CHAPTER NINE

Insights into mammalian morphogen dynamics from embryonic stem cell systems

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Abstract

Morphogens play an essential role in cell fate specification and patterning including in laying out the mammalian body plan during gastrulation. *In vivo* studies have shed light on the signaling pathways involved in this process and the phenotypes associated with their disruption, however, several important open questions remain regarding how morphogens function in space and time. Self-organized patterning systems based on embryonic stem cells have emerged as a powerful platform for beginning to address these questions that is complementary to *in vivo* approaches. Here we review recent progress in understanding morphogen signaling dynamics and patterning in early mammalian development by taking advantage of cutting-edge embryonic stem cell technology.

1. Introduction

During development, extracellular signaling molecules are essential for creating spatial patterns of cell fates. In 1952, Alan Turing introduced the term morphogen to define "a *chemical signal that could produce a form*" (as the name suggests) (Turing, 1952). It was decades before such molecules were actually discovered and, since then, much has been learned about the mechanisms by which morphogens pattern tissues. However, much remains obscure, particularly in mammals where development is difficult to observe and to manipulate.

In Turing's time, a broad definition of a morphogen was appropriate as nothing was known about the molecular underpinnings of development. In this review, we will use more recent definitions which add the requirement of a morphogen producing more than two fates, to exclude simpler cases in which the molecule acts as a simple switch, where cells adopt one fate in its absence and a different one in its presence (Dyson & Gurdon, 1998; Wolpert, 1969). Here we focus on mammalian development and only briefly summarize general concepts in morphogen gradient formation and interpretation. For more details about these processes we refer the reader to several recent reviews (Briscoe & Small, 2015; Green & Sharpe, 2015; Kondo & Miura, 2010; Rogers & Schier, 2011; Sagner & Briscoe, 2017).

The simplest models for morphogen gradient generation rely on localized production, and diffusion and degradation throughout the tissue (Crick, 1970; Lawrence, 1966; Stumpf, 1966; Wolpert, 1969). This creates a gradient that provides positional information to cells which differentiate to different fates according to its concentration (Wolpert, 1969). This synthesis-diffusion-degradation (SDD) model predicts an exponential decay in the morphogen concentration (Driever & Nusslein-Volhard, 1988; Drocco, Wieschaus, & Tank, 2012; Gregor, Wieschaus, Mcgregor, Bialek, & Tank, 2007), and has been supported by measurements of Bicoid in *Drosophila* and Sonic-Hedgehog in the murine neural tube, which produce a gradient with this property (Chamberlain, Jeong, Guo, Allen, & McMahon, 2008; Gregor, Tank, Wieschaus, & Bialek, 2007; Gregor, Wieschaus, et al., 2007).

The SDD model assumes localized production, but many developmental processes are self-organized without a preexisting source. Turing showed mathematically that, under particular conditions, diffusion causes the state in which the morphogen is homogenous in space to become unstable, and results in the development of a pattern of morphogen concentration (Turing, 1952). This is known as a diffusion-driven or Turing instability (Kondo & Miura, 2010; Meinhardt, 1978; Turing, 1952). This pattern of morphogen could then serve as the gradient which conveys positional information to the cells (Green & Sharpe, 2015). Turing patterns can be generated through the coupling of two molecules: a short-range activator that activates itself as well as its own long-range inhibitor (Gierer & Meinhardt, 1972), and several biological examples have been demonstrated (Economou et al., 2012; Green & Sharpe, 2015; Raspopovic, Marcon, Russo, & Sharpe, 2014).

As the morphogen diffuses through the tissue, it interacts with the field of cells by binding to cell-surface receptors and initiating signaling cascades within the cells. Let us use the TGF- β pathway as an example, since we will focus on this pathway below. TGF- β ligand binding leads to the assembly of heteromeric complexes of type I and type II receptors, which causes the type II receptor to activate the type I by phosphorylation. It has been suggested that the absolute number of occupied receptors is the signal that is conveyed to the nucleus (Dyson & Gurdon, 1998), although, as we discuss below, this simple formulation ignores the time-dependence of signaling. The type I receptor activates a class of signal transducers known as receptor-associated Smads (R-Smads). R-Smads then form a complex with another signal transducer, Smad4, and this complex moves into the nucleus where it regulates transcription. There are two branches of this pathway, the BMP branch and the Activin/Nodal branch, which share some components, including Smad4, but utilize different type I receptors and R-Smads. Thus, the signaling pathway relays information from outside the cell to its nucleus.

In the following sections, we summarize our current understanding of morphogens in early mammalian development, and opportunities to make progress in ESC systems. We first review what is known from *in vivo* experiments in the mouse, and discuss remaining open questions. We then focus on progress to date, and opportunities for the future, in addressing these questions using ESCs. The ESC systems allow for experiments that are currently impossible in the embryo, but the findings must be tested to ensure that that they reflect what occurs *in vivo*. They also allow for investigation of development directly in human cells, providing a powerful complement to ongoing research using model organisms.

2. The role of morphogens in mammalian gastrulation

Gastrulation is the first differentiation event of the embryo proper, when the pluripotent epiblast differentiates into the three germ layers of the embryo: ectoderm, mesoderm, and endoderm. Its initiation relies on three signaling pathways: the two branches of the TGF- β pathway, BMP and Activin/Nodal, and the Wnt pathway. As discussed above, BMP and Activin/Nodal signal through complexes of R-Smads (Smad1/5/8 and Smad2/3, respectively) and Smad4, while Wnt signals are transduced by β-catenin. In the mouse, Nodal signaling from the epiblast promotes BMP4 expression in the extra-embryonic ectoderm which activates WNT in both the visceral endoderm and the epiblast (Ben-Haim et al., 2006; Tortelote et al., 2013; Yoon et al., 2015). Wnt, in turn, increases Nodal signaling in the epiblast. Expression of Wnt and Nodal inhibitors in the anterior visceral endoderm (AVE) restricts these signals to the posterior of the embryo, where the primitive steak, the site of gastrulation, forms (Arnold & Robertson, 2009; Shahbazi & Zernicka-Goetz, 2018). Here, we review what has been learned in vivo regarding the identity and function of these morphogen ligands, focusing on Nodal signaling as an example, and highlight open questions that have remained difficult to address.

The most direct evidence for the function of TGF-beta ligands as morphogens in vertebrates comes from experiments with dissected Xenopus animal caps. These cells will adopt dorsal ectodermal fates when cultured in isolation, however, when exposed to low doses of Activin, they adopt a ventral mesodermal fate, while high doses lead to increasing dorsalization (Green, New, & Smith, 1992). Activin is also capable of spatially patterning a tissue in vitro, as an Activin bead placed within an animal cap will induce localized expression of Goosecoid, which requires high Activin, and a ring of Brachyury (BRA), which requires lower signaling, at a distance from the bead (Gurdon, Harger, Mitchell, & Lemaire, 1994). Similarly, exposure of dissociated animal caps to BMP4 induces increasingly ventral fates within the ectoderm with the highest doses giving rise to epidermis (Wilson, Lagna, & Hemmati-Brivanlou, 1997). While Suzuki, these experiments demonstrate the ability of these ligands to induce multiple fates at different concentrations, they do not demonstrate that they function in this manner in vivo. Notably, Activin plays a limited role in embryonic patterning, while Nodal, which activates the same pathway, is central to patterning, but signals over a shorter range (Jones, Armes, & Smith, 1996). Moreover, while these experiments in which ligand is suddenly added to culture media are consistent with ligand concentration as the central factor in determining cell fate, they are also consistent with other factors, such as ligand rate of change (Heemskerk & Warmflash, 2016). Finally, as discussed below, similar experiments with mammalian pluripotent cells yield different results

(Nemashkalo, Ruzo, Heemskerk, & Warmflash, 2017; Yoney et al., 2018), calling into question whether these ligands can function as classical morphogens in these systems.

It is instructive to consider the case of Nodal signaling in mammalian embryos. While there are six Nodal ligands in Xenopus and two in Zebrafish, mammals have only a single ligand, and deletion of this ligand leads to severe defects in embryogenesis shortly after implantation (Brennan et al., 2001; Conlon et al., 1994), consistent with the roles of Nodal signaling both in maintaining epiblast pluripotency (James, Levine, Besser, & Hemmati-Brivanlou, 2005; Vallier, Alexander, & Pedersen, 2005), and primitive streak induction (Brennan et al., 2001). Understanding of the role of Nodal in the mammalian embryo has come primarily from two types of experiments, measurements of expression patterns of Nodal, its inhibitors, and its targets, and assessments of the phenotypes of modified alleles of the genes that code for these extracellular molecules or for components of the intracellular signaling pathway (Arnold & Robertson, 2009; Dunn, Vincent, Oxburgh, Robertson, & Bikoff, 2004) (Fig. 1). Nodal is initially expressed throughout the epiblast shortly after implantation, while during gastrulation its expression is highest in the proximal posterior epiblast. At late gastrulation, it becomes restricted to the node, which forms at the anterior end of the primitive streak (Zhou et al., 1993).

Intracellularly, Nodal signals are transduced from the receptor to the cell nucleus by two very similar proteins known as Smad2 and Smad3. Surprisingly, while Nodal knockout mice rarely form mesoderm (Conlon et al., 1994), in Smad2 knockout mice, the entire embryo proper is converted to mesodermal cells (Brennan et al., 2001; Waldrip, Bikoff, Hoodless, Wrana, & Robertson, 1998). This results from loss of anterior identity due to a failure to form the anterior visceral endoderm signaling center within the extraembryonic endoderm. Epiblast specific Smad2 knockouts do form this signaling center, however, they have severe defects in forming anterior portions of the embryo which originate in a failure to specify the anterior most mesoderm and endoderm in the primitive streak during gastrulation (Vincent, Dunn, Hayashi, Norris, & Robertson, 2003). This phenotype is very similar to that observed in embryos which are heterozygous at the Nodal locus with one allele representing a null mutation and the other lacking an enhancer which drives Nodal expression in the primitive streak, which led the authors to conclude that the defects in epiblast specific Smad2 knockouts result from a general reduction in Nodal signaling levels (Vincent et al., 2003). In these embryos, the node and midline structures still form, however, removal of one copy of



Fig. 1 Schematics of the mouse embryo at gastrulation stage. Schematics show the expression of Nodal mRNA and the fate map of cells arising from the primitive streak (PS) in the early gastrulation (E6.5, top left and center), and late gastrulation (E7.5, bottom left and center) staged mouse embryo. Also shown are the Nodal gradient at E6.5 (top right), and the requirement for Nodal signaling to differentiate to primitive streak fates as determined from genetic experiments (bottom right). The phenotypic thresholds (a), (b) and (c) correspond to gradual decrease in Smad2/3 dosage, obtained from Dunn et al. (2004). The first eliminates definitive endoderm and anterior axial mesoderm (AME), followed by the node in (b), and finally the paraxial mesoderm (PXM) and lateral plate mesoderm (LPM) in (c). Adapted from Brennan, J., Lu, C.C., Norris, D.P., Rodriguez, T.A., Beddington, R.S.P., Robertson, E.J. 2001. Nodal signalling in the epiblast patterns the early mouse embryo. Nature, 411, 965–969; Dunn, N.R., Vincent, S.D., Oxburgh, L., Robertson, E.J., & Bikoff, E.K. 2004. Combinatorial activities of Smad2 and Smad3 regulate mesoderm formation and patterning in the mouse embryo. Development, 131, 1717–1728; Martinez Arias, A. & Steventon, B. 2014. On the nature and function of organizers. Development, 2018, 145; Robertson, E.J. Dose-dependent nodal/Smad signals pattern the early mouse embryo. Seminars in Cell & Developmental Biology, 32, 73-79; Stern, C.D. 2004. Gastrulation: From cells to embryo; Cold Spring Harbor Laboratory Press; Tam, P.P.L., & Behringer, R.R. 1997. Mouse gastrulation: The formation of a mammalian body plan. Mechanisms of Development, 68, 3–25; Zhou, X., Sasaki, H., Lowe, L., Hogan, B.L.M., & Kuehn, M.R. 1993. Nodal is a novel TGF- β -like gene expressed in the mouse node during gastrulation. Nature, 361, 543-547.

Smad3 in this context causes a loss of both structures, while embryos lacking both Smad2 and 3 fail to form mesoderm altogether (Dunn et al., 2004; Vincent et al., 2003). Taken together, these results have been interpreted to indicate a dose-dependent patterning of the mesendoderm by Nodal signals (Robertson, 2014).

There remain, however, several points of confusion. First, there is not a clear correspondence between the expression patterns of Nodal and the phenotypes induced. During gastrulation, Nodal is highest in the posterior proximal primitive streak, while reduction in Nodal levels results in a loss of the anterior most derivatives (Fig. 1). Similarly, Nodal expression is widespread at earlier stages so it is unclear what restricts the position of the DVE/AVE signaling center to the distal and anterior side, although it has been suggested to be due to a balance between Nodal and BMP signaling (Yamamoto et al., 2009). Second, we largely lack measurements of signaling activity, such as the amount of phosphorylated, nuclear-localized Smad proteins, and so interpretations of phenotypes must assume what the effect on signaling has been, and these can often be counterintuitive. The case of global knockout of Smad2 discussed above provides a good example. Unexpectedly, mesoderm is drastically expanded due to the loss of the AVE and its inhibitors. As the pathway contains multiple levels of feedback, effects such as this may be common. Further, some perturbations may change the spatial or temporal patterns of signaling in a manner which is difficult to predict. Well-studied examples include changes in the expression of molecules which affect the range of signaling gradients such as inhibitors or receptors (Wang & Ferguson, 2005). Changing expression of molecules to reduce diffusion can have the effect of sharpening the gradient—increasing levels close to the source while reducing those at a distance. Existing measurements of ligand and inhibitor expression, while informative, are largely non-quantitative and interpreting these requires determining how signaling activity is determined as a function of both activating and inhibiting influences. In the next section, we will return to these questions and discuss how in vitro experiments may provide an inroad into these issues.

3. ESC studies: Insights and opportunities

The difficulty in addressing questions about how signaling pathways pattern the early mammalian embryo in space and time has led to the development of ESC cultures that are complementary to *in vivo* models. These are particularly critical for studying early human development as ethical and practical considerations limit availability of embryos between approximately 2 weeks, when the 14-day rule takes effect, and 5 weeks when the earliest tissue from elective terminations is available. In this section, we briefly review ESC-based systems for studying early embryonic patterning. The basic features of most of these systems have been covered in more detail in previous reviews (Heemskerk & Warmflash, 2016; Shahbazi, Siggia, & Zernicka-Goetz, 2019; Siggia & Warmflash, 2018). In this review, we will not discuss organoids, which recapitulate some of the patterning of particular organs at later developmental stages. The reader is directed to recent reviews that focus on these (Brassard & Lutolf, 2019; Simunovic & Brivanlou, 2017).

3.1 ESC systems for studying gastrulation

Organized two-dimensional ESC patterns were first reported in Warmflash, Sorre, Etoc, Siggia, and Brivanlou (2014). That study took advantage of micropatterning technology to control the geometry of hESC cultures and showed that this was sufficient to overcome the hurdles of inconsistency and lack of reproducibility in regular culture. This micropatterning technology creates complementary patterns of adhesive and non-adhesive territories on the culture surface. This can be accomplished by coating the surface with a non-adhesive "passivating" material such as PLL-PEG, and then burning this material off the surface in defined regions using a mask and UV light (Azioune, Storch, Bornens, Th, & Piel, 2009). Alternative approaches based on stamping are also possible (Théry & Piel, 2009). Remarkably, simple confinement and stimulation with BMP4 led to the emergence of patterns which contained all three germ layers and extra embryonic cells. In what follows, we refer to these micropatterned colonies treated with BMP4 as "2D gastruloids". Treatment of micropatterned colonies with Wnt ligands also produces cell fate patterns, but without the extra-embryonic population at the edge (Martyn, Brivanlou, & Siggia, 2019).

While it is impossible to directly compare these micropatterns with human embryos, a recent study developed an analogous platform with mESCs, and compared the resulting patterns to the mouse embryo (Morgani, Metzger, Nichols, Siggia, & Hadjantonakis, 2018). They showed that the patterning process of Epiblast like cells (EpiLCs) grown in two-dimensional micropatterns mimics that observed *in vivo* (E5.0–E8.0), with cell differentiation following similar developmental paths. Induction of EpiLCs by FGF, BMP, Wnt and Nodal (signals observed at the posterior end of the embryo) resulted in a pattern comprised of posterior cell types

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including posterior epiblast, primitive streak, mesoderm, and extra-embryonic mesoderm. In agreement with *in vivo* observations, if BMP is not present in the induction media, the pattern is composed of anterior and distal cell types, such as anterior epiblast, anterior primitive streak, and definitive endoderm. These findings support the idea that organized two-dimensional ESC systems can mimic some of the pattern formation that occurs *in vivo*.

Two-dimensional systems are inherently limited in their ability to reproduce the morphogenesis and symmetry breaking that is observed in vivo. Several three-dimensional ESC systems to mimic early development have been devised. The closest analogue of the 2D systems are three-dimensional gastruloids which are created from mESCs. These systems are built by pulsing aggregates of mESCs of a defined size with a WNT activator 2 days into their differentiation (Turner et al., 2017; Van Den Brink et al., 2014). They subsequently develop an anterior-posterior axis that elongates, and even shows sequential and regionalized activation of Hox genes (Beccari et al., 2018). Other systems are generated by combining multiple cell types of the early embryo together. This is most easily done with mouse cells, because there are defined cell lines for all three lineages of the early embryo: epiblast, visceral endoderm (known as XEN cells), and trophoblast. Combining mESCs with trophoblast stem cells (TSCs) can produce configurations which look remarkably similar to the mammalian blastocyst (mouse E3.5) (Rivron et al., 2018), while in a slightly different configuration it can also lead to the development of structures resembling the egg cylinder staged mouse embryo (E5) (Harrison, Sozen, Christodoulou, Kyprianou, & Zernicka-Goetz, 2017), known as ETS embryos. These ETS embryos go on to show some early signs of gastrulation including regionalized induction of BRA close to the boundary between the ESCs and TSCs. Combining XEN cells with trophoblasts and ESCs forms structures known as an ETX embryos, and further improves this process (Sozen et al., 2018), with more robust symmetry breaking and primitive streak differentiation.

For human cells, it has been shown that culturing hESCs in Matrigel on top of a soft gel leads to differentiation to a hollow sphere of amniotic ectodermal cells (Shao, Taniguchi, Gurdziel, et al., 2017). Interestingly, in some fraction of cases, the initial spherical symmetry is broken and only half of the aggregate becomes amnion while the rest remains pluripotent epiblast, a configuration which resembles the amniotic sac *in vivo*(Shao, Taniguchi, Townshend, et al., 2017). By varying the gel composition, colonies of hESCs can also be grown as three-dimensional spheres of pluripotent cells. When treated with low doses of BMP, these also break symmetry, but instead divide into SOX2 expressing ectoderm, and BRA expressing mesoderm, which could be indicative of an anterior-posterior axis (Simunovic et al., 2019).

3.2 Do ligands function as morphogens in mammalian gastrulation?

BMP signal is essential for mammalian gastrulation (Arnold & Robertson, 2009; Winnier, Blessing, Labosky, & Hogan, 1995) and has been shown to act as a morphogen in other organisms. In particular, Dpp, the BMP4 homolog in *Drosophila*, was shown to function as a morphogen to control dorsal-ventral patterning of the early embryo and anterior-posterior patterning and growth of the imaginal disk (Ferguson & Anderson, 1992; Lecuit et al., 1996; Matsuda, Harmansa, & Affolter, 2016; Nellen, Burke, Struhl, & Basler, 1996). Treating dissociated cells from the Xenopus animal cap with increasing doses of BMP4 causes them to adopt progressively more ventral fates (Wilson et al., 1997). Simple treatment of mammalian ESCs by BMP ligands leads to mixtures of trophectodermal (Horii et al., 2016; Li et al., 2013; Xu et al., 2002), extra-embryonic (Morgani et al., 2018), and mesodermal fates (Etoc et al., 2016; Warmflash et al., 2014), while as noted above, in the 2D gastruloid system, BMP4 treatment causes ordered differentiation to all three germ layers.

While some studies have suggested that the patterns in 2D gastruloids reflect cells reading levels of BMP4 to give rise to multiple different fates (Tewary et al., 2017), there is evidence to suggest that this is not the case. First, measuring the time-dependent response to BMP treatment shows that BMP initially activates SMAD1/5/8 throughout the colony and this later becomes restricted to the edge (Etoc et al., 2016; Heemskerk et al., 2019). This response is binary rather than graded along the radial axis: cells at the edge show a sustained response and differentiate to extra-embryonic fates, while the remainder of the cells show a transient response. Termination of BMP signaling at the colony center depends on both secreted inhibitors and polarization of BMP receptors to the basal side of the cells (Etoc et al., 2016; Zhang, Zwick, Loew, Grimley, & Ramanathan, 2019), and is highly synchronous between cells (Heemskerk et al., 2019). Thus, there is not sufficient diversity in the direct response to BMP to explain the cell fates that arise. Further, a separate study used isolated colonies containing fewer than 10 cells to show that there is a sharp transition between pluripotency and extra-embryonic fates as the BMP concentration is raised, and BMP alone never gives rise to intermediate fates

(Nemashkalo et al., 2017). Finally, in larger colonies, application of intermediate BMP concentrations does yield mesoderm differentiation, however, this effect is lost upon either WNT or NODAL inhibition (Nemashkalo et al., 2017). Similarly, in micropatterned colonies, inhibiting endogenous WNT or NODAL signals results in a loss of the rings of mesoderm and endoderm (Chhabra, Liu, Goh, Kong, & Warmflash, 2019; Martyn, Kanno, Ruzo, Siggia, & Brivanlou, 2018; Tewary et al., 2017; Warmflash et al., 2014). Thus, evidence from stem cells suggests that in this context, BMP does not function as a morphogen, but rather as a switch which both activates extra-embryonic differentiation and WNT/ NODAL activity, and it is the combinatorial effect of these pathways that gives the appearance of a morphogen effect and patterns the 2D gastruloids.

We illustrate how two signals could lead to an apparent morphogen effect as well as pattern the radial axis of 2D gastruloids with a mathematical model. We will compare this two-signal model with the classic model in which the concentration of a single signal is utilized for patterning (Fig. 2A). Details of both models can be found in Appendix. In both cases, the concentration of the signal is interpreted by a network of three mutually repressive transcription factors, which for simplicity we label CDX2, BRA, SOX2, well established markers of the trophoectodermal, mesodermal, and pluripotent states, respectively. We note that this choice is not meant to model actual interactions between these genes, but, as we are focused on signaling, this is the simplest gene regulatory model that implements a three-way decision. A gene regulatory network with a similar architecture has been shown to govern patterning of the neural tube in response to Sonic hedgehog (Balaskas et al., 2012) and the anterior-posterior axis of the drosophila embryo (Chen, Xu, Mei, Yu, & Small, 2012; Jaeger et al., 2004). In the classic model in which BMP acts as a morphogen, the cell remains in a default SOX2-positive pluripotent state in the absence of signal (Fig. 2A–C). When the concentration of the morphogen is sufficiently high, CDX2 is strongly induced and represses both SOX2 and BRA (Fig. 2A-C). However, at intermediate morphogen concentrations, CDX2 expression is not sufficient to repress BRA, and once BRA is activated, it suppresses both CDX2 and SOX2 (Fig. 2A-C). Thus, in this model, the concentration of a single morphogen yields three different fates as in the French Flag model (Fig. 2C).

We asked now if two signals, neither of which acts as a morphogen on its own, could produce the same phenomena. In this model, the gene regulatory network remains the same but, the upstream signaling molecule, BMP,



Fig. 2 Mathematical models for signal induction. (A) Classic model for morphogen induction: three mutually repressive transcription factors (CDX2, BRA, SOX2) are activated at different rates by the morphogen ligand, in this case BMP. (B) Schematic representation of cell fate dependence on BMP signal concentration. High BMP signal induces pluripotent (green) cells to adopt CDX2 (yellow) fate (top). At intermediate concentrations of the morphogen, BRA (blue) expression is induced (middle). However, if BMP concentration is low, SOX2 (green) expression remains active (bottom). (C) Morphogen signaling gradient (top) and the corresponding TF expression profile (bottom) as functions of the distance from the source. For example, a cell that is at a *(Continued)*

would activate both the expression of the transcription factor CDX2, and the production of a secondary signaling molecule, WNT, which activates the second transcription factor BRA (Fig. 2D-F). At low concentrations of BMP, WNT is also not expressed, and cells remain in the default (pluripotent) state expressing SOX2. At intermediate concentrations, BMP activates the WNT signal, but does not override it, and cells differentiate to mesoderm. When BMP is sufficiently high, WNT is expressed but CDX2 is expressed too strongly and the cells differentiate to trophectoderm. A similar effect can occur in space when BMP activates a diffusible WNT signal (Fig. 2I and J). Thus, an identical transcriptional network could interpret either a single signal via a classic morphogen effect or two signals, in which cells do not sense the absolute concentration of either one. These interactions could also explain the pattern observed 2D gastruloids (Fig. 2I), where the BMP signal is restricted to the edge but induces WNT signaling that propagates toward the center of the colony (Chhabra et al., 2019), resulting in a ring of mesendoderm differentiation.

It is important to note that while the two models have very similar networks, the logic at the level of signaling is different, and they make different predictions regarding experiments in which ligand levels are varied. In the single-signal model, cells autonomously decide their fate regardless of

Fig. 2—Cont'd distance from the source will be exposed to an intermediate BMP level and will express a high level of BRA (blue). (D) Pattern created in a row of cells exposed to the gradient signal showed in (C). (E) Transcriptional network mimicking morphogen induction by induction of a secondary signal: three mutually repressive transcription factors (CDX2, BRA, SOX2) are controlled by a balance of an upstream signal (BMP) and a secondary signal (WNT). (F) Schematic representation of cell fate dependence on the combination of BMP and WNT signals. High BMP signal induces pluripotent (green) cells to adopt CDX2 (yellow) fate (top). At intermediate BMP concentrations, the secondary signal, WNT, is induced and BRA (blue) expression is induced (middle top). If at middle concentrations of BMP, the secondary signal was inhibited, CDX2 (yellow) fate would be induced instead (middle bottom). Low BMP concentration would not affect SOX2 (green) expression, which remains active in this case (bottom). (G) Signaling levels (top) and the corresponding TF expression profile (bottom) as functions of the distance from the source of BMP. (H) Pattern created in a row of cells exposed to the signaling levels showed in (G). The blue cells are only present due to the activation of the secondary signal and not to the BMP gradient directly. (I) Sketch of the signaling dynamics of a micropattern exposed to BMP signal. First, BMP signal is restricted to the edge of the colony. Second, BMP signal activates the secondary signal WNT at a distance from the edge, which expands inwards in the colony. (J) The signaling dynamics result into a pattern with three regions: extra-embryonic (yellow), mesendodermal (blue) and ectodermal (green).

secondary signaling, while in the two-signal model, interactions between cells through the downstream pathway are required to specify the intermediate fate. Thus, if one grows cells under conditions where secondary signaling is reduced, such as in small colonies or at low density, the one-signal model predicts that the fate decision will remain the same, while the two-signal model predicts a loss of the intermediate fate. Similarly, the two-signal model predicts a loss of the intermediate fate when secondary signals are inhibited, while the one-signal model does not. All of these predictions were tested by varying BMP dose in hESC colonies of controlled size or density (Nemashkalo et al., 2017), and the results strongly support the two-signal model.

While BMP does not function directly as a morphogen in human pluripotent cells, whether WNT or NODAL can remains an open question. ACTIVIN alone is not sufficient to induce primitive streak differentiation, however, hESCs pretreated with WNT3A for 24h before ACTIVIN differentiate into mesendodermal fates (Yoney et al., 2018). Thus, whether or not these signals function as morphogens, combinatorial effects will also be essential in understanding their function during germ layer specification.

3.3 How are changing morphogen concentrations interpreted in time?

While there is a wealth of information on the phenotypes of mouse embryos when signaling components have been removed, very few direct measurements of signaling activity have been performed due to the challenge of following subcellular events in mammalian embryos. Fluorescently tagging endogenous signal transducers in ESCs and imaging them during differentiation and patterning provides a window into how cells interpret dynamic signals (Heemskerk et al., 2019; Massey et al., 2019; Nemashkalo et al., 2017; Yoney et al., 2018). These studies have led to the conclusion that each pathway performs its own unique signal processing.

hESCs respond stably to stimulation by BMP, and the sustained response is necessary for differentiation to extra-embryonic fates (Nemashkalo et al., 2017). In contrast, hESCs exposed to WNT or ACTIVIN signals show an adaptive response which decays even under constant stimulation (Heemskerk et al., 2019; Massey et al., 2019; Yoney et al., 2018). ACTIVIN treated cells show a decay on the time scale of about 4 h to level slightly above the baseline independently of ligand concentration. In contrast, in response to WNT, adaptation is slower (taking around 12 h to reach baseline) and dose-dependent, such that it is nearly complete at lower doses of WNT, and partial at higher doses (Massey et al., 2019). Adaptation allows the cells to read the rate of change of the signal in time instead of the absolute concentration (Heemskerk et al., 2019; Sorre, Warmflash, Brivanlou, & Siggia, 2014), and changing the ligand concentration slowly abrogates the response to ACTIVIN but not to BMP. Interestingly, the cells of the neural tube have also been shown to respond adaptively to Sonic hedgehog (Dessaud, McMahon, & Briscoe, 2008), and it will be interesting to determine whether the mechanisms by which different adaptive signals are interpreted are similar.

Stem cell differentiation protocols can be optimized based on this dynamic information. In the case of ACTIVIN, pulsing ligand is more efficient that constant exposure even though the integrated exposure is lower (Heemskerk et al., 2019). Thus, ESCs have allowed for direct measurement of signaling dynamics in pluripotent cells and in response to controlled concentrations and dynamic presentation of ligand. Whether these dynamics are relevant to the embryo remains an open question, however, insights are beginning to emerge from the patterning systems described in the next section.

3.4 How do morphogen systems result in spatial patterns of cell fates?

During development, the signaling dynamics described above lead to the formation of spatial patterns. The 2D gastruloid system has allowed for the direct observation of signaling dynamics during self-organized patterning. These results show that initially the entire colony responds to BMP before its restriction to the edge approximately 12h later (Heemskerk et al., 2019). This pattern of BMP signaling, that is high only at the edge, is then stable for the remainder of patterning. BMP signaling at the edge triggers a wave of WNT signaling which initiates around 24h after BMP treatment near the edge and moves inward at a constant rate (Fig. 3A) (Chhabra et al., 2019). Slightly later, WNT initiates NODAL signaling near the colony edge, and NODAL also expands inwards at a constant rate (Fig. 3B) (Heemskerk et al., 2019). The wave of WNT signaling is initiated earlier than the wave of NODAL, but the NODAL wave travels faster allowing it to overtake the WNT wave (Chhabra et al., 2019).

Importantly, mesodermal differentiation requires both WNT and NODAL, however, the spatial extent of differentiation does not map to any particular level of these pathways (Chhabra et al., 2019). As the signaling travels in a wave, the timing of signaling onset is much more variable than



Fig. 3 Nodal and WNT signaling dynamics in BMP treated micropatterned hESC colonies. (A) Pie sections of a live-imaged micropatterned GFP-beta-catenin hESC colony stimulated with BMP4 showing a clockwise time evolution (17-47 h) of non-membrane beta-catenin expression. Each section is a snapshot from time-lapse imaging at clockwise increasing time points post BMP treatment. The outer orange circumference highlights the pinned back end of the WNT wave while the white spiral approximates the inward movement of the front end of the WNT wave. (B) Pie sections of micropatterned hESC colonies stimulated with BMP4 showing a clockwise time evolution (24-48h) of SMAD1 (red) and SMAD2 (cyan) expression. Each section corresponds to a micropatterned colony fixed at the indicated time point post BMP treatment. The red circumference highlights that SMAD1 stays restricted to the edge at all time points. The white spiral approximates the front end of the SMAD2 wave that moves inward in time. Panel (A): Data from reference Chhabra, S., Liu, L., Goh, R., Kong, X., & Warmflash, A. 2019. Dissecting the dynamics of signaling events in the BMP, WNT, and NODAL cascade during self-organized fate patterning in human gastruloids. PLoS Biology, 17, 2019, e3000498; Panel (B): Data from reference Heemskerk, I., Burt, K., Miller, M., Chhabra, S., Guerra, M.C., Liu, L., et al. 2019. Rapid changes in morphogen concentration control self-organized patterning in human embryonic stem cells. eLife, 8, e40526.

the level of signaling, and it is possible that it is this timing what cells sense when determining their fate. With the exception of the edge cells, each cell inside the colony experiences three events: the attenuation of the initial BMP signal, the WNT signal, and the NODAL signal. We speculate that the relative timing of these events is what determines the final pattern. As noted above, differentiation in response to NODAL signal requires previous WNT signaling (Yoney et al., 2018), so one hypothesis is that the point in space where NODAL signaling overtakes WNT marks the edge of the territory of mesodermal differentiation. As this mechanism differs from previously proposed models of spatial patterning, it will be interesting to

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determine how the gene regulatory network, which determines cell fates downstream of signaling, functions to implement this decision.

A WNT wave also forms when hESCs colonies are treated with WNT3A, this time patterning the colonies into two regions: an outer ring of mesendodermal cells and an inner region of ectodermal cells (Martyn et al., 2019, 2018). The initial response to WNT is limited to the colony edge by cell-cell contacts through *E*-cadherin (E-CAD). The wave is formed by a *domino effect* where response to WNT ligands causes the cell to undergo EMT and downregulate E-CAD at the junctions with its neighbors, thereby freeing more cells to respond to the WNT signal. This process continues until a sufficient amount of the secreted feedback inhibitor DKK1 builds up and halts the EMT wave.

The WNT waves created by BMP or WNT3A, although similar in appearance, are mechanistically distinct. In the case of WNT3A induction, the wave created is due to a wave of competence to respond to exogenous WNT signaling, and it is not inhibited even if IWP2, an inhibitor of WNT ligand secretion, is present in the media the entire time (Martyn et al., 2019). In the case of BMP induction, the wave results from endogenous signaling, and does not start at the edge of the colony, where *E*-CAD expression is low. It also does not require EMT as the wave propagates farther into the colony than the territories where differentiation and EMT are observed (Chhabra et al., 2019).

An interesting open question is what separates the mesoderm from the endoderm. Knockout of CER1 during micropatterned differentiation, causes upregulated NODAL signaling and promotes endoderm at the expense of mesoderm differentiation (Yoney et al., 2018). However, how the NODAL signal is different upon CER1 knockout is unclear. Are its dynamics or spatial extent perturbed or only its level? Recent results with a reporter system in mouse embryos and stem cells suggest that the endoderm arises in randomly distributed cells within the anterior primitive streak and then segregates from the mesoderm (Pour et al., 2019) but, how does this observation relate to the Nodal levels or distribution? One hypothesis is that the fate choice is stochastic with a bias towards endoderm that depends on the level of Nodal signaling that a cell receives.

3.5 How are symmetries broken in the early embryo?

Two-dimensional models are very useful for studying signaling dynamics and patterning, however, they do not recapitulate the symmetry breaking or morphogenesis which is observed *in vivo*. This issue has driven the development of the 3D models described above. Most studies to date have concentrated on determining to what degree these models resemble *in vivo* embryos, however, they have provided some insights about the signals that drive patterning during early mammalian development and have set a course for future investigations.

Several of the three-dimensional models described above break symmetry from an initial spherical configuration. In the case of the human models of amnion (Shao, Taniguchi, Gurdziel, et al., 2017; Shao, Taniguchi, Townshend, et al., 2017), it is BMP signaling which separates the amnion from the epiblast. The ETS (Harrison et al., 2017) and ETX (Sozen et al., 2018) embryos, as well as 3D hESCs treated with low doses of BMP4 (Simunovic et al., 2019), localize expression of BRA to one side of the epiblast compartment. This is particularly surprising in the case of ETS embryos as they lack the visceral endoderm which places a role in positioning the primitive streak to the posterior side of the embryo. In all of these cases, symmetry breaking depends on Wnt signaling, and in the hESC model it has been shown that removing DKK1 causes the entire sphere to adopt a mesodermal fate (Simunovic et al., 2019). Some caution in interpretation is warranted, however, as the markers used such as Sox2 and Bra delineate germ layers rather than the AP axis specifically. Further, in the case of the hESC model, the final object contains one axis of rotational signaling, the same as the 2D micropatterned colonies, so it is not clear that one model represents a true AP axis more than the other (Heemskerk & Warmflash, 2016). Nonetheless, these are useful models for how morphogen pathways are deployed to break spherical symmetry, and it will be interesting to use them to study the dynamics of the BMP and WNT pathways in these events.

The mouse gastruloid system develops a more complete AP axis, including axial elongation and the temporal and spatial emergence of Hox genes (Beccari et al., 2018; Turner et al., 2017). A pulse of the Wnt agonist CHIR99021 (CHIR) is essential for these events, however, little is known about the response to this pulse or how downstream signals ultimately lead to patterning. In the future, this represents an exciting system to begin to explore mechanistically how signals pattern the AP axis.

4. Conclusions

In the previous sections we have reviewed recent insights on morphogen dynamics and patterning that have come from newly developed stem cell systems. Recent results indicate that the BMP pathway does not function as a morphogen in hESCs (Nemashkalo et al., 2017). Analogous experiments have not been performed for Wnt and Nodal, however, the mechanisms uncovered in hESC demonstrate that these pathways are not functioning as classical morphogens during self-organized fate patterning in micropatterned systems. It will be interesting to test more directly whether these pathways are capable of functioning as morphogens in mammalian ESCs.

The potential of *in vitro* systems for studying early development has only begun to be realized, and many new and exciting questions are yet to be explored. In the above sections, we highlighted several questions that are currently being addressed, including understanding precisely how the position of mesendodermal differentiation is established by the combinatorial effects of BMP, Wnt, and Nodal, how mesoderm and endoderm are separated, and what signaling dynamics lead to the symmetry breaking that underlies axis formation in the mammalian embryo. The combination of the stem cells systems and live cell reporters that have been developed should allow rapid progress on these longstanding questions. For example, DKK1 knockouts have been shown to cause defects in symmetry breaking in hESC spheroid models, and CER1 knockouts have been shown to lead to increased endoderm differentiation. Creating these knockouts directly in WNT or NODAL reporter lines, respectively, will allow for direct observation of the signaling dynamics, and how they lead to the observed phenotypes.

Here we have focused on signaling, however, much also remains to be learned about how these signals are interpreted by transcription factor networks inside of cells to implement cell fate decisions, the subject of intense study in other systems (Briscoe & Small, 2015; Driever, Thoma, & Nusslein-Volhard, 1989). The simplest model is that different binding sites have different affinities for the transcription factors induced by the morphogen (Driever et al., 1989; Struhl, Struhl, & Macdonald, 1989), however, recent studies have failed to show that there is a correlation between binding affinities and the range of morphogen induction in systems such as the vertebrate neural tube and AP patterning in fly (Hannon, Blythe, & Wieschaus, 2017; Oosterveen et al., 2012; Peterson et al., 2012). Further, it is clear that understanding the interplay between morphogen dynamics and their interpretation by the individual promoters of target genes is important for understanding patterning (Dubrulle et al., 2015). Directly studying these issues in mammalian stem cells could shed light on the gene regulatory network that separates germ layers during gastrulation.

A major remaining challenge is comparing the results from *in vitro* systems with actual embryos, particularly for human. Recently, a system for culturing post-implantation human embryos has been developed, and the different cell types present at these early stages were characterized (Deglincerti et al., 2016; Shahbazi et al., 2016), however, these are still limited by the 14-day rule, and it is also still unclear to what degree the cultured embryos mimic processes that occur *in vivo*.

Interspecies comparisons can serve as tool for validating in vitro systems and for understanding differences between humans and model organisms. Mouse ESC systems have begun to be compared to mouse embryos (Beccari et al., 2018; Harrison et al., 2017; Morgani et al., 2018), and validating in vitro systems for mouse would yield more confidence in human in vitro systems. There are a number of known differences between mouse and human including the geometry of the embryo (Behringer, Wakamiya, Tsang, & Tam, 2000), the positioning of the extra embryonic tissues (Dobreva et al., 2010), and molecular differences that are beginning to be probed by single cell RNA-Seq studies (Blakeley et al., 2015; Petropoulos, Edsga, Reinius, & Linnarsson, 2016). In vitro systems have the potential to examine these differences in more controlled settings by growing ESCs from the same developmental stages in the same conditions. Continuing to develop insights from stem cell systems and validating them in vivo is providing windows into previous inaccessible times during human development and into understanding developmental differences between humans and other species.

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Appendix

Mathematical model for fate patterning by a single morphogen

The dynamics of a gene regulatory network comprised of three mutually repressive transcription factors (TF1, TF2 and TF3) controlled by a

morphogen signal (S), as in Fig. 2A, can be described by the system of differential equations:

$$\frac{d\mathrm{TF1}}{dt} = a_1 \frac{(\mathrm{S}/k_{S1})^{n_{S1}}}{1 + (\mathrm{S}/k_{S1})^{n_{S1}}} \frac{1}{1 + (\mathrm{TF2}/k_{21})^{n_{21}} + (\mathrm{TF3}/k_{31})^{n_{31}}} - \delta_1 \mathrm{TF1}$$
$$\frac{d\mathrm{TF2}}{dt} = a_2 \frac{(\mathrm{S}/k_{S2})^{n_{S2}}}{1 + (\mathrm{S}/k_{S2})^{n_{S2}}} \frac{1}{1 + (\mathrm{TF1}/k_{12})^{n_{12}} + (\mathrm{TF3}/k_{32})^{n_{32}}} - \delta_2 \mathrm{TF2}$$
$$\frac{d\mathrm{TF3}}{dt} = a_3 \frac{1}{1 + (\mathrm{TF1}/k_{13})^{n_{13}} + (\mathrm{TF2}/k_{23})^{n_{23}}} - \delta_3 \mathrm{TF3}$$

where the variables TF1, TF2 and TF3 represent the expression values of the corresponding transcription factors. In the main text these transcription factors are CDX2, BRA and SOX2, respectively. The parameter *S* corresponds to the concentration of the morphogen, which in the main text is BMP. The parameters n_{ij} , k_{ij} (with $i \in \{S, 1, 2, 3\}$, $j \in \{1, 2, 3\}$) are the Hill coefficients and dissociation constants respectively, and a_i , δ_i with $i \in \{1, 2, 3\}$ are the production and degradation rates, respectively.

In Fig. 2C of the main text, the system of differential equations was simulated with initial condition TF1=0, TF2=0 and TF3=2, the parameter values given in Table A1, and S decreased exponentially as a function of the distance from the source.

Parameter	Value	Parameter	Value	Parameter	Value
<i>a</i> ₁	8	<i>a</i> ₂	4	<i>a</i> ₃	2
δ_1	1	δ_2	1	δ_3	1
k_{S1}	7	k_{S2}	0.5	k ₁₃	0.1
n _{S1}	1	n _{S2}	5	<i>n</i> ₁₃	1
<i>k</i> ₂₁	0.4	<i>k</i> ₁₂	0.3	k ₂₃	0.1
<i>n</i> ₂₁	1	<i>n</i> ₁₂	1	n ₂₃	1
k ₃₁	0.5	k ₃₂	1		
<i>n</i> ₃₁	1	n ₃₂	1		

Table A1 Table of parameter values used in the simulations shown in Fig. 2C.

Mathematical model for patterning multiple fates with two signals

The dynamics of a gene regulatory network comprised of three mutually repressive transcription factors (TF1, TF2 and TF3), this time controlled by a balance of an upstream signal (S1) and a secondary signal (S2), as in Fig. 2E, can be described by the system of differential equations:

$$\frac{dS2}{dt} = a_{S2}S1 - \delta_{S2}S2$$

$$\frac{dTF1}{dt} = \frac{a_1 + b_1S1}{1 + (TF2/k_{21})^{n_{21}} + (TF3/k_{31})^{n_{31}}} - \delta_1 TF1$$

$$\frac{dTF2}{dt} = \frac{a_2 + b_2S2}{1 + (TF1/k_{12})^{n_{12}} + (TF3/k_{32})^{n_{32}}} - \delta_2 TF2$$

$$\frac{dTF3}{dt} = \frac{a_3}{1 + (TF1/k_{13})^{n_{13}} + (TF2/k_{23})^{n_{23}}} - \delta_3 TF3$$

where the variables S2, TF1, TF2 and TF3 correspond to the expression values of the secondary signal and the corresponding transcription factors. In the main text, the secondary signal is WNT and the transcription factors are CDX2, BRA and SOX2, respectively. The parameter S1 is the concentration of the upstream signal, BMP in the main text. The parameters n_{ij} , k_{ij} (with $i,j \in \{1,2,3\}$) are the Hill coefficients and dissociation constants and a_i , δ_i with $i \in \{S2,1,2,3\}$ are the production and degradation rates, respectively.

Parameter	Value	Parameter	Value	Parameter	Value
<i>a</i> _{<i>S</i>2}	2	<i>a</i> ₂	0.05	<i>a</i> ₃	5
δ_{S2}	0.5	b_2	14	δ_3	0.1
<i>a</i> ₁	0.7	δ_2	0.5	k ₁₃	1
b_1	5	k_{12}	0.1	<i>n</i> ₁₃	1
δ_1	0.5	<i>n</i> ₁₂	1	k ₂₃	0.01
k_{21}	0.8	k ₃₂	0.5	n ₂₃	1
<i>n</i> ₂₁	1	n ₃₂	1		
k ₃₁	0.5				
<i>n</i> ₃₁	1				

 Table A2
 Tables of parameter values used in the simulations shown in Fig. 2G of the main text.

In Fig. 2G of the main text, the system of differential equations was simulated with initial condition S2=0, TF1=0, TF2=0 and TF3=3, the parameter values given in Table A2 and the main signal, and S1 decreased exponentially as a function of the distance from the source.

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