., 2016). Although currently limited in three dimensions to tissues less than 100 μm thick, in two dimensions it offers single-cell spatial resolution. Improved recent methods have also made it much more accessible to new users and less reliant on specialized microarray spotting technology (Scheideler et al., 2020). Among its applications, Liu and colleagues used it to create defined mosaics of H-Ras activated MCF10A cells and show that these cells only extrude and invade when at a boundary

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occupied by wild type cells (Liu et al., 2012). In another example, Hughes and colleagues assembled fibroblasts in specific patterns so that they in turn generated particular patterns of stress and compaction that directed the folding of a tissue in programmable ways (Hughes et al., 2018). Inspired by the Japanese art of origami, Hughes and colleagues have also recently taken on trying to create more complex 3D shapes, such as the branching hierarchy of the embryonic mouse ureteric epithelium (Viola et al., 2020).

4.4. Programming cadherins and synthetic morphogen gradients

Genetic engineering also offers the promise of creating and investigating living boundaries. Synthetic Notch (SynNotch) receptors (Morsut et al., 2016) allow cells to communicate and perform logic across cell-boundaries using genetic circuits. The advent of SynNotch enabled coupling the output of cell-cell communication networks to adhesion molecule expression, thereby encoding boundary formation through well-understood cell sorting mechanisms (Foty and Steinberg, 2004, 2005; Cachat et al., 2016). Toda and colleagues used this concept to program 3D structures that self-organize to form new living boundaries through hierarchical self-organization (Toda et al., 2018). They developed a two-step genetic program by first having one cell type present a CD19 ligand that was detected by another cell types expressing an anti-CD19 SynNotch receptor. Activation of anti-CD19 SynNotch triggered cell-surface expression of a GFP molecule together with high levels of E-cadherin, the latter causing it to sort into the core of the tissue so as to maximize its contact with other E-cadherin expressing cells. In the second step, the membrane-bound GFP signalled back to the first cell type through an anti-GFP SynNotch to induce intermediate levels of E-cadherin. The resulting synthetic tissue had three “differentiation states” defined by low, intermediate, and high E-cadherin expression, resulting in three stable concentric shells ordered by the three different cadherin level expressions. Several other genetic programs were also demonstrated in this study, each leading to the formation of a unique constellation of tissue structures. It is worth noting that the differential interfacial tension hypothesis would predict many of these structures purely based on cell sorting without the need for hierarchical programming, but the general framework presented by this study provides a unique context to probe for genetic programs in which hierarchy does matter, and to ask how temporal ordering of self-organizing events can alter the path of an integrated developmental program. Moreover, the living nature of the boundaries formed in these studies was highlighted by their ability to “heal” after injury, a feature that will likely prove critical if synthetic embryologists are ever to engineer more complex and functional tissues. Finally, an exciting idea for future studies is that these living boundaries might be used to program the morphogenesis of wild-type embryonic tissues, then selectively removed at a later time-point by inducing their death. In this way, the authors have laid the groundwork for engineered living boundaries that can have wide applications in organoid and regenerative engineering.

Illustrating the versatility of the SynNotch system, the same group combined it with secreted fluorescent proteins to build two-dimensional gradients. They noted three important aspects of the boundaries formed by this system (Toda et al., 2020). First, the shape of the gradient depended on the position and density of “morphogen” secreting cells; second, non-functional receptors expressed by the receiving cells increased the affinity of the synthetic

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morphogen for the monolayer surface and thus biased diffusion laterally, and third, additional lateral bias in the form of a hydrogel overlay that limited convection was also required. The need for a hydrogel overlay was especially interesting as other recent investigations of synthetic gradients do not share this requirement. Engineered Hedgehog gradients for example can form in similar monolayers even when convection currents are deliberately introduced with an orbital rocker (Li et al., 2018). Similarly, BMP, Noggin, and DKK1 gradients can form in apically-basally polarized monolayers without a hydrogel layer as well (Etoc et al., 2016; Martyn et al., 2019). Future experiments with the SynNotch system may attempt to dissect what accounts for the difference among these systems.

The different mechanisms by which boundaries can shape signalling gradients has been demonstrated with several synthetic *in vivo* systems. For example, Stapornwongkul and colleagues built a synthetic developmental system in the context of the developing fly wing disc by replacing the native dpp morphogen (expressed at the midline of fly wing disc) with a secreted GFP (Stapornwongkul et al., 2020). They also added anti-GFP nanobody receptors to the cells adjacent to the midline that would normally respond to dpp. In the absence of the nanobody coupled GFP receptors, no GFP was observed beyond those cells that produced it. In the presence of receptors, a gradient was observed, but the gradient had a long flat tail even at distances far from the midline. Reasoning that another boundary was needed, they found that expressing extra receptors in the fly fat pad—a layer of cells underneath the hemolymph layer—or non-functional receptors in the wing-disc, eliminated this tail. These extra receptors in neighboring tissue layers acted as sinks to sculpt the GFP gradient. The behavior of this system is further complicated by the fact that morphogens can be secreted either apically or basolaterally (Harmansa et al., 2017). Patterns of morphogen secretion, transport, and diffusion *in vivo* can be dizzying complex. For example the morphogen wg is first secreted apically and then reabsorbed and transcytosed to diffuse basal-laterally where diffusion is dependent on glypicans (Yamazaki et al., 2016; Mcgough et al., 2020), and similar behaviour has been preliminarily obsered with Noggin in hESC epithelia (Phan-Everson et al., 2020). How these complex behaviors can further shape gradients will be an exciting area for future investigation. For example, one might speculate how boundaries engineered in these ways might sculpt that patterns that emerge in reaction diffusion systems (Hiscock and Megason, 2015). Taken together, these studies demonstrate that establishing signalling gradients is highly dependent on the shape and property of nearby boundaries. One can easily imagine utilizing these new genetic tools in other processes in development to discover properties of the boundaries involved there.

5. Conclusions and outlook

The emerging tools of synthetic embryology are providing a rich landscape for investigating how the chemical, mechanical, and electrical properties of boundaries influence self-organization. One important emerging area is the need for a “chassis” of mammalian development for investigating the role of different types of boundaries in shaping morphogenesis. One potential chassis is the gastruloid system, which produces many of the hallmarks of anterior posterior patterning with only minimal constraining boundaries (Turner et al., 2017; van den Brink et al., 2014). Recent work has demonstrated how layering additional constraints on this system in the form of new boundaries can sharpen and alter the

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outcome of self-organization (van den Brink et al., 2020; Veenvliet et al., 2020). By using a single chassis to investigate multiple developmental events in a synthetic context, the system has the potential to identify unifying and integrated principles. Another important area for investigation are living boundaries. *In vivo*, boundaries are dynamic, themselves changing shape and properties in response to neighboring tissues. Genetic circuits like synNotch hold the potential to provide the necessary feedback, and "growing" synthetic boundaries will be an exciting area for future investigation.

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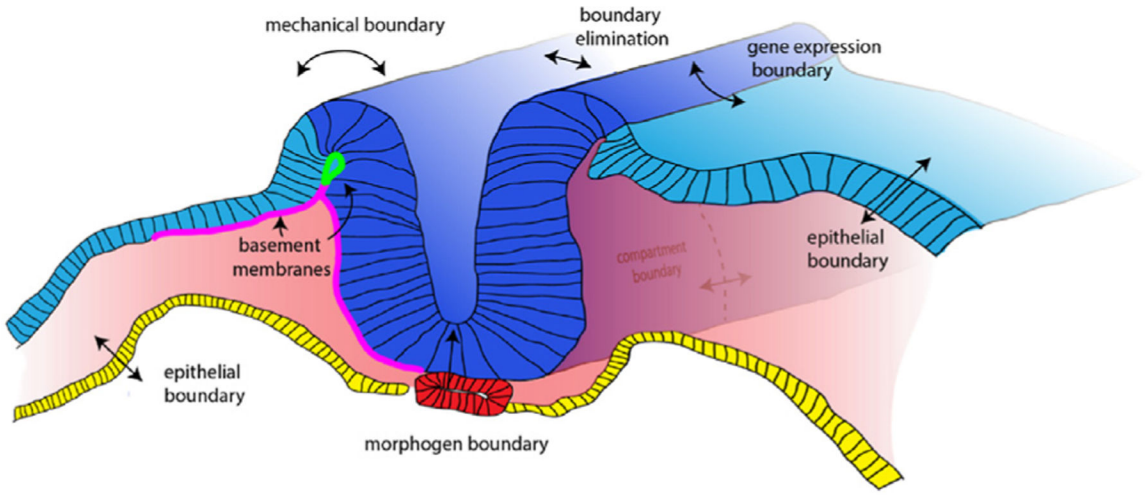

Fig. 1. Boundaries present during primary neurulation.

Cross-section of chick primary neurulation. Recreating features of the vertebrate neural tube *in vitro* is a goal of many synthetic embryologists, yet the neural tube is impacted by many different changing boundaries during its development. Here we indicate a few different types of boundaries present that researchers may wish to consider for investigation or for engineering.

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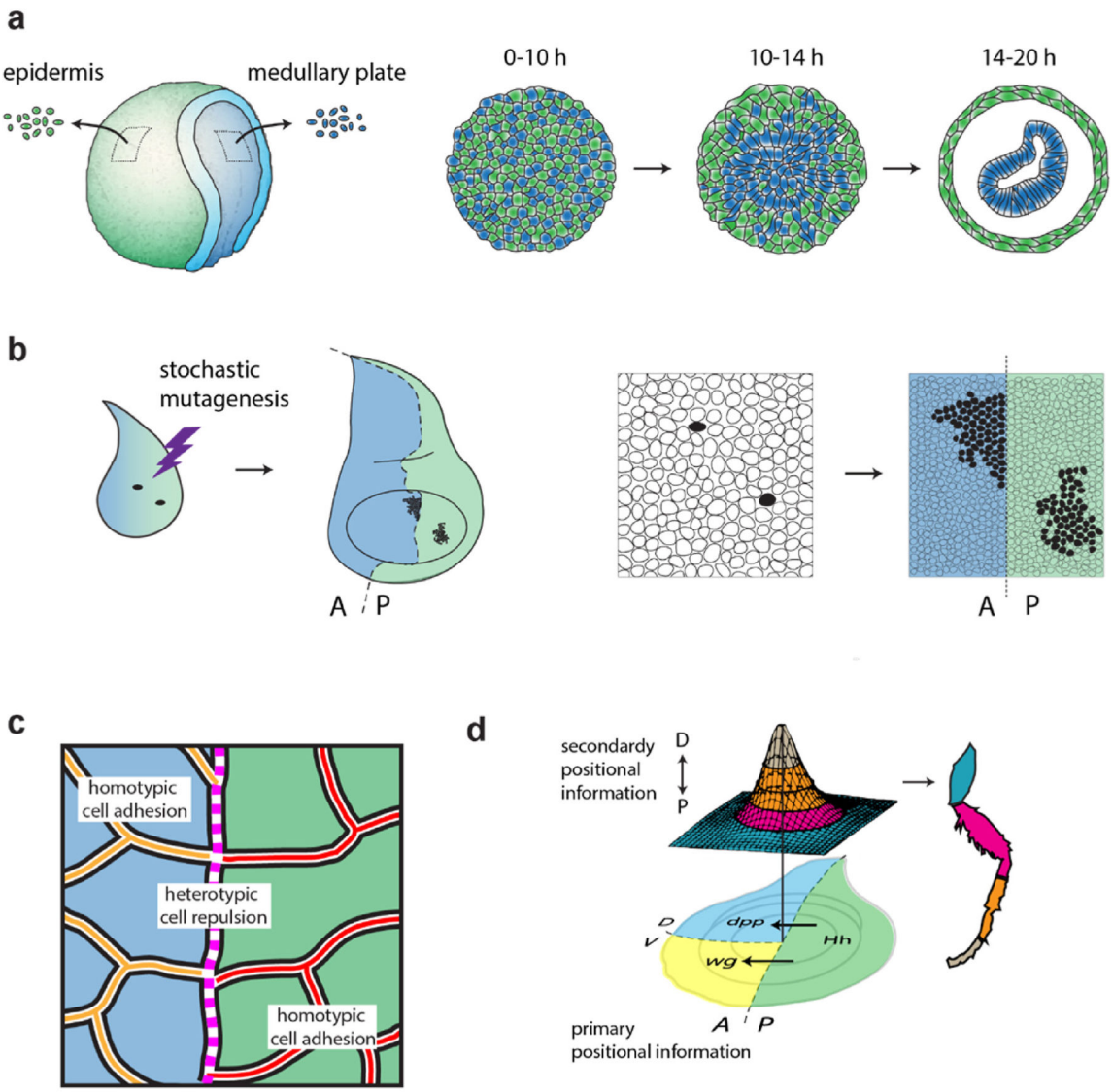

Fig. 2. Classical embryological boundaries.

(a) Example cellular disaggregation-reaggregation experiment with a neurulation stage amphibian embryo. Regions of the medullary plate (neuroectoderm) and epidermis (non-neural ectoderm) are excised and disaggregated into single cells and then mixed together. Over a period of a day the cells sort themselves back out into concentric self-consistent epidermal and neural layers. (b) Discovery of compartment boundaries in the fly wing disc. At the onset of wing disc formation stochastic mutations are introduced that result in clonally marked cells. As the wing disc develops, these cells proliferate and their descendants can become noticeably restricted to one side of a compartment boundary. (c) Example molecules involved in tissue sorting and boundary formation. Differentially expressed cadherins (orange and red) allow cells to pair with similar cadherin expressing cells, while complementary expressing Eph-Ephrins (pink) allow emergence of a sharp boundary. (d) Patterning of the fly leg. Morphogens created by primarily determined anterior-posterior (AP) and dorsal-ventral (DV) regions combine at their intersection point to

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create a new morphogen gradient that results in the patterning of a new proximal-distal (PD) axis.

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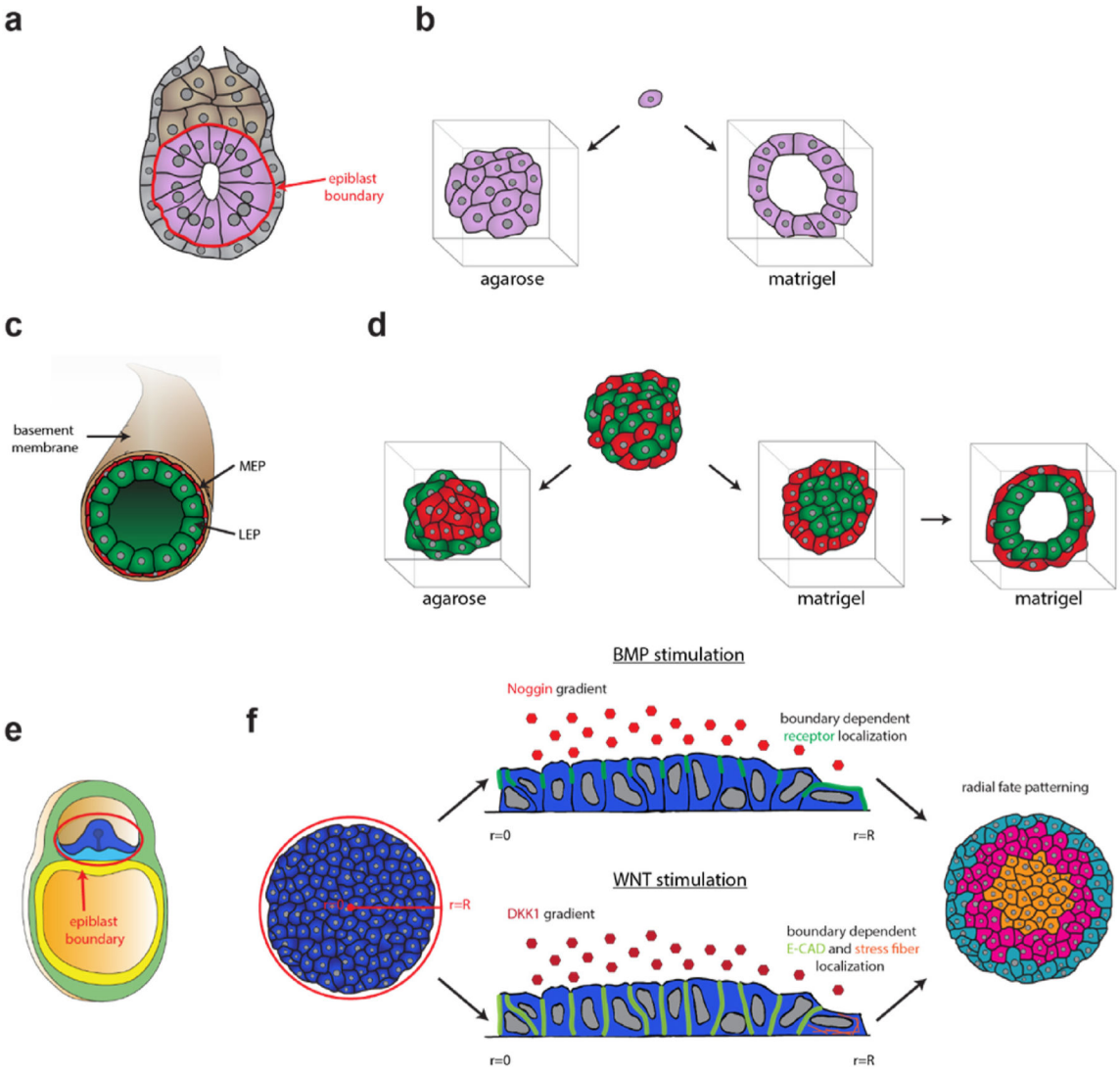

Fig. 3. Recent boundary investigations from synthetic embryology.

(a) E5.25 mouse embryo. ECM from the primitive endoderm and trophectoderm provide polarization cues to the epiblast cells that leads to establishment of apical-basal polarity, rosette formation, and subsequent lumenization. (b) Mouse stem cells, either taken directly from the pre-implantation embryo or from mES lines, can be embedded as single cells in either agarose or Matrigel. In agarose they grow as unpolarized clumps, but the interface with Matrigel leads to successful polarization and creation of a central lumen. (c) Cross-section of a mammary duct showing internal luminal epithelial cells (LEPs) surrounded by myoepithelial cells (MEPs) which are further surrounded by a basement membrane. (d) Disaggregation and reaggregation of MEP/LEP mixtures in agarose versus Matrigel leads to development of different tissue architectures. (e) Cross section of primitive streak stage human embryo. The epiblast is an epithelial monolayer of embryonic stem cells suspended between the amniotic cavity and yolk sac. (f) Micropatterning technology allows for the *in vitro* modelling of the pre-primitive streak stage human epiblast with human embryonic stem cells (hESCs). Stimulation with BMP or WNT results in the induction of morphogen

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specific secreted inhibitors, which, due to the geometry of the model epiblast, leads to a morphogen gradient and subsequent radial fate patterning. The gradient is further compounded by other boundary determined effects such as differential receptor and cadherin junction localization and intercellular tension.