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Reassembling gastrulation

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ABSTRACT

During development, a single cell is transformed into a highly complex organism through progressive cell division, specification and rearrangement. An important prerequisite for the emergence of patterns within the developing organism is to establish asymmetries at various scales, ranging from individual cells to the entire embryo, eventually giving rise to the different body structures. This becomes especially apparent during gastrulation, when the earliest major lineage restriction events lead to the formation of the different germ layers. Traditionally, the unfolding of the developmental program from symmetry breaking to germ layer formation has been studied by dissecting the contributions of different signaling pathways and cellular rearrangements in the *in vivo* context of intact embryos. Recent efforts, using the intrinsic capacity of embryonic stem cells to self-assemble and generate embryo-like structures *de novo,* have opened new avenues for understanding the many ways by which an embryo can be built and the influence of extrinsic factors therein. Here, we discuss and compare divergent and conserved strategies leading to germ layer formation in embryos as compared to *in vitro* systems, their upstream molecular cascades and the role of extrinsic factors in this process.

1. The toolbox of gastrulation

Upon fertilization, an organism is faced with the task of generating a differentiated multicellular structure from a single cell. To this end, it has to amplify the number of cells, specify their fate and rearrange them to eventually form fully differentiated tissues and organs arranged in a stereotypical manner along the main body axes. A key process in the transition from a seemingly uniform cluster of cells to a multi-layered embryo is the process of gastrulation (Fig. 1A). While the start point of gastrulation – a rather undifferentiated group of cells called blastula – and the endpoint – a more complex embryo with established germ layers and main body axes – are highly similar in different animals, the implementation of the gastrulation process as such can be rather variable (Leptin, 2005). Still, an evolutionarily conserved set of cell movements and signaling pathways are re-employed in different combinations and contexts for germ layer specification, patterning and morphogenesis (Solnica-Krezel, 2005).

Among Eumetazoans, two main gastrula types can be distinguished: diploblastic animals, such as cnidarians and ctenophores, forming two germ layers (ectoderm and endoderm), and triploblastic animals, including all bilaterians, forming three germ layers (ectoderm, endoderm and mesoderm). Generally, the germ layers are rearranged so that mesodermal and/or endodermal progenitors are located on the inside of the embryo and the ectoderm on the outside (Fig. 1A). In the remainder of this review, we will focus on gastrulation processes in vertebrates and thus the processes leading to the formation of all three germ layers.

The fundamental developmental step of generating cell type diversity in gastrulation, i.e. adopting the specific germ layer fates, is regulated by the activity of several signaling pathways, most notably the Nodal/TGFβ, Wnt, BMP, and FGF signaling pathways (reviewed in Kiecker et al., 2016; Morgani and Hadjantonakis, 2020). Nodal/TGFβ, BMP, Wnt and FGF ligands are thought to act in this process as morphogens, molecules that move from the place of their production and elicit a concentration-dependent response within the target tissue away from the source (Fig. 1B) (reviewed in Rogers and Schier, 2011). Nodal signaling is needed for specification and lineage segregation of mesoderm and endoderm (collectively called mesendoderm) progenitors from ectoderm progenitors in a variety of organisms (reviewed in Shen, 2007). Its loss results in the absence of most mesendodermal tissues in zebrafish embryos and prevents primitive streak formation in mouse embryos, while ectopic Nodal activation is sufficient to induce ectopic mesendoderm formation (reviewed in Schier, 2009). FGF and BMP signaling also play crucial roles in mesendoderm induction and patterning, but in contrast to Nodal signaling, do not seem to be sufficient to induce mesendodermal cell fates (reviewed in Kiecker et al., 2016). Finally, Wnt/β-catenin signaling has been shown to be important during the early steps of

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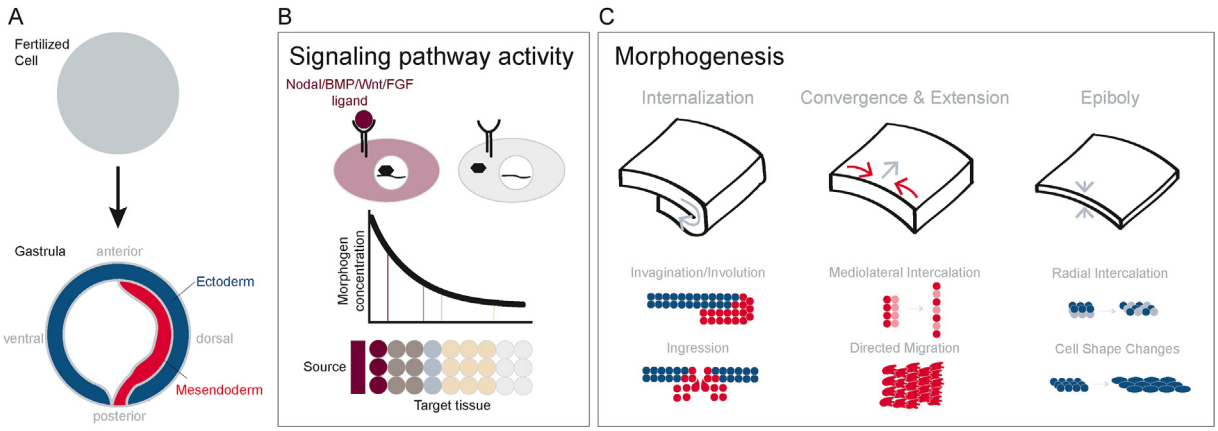

Fig. 1. The toolbox of gastrulation.
(A) Schematic representation of the transition from a fertilized oocyte to a multilayered gastrula, where the different germ layer fates (ectoderm in blue, mesendoderm in red) are formed and the main body axes are established. (B) Schematic representation of tissue patterning as a function of ligand concentration within the target tissue. An extracellular ligand (ruby colored) binds to a cell-surface bound receptor, thereby inducing an intracellular signaling response, which in turn results in the nuclear localization of a downstream transcription factor (black) and associated cell fate changes. The morphogen is secreted from a source (ruby box) and travels within a tissue, giving rise to different cell fates in a concentration- and time-dependent manner, so that cell fates which require the highest dose of morphogen-induced signaling are positioned closest to the source. (C) Schematic representation of major large-scale cellular rearrangements and associated cellular processes leading to the formation and rearrangement of the germ layers during gastrulation (based on (Solnica-Krezel, 2005)).

mesendoderm induction in several species and subsequently for patterning of the mesendoderm and ectoderm (reviewed in Kiecker et al., 2016; Morgani and Hadjantonakis, 2020).

Importantly, only the combined action of all these different signaling pathways (Nodal/TGFβ, BMP, FGF and Wnt) allows proper and robust germ layer patterning *in vivo*. Moreover, signaling pathway activity, elicited by morphogen binding to their respective receptors, has to be tightly controlled in time (length of signal exposure, dynamics of signaling activity) and space (dimensions of signaling domain) (reviewed in Ashe and Briscoe, 2006). This is achieved through an array of regulatory mechanisms functioning at different levels of the pathway, such as transcriptional control and post-translational processing of pathway components, crosstalk between signaling pathways and intracellular and extracellular feedback mechanisms (reviewed in Freeman and Gurdon, 2002). More recently, also ligand-independent signaling mechanisms, such as mechanical forces, were implicated in modulating downstream signaling pathways involved in germ layer formation (reviewed in Fernandez-Sanchez et al., 2015).

In addition to acquiring different fates, germ layer progenitor cells undergo large-scale rearrangements during gastrulation. Three main types of cellular rearrangements can be distinguished (Fig. 1C) (reviewed in Solnica-Krezel and Sepich, 2012): (i) Internalization or emboly refers to the movement of mesendodermal progenitors to the inside of the embryo. The underlying cellular mechanism leading to internalization can vary between different species - cells can either internalize as a coherent sheet, a movement commonly termed 'invagination' or 'involution', or as single cells in a process called 'ingression' (reviewed in Solnica-Krezel and Sepich, 2012). For instance, during chick and mouse gastrulation, mesendodermal progenitors move to the inside of the embryo as individually ingressing cells (Harrisson et al., 1991; Tam et al., 1993), while during frog gastrulation they involute as a coherent sheet (Shih and Keller, 1994). (ii) Epiboly is another major type of cell rearrangement during gastrulation, referring to the spreading and thinning of the germ layers through cell intercalation, e.g. blastoderm spreading over a large yolk cell in teleost embryos (Bruce and Heisenberg, 2020; Trinkaus, 1984) or ectoderm expansion and thinning during blastopore closure in amphibian gastrulation (Keller, 1980). Finally, there are (iii) convergence and extension (C&E) movements, which typically begin once internalization and epiboly movements are underway, and lead to germ layer extension along their anterior-posterior axis and concomitant narrowing along their mediolateral extent (reviewed in Solnica-Krezel and Sepich, 2012). Several types of cell behaviors have been implicated in C&E movements, such as collective cell migration and mediolateral cell intercalation (reviewed in Tada and Heisenberg, 2012). A classic example of C&E movements predominantly driven by mediolateral cell intercalation can be found in *Xenopus* gastrulation (Keller et al., 1985; Shih and Keller, 1992a), while in zebrafish, for instance, mesendoderm C&E movements rely on both collective migration and mediolateral intercalation (Sepich et al., 2005). Importantly, the different gastrulation movements occur largely in parallel and thus have to be precisely coordinated in space and time to properly shape the embryo. Moreover, these movements are interdependent and can be regulated by overlapping signaling pathways.

Notably, cell signaling, fate specification and rearrangement do not occur in isolation, but are tightly interconnected: cell signaling not only affects cell fate specification and tissue patterning, but also cell motility and adhesion, thereby controlling a cell's capacity for undergoing rearrangements. Conversely, cell rearrangements are crucial for cells to be located in the right position for receiving the signals required for their proper specification (reviewed in Chan et al., 2017; Gilmour et al., 2017; Heisenberg and Solnica-Krezel, 2008; Pinheiro and Heisenberg, 2020).

While the basic toolbox of gastrulation - the major signaling pathways and cellular rearrangements - is conserved amongst different vertebrate species, many other aspects of the gastrulation process can vary considerably, such as the speed of developmental processes, embryo size and shape, the presence of extraembryonic structures, and the formation of a blastocoel, all of which actively and/or passively affect gastrulation (Fig. 2A). This raises important questions as to how the same basic toolbox is re-used and adjusted under different conditions in order to implement robust germ layer formation.

2. Germ layer patterning *in vitro*

It has long been known that when taken out of their endogenous context, embryonic cells and substructures still retain a high capacity to self-assemble and give rise to the structures they were primed to form (reviewed in Moris et al., 2020b). Classic examples for this are studies by Townes and Holtfreter from the middle of last century, demonstrating that upon dissociation and reaggregation of an amphibian gastrula, germ layer progenitors could retain their identity and even sort into distinct domains (Townes and Holtfreter, 1955). Consistent with this, dorsal mesendodermal and ectodermal explants from gastrulating *Xenopus*

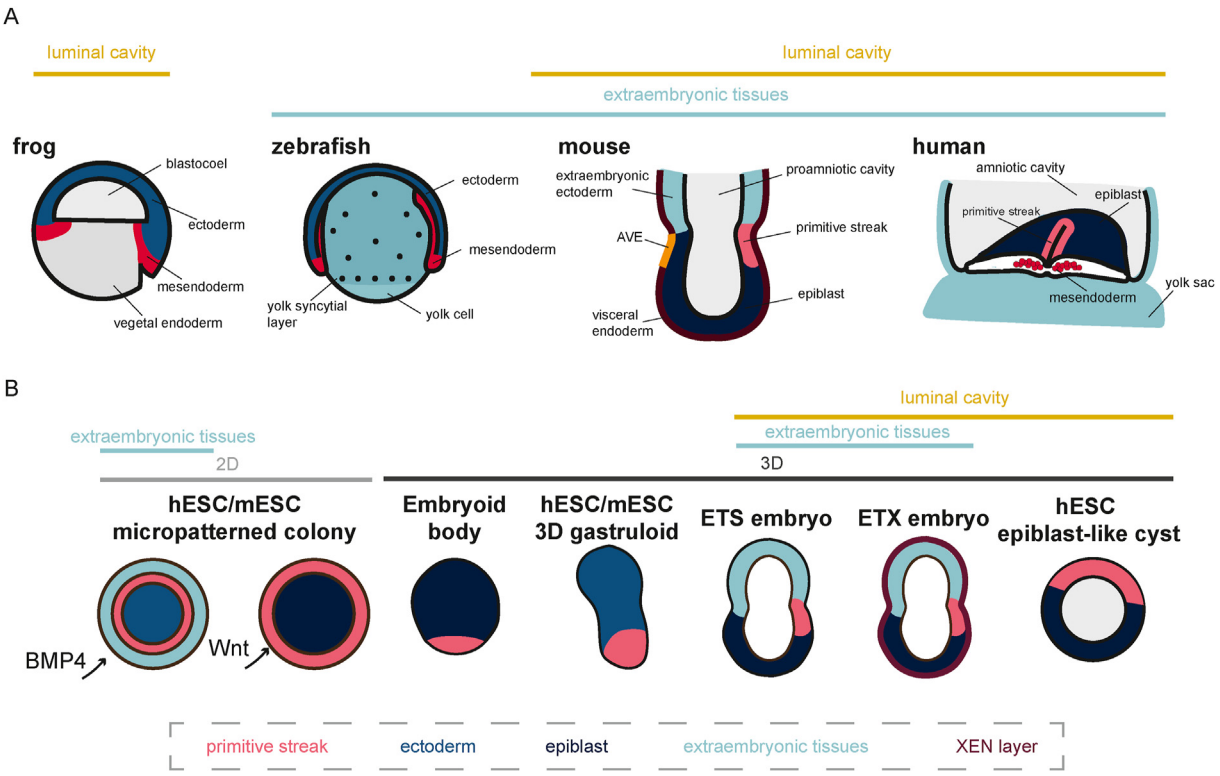

Fig. 2. The shapes and forms of the gastrula *in vivo* and *in vitro*.
(A) Schematic representation of gastrula-stage embryos of different vertebrate species. For each embryo, major morphological features associated with gastrulation are indicated. Common species-specific features (presence of extraembryonic tissues, luminal cavity) are indicated on the top. (B) Schematic representation of selected *in vitro* gastrulation models. All systems are generated from embryonic stem cells (ESCs). Common system-specific features (presence of extraembryonic tissues, luminal cavity, 2D, 3D system) are indicated on the top. Micropatterned colonies are confined on 2D circular surfaces and treated with signaling molecules (BMP4 or WNT) leading to specific patterning outcomes. For 3D embryoid bodies and gastruloids, ESC aggregates are cultured unconstrained in liquid culture and undergo symmetry breaking to establish a primitive streak-like domain. Gastruloids undergo axis extension and axial patterning. ETS embryos consist of mESCs and TSCs and are embedded in extracellular matrix. ETX embryos consist of mESCs, TSCs and XEN cells. ETS and ETX embryos form a luminal cavity and display patterning of the primitive streak. hESC epiblast-like cysts are cultured in Matrigel, undergo symmetry breaking to form a domain of primitive streak marker expression and also form a luminal cavity. Abbreviations: AVE = anterior visceral endoderm, hESC = human embryonic stem cells, mESC = mouse embryonic stem cells, XEN = extraembryonic endoderm stem cells, TSC = trophoblast stem cells.

embryos, so-called 'Keller explants', can undergo extensive morphogenesis *in vitro*, highly reminiscent of their behavior *in vivo* (Keller and Danilchik, 1988; Keller et al., 1985; Shih and Keller, 1992a, 1992b; Wilson and Keller, 1991).

Building on those seminal observations, more recent efforts using embryonic stem cell (ESC) cultures were successful in recreating a large

Box 1
Morphogenesis *in vitro*.

In addition to specifying germ layer cell fates, mouse and human ESC gastrulation models also show some characteristics of morphogenetic movements, typically associated with gastrulation in intact embryos (Beccari et al., 2018; Marikawa et al., 2009; Martyn et al., 2018; 2019a; 2019b; Morgani et al., 2018; Moris et al., 2020a; Simunovic et al., 2019; Sozen et al., 2018; ten Berge et al., 2008; Turner et al., 2017; van den Brink et al., 2014; Warmflash et al., 2014). Most prominently, gastruloids form a polarized extension in the domain of mesendodermal marker expression (Beccari et al., 2018; Marikawa et al., 2009; Moris et al., 2020a; Turner et al., 2017; van den Brink et al., 2014), reminiscent of the behavior of Keller explants, Activin-treated *Xenopus* animal caps and aggregates, and zebrafish explants (Fulton et al., 2020; Green et al., 2004; Keller and Danilchik, 1988; Keller et al., 1985; Ninomiya et al., 2004; Schauer et al., 2020; Williams and Solnica-Krezel, 2020; Xu et al., 2014). Axis elongation in gastruloids occurs despite incomplete induction of the most anterior embryonic structures (Beccari et al., 2018; Moris et al., 2020a; Turner et al., 2017; van den Brink et al., 2014), raising questions as to how axis patterning is translated into large-scale axis extension, and what minimal polarity information and domain coherence have to be provided for axis elongation to occur. Additionally, cells are extruded from the mesendodermal domains of the extension in gastruloids (van den Brink et al., 2014), and movement of cell islands with endodermal identity was suggested to exhibit fragmentation and sorting dynamics rather than transitioning to a mesenchymal state (Hashmi et al., 2020; Vianello and Lutolf, 2020). In ETX embryos, consisting of mESCs, trophoblast stem cells and XEN cells, primitive streak cells even undergo an epithelial-to-mesenchymal transition (EMT), reminiscent of the internalization movements of their *in vivo* counterparts (Sozen et al., 2018). Also 2D micropattern colonies, when differentiated towards germ layer fates, show some signatures of morphogenesis associated with gastrulation (Martyn et al, 2018, 2019a, 2019b; Morgani et al., 2018; Warmflash et al., 2014). For instance, primitive streak-like cells express EMT markers and progressively move more centrally over time (Morgani et al., 2018; Warmflash et al., 2014).

extent of germ layer patterning and even morphogenesis *in vitro* (Box 1) (reviewed in Baillie-Benson et al., 2020; Brickman and Serup, 2017; Fu et al., 2020; Hadjantonakis et al., 2020; Heemskerk, 2020; Metzger et al., 2018; Moris et al., 2020b; Shahbazi et al., 2019; Shahbazi and Zernicka-Goetz, 2018; Siggia and Warmflash, 2018). Importantly, the degree to which these stem cell models can pattern germ layers and trigger morphogenesis critically depends on the nature of the source cells, extrinsic signals as well as geometric and mechanical inputs (reviewed in Fu et al., 2020; Heemskerk, 2020; Metzger et al., 2018; Shahbazi et al., 2019; Shahbazi and Zernicka-Goetz, 2018; Simunovic and Brivanlou, 2017). For instance, when colonies of human (h) or mouse (m) ESCs are confined in two dimensions (2D) on micropatterns and uniformly exposed to signaling molecules, they establish distinct, radially-organized expression domains of germ layer and extraembryonic tissue markers (Fig. 2B) (Martyn et al, 2018, 2019a; Morgani et al., 2018; Warmflash et al., 2014). More specifically, upon treatment with BMP4 ligand, micropatterned colonies form ring-like expression domains of extraembryonic tissues, endoderm, mesoderm and ectoderm arranged along the radial axis from the colony rim towards its center (Warmflash et al., 2014). In contrast, exposure to Wnt signaling was shown to be sufficient to induce the expression of genes marking the primitive streak, such as *brachyury*, at the rim of the colony, but failed to specify extraembryonic cell fates (Fig. 2B) (Martyn et al, 2018, 2019a). Recently, single-cell transcriptomics has been used to characterize the gene expression profile of hESCs in micropatterned colonies, leading to the characterization of various epiblast, ectoderm, mesoderm, endoderm derivatives and extraembryonic cell types by comparison with other mammalian species (Minn et al., 2020).

Approaches using three-dimensional (3D) aggregates of mESCs, commonly called 'embryoid bodies', also showed that a polarized expression domain of primitive streak markers can spontaneously emerge when signaling molecules, such as Wnt, BMP or Nodal, are uniformly supplied in the culture medium (Fig. 2B) (ten Berge et al., 2008). Follow-up studies could further demonstrate that different cell lines possess a high intrinsic capacity for generating gastrula-like features under specific culture conditions, thereby highlighting the importance of extrinsic factors in the ESC cultures to direct germ layer formation and positioning (Baillie-Johnson et al., 2015; Marikawa et al., 2009; Poh et al., 2014; Turner et al., 2017; van den Brink et al., 2014). In particular, so-called 'gastruloids' were shown not only to break symmetry with remarkable robustness from initially homogeneous 3D aggregates of mESCs, but also to form multiple body axes, undergo axis elongation (Box 1) and even show signatures of somite development and cardiogenesis under appropriate culture conditions, highly reminiscent of their *in vivo* counterparts (Fig. 2B) (Beccari et al., 2018; Rossi et al., 2020; Turner et al., 2017; van den Brink et al., 2014, 2020; Veenvliet et al., 2020). This points at an extraordinary self-organizing capacity of mESCs to recapitulate germ layer formation and morphogenesis under suitable culture conditions. Interestingly, gastruloids typically do not express markers of extraembryonic cell types and also show deficiencies in brain and anterior head structure formation, suggesting that cell fate specification is incomplete (Beccari et al., 2018; Turner et al., 2017; van den Brink et al., 2014). Similarly to mESCs, hESCs display a high capacity to self-organize germ layer patterning and undergo axis elongation (Fig. 2B) (Moris et al., 2020a). Moreover, 3D epiblast models of hESCs embedded in Matrigel were shown to break radial symmetry to specify a domain of primitive streak marker expression, and to form a luminal cavity (Fig. 2B) (Simunovic et al., 2019).

Given the importance of extraembryonic tissues for proper mammalian development (reviewed in Rossant and Tam, 2009), ESCs were also co-cultured with extraembryonic stem cells (trophoblast stem cells and extraembryonic endoderm/XEN cells) to more closely recapitulate early mammalian development (Harrison et al., 2018, 2017; Rivron et al., 2018; Sozen et al., 2018; S. Zhang et al., 2019). Strikingly, co-culturing mESCs and trophoblast stem cells in the presence of extracellular matrix (ETS embryos) can trigger the formation of aggregates with patterned mesendodermal progenitors, the geometry of which closely resembles a gastrulating embryo (Fig. 2B) (Harrison et al., 2017). Moreover, in such co-cultures, Nodal signaling leads to the formation of a cavity (Harrison et al., 2017), and primitive streak cells even undergo an epithelial-to-mesenchymal transition (EMT) in the presence of XEN cells (ETX embryos) (Fig. 2B) (Box 1) (Sozen et al., 2018; S. Zhang et al., 2019).

Collectively, extending the seminal observations that embryonic substructures can retain their developmental potential when taken out of their embryo-context, *in vitro* approaches revealed that mESCs and hESCs aggregates are capable of closely recapitulating developmental processes that occur in embryo gastrulation. This provides a unique opportunity to study gastrulation by trying to reconstitute this process *in vitro*.

3. Symmetry breaking upstream of germ layer formation

Before germ layer formation begins, the initial symmetry of the embryo has to be broken in order to specify the main body axes and define the site of gastrulation initiation. How this occurs can vary, ranging from asymmetries on the single cell level to tissue-level symmetry breaking events (Fig. 3A). In some embryos, such as the frog embryo, an asymmetry is already intrinsic to the oocyte through the localization of maternal determinants (reviewed in Heasman, 2006). More specifically, during *Xenopus* development, a process known as cortical rotation establishes the dorsoventral axis by transporting cortical vegetal cytoplasm, which contains mRNAs coding for signaling molecules (e.g. *Wnt11*, *Vg1*) and other factors, to the future dorsal side (reviewed in Heasman, 2006). An inductive signaling cascade on the dorsal side, together with TGFβ signaling pathway activation originating from the vegetal cells, then leads to mesoderm induction and the formation of the so-called Spemann organizer on the dorsal side of the gastrula (reviewed in De Robertis et al., 2000). Supporting the importance of this early dorsal center, classical experiments by Spemann showed that when dividing an amphibian gastrula into a dorsal and ventral half, only the dorsal half will give rise to a full embryo, while the ventral half does not form axial structures (Spemann, 1938). A different strategy to break symmetry is used in mammals, such as mouse, where inductive interactions between the epiblast, giving rise to the embryo proper, and the extraembryonic ectoderm (ExE) and visceral endoderm (VE) are essential (reviewed in Stern and Downs, 2012; Tam and Loebel, 2007). In mouse, positioning of the site of gastrulation, the primitive streak, and concomitantly of the anterior-posterior axis of the embryo, requires secretion of inhibitors of Nodal and Wnt signaling from the anterior visceral endoderm (AVE), suppressing posterior fates (reviewed in Arnold and Robertson, 2009). In some species, such as zebrafish, also a combination of maternal pre-patterning, as in frogs, and signaling from extraembryonic tissues, as in mice, are required for robust mesendoderm formation (Carvalho and Heisenberg, 2010; Chen and Kimelman, 2000; Fan et al., 2007; Gagnon et al., 2018; Hong et al., 2011; Langdon and Mullins, 2011; Marlow, 2020; Mizuno et al, 1996, 1999; Ober and Schulte-Merker, 1999; Veil et al., 2018; Xu et al., 2012).

In systems recapitulating mouse germ layer formation with mixtures of embryonic and extraembryonic stem cells, symmetry breaking, as assayed by asymmetric *brachyury* expression, occurs at the boundary between embryonic and extraembryonic cells, like in intact embryos (Fig. 3B) (Harrison et al., 2017; Sozen et al., 2018; S. Zhang et al., 2019). In particular, the presence of trophectoderm stem cells appears important to promote mesoderm induction, while the presence of both trophectoderm and XEN cells confers the highest capacity to ESCs to form a primitive streak-like domain (Harrison et al., 2017; Sozen et al., 2018; S. Zhang et al., 2019). Notably, a domain of AVE marker expression can form within the XEN-layer in such gastrulation models, albeit at variable frequency (Sozen et al., 2018; S. Zhang et al., 2019). These findings raise questions as to how the XEN layer itself is patterned, and how AVE specification and symmetry breaking are connected in such reconstituted systems.

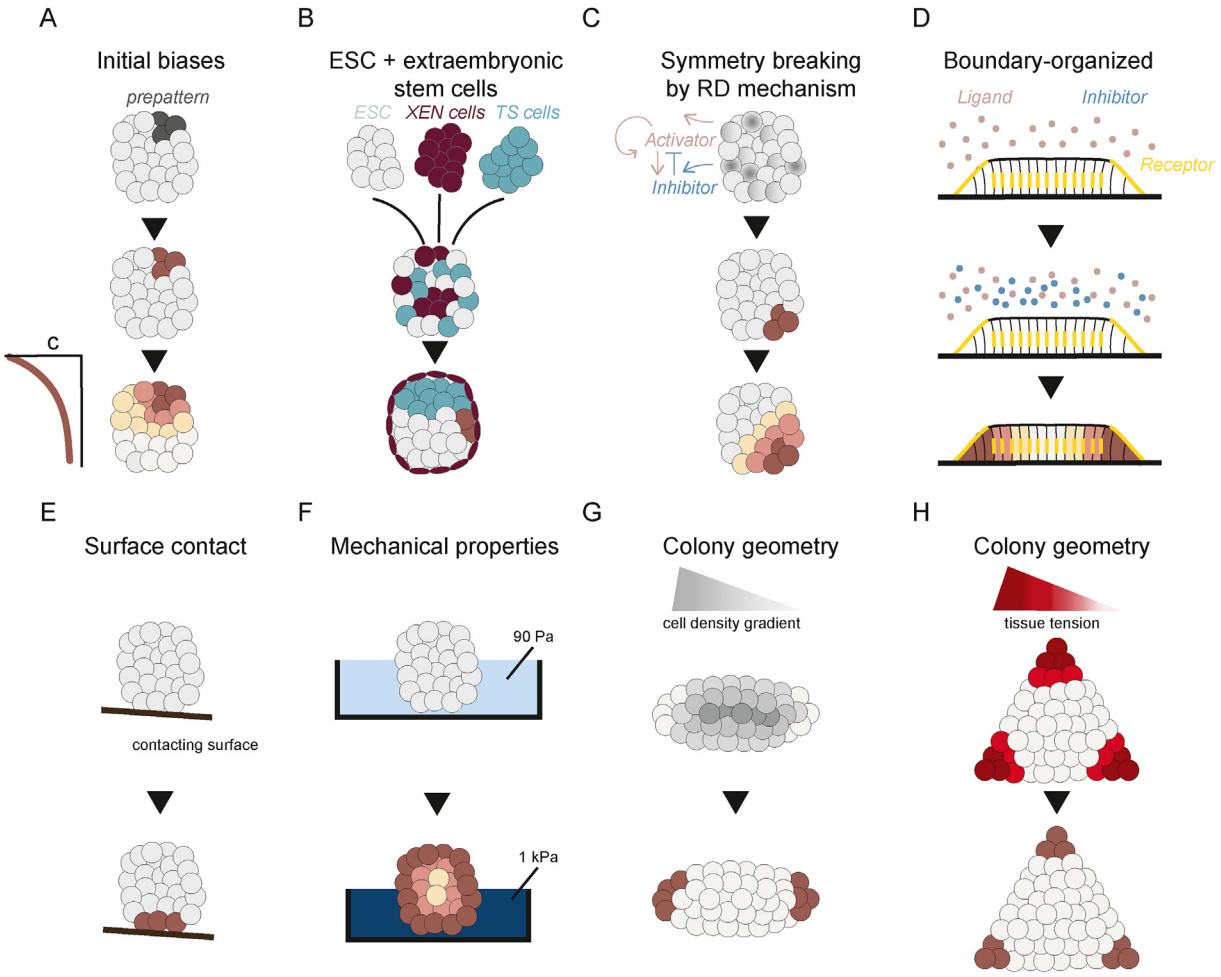

Fig. 3. Symmetry breaking and extrinsic factors.
(A) Schematic representation of a cell cluster with an initial bias to form an organizing/patterning center, as frequently found in embryos. Prepattern (dark grey cells) induces expression of signaling molecules leading to signaling gradient formation and patterning of the cell cluster. Brown cells indicate the symmetry breaking center. (B) Schematic representation of combinations of ESCs, XEN cells and TSCs sorting into different domains and inducing distinct cell fates at the forming boundary of ESC and TS cells. (C) Schematic representation of symmetry breaking by cell-to-cell variability and reaction-diffusion mechanisms in cell clusters. Fluctuations in the cell state within a seemingly homogeneous cell cluster are indicated in different shades of grey. Interactions between a diffusible activator and inhibitor lead to the formation of a signaling center that gives rise to a signaling gradient and leads to patterning of the cell cluster. (D) Schematic representation of a 2D micropatterned colony with polarized receptor localization. Receptors are found at the basolateral side in the middle of the colony and on the apical side at the colony rim. Signaling molecules are therefore predominantly sensed at the rim, triggering the expression of a fast-diffusing inhibitor to further promote the signaling response at the rim and subsequent pattern establishment from the rim of the colony to its center. (E) Schematic representation of a 3D aggregate contacting a surface on one side. Surface contact biases the formation of the asymmetric pattern on this side. (F) Schematic representation of patterning of a cell cluster on substrates of different mechanical properties. Different soft substrates promote patterning within the cell cluster. (G) Schematic representation of geometry-dependent cell density gradients affecting cell fate specification and differentiation. (H) Schematic representation of geometry-dependent differential tissue tension biasing cell fate specification and differentiation. Abbreviations: RD = reaction-diffusion, TSC = trophoblast stem cells, XEN = extraembryonic endoderm stem cells, ESC = embryonic stem cells.

Observations in other *in vitro* models, such as 3D mESC and hESC gastruloids and hESC cysts, suggest that symmetry breaking can also occur in the absence of extraembryonic cell fate specification (Beccari et al., 2018; Moris et al., 2020a; Simunovic et al., 2019; Turner et al., 2017; van den Brink et al., 2014). Interestingly, while in those cases, symmetry breaking requires uniform exposure to Nodal, Wnt or BMP activators (Box 2), no initial spatial signaling bias seems to be needed (Beccari et al., 2018; Moris et al., 2020a; Simunovic et al., 2019; ten Berge et al., 2008; Turner et al., 2017; van den Brink et al., 2014). This suggests that additional mechanisms must be in place to translate an initial uniform exposure to signaling factors into distinct patterns of signaling pathway activities and associated fate specification.

One possible explanation for such mechanism(s) is given by reaction-diffusion models, pioneered by Turing, which provide a theoretical framework of how spatiotemporal concentration heterogeneities and subsequently patterns can arise from an initially uniform system through reactions of differentially diffusing morphogens (Turing, 1952). Extensions of this model suggest that initial signaling fluctuations can be amplified by a diffusible signaling activator that locally enhances its own production and the production of an antagonist with higher diffusivity, inhibiting signal activation at a larger distance (Fig. 3C) (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 2000). Such systems can give rise to various self-organized patterns *de novo* depending on the reaction and diffusion parameters of the specific morphogen and inhibitor pair (reviewed in Landge et al., 2020). In gastrulating zebrafish embryos, for instance, the morphogen Nodal and its antagonist Lefty were proposed to trigger localized mesendoderm specification by functioning as an activator-inhibitor pair with differential diffusivity engaged in a Turing-like reaction-diffusion mechanism (Chen and Schier, 2001; Müller et al., 2012). It is thus conceivable that an intrinsic variability within the initial stem cell population could promote signaling fluctuations, which in turn lead to symmetry breaking via a reaction-diffusion

Box 2
Signaling dynamics for *in vitro* germ layer patterning.

In the gastrulating embryo, maternal pre-patterning and/or interactions with extraembryonic tissues are required for the proper expression of signaling molecules involved in germ layer formation, while in *in vitro* stem cell cultures these signaling molecules are typically supplemented to the medium. A characteristic feature of 3D *in vitro* gastrulation models is the formation of a Wnt signaling domain that is required for the expression of primitive streak markers (Harrison et al., 2017; Moris et al., 2020a; Simunovic et al., 2019; ten Berge et al., 2008; Turner et al., 2017; van den Brink et al., 2014). Consistent with this, asymmetric *brachyury* expression is abolished by exposure to the Wnt antagonist DKK, independently of whether symmetry breaking is triggered by the addition of Activin, Wnt or BMP ligands (ten Berge et al., 2008). Furthermore, the interplay of Wnt and Nodal signaling, and the timing of exposure to the Wnt agonist Chiron are critical for robust symmetry breaking in gastruloids (Turner et al., 2017). In 2D micropatterned hESC colonies, exposure to BMP4 leads to activation of BMP, Nodal/Activin and Wnt signaling, and subsequent extraembryonic, ectodermal, mesodermal and endodermal cell fate specification (Chhabra et al., 2019; Warmflash et al., 2014). Moreover, when Nodal and Wnt signaling are inhibited, differentiation of mesendodermal progenitors is defective in those cultures (Chhabra et al., 2019; Martyn et al., 2018; Morgani et al., 2018; Tewary et al., 2017; Warmflash et al., 2014). Notably, varying the composition of supplemented signaling molecules has profound effects on the types of tissues which can form (Martyn et al, 2018, 2019a; Morgani et al., 2018; Warmflash et al., 2014). For instance, while exposure to Wnt signaling activators promotes the induction of primitive streak fates at the rim of the colony, adding both Wnt and Activin to the culture medium even leads to the formation of a functional organizer (Martyn et al, 2018, 2019a). This suggests that the repertoire of possible cell fate decisions *in vitro* depends on the combined activation of different signaling pathways in response to the ligands supplied. Finally, cell fate decisions have long been suggested to depend on the duration and level (dosage) of signaling, and the interactions with other signaling pathways, properties that are typically intertwined in intact embryos and thus can be best untangled using *in vitro* systems, such as gastruloids. For instance, in hESC 2D micropatterned colonies, a stable transcriptional response to Nodal signaling was shown to rely on prior Wnt signaling, and target gene expression to depend on signaling dynamics, i.e. Nodal target gene expression is determined by changes in ligand concentration, while BMP-induced gene expression depends on the absolute amount of the upstream signal (Heemskerk et al., 2019; Massey et al., 2019; Yoney et al., 2018).

mechanism that is stabilized and amplified through feedback loops and acts as a reference point for further patterning (Turner et al., 2016; Xavier da Silveira dos Santos and Liberali, 2019). Such cell-to-cell heterogeneities can for instance originate from differences in the cell cycle phase or the surrounding microenvironment and were, indeed, observed in undifferentiated cultures of ESCs, which exhibit fluctuations in the expression of pluripotency factors, such as Nanog (reviewed in Xavier da Silveira dos Santos and Liberali, 2019). Yet, whether and how such fluctuations affect symmetry breaking is still unclear.

While reaction diffusion models coupled to random signaling fluctuations (Fig. 3C) provide a plausible explanation for *in vitro* symmetry breaking in 3D models in the absence of extraembryonic tissues, supporting evidence from experiments is still scarce: symmetry breaking in hESC cysts upon BMP4 exposure depends on both Wnt ligands and Wnt signaling inhibitors (DKK-1) expressed within the same cells, suggesting that Wnt and DKK-1 could in principle function as a Turing-like activator-inhibitor pair for symmetry breaking (Simunovic et al., 2019). However, a systematic analysis of their physical properties and interactions to support such a mechanism is still lacking. Furthermore, live-imaging of mESC and hESC gastruloids undergoing symmetry breaking showed that a dominant patch of *brachyury* expression forms from an initially transient uniform expression domain and further expands, a behavior indicative of reaction diffusion models (Moris et al., 2020a; van den Brink et al., 2014). Yet, to show that symmetry breaking is indeed achieved in such *in vitro* models through a reaction-diffusion mechanism, dynamic changes in signaling pathway activity and ligand/antagonist expression during symmetry breaking need to be monitored in more detail.

In addition, or alternative to reaction-diffusion mechanisms, a combination of biochemical signals, such as Wnts, and contact with external surfaces, was proposed to influence primitive streak formation in embryoid bodies (Fig. 3E) (Sagy et al., 2019). When a source of Wnt or DKK is close to a contact point with external surfaces, the *brachyury* expression domain within the embryoid body is shifted relative to the contact point, suggesting that biochemical and mechanical signals can function together in determining the localization of the primitive streak (Sagy et al., 2019). Further studies will need to determine to what extent such potential mechanochemical feedback loops promote symmetry breaking both *in vitro* and *in vivo*.

In contrast to 3D embryoids/gastruloids, micropatterned 2D ESC colonies do not break radial symmetry upon uniform ligand exposure. Yet, they still form asymmetric expression domains from the rim of the colony towards its center, when uniformly exposed to, for instance, BMP signals (Morgani et al., 2018; Tewary et al., 2017; Warmflash et al., 2014). This is also reflected by the formation of an activity gradient of the BMP and Nodal intracellular signaling mediators pSMAD1 and pSMAD2 along the radial axis of the colony (Chhabra et al., 2019; Etoc et al., 2016; Morgani et al., 2018; Tewary et al., 2017; Warmflash et al., 2014). Notably, such 2D colonies are apically polarized, and self-organized patterning in response to BMP4 exposure relies on a cell density-dependent re-localization of the BMP receptors to the basolateral side in the colony center, and induction of Noggin, a secreted BMP inhibitor (Fig. 3D) (Etoc et al., 2016). This points at the intriguing possibility that a reaction-diffusion mechanism (with BMP and Noggin as an activator-inhibitor pair), in conjunction with a pre-pattern of differential receptor localization, restricts BMP signaling activity to the rim of the colony (Etoc et al., 2016). Interestingly, while this initial gradient of BMP signaling activity remains restricted to the rim of the colony, gradients of signaling activity downstream of BMP, in particular Wnt and Nodal signaling gradients, propagate to the inside of the colony in a wave-like manner, rather than forming stable signaling gradients (Chhabra et al., 2019; Heemskerk et al., 2019). This behavior cannot be explained by a Turing-like reaction-diffusion mechanism alone and has important implications for patterning of the colonies (Chhabra et al., 2019).

Similar to the observations made on BMP-mediated symmetry breaking, micropatterned ESC colonies, which are treated with Wnt ligands to induce primitive streak fate at the rim, also show a cell density dependent restriction of Wnt signaling activation to the colony rim (Martyn et al., 2019a). Moreover, this rim-restriction of Wnt signaling activity does not occur when cells are treated with the intracellular Wnt signaling activator Chiron, while it depends on both Wnt-dependent activation of the Wnt antagonist DKK and E-cadherin-mediated cell-cell adhesion (Martyn et al., 2019a). This suggests that reaction-diffusion systems, combined with rim sensing, i.e. the competence of a cell to respond to a signal being dependent on the distance from the colony rim, can function as a universal mechanism to promote radial patterning of initially uniform 2D micropatterned colonies in response to different inducing signals. The capacity of symmetry breaking in 2D colonies was

also highlighted by recent studies, demonstrating that asymmetrically providing the ligand using microfluidics can break the radial symmetry of the colony and induce asymmetric patterning (Manfrin et al., 2019).

4. The role of boundary conditions and extrinsic factors for robustness of germ layer formation

While the main signaling pathways and morphogenetic movements leading to germ layer formation appear to be evolutionarily conserved, embryos from different species vary substantially in size, presence of extraembryonic tissues and overall morphology (Fig. 2A). The impact of such differences for embryo patterning and morphogenesis is difficult to dissect *in vivo* given the inherent complexity of the developing embryo and the interdependence of these various processes. *In vitro* models, however, provide a more controlled environment to systematically dissect how these differences impact on gastrulation.

4.1. The role of extraembryonic structures

A key part of embryo development in many species is the formation of extraembryonic tissues, which play crucial roles for signaling and morphogenesis during gastrulation. Yet, the observation that embryoid bodies and gastruloids can break their radial symmetry and give rise to primitive streak fate in absence of extraembryonic tissues (Beccari et al., 2018; Moris et al., 2020a; Simunovic et al., 2019; Turner et al., 2017; van den Brink et al., 2014) raises questions as to the importance and actual role of extraembryonic tissues in these processes.

Not only mouse and human gastruloids (Beccari et al., 2018; Moris et al., 2020a; Turner et al., 2017; van den Brink et al., 2014), but also zebrafish embryonic explants were recently shown to undergo symmetry breaking and germ layer specification in the absence of extraembryonic structures or exogenously supplied signaling factors (Fulton et al., 2020; Schauer et al., 2020). Zebrafish embryos consist of the blastoderm, which gives rise to the embryo proper, sitting on top of an extraembryonic yolk cell, over which the blastoderm spreads during gastrulation (Kimmel et al., 1995). Previous studies had shown that the yolk cell, in addition to its function as a substrate for gastrulation movements, is crucial for germ layer induction. Specifically, signaling molecules, such as Nodal and BMP ligands are thought to be secreted from the yolk syncytial layer (YSL), a thin cytoplasmic layer at the surface of the yolk cell, thereby initiating and promoting mesendoderm induction and patterning of the three germ layers (Carvalho and Heisenberg, 2010; Chen and Kimelman, 2000; Erter et al., 1998; Fan et al., 2007; Feldman et al., 1998; Gagnon et al., 2018; Hong et al., 2011; Mizuno et al, 1996, 1999; Ober and Schulte-Merker, 1999; Rebagliati et al., 1998; Sun et al., 2014; Veil et al., 2018; Wilkins et al., 2008; Xu et al., 2012). Nonetheless, when explants of the entire zebrafish blastoderm are cultured without the yolk cell, derivatives of all three germ layers are formed and patterned, suggesting that formation of a proper YSL could be dispensable for these processes (Fulton et al., 2020; Schauer et al., 2020).

While these findings suggest that germ layers can be induced in absence of extraembryonic structures, neither zebrafish explants nor mESC- or hESC-based gastruloids can robustly recapitulate the full patterning and morphogenetic potential of the intact embryo. In zebrafish explants, for instance, mesendoderm internalization is defective giving rise to an 'exogastrula', where the mesoderm, instead of internalizing below the ectoderm, forms an extension (Box 1) (Fulton et al., 2020; Schauer et al., 2020; Williams and Solnica-Krezel, 2020; Xu et al., 2014). Moreover, the spatiotemporal dynamics of Nodal signaling activity are altered and subsequently the formation of progenitor types that require the highest levels of Nodal signaling activity, namely head mesoderm and endoderm (Dougan et al., 2003; Gritsman et al., 2000; Schier et al., 1997; Thisse and Thisse, 1999), is highly variable (Schauer et al., 2020). Interestingly, this is similar to embryos where the production of Nodal signals is reduced specifically within the YSL, suggesting that the zebrafish YSL is critical to reach peak levels of Nodal signaling activity required for robust head mesoderm and endoderm formation (Fan et al., 2007; Schauer et al., 2020). Whether other signaling pathways might be affected by the loss of the extraembryonic YSL, thereby further contributing to the observed mesendoderm patterning variability, remains to be tested. Moreover, how mesendoderm cells adopt the morphogenetic program to form an exogastrula in explants, instead of internalizing below the presumptive ectoderm, is currently unclear.

Notably, mESC- and hESC-based gastruloids, similar to zebrafish explants, have been shown to be deficient in the formation of the most anterior structures, presumably due to the absence of the extraembryonic AVE in gastruloids (Beccari et al., 2018; Moris et al., 2020a; Turner et al., 2017; van den Brink et al., 2014), which in the intact mouse embryo secretes various signaling inhibitors and is required for primitive streak formation and induction of anterior neural structures (reviewed in Rossant and Tam, 2009; Srinivas, 2006). Interestingly, a recent report describing post-implantation epiblast-like aggregates (EPI aggregates) shows that uniformly inhibiting Wnt signaling leads to a seeminlgy more complete patterning of the anterior-posterior axis, now including anterior neural tissues, suggesting that proper modulation of Wnt signaling levels might be key for proper patterning of the anterior-posterior body axis *in vitro* (Girgin and Lutolf, 2020). Moreover, ETX embryos, which consist of embryonic and extraembryonic stem cells, also show signatures of most anterior fates, in addition to mimicking the morphology of the intact embryos more closely by e.g. forming a proamniotic cavity and a primitive streak where cells undergo EMT (Sozen et al., 2018). A systematic comparison of ETX models with gastruloids that are cultured under different signaling regimes and thus exhibit different patterning capacities, and intact embryos will be needed to fully understand the various contributions of extraembryonic tissues for robust symmetry breaking, embryo patterning and morphogenesis.

4.2. The role of size, scaling and cell density

During gastrulation, correct proportions of ectodermal, mesodermal and endodermal progenitor cell populations have to be formed. Recent findings in zebrafish have suggested that Nodal signaling, a key factor in mesendoderm induction and patterning, plays an important role in adjusting germ layer dimensions to embryo size by scaling the amount of the highly diffusible Nodal antagonist Lefty with the size of the blastoderm (Almuedo-Castillo et al., 2018). Intriguingly, embryos display a remarkably high capacity to scale patterns even when their size is experimentally altered (reviewed in Čapek and Müller, 2019). In amphibians, for instance, cutting the embryo in two leads to the formation of smaller, but viable and well-proportioned tadpoles, as long as both halves retain some part of the dorsal organizer (Spemann, 1938). Different mechanisms, such as feedback interactions, input from opposing morphogen gradients and adjusted boundary conditions, were proposed to explain how embryo patterning can scale with changes in embryo size (reviewed in Čapek and Müller, 2019; Kicheva and Briscoe, 2015).

Strikingly, controlling the initial size of stem cell aggregates is critical for conferring robustness to patterning (Bauwens et al., 2008), with only gastruloids made from approximately 300 cells being able to reliably form one single elongating axis (van den Brink et al., 2014). Yet, how aggregate size affects the likelihood of having one single symmetry breaking event is still unclear. Interestingly, in 2D hESC micropatterned colonies, the size of progenitor domains, measured from the colony rim to its center, does not scale with the size of the colony, resulting in cell fates from the middle of the domain being progressively lost when the colony size is reduced (Tewary et al., 2017; Warmflash et al., 2014). This is consistent with the proposed mechanism of boundary-organized cell fate induction, whereby increased cell density in the colony center restricts signaling responses to the rim of the colony by promoting differential localization of the corresponding receptors, a mechanism not directly dependent on colony size (Etoc et al., 2016). Moreover, the length-scale of the signaling response from the rim towards the colony center depends on the concentration of BMP4 ligand in the medium to

differentiate ESC colonies (Etoc et al., 2016; Tewary et al., 2017). This suggests that for a given BMP4 concentration a minimal colony size is necessary to establish gradients of signaling activity that allow patterning of multiple cell fates (Tewary et al., 2017). Thus, while micropatterned colonies appear to lack an intrinsic scaling capacity, adjusting the signaling gradients from the rim to the colony center with the size of the colony represents a possible mechanism promoting induction of the full complement of germ layer cell types in colonies of variable sizes.

Collectively, the apparent inability of 3D gastruloids and 2D micropatterned colonies to directly scale patterning with size (Heemskerk, 2020; Simunovic and Brivanlou, 2017) raises important questions as to why scaling is present in embryos, but not in those *in vitro* models, and what roles e.g. initial asymmetries by pre-patterning in the embryo might play in this process.

4.3. The role of geometry, morphology and mechanics

Increasing evidence shows that tissue geometry, mechanics and patterning are interconnected processes in embryogenesis and organogenesis (reviewed in Hannezo and Heisenberg, 2019). Gastrulating embryos can display a variety of different shapes and structures, such as the blastocoel, which forms in some species (e.g. frogs and mice), but not in others (e.g. zebrafish). These differences in embryo and tissue geometry can have profound influences on processes guiding embryo patterning and morphogenesis by, for instance, affecting tissue mechanics or morphogen gradient establishment (reviewed in Hannezo and Heisenberg, 2019; Nelson, 2009).

Experiments varying the shape of micropatterned surfaces provide insight into the mechanisms by which 2D ESC colony geometry affects positioning of *brachyury* expressing cells giving rise to the primitive streak (Blin et al., 2018; Chhabra et al., 2019; Muncie et al., 2020; Smith et al., 2018). In colonies with ellipsoid shape, for instance, *brachyury* positive cells preferentially localize to the tips of colonies, an effect that was attributed to shape-dependent differences in local cell densities (Fig. 3G) (Blin et al., 2018). The specific geometry of stem cell colonies was also proposed to affect colony patterning by inducing differential tension along the colony boundary, with high tension promoting *brachyury* expression (Fig. 3H) (Muncie et al., 2020; Smith et al., 2018). In line with this, E-cadherin-mediated tissue tension was suggested to induce *brachyury* expression in ESC colonies through tension-dependent nuclear translocation of the transcriptional coactivator β-catenin (Muncie et al., 2020), similar to the proposed role of mechanosensitive β-catenin in germ layer formation *in vivo* (Brunet et al., 2013; Pukhlyakova et al., 2018). Finally, geometry-specific patterning in micropatterned ESC colonies can also be predicted solely based on the inherent signaling dynamics, independent of tissue mechanics (Chhabra et al., 2019).

Not only global shape, but also compartmentalization of the gastrulating embryo is critical for proper patterning. Recent studies in mouse embryos revealed that the specific basolateral localization of receptors in cells facing the proamniotic cavity is important for the robust formation of a BMP-pSMAD1 signaling gradient and subsequently primitive streak formation (Z. Zhang et al., 2019). Specifically, BMP4 ligands released into the proamniotic cavity need to pass through a channel at the boundary between the epiblast and extraembryonic ectoderm in order to reach the receptors localized on the basolateral side of cavity lining cells. This channeling mechanism of BMP ligands not only allows robust gradient formation from uniform ligand distributions, but also buffers the system for fluctuations in ligand concentrations by providing a large (proamniotic) cavity into which the ligand diffuses (Z. Zhang et al., 2019). Comparing primitive streak formation between mouse gastrulation models containing extraembryonic tissues and forming a clearly discernible central lumen (ETS and ETX embryo models) with those lacking these structures (mESC gastruloids) (Harrison et al., 2017; Sozen et al., 2018; van den Brink et al., 2014; S. Zhang et al., 2019), might provide further insight into the role that tissue compartmentalization and fluid-filled cavities play for robust primitive streak formation and germ layer patterning.

Besides embryo shape and compartmentalization, tissue mechanics were also implicated in germ layer patterning *in vitro* (Muncie et al., 2020; Poh et al., 2014; Smith et al., 2018). For instance, it was found that culturing ESCs under differentiation conditions on softer substrates promotes mesoderm and endoderm formation (Chen et al., 2020; Muncie et al., 2020; Przybyla et al., 2016), and moving 3D stem cell colonies from soft (90-Pa stiffness) to less soft (1-kPa stiffness) substrates can trigger the formation of radially arranged germ layer progenitors in absence of differentiation factors (Fig. 3F) (Poh et al., 2014). Moreover, the mechanical environment was found to be critical for the organization of *in vitro* models of the post-implantation human amniotic sac (Shao et al., 2017). These findings suggest that germ layer induction and patterning in ESC models is affected by both geometrical and mechanical factors, pointing at the importance of incorporating mechanical signals in the processes leading to germ layer patterning and morphogenesis.

5. Conclusions

Embryogenesis reliably occurs, irrespective of whether the embryo develops internally or externally, in water or on land, and independent of the final embryonic morphology. Recent studies demonstrated that clusters of embryonic stem cells, when supplied with the right external input, can form structures closely resembling embryos. This shows a remarkable capacity of ESCs for self-organization, but also raises key questions as to how closely such stem cell models mimic the decision making and patterning processes of an intact embryo. That said, *in vitro* models provide a powerful platform to advance our understanding of early developmental processes by showing the many ways by which embryogenesis can be achieved, even if not necessarily how it is achieved in the intact organism. Ultimately, comparing *in vitro* models and embryos can provide critical insight into developmental robustness and the capacity of each developmental step to self-organize depending on the specific upstream input. In particular, the unique ability to precisely control cell signaling and boundary conditions, such as geometry, *in vitro* provides a great opportunity for teasing apart the specific contributions of those processes to embryogenesis, something much more difficult to achieve in the intact organism.

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