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The dynamics of morphogenesis in stem cell-based embryology: novel insights for symmetry breaking

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Abstract

Breaking embryonic symmetry is an essential prerequisite to shape the initially symmetric embryo into a highly organized body plan that serves as the blueprint of the adult organism. This critical process is driven by morphogen signaling gradients that instruct anteroposterior axis specification. Despite its fundamental importance, what triggers symmetry breaking and how the signaling gradients are established in time and space in the mammalian embryo remain largely unknown. Stem cell-based *in vitro* models of embryogenesis offer an unprecedented opportunity to quantitatively dissect the multiple physical and molecular processes that shape the mammalian embryo. Here we review biochemical mechanisms governing early mammalian patterning *in vivo* and highlight recent advances to recreate this *in vitro* using stem cells. We discuss how the novel insights from these model systems extend previously proposed concepts to illuminate the extent to which embryonic cells have the intrinsic capability to generate specific, reproducible patterns during embryogenesis.

Introduction

Mammalian embryonic patterning and axis formation is highly intricate and not easily dissected into its component parts. During gastrulation, the entire embryo undergoes coordinated morphogenesis to establish the future body plan, all seemingly relying on sophisticated interactions within and between tissues (Tam and Behringer, 1997; Tam et al., 1993). This process requires complex networks of signaling interactions at the level of single cells, as well as geometric and topological transformations at the level of the embryo (Arnold and Robertson, 2009; Tam and Loebel, 2007). This complexity is amplified at gastrulation, where embryonic radial symmetry is broken to establish the anterior-posterior (AP) axis and germ layers are subsequently specified (Tam et al., 1993).

How the early embryo can undertake this complex transformation with such fidelity and precision has remained for the most-part a mystery. This is primarily because decoupling the cellular movements in different parts of the developing embryo is prohibitively difficult given limited possibilities of *in vivo* manipulations. These difficulties have prompted efforts to model

embryogenesis *in vitro* using stem cell lines, which are more amenable and tractable for experimentation. Reconstructing mammalian gastrulation *in vitro* enables the control of biophysical and biochemical properties of the cells and tissues as they form, providing novel insights into the principles of axis patterning.

Here, we discuss the utility of *stem cell-based embryology*, in answering one of the most contested questions in early mammalian development: how embryonic symmetry is broken. By outlining recent advances made in stem cell-based embryology, we discuss how simplified stem cell models have contributed to our understanding of the dynamics of signaling that underpin symmetry breaking and how the manipulability of form in these model systems points towards a role for geometry and size in governing mammalian gastrulation.

i) Signaling interactions between embryonic and extra-embryonic tissues in the regulation of symmetry breaking

On the fourth day after fertilization (E4.5), the mouse blastocyst implants into the uterus of the mother and undergoes a series of morphological changes that lead to formation of the post-implantation egg cylinder within 24 hours. The egg cylinder is hollow, consisting of two juxtaposed tissues; the pluripotent epiblast and the polar trophectoderm-derived extraembryonic ectoderm (ExE). Both of these tissues will become enveloped by another extra-embryonic tissue, the primitive endoderm-derived visceral endoderm (VE) **(Fig. 1A)** (Arnold and Robertson, 2009; Bedzhov et al., 2014). In synchrony with a transition in stem cell potential within the epiblast and ExE (Neagu et al., 2020; Shahbazi et al., 2017), lumenogenesis within these inner two tissues is promoted by the signals from the newly deposited basement membrane formed by the VE (Bedzhov and Zernicka-Goetz, 2014). This leads to formation of the pro-amniotic cavity that spans the whole length of the egg cylinder, and requires the induction of cell polarization triggered by β1-integrin signaling (Bedzhov and Zernicka-Goetz, 2014; Christodoulou et al., 2018; Li et al., 2005). Collectively, these developmental steps establish the pre-gastrula stage mouse embryo **(Fig. 1A)**.

Such morphological transformations during pre-gastrula development set the stage for breaking the mouse embryo's cylindrical symmetry by placing epiblast, ExE and VE tissues in the correct configuration along the proximal-distal axis and ensuring cells are in the appropriate transcriptional state. This configuration is crucial as it facilitates signaling interactions of NODAL, Wingless-INT (WNT) and Bone Morphogenetic Protein (BMP) between the tissues (Brennan et al., 2001; Rivera-Perez and Magnuson, 2005; Robertson, 2014; Winnier et al., 1995). As development progresses, these signals become graded in the epiblast towards the future posterior, facilitating the breaking of symmetry **(Fig. 1B)**. To achieve this, a group of VE cells appears at the distal tip of the embryo at E5.25/5.5, the Distal VE (DVE), displaying a distinct transcriptional signature of transcription factors and secreted molecules (Takaoka et al., 2011; Thomas and Beddington, 1996). Importantly, by E5.75/6.0, these cells move proximally, to form the so-called Anterior VE (AVE), which is located prominently at the boundary between the epiblast and ExE (Rosenquist and Martin, 1995; Yamamoto et al., 2004). The movement of the DVE is considered to be instrumental in the acquisition of AP polarity of the mouse embryo, as the AVE expresses and secretes the NODAL and BMP antagonists, Cerberus-like protein 1 (Cer1) and Left-right determining factor 1 (Lefty1), as well as the canonical WNT antagonist, Dikkopf1 (Dkk1) (Rivera-Perez et al., 2003). While the AVE represses the activity of NODAL, BMP and WNT in the anterior epiblast, a BMP4 signal from the ExE induces these factors in the adjacent epiblast establishing a signaling gradient that specifies posterior identity (Winnier et al., 1995). Observations of the coordinated signaling, through these multiple pathways have led to a model whereby a positive feedback loop between the epiblast (WNT and NODAL) and the ExE (BMP4) is responsible for the robust specification of proximo-posterior epiblast molecular

identity (Arnold and Robertson, 2009; Ben-Haim et al., 2006; Tam and Loebel, 2007) **(Fig. 1B)**. This culminates in the formation of the primitive streak at E6.5 at the proximo-posterior side of the epiblast where it joins the ExE, marking the onset of gastrulation in amniotes **(Fig. 1A-B)**. The formation of the primitive streak is marked by the expression of early mesendodermal markers such as the T-box transcription factor *Brachyury* (T/Bra) (reviewed in (Balmer et al., 2016).

While the biochemical and molecular interactions necessary for axial establishment are fairly well elucidated *in vivo,* the mechanism of spatiotemporal coordination is still not clear. To gain comprehensive understanding of axis formation in early embryogenesis, it has been suggested that a mechanism is required to integrate interactions across multiple scales, whereby the intrinsic properties of embryonic cells can initiate spontaneous symmetry breaking (Gierer and Meinhardt, 1972; Juan and Hamada, 2001; Meinhardt, 2008).

Emerging view: spontaneous symmetry breaking by a self-organizing mechanism

To date, studies that combine genetic approaches with live-embryo imaging techniques have emphasized an instructive role for the AVE in establishing AP asymmetry in the epiblast (Stower and Srinivas, 2014; Yamamoto et al., 2004). While the positioning of the AVE is seemingly sufficient to explain the site of posterior formation, several studies have indicated that alternative mechanisms may also play a role in axis patterning. First, it was found that when the AVE is grafted to lateral regions of the late-streak embryo, no additional neural tissues are induced, suggesting AVE alone is not sufficient for anterior development in mouse (Tam and Steiner, 1999). Second, genetic ablation of DVE/AVE activity still permitted symmetry breaking of the epiblast, where in approximately 1/3 of cases a single primitive streak formed and embryos completed gastrulation (Perea-Gomez et al., 2002; Stower and Srinivas, 2014). The remaining embryos, showed phenotypes with primitive streak duplication in the absence of the DVE/AVE (Perea-Gomez et al., 2002). This capacity to self-organize AP axial identity in the absence of DVE/AVE is perhaps best illustrated in recent stem cell-based *in vitro* model systems, which we elaborate in detail in the following sections.

An intriguing hypothesis proposes that symmetry breaking may potentially be explained by self-organization properties of the epiblast itself, which is still linked to but distinct from extra-embryonic positional information. This hypothesis stems from one of the most influential concepts for biological patterning in developmental biology, put forward by Alan Turing in 1952. Turing proposed a simple model demonstrating that the self-regulating, repeating patterns of cell fate can be explained by the reaction between two morphogens diffusing through a tissue (Turing, 1952). In this model, the most well-known Turing mechanism is the activator-inhibitor reaction-diffusion circuit. This mechanism proposes that an activator stimulates its own production (*local-activation*) as well as that of its own inhibitor, allowing stable patterns to emerge provided the inhibitor diffuses faster than the activator *(long-range inhibition)*. Many other Turing circuits can also yield stable biological patterns, all relying on an underlying logic of local-activation and long-range inhibition (Gierer and Meinhardt, 1972; Hiscock and Megason, 2015). Turing models have been successfully applied to explain achieving robust and reproducible pattern formations in numerous developmental contexts (Cornwall-Scoones and Hiscock, 2020; Economou et al., 2012; Raspopovic et al., 2014; Sick et al., 2006).

Turing-like mechanisms have been speculated to play important roles in regulating cell fate in multiple contexts within the mammalian body, with many proposing such mechanisms may account for AP patterning in embryogenesis (Juan and Hamada, 2001; Meinhardt, 2008; Simunovic et al., 2019). Unlike mechanisms of patterning that rely on graded morphogens, Turing-like mechanisms propose that interactions between cells are sufficient to generate patterns of gene expression, without external cues (Gierer and Meinhardt, 1972). In this way,

Turing mechanisms can explain cases of *self-organized* and *spontaneous* symmetry breaking in the absence of DVE/AVE, where patterns emerge by virtue of local interactions rather than an upstream trigger (Turing, 1952). Further evidence accrued over the past decades points towards a potential mechanistic basis for local-activation and long-range inhibition in AP symmetry breaking during embryogenesis and could explain how initially identical cells of the epiblast can spontaneously become different. For example, both key signaling molecules that drive mesoderm formation and axial development — WNT and NODAL — and their antagonists are expressed in the same region of posterior mouse epiblast, as shown earlier (Arkell and Tam, 2012; Meno et al., 1999). Moreover, recent advances in spatial transcriptomics has made it possible to analyze the polarized distribution of gene expression in mouse epiblast, revealing restricted posterior joint-expression of WNT, NODAL, and their respective antagonists (Peng et al., 2019). A recent study also showed that a 3D model of epiblast with human embryonic stem cells (ESCs) alone can break the symmetry in the absence of extra-embryonic tissues and asymmetric signaling activity (Simunovic et al., 2019). This was explained by the feedback between a morphogen activity (WNT) and its inhibitor (Dkk1) in this 3D epiblast model (Simunovic et al., 2019). These results are consistent with Turing's activator-inhibitor circuit whereby these morphogens can drive the expression of their own antagonists. Turing-like mechanisms of symmetry breaking can produce repeating patterns (with formation of more than one domain) when freed from geometric or signaling constraints. Consistently, the duplication of the primitive streak, observed in 2/3 of embryos where the AVE's functionality is disrupted, provides support for the production of repeating patterns by a mechanism of spontaneous symmetry breaking (Perea-Gomez et al., 2002). Similarly, patterning still occurs following ablation of the functionality of chick hypoblast, homologous to the mouse AVE, with the formation of multiple streaks (Bertocchini and Stern, 2002).

Taken together, these examples suggest that Turing-like mechanisms may play a part in symmetry breaking events during embryogenesis *in vivo* and *in vitro*. While it can be debated whether a Turing model is indeed in operation here, this guiding principle of local-activation and long-range inhibition may offer a useful hypothesis to interrogate the mechanism of AP symmetry breaking. Under this hypothesis, the establishment of AP axial asymmetry can be coordinated by signaling interactions between epiblast cells, which although capable of undergoing spontaneous symmetry breaking alone, are likely to be guided by graded cues from the AVE and ExE.

ii) New insights into AP symmetry breaking: modelling mammalian embryogenesis and gastrulation *in vitro*

In vivo approaches have made substantial headway in unravelling the principles of AP symmetry breaking, from molecules to mechanism. As eluded to above, this process requires a series of complex, yet perfectly orchestrated, signaling interactions. How the concentration gradients of signaling ligands required for these processes are established remains a notoriously difficult question to answer *in vivo.* To uncover the impeccable interplay between cell morphogenesis, fate specification, proliferation, migration and growth that guides *in vivo* embryogenesis necessitates a different approach, whereby these multiple factors can be decoupled.

Stem cell-based embryology relies on culturing cell lines derived from pre-implantation embryos, in isolation or in combination **(Fig. 2)** with an aim to investigate each factor involved in embryonic patterning. Rather than being perfect replicates of natural embryos with full developmental capacity, these stem cell-based embryos are relatively simple systems, lacking some features, yet effectively recapitulating key aspects of mammalian embryogenesis leading to symmetry breaking and gastrulation. They thus help to understand embryogenesis and offer the potential to open up new avenues and challenge preconceptions underpinning poorly

understood developmental events. While all strategies of *in vitro* stem cell-based platforms follow a similar goal, the approaches are varied and provide model for different stages of developmental progression. Here we describe the numerous *in vitro* embryogenesis models, illustrating how the insights they provide are complementary in nature.

Modelling gastrulation in 2D micropatterns

In these systems, primed pluripotent state embryonic stem cells (PSCs), the *in vitro* counterparts of the post-implantation mammalian epiblast, are confined to disk-shaped, sub-millimeter colonies that create a spatially organized signaling environment, named *micropatterned colonies* (Etoc et al., 2016; Morgani et al., 2018; Warmflash et al., 2014). Geometrically constrained mouse or human PSC colonies spontaneously self-organize into germ layers with remarkable reproducibility, therefore acting as a powerful quantitative tool to functionally investigate signaling feedback mechanisms within cell populations to assess embryonic patterning. In this 2D system, cells undergo radial patterning of cell fates when exposed to homogenous sources of signaling factors, with each ring corresponding to a distinct germ layer **(Fig. 2).** Specifically, the combination of BMP, WNT, Activin (NODAL) and FGF directs cells to posterior fates with radial symmetry in mouse PSCs (Morgani et al., 2018). Conversely, when BMP is removed from this cocktail of external cues, cells are directed toward anterior identities (Morgani et al., 2018). Hence this system shows parallels with the mouse gastrula signaling patterns *in vivo*, offering a description of signaling dynamics and cell-cell interactions in a flat-disc geometry. This becomes in particular useful for human studies (Martyn et al., 2019; Martyn et al., 2018), given the current guidelines forbidding the culture of human embryos after 14 days (the 14-day rule) when gastrulation begins. In human micropatterns, the durations of WNT and NODAL is shown to control mesoderm fate, while the duration of BMP directs the CDX2-positive extra-embryonic cell specification (Chhabra et al., 2019). This study further shows that BMP signaling initiates waves of WNT and NODAL signaling activity in human PSCs. Mathematical modelling predicts that this signaling behavior in 2D human micropatterns lies outside the Turing instability regime where spatial gradients of signaling activities can autonomously self-organize (Chhabra et al., 2019). However, an *in vivo* validation for how this interpretation is mirrored during development remains to be addressed. Despite their utility, these 2D micropattern systems do not overcome the challenges of correctly mimicking tissue density, mechanical properties, dimensionality and developmental timing (Siggia and Warmflash, 2018). Another specific difficulty with these 2D micropatterned colonies is that they do not contain a signaling center, such as DVE/AVE, and so fail to recapitulate the symmetry breaking events observed in natural embryos. That said, a recent microfluidic approach was able to engineer an artificial signaling center that exposes human PSC colonies to microfluidically generated BMP4 gradients, serving as a proxy for the asymmetric BMP signaling activity, and resulting in an asymmetric cell fate pattern within the colonies (Manfrin et al., 2019). Thus, by using this approach, cell colonies are seen to break radial symmetry, as opposed to conventional micropattern platforms that cause concentric rings of cell types as followed by a uniform exposure to morphogens. It thereby provides a tool to investigate morphogen gradient-driven bias in germ layer positioning.

Modelling gastrulation in 3D culture platforms

Despite considerable progress in utilizing PSCs to study post-implantation embryonic patterning, the inability to capture embryonic architecture provided the impetus to generate 3D *in vitro* culture protocols, which recapitulate aspects of both the morphogenesis and cell-fate decisions associated with various stages of development. Thus, in recent years much effort has gone into designing 3D *in vitro* models, which are becoming increasingly successful in mimicking different aspects of mammalian embryogenesis and in interrogating the signaling

interactions that underpin these processes (Beccari et al., 2018; Harrison et al., 2017; Moris et al., 2020; Rivron et al., 2018; Sozen et al., 2018; Sozen et al., 2019; van den Brink et al., 2020; Zheng et al., 2019).

Initial attempts to model early post-implantation mouse embryogenesis with ESCs alone resulted in the concept of 3D multi-cellular aggregates, so called *embryoid bodies* (EBs) or *gastruloids* (Desbaillets et al., 2000; ten Berge et al., 2008; Turner et al., 2017; van den Brink et al., 2014). Remarkably, these platforms permit ESCs to show an extraordinary capacity of self-organization *in vitro,* mimicking the patterning of mammalian gastrulation **(Fig. 2)** (Beccari et al., 2018; Turner et al., 2017; van den Brink et al., 2014). One major benefit of these platforms is the reliance on a single stem cell type, relatively reducing potential variability in generating these structures and the developmental outcomes, hence allowing for high reproducibility. Even though these structures vary in size, shape and pattern, they provide an excellent opportunity to study the influence of biochemical and physical cues on cell fate transitions.

Since both EBs and gastruloids show robust AP axis formation, one important question is whether necessary signals and morphogen gradients are properly established in these platforms in the absence of extraembryonic cues. It has been shown that exogenous Wnt3a establishes posterior identity in EBs, resulting in a localized WNT signaling gradient with polarized T/Bra expression (marking mesoderm specification) at one pole (ten Berge et al., 2008). The principle of building gastruloids differs from EBs by using a simple pulse of the Wnt agonist CHIR99201 (Chi) 48h after EB aggregation (van den Brink et al., 2014). During the early period of their development (0-72h), gastruloids display uniform WNT and NODAL signal expression in the entire structure and show no detectable BMP activity (Turner et al., 2017). However, a detailed study of whether signaling gradients emerge, and if so how, is lacking. Given that gastruloids correspond to the E8.5 somitogenesis staged embryo (Beccari et al., 2018; van den Brink et al., 2020), which differs from the other platforms modelling the gastrula stages, the initiation of symmetry breaking dynamics in gastruloids system at earlier time-points needs further investigation. Nevertheless, the Chi pulse appears to function in promoting a polarized T/Bra-expressing posterior pole in gastruloids and their subsequent axial elongation (Turner et al., 2017). While 3D strategies relying on ESCs alone are helping to elucidate the molecular circuits in AP patterning, they lack crucial native interactions between the epiblast and extra-embryonic tissues. Thus, the origin of AP patterning in EBs and gastruloids remains unclear.

Combining extraembryonic stem cells with ESCs in a 3D platform leads to spontaneous formation of structures, named *ET* or *ETX embryos*, which are more akin to the natural mammalian embryo and are able to undertake early gastrulation events (Harrison et al., 2017; Sozen et al., 2018). Importantly, in these platforms, embryonic and extra-embryonic cells self-assemble into the appropriate configuration, mirroring that of the natural early post-implantation embryo **(Fig. 2)**. Thus, this 3D platform stands out among stem cell–based model systems for two main reasons: *(1)* it includes not only embryonic but also extra-embryonic tissues, and therefore, remarkably recapitulates the architecture and gene expression signatures of the early-streak stage natural gastrula; and *(2)* self-organization and patterning occurs organically through embryonic/extra-embryonic cellular interactions. Despite the unprecedented advantages, these 3D platforms involving both embryonic and extra-embryonic stem cells have low efficiency, as this aggregation design relies on interactions of multiple stem cells in the right number and ratio. This technical challenge has yet to be overcome, demanding future innovative micro-engineering approaches to increase efficiency of self-assembly. In spite of this, approximately half of the correctly assembled ET/ETX embryos can break symmetry and establish AP axis in the current designs (Harrison et al., 2017; Sozen et al., 2018).

Patterns of symmetry breaking in ET and ETX embryos show similarities with phenotypes observed in DVE/AVE mutant embryos. ET embryos, constituted from ESCs and trophoblast stem cells (TSCs) in an extracellular matrix scaffold, do not possess a VE-like layer, and therefore should lack an AVE-induced a NODAL signaling gradient within the ESC-derived epiblast-like embryonic compartment. In spite of this, ET embryos can break symmetry and display a WNT gradient across the embryonic compartment, and later, polarized T/Bra expression at the posterior pole (Harrison et al., 2017). Incorporation of extra-embryonic endoderm (XEN) stem cells resulting in the formation of ETX embryos can induce AVE-like domain specification, albeit at low efficiency and its full-functionally still debatable (Sozen et al., 2018). The majority of ETX embryos establish WNT and NODAL signaling gradients across the embryonic compartment that corresponds with the polarized T/Bra-expressing posterior domain (Sozen et al., 2018). Importantly, both ET and ETX embryos display the asymmetric, spatio-temporal formation of the posterior, whether or not DVE/AVE organizer functions are provided within the structure (Harrison et al., 2017; Sozen et al., 2018). BMP4 production from TSCs is required to establish posterior identity and notably, neither ET nor ETX embryos break symmetry when TSCs are excluded from the 3D culture (Harrison et al., 2017; Sozen et al., 2018). Thus, these studies provide support for the potential crucial role of the ExE tissue *in vivo* and moreover, provide a further tool to interrogate the function of the AVE signaling center.

Whereas the mechanism of proper posterior formation in DVE/AVE-double mutant natural embryos and in ET/ETX embryos is yet unclear, these studies challenge the notion of the anterior organizing center as an absolute sole necessity for symmetry breaking. This suggests that alternative mechanisms can contribute to axis patterning. It is important to note that, since AP patterning takes place with varying frequencies *in vitro,* the presence of an anterior organizing center may function in ensuring the timing and robustness of symmetry breaking mechanism, as previously hypothesized (Stern and Downs, 2012). Together, the accumulating data is now revitalizing a prominent question in mammalian developmental biology: what are the precise drivers of symmetry breaking?

iii) Stem cell-based embryology as a new solution to an old question: the threshold hypothesis for the onset of mammalian gastrulation

One pervading question in the study of AP symmetry breaking is the regulation of its developmental timing. Studies so far have indicated that there may be various intrinsic properties in the developing embryo, such as embryonic tissue size or geometry, and that these intrinsic properties may act as an internal threshold for initiating symmetry breaking. Below we describe how these hypotheses emerged through classical embryology and how, more recently, they have been expanded as a result of novel technological advances in stem cell-based embryology.

The first hypothesis proposes the role of an internal threshold to explain the developmental timing of embryo patterning, specifically the role of ***embryonic tissue size and mass*** **(Fig. 3A)**. This hypothesis is in agreement with the results of experiments performed more than three decades ago (Power and Tam, 1993; Snow and Tam, 1979; Tam, 1988). At the time symmetry breaking commences in the post-implantation mouse embryo at E6.0/6.5, the epiblast contains approximately 650-1000 cells, the products of at least 12 rounds of cell doublings after fertilization (Kojima et al., 2014; Power and Tam, 1993). Embryos in which cell numbers have been artificially reduced, either as a result of decreased cell proliferation or increased cell death, are delayed in undertaking gastrulation until their epiblast attains a critical threshold cell number (Power and Tam, 1993; Snow and Tam, 1979; Tam, 1988). Further insight came from embryos that had been experimentally manipulated to increase or decrease their size. Removing one blastomere from a 2- or 4-cell stage embryo, for example, results in the development of post-implantation embryos that have fewer than 650 cells in the epiblast by

E6.5, the stage when symmetry is normally broken. However, symmetry breaking in these undersized embryos is delayed until E7.0, the time at which these embryos attain the threshold epiblast cell number (Power and Tam, 1993; Rands, 1986b). Further studies have revealed that it is not cell number *per se* but rather embryonic size that regulates the timing of gastrulation. Tetraploid embryos that contain half number of cells, which undergo hyperploidy to almost double their volume, initiate gastrulation at the same age as their non-manipulated peers (Henery et al., 1992). On the other hand, quadruplet embryos which are 2-2.5 times greater in volume than their normal peers at E5.5 do not show expedited developmental timing, and still initiate gastrulation at E6.5, the same age as wild-type embryos (Rands, 1986a). Overall, these *in vivo* findings are in general support of the proposed concept that the initiation of symmetry breaking and pattern formation requires attainment of a threshold of tissue volume or cell number, instead of being entirely governed by chronological age or some intrinsic cellular clock. These findings also accord with several recent *in vitro* models of early development. The concentric patterns of 2D ESC colonies, for example, show size-dependence of their patterning, in which there is a progressive loss of the inner-most rings of cell fates as the colony radius is reduced and a disruption of the signaling response at low cell density (Morgani et al., 2018; Warmflash et al., 2014). In a similar vein, gastruloids seeded with critically few cells (<200) do not break symmetry (van den Brink et al., 2014). Similarly, matrigel-embedded EBs only show polarized T/Bra expression when they attain a sufficiently large diameter (>240 μm) (Sagy et al., 2019). However, the mechanism explaining this spontaneous symmetry breaking remains unclear, requiring further research to unveil the underlying processes.

A second hypothesis proposes the role of intrinsic properties of the embryo to explain the drivers of patterning at the onset of gastrulation, specifically the role of ***embryonic geometry and receptor positioning*** **(Fig. 3B).** This hypothesis is in the agreement with experiments showing that asymmetric receptor localization restricts signal receptivity to one surface of a cell, affecting the sensing of morphogens and subsequent signal transduction between neighboring compartments (Hobert and Carlin, 1995; Saitoh et al., 2013). Indeed, patterning established in 2D micropatterned colonies suggests the importance of receptor localization for establishing discrete domains of gene expression (Etoc et al., 2016; Murphy et al., 2004; Stull et al., 2007). This is because, although 2D colonies are homogenously exposed to external signaling factors (e.g. Activin, BMP4), their cells respond in a spatially segregated manner due to their restricted spatial ability to detect morphogens: the basolateral localization of receptor in central cells precludes any response to apically located morphogens, resulting in graded signaling that is greatest at the periphery (Camacho-Aguilar and Warmflash, 2020; Etoc et al., 2016; Morgani et al., 2018; Warmflash et al., 2014). In human PSC micropatterned colonies, this effect is further promoted by the apical localization of BMP receptors in the outer-most cells, and by a self-organized counter positioned gradient of BMP antagonist Noggin (Etoc et al., 2016). Subsequently, a WNT3A response, operating downstream of BMP4, is also shown to be localized at the edge and density dependent (Martyn et al., 2019). However, unlike in the case of BMP patterning (via asymmetric receptor localization), WNT patterning is controlled by β-Catenin mechanosensation via E-Cadherin and the cytoskeleton, which is biased peripherally due to the circular geometry (Martyn et al., 2019). In addition to these models of radial patterning, a recent study in mouse showed that the geometrical confinement of ESC colonies leads to asymmetric organization of cell fates and controls the positioning of a pre-streak cell population, marked by T/Bra in 2D micropatterns (Blin et al., 2018). While this is intriguing, it is important to note this system involves spontaneous differentiation of cells rather than via the morphogen exposure as applied in other 2D micropatterned platforms. In addition, the spontaneous onset of T/Bra-expression and the generation of a single or multiple polarized T/Bra-expressing domains in EBs is reported to be dependent on the geometry of the surrounding surfaces (Sagy et al., 2019). These findings provide important insight into

mechanisms of symmetry breaking in 3D model systems which lack extra-embryonic tissues, demonstrating how physical/mechanical contact and biochemical signals can together drive posteriorization (Sagy et al., 2019). Moreover, recent study in the mouse embryo shows that a robust BMP signaling gradient depends on the restricted, basolateral localization of BMP receptors, where apical receptor localization results in ectopic BMP signaling across the epiblast (Zhang et al., 2019). Mathematical modelling illustrates the significance of this localization *in vivo,* indicating that the proamniotic cavity can act as an *entropic buffer* to ensures the robust establishment of a BMP gradient along the proximo-distal axis in the epiblast (Zhang et al., 2019). Together, these experimental and theoretical studies highlight a role for both cell and tissue geometry in shaping morphogen signaling and in controlling cell fate specification for axis determination and subsequently gastrulation.

Conclusions and Perspectives

Breaking the embryonic symmetry is perhaps one of the most important events in the life of the embryo as it allows the establishment of the whole body plan. Decades of molecular and genetic experimentation in the mouse have shed substantial light on the signaling interactions that instruct this critical event. Furthermore, *in vivo* studies have highlighted roles for the localization of morphogens in spatial domains, and proliferation dynamics of the embryo as a whole. The outstanding robustness of embryogenesis suggests multiple layers of regulation are required to reinforce patterning. However, how these layers are tightly coordinated remains in many ways unclear.

It is becoming increasingly apparent that the intrinsic properties of embryonic cells mediate the self-organization capacity of the mammalian embryo. Embryo-like structures form through similar self-organizational processes reflecting the intrinsic properties of stem cells. Although these *in vitro* platforms contain initially relatively homogenous populations of stem cells, they spontaneously break symmetry and undertake embryo-like pattern formation. Considerable progress in refining such *in vitro* models, as discussed throughout this review, demonstrates the potential of synthetic systems for studying development. To date, these systems ably mimic the key biochemical features of gastrulation. Future investment into studies of the biophysical mechanisms of tissues and the coupling of these mechanisms to biochemical properties should enable the multiple levels of regulation in embryonic development to be deciphered. One captivating hypothesis is that biophysical mechanisms operating within the embryonic tissue may play an important default role in coordinating patterning of the AP axis and timing of its formation. In Turing-like systems, pattern formation has an intrinsic wavelength, a length scale that is contingent on reaction-diffusion parameters independent of external cues. If a Turing-like mechanism takes part in governing symmetry breaking, the axis would be predicted to emerge when there is a sufficient separation in space between the embryonic tissue and the pattern-forming wavelength, potentially explaining dependence of symmetry breaking on embryo volume and geometry. The modelling of symmetry breaking in silico and *in vitro*, where the many moving parts of the symmetry breaking machinery can be decoupled and manipulated, can provide the scope to both frame and test such hypotheses. Understanding symmetry breaking and AP axis formation in humans may also benefit from this outlook. As human embryos adopt a radically different elliptical geometry from the cylindrical geometry of mouse embryos and with a different juxtaposition of tissues, controlled stem cell models may provide useful tools to dissect similarities and differences in axis establishment between the two species.

In conclusion, experimental manipulations carried out *in vivo* can only go so far and may be currently seem unable to fully disentangle the myriad complex processes that occur in the developing embryo. Stem cell-based embryology, therefore, promises to provide better understanding of the potential mechanisms of embryonic patterning. No single approach is

perfect but their utility lies in their simplicity. Each system can act as a complementary tool for studying distinct stages of early embryo development. Therefore, while an integrative view remains far from complete, these emerging tools provide potential for piecing the building blocks together.

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Figure Legends

Figure 1. A. Remodeling of the mouse pre-implantation blastocyst is concomitant with the onset of gastrulation. In the remodeling process, the initially amorphous epiblast at E4.5 develops into a distally positioned epithelial cup; the polar trophectoderm (TE) forms the proximally located extra-embryonic ectoderm (ExE); and both tissues become enveloped by the primitive endoderm (PE)-derived visceral endoderm (VE). Transformation of the epiblast requires polarization signals from the VE to initiate lumenogenesis. The ExE also undergoes cavitation and the embryonic and extra-embryonic cavities unite into the pro-amniotic cavity, defining the pre-gastrula stage embryo at E5.5. A specialized population of VE cells at the distal tip of the embryo, named the distal visceral endoderm (DVE) appear at the pre-gastrula stage, and collectively migrate towards the future anterior of the embryo, becoming anterior visceral endoderm (AVE) by the onset of gastrulation. Cells at the opposite side, proximo-posterior epiblast, begin to form nascent mesoderm which defines the posterior pole. As a result, the formation of primitive streak marks the symmetry breaking. **B. The positive feedback loop of signaling interactions in the post-implantation epiblast.** The DVE/AVE act as the source of inhibitors, expressing a number of secreted molecules including NODAL and BMP antagonists Cer1 and Lefty1, plus the canonical WNT antagonist, Dkk1. Conversely to DVE/AVE activity, a BMP4 signal from the ExE promotes these signals in the adjacent epiblast establishing a signaling gradient. With the movement of DVE cells to their new anterior location, the source of inhibitors (AVE) converts morphogen gradients across the epiblast into the AP axis (future head-tail axis), and thus triggers symmetry breaking by E6.5. Black arrows indicate the expansion of proamniotic cavity, yellow arrows indicate the direction of cell migration. Pr, proximal; D, distal; A, anterior; P, posterior.

Figure 2. Summary of stem cell models of mouse gastrulation. Prior to the onset of implantation at E4.5 the blastocyst comprises three lineages: the epiblast, and the extra-embryonic trophectoderm (TE) and primitive endoderm (PE), that are the sources of embryonic or pluripotent stem cells (ESCs, PSCs), trophoblast stem cells (TSCs) and extra-embryonic endoderm (XEN) stem cell lines, respectively. Studies using ESCs alone or in combination with extra-embryonic stem cells (ExSCs) led the emergence of various simplified *in vitro* models, on 2-dimensional (2D) or 3-dimensional (3D) platforms that mimics different stages of development and are complemented to the mammalian embryos. Distinct features of each system are listed below each illustration (for a detailed discussion please see the text in section ii).

Figure 3. Summary of studies that indicate an internal threshold to initiate symmetry breaking within the epiblast. A. Insights obtained from experimentally manipulated mouse embryos suggest that embryonic tissue size and mass may act as an intrinsic property for initiating symmetry breaking. In normal mouse embryo development, symmetry becomes broken at E6.0/6.5, when the epiblast contains approximately 650 cells. Following one blastomere removal at 2-cell or 4-cell pre-implantation stage embryos develop undersized to post-implantation, attaining less number of cells within the epiblast, and they show delayed symmetry breaking until they attain the threshold epiblast cell number at E7.0. Interestingly, while tetraploid embryos do not contain this threshold epiblast cell number, cells undergo hyperploidy to almost double their volume and thus epiblast reaches similar size as their non-manipulated peers. These tetraploid embryos initiate gastrulation at the same age as their non-manipulated peers, indicating a role for embryonic size in regulation of symmetry breaking timing. However, while quadruplet embryos, formed by aggregation of four 8-cell stage pre-implantation embryos, are 2-2.5 times greater in total volume in the epiblast tissue, they do not show expedited developmental timing for symmetry breaking. **B. (i)** More recently it was shown that embryonic geometry and receptor positioning act as a contributing factor in regulation of symmetry breaking. According to these studies, differential receptor positioning restricts signal receptivity to one surface of a cell, affecting the sensing of morphogens and subsequent signal transduction between neighboring compartments in both 2D micropattern colonies and the mouse post-implantation embryo. **(ii)** Additionally, tissue geometry can play roles in shaping morphogen signaling and thus can control asymmetric patterning of cell fates, as shown in recent stem cell-based embryology platforms.

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Figure 1

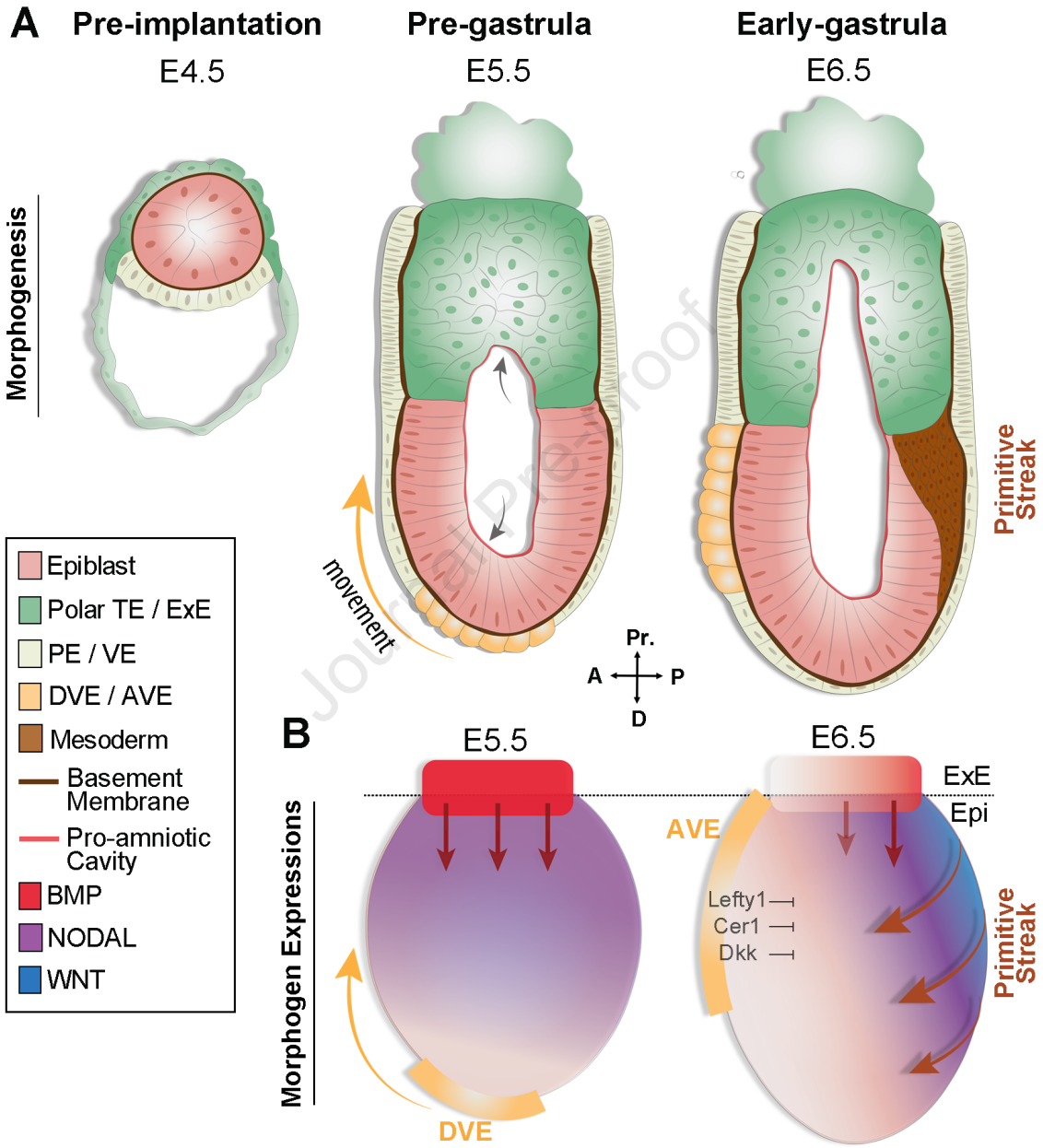
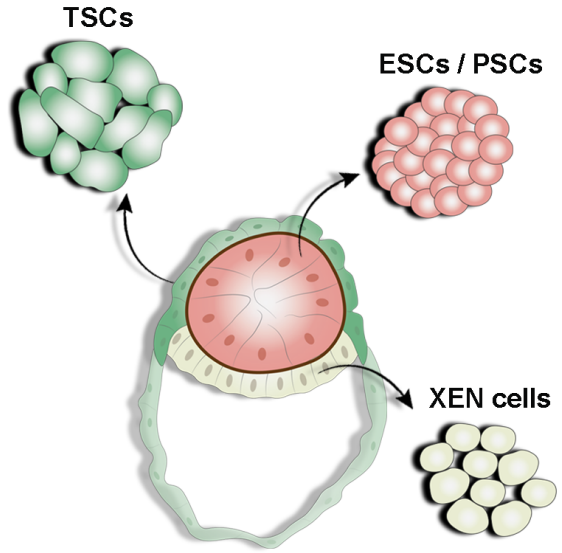
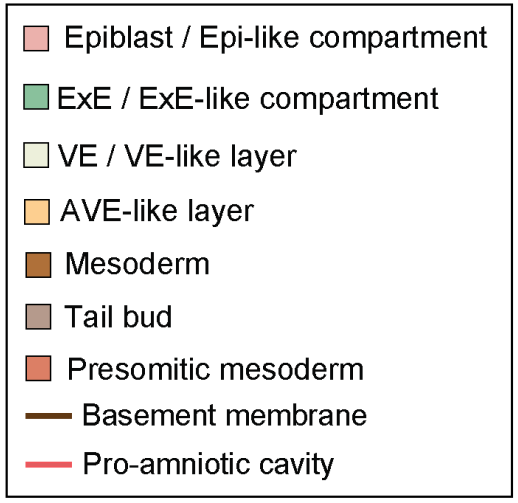


Figure 2





only

2D micropattern colonies

self-organisation
symmetric/radial patterning
receptor asymmetry

Embryoid Bodies (EBs)

3D

cross section

+ external Wnt3a pulse
self-organisation
AP axis patterning

A ⟷ P

3D Gastruloid

cross section

elongation

+ external Chi pulse
self-organisation
axial elongation
AP+DV+LR axis patterning

R D
A P
V L

models with ESCs and ExSCs

ET Embryos
ESCs + TSCs

3D

cross section

self-assembly
self-organisation
limited morphogenesis
AP axis patterning

Pr.
A P
D

ETX Embryos
ESCs + TSCs + XEN cells

3D

?

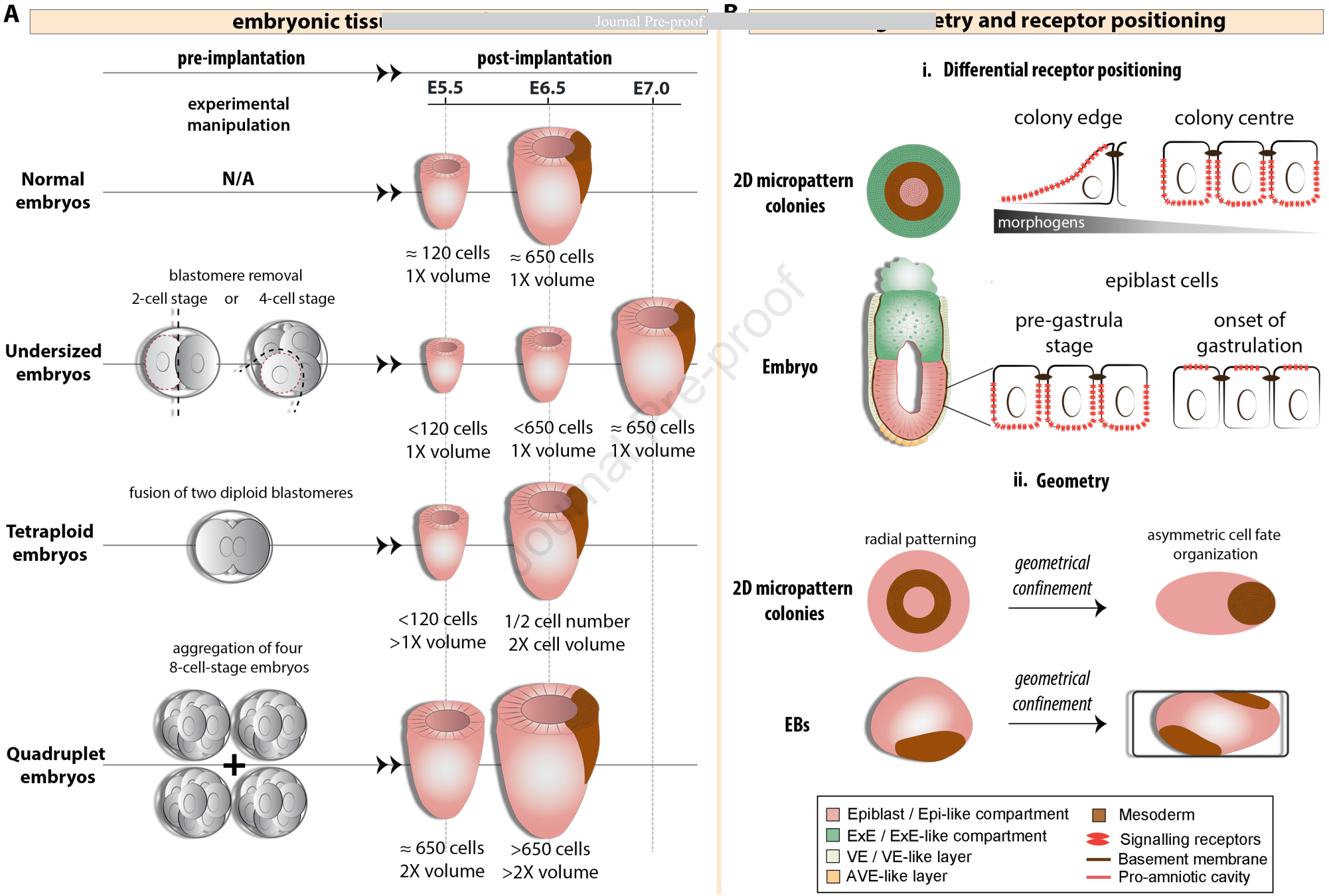
cross section

?

self-assembly
self-organisation
morphogenesis
AP axis patterning

Pr.
A P
D

Figure 3



Highlights

- We review decades of experimentation in the mouse for biochemical and biophysical mechanisms underlying embryo patterning
- We discuss advances in stem-cell based embryo models with a particular focus on symmetry breaking
- The role for embryonic geometry and size in governing mouse gastrulation is discussed
- We question yet unknown contributing factors to symmetry breaking events in mammalian embryos