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Pluripotent Stem Cells as a Model for Embryonic Patterning: From Signaling Dynamics to Spatial Organization in a Dish

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Abstract: **In vivo studies have identified the signaling pathways and transcription factors involved in patterning the vertebrate embryo, but much remains unknown about how these are organized in space and time to orchestrate embryogenesis. Recently, embryonic stem cells have been established as a platform for studying spatial pattern formation and differentiation dynamics in the early mammalian embryo. The ease of observing and manipulating stem cell systems promises to fill gaps in our understanding of developmental dynamics and identify aspects that are uniquely human. *Developmental Dynamics 000:000–000, 2016.* © 2016 Wiley Periodicals, Inc.**

Key words: human embryonic stem cells; morphogen; gastrulation; dynamics; signaling

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Introduction

Despite its importance, relative to other vertebrate classes, much remains unknown about mammalian development in general, and human development in particular. Knowledge of human development has medical applications ranging from directed stem cell differentiation for regenerative medicine (Trounson and McDonald, 2015) to treatment of childhood cancers (Hatten and Roussel, 2011). Moreover, an anthropocentric worldview makes our own development a fascinating subject of fundamental research. Gaps in our knowledge result, to a large extent, from the difficulty in studying mammalian development: It is slow and takes place in utero. Human development poses a particular challenge because ethical considerations prevent experimentation on human embryos past the blastocyst stage.

Although mammals may be challenging to study in vivo, this is balanced by the fact that pluripotent stem cells derived from mammalian embryos can be maintained in culture, enabling in vitro study of early development. Embryonic stem cells (ESCs) provide a powerful complement to in vivo studies of development, as well as a unique model for human development past the blastocyst stage. However, the strengths of the stem cell model go far beyond the insight it may provide into specifically mammalian traits. The possibilities that cell culture provides for direct observation and experimental manipulation allow us to address general questions about embryonic development that are difficult to answer in any in vivo model, and improve our understanding more broadly.

In the early embryo, pluripotent cells differentiate into ectoderm, mesoderm, and endoderm, and organize into a trilaminar structure, a process known as gastrulation. Gastrulation is orchestrated by morphogens thought to form gradients across the embryo and specify the germ layers in a concentration-dependent manner. Much remains uncertain about the dynamics and shape of these gradients, the way in which they determine cell fate and how they relate to the size and shape of the embryo. It is also unclear whether findings from model systems such as the mouse can be applied to human development, or, more generally, which aspects of mammalian development are conserved.

In this article, we will review what is known about the dynamics of differentiation and morphogen gradient formation in the early embryo, in particular during gastrulation. Although our focus is on understanding mammalian embryogenesis, we also review studies of embryonic patterning in non-mammalian models, as quantitative studies of signaling and patterning dynamics have not been performed in mammalian systems, and many aspects are conserved across vertebrates or even bilaterians. We will discuss general gaps in our understanding of vertebrate patterning as well as those that are specific to mammals or to humans. After expanding on the challenges of studying development in vivo, the strengths of the stem cell model, and the relation between stem cells and the embryo, we will discuss differentiation, spatial patterning, and interspecies differences.

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Our emphasis will be on the importance of dynamics in morphogen gradient interpretation; the need for studying endogenous morphogens to understand pattern formation; and the significant differences that exist between model organisms despite the conservation of core pathways. We will discuss how stem cell models have contributed to our understanding of development to date, and examine the opportunities that they provide to complement in vivo studies and answer open questions in developmental biology.

Background and Perspective

Difficulties With In Vivo Models

Unraveling the complexity of embryonic development is a daunting task. In vivo, patterning, growth, and morphogenesis of both embryonic and extraembryonic tissues all happen simultaneously with little ability to separate processes in space or time. Imaging of three-dimensional (3-D) embryos and quantitative analysis of the resulting data is technically challenging. Moreover, quantitative understanding of development would be greatly aided by making subtle perturbations, such as changing the shape of a tissue or inhibiting and activating signaling in sequence over relatively short timescales, but these types of perturbations are impossible in vivo. Much has been learned from genetic studies, but there is often difficulty in understanding the role of particular genes due to partially penetrant phenotypes, even among littermates. For example, mouse mutants for the TGFβ family ligand BMP4 (Winnier et al., 1995) and the Nodal inhibitors Lefty and Cerberus (Perea-Gomez et al., 2002) show a wide range of morphological phenotypes, and even vary in whether gastrulation takes place. Although such phenotypic diversity is hard to interpret when it concerns the overall development of an embryo, it may contain additional information about the gene network when intermediate steps such as the abundance of particular cell fates and expression of target genes are quantitatively evaluated (Corson and Siggia, 2012; Raj, et al., 2010).

Mammalian development poses special challenges because it takes place in utero. The mouse is the mostly widely studied mammalian model system due to several advantages: relatively large litter sizes, short generation times, and straightforward genetic manipulation. Nonetheless, technical difficulties stand in the way of rapid progress, especially in going beyond identifying genes and pathways to a quantitative, systems-level understanding of differentiation and patterning. In vivo imaging is essentially impossible, and live imaging ex vivo is complicated by high sensitivity to phototoxicity (Piliszek et al., 2011). Moreover, keeping embryos healthy ex vivo for an extended period of time is extremely challenging. Although major achievements were made in embryo culture nearly 30 years ago, these predated the live-imaging era and were never widely adapted (Chen and Hsu, 1982; Hsu, 1979; Tam, 1998). Recently, a technique allowing observation of embryos as they progress through implantation in vitro has been reported for both mouse and human (Bedzhov and Zernicka-Goetz, 2014; Deglincerti et al., 2016; Shahbazi et al., 2016), which is a great step forward in the understanding of this obscure stage of development. However, whether these embryos will proceed through gastrulation remains unclear, and even if they can, in the human case they will not be permitted to do so, so alternative models remain essential.

In addition to experimental challenges, the mouse has limitations as a model for human development, as early mammalian development is diverse (Eakin and Behringer, 2004) and differences between human and mouse are likely significant. For example, the development of extraembryonic tissues, and consequently the signaling environment of the embryo proper, is qualitatively different; also, the mouse embryo is cup-shaped whereas the human embryo is disc-shaped (Behringer et al., 2000; Dobreva et al., 2010; Rossant, 2015).

Stem Cell Culture to Complement In Vivo Studies

The flip side of slow in utero development is the possibility of maintaining ESCs in vitro. Moreover, unlike other model systems, in mammals the formation of the embryonic axes and germ layer segregation is independent of maternal transcripts. These features enable an alternative approach to the study of development. Furthermore, it has recently been shown that axis formation not only is independent from maternal transcripts, but takes place without any maternal cues, which is encouraging for attempts to reproduce this process in vitro (Bedzhov and Zernicka-Goetz, 2014; Bedzhov et al., 2015; Morris et al., 2012).

ESCs are pluripotent, meaning they are capable of differentiating to all the cell types of the body. Traditionally, ESCs have served as a model for lineage specification but not patterning due to the spatial disorganization that results from the majority of ESC differentiation methods. However, in recent years they have been made to reproduce spatial processes ranging from early embryonic patterning (ten Berge et al., 2008; van den Brink et al., 2014; Warmflash et al., 2014) to morphogenesis of organs like the optic cup (Eiraku et al., 2011; Nakano et al., 2012). Whereas ESCs are derived from blastocyst embryos (Martin, 1981; Thomson et al., 1998), it is also possible to derive pluripotent cells by reprograming somatic cells (Takahashi and Yamanaka, 2006) (reviewed in Hochedlinger and Jaenisch, 2015). Such induced pluripotent stem cells (iPSCs) are important for clinical applications (Robinton and Daley, 2012), but we shall focus on ESCs as both cell types behave similarly (Choi et al., 2015), with iPSCs offering no clear advantage in studying early development.

ESCs ameliorate many of the in vivo challenges to studying early development. Clonal cell lines give reproducible phenotypes, or at least exclude genetics as the source of variation. The environment of the cells can be controlled and systematically varied in many ways. Micropatterns that restrict cell growth to designated areas allow control over tissue shape and bring high reproducibility to spatial distributions of cells, and therefore to patterning (Ma et al., 2015; Warmflash et al., 2014). Microfluidics allow precise spatial and temporal control of external signaling molecules (Keenan and Folch, 2008; Moledina et al., 2012; Przybyla and Voldman, 2012; Sorre et al., 2014; Tay et al., 2010). Moreover, protocols to maintain cells in particular states allow us to pause development at particular stages along a single lineage, e.g., mouse ESCs (mESCs), epiblast cells (mEpiSCs) (Brons et al., 2007; Tesar et al., 2007), and mesenchymal stem cells (mMSCs)(Bianco, 2014), or to study interaction between distinct lineages in a controlled manner, e.g. mESCs and extraembryonic endoderm (XEN) (Toh et al., 2011). From this perspective, two important open questions are which intermediate states are sufficiently stable to be maintained by the right culture conditions, and whether this stability is related to naturally occurring

checkpoints in development such as diapause in the mouse blastocyst. (Nichols et al., 2001; Scognamiglio et al., 2016)

Combining these technologies to control ESCs allows for a synthetic approach to embryogenesis and organogenesis. The field of synthetic biology has approached the complexity of genetic networks by rebuilding functional circuits out of minimal components (Sprinzak and Elowitz, 2005), and multicellular circuits involving distinct bacterial (Chen et al., 2015) or mammalian (Morsut et al., 2016; Roybal et al., 2016) cell types have recently been constructed. Similarly, one could imagine engineering minimal versions of multicellular circuits such as those that exist between embryonic and extraembryonic tissues to deconstruct the complex interactions involved in lineage specification. Extraembryonic signals could be supplied to ESCs by extraembryonic cell lines, engineered cell lines with the required circuits inserted, or a completely artificial substitute, supplying the right signals at the right place and time through microfluidics. The ability to reconstruct and manipulate these circuits in vitro would provide a level of understanding impossible to achieve in a developing embryo.

Comparing Stem Cells to Embryos

To translate between the lessons learned in stem cells and embryos, it is necessary to have a precise understanding of the in vivo counterparts of stem cell lines. Mammalian embryogenesis involves the extensive development of extraembryonic tissues that are crucial for supplying both nutrition and patterning signals to the embryo proper. The first lineage segregation to take place is between inner cell mass (ICM) and trophectoderm (TE). Next, the inner cell mass forms a double layer consisting of hypoblast or visceral endoderm (VE) and epiblast. It is predominantly the epiblast that gives rise to the embryo, although in the mouse the VE has been found to contribute as well (Kwon et al., 2008). During gastrulation, the epiblast differentiates into ectoderm, mesoderm, and endoderm. This process is mainly orchestrated by the BMP and Activin/Nodal branches of the TGFβ pathway, through interplay with Wnt and FGF (Arnold and Robertson, 2009). BMP and Nodal are morphogens (Green et al., 1992; Wilson et al., 1997) thought to specify the germ layers in a concentration-dependent manner through gradients across the embryo.

Human ESCs (hESCs) resemble the epiblast, which, based on the mouse model, is believed not to contribute to the TE and VE in vivo. In vitro, hESCs still possess the potential to contribute to extraembryonic lineages. The vast majority of evidence now indicates the potential for hESCs to differentiate to trophectoderm upon treatment with BMP4 (Li et al., 2013; Sudheer et al., 2012; Xu et al., 2002), although this remains controversial (Bernardo et al., 2011) (reviewed in Li and Parast, 2014). It is also possible to revert hESCs to a more ICM-like state. (Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014), and it has recently been shown that it is possible to derive such naïve human stem cells directly from the ICM (Guo et al., 2016). In contrast to hESCs, mESCs are equivalent to ICM cells (Hanna et al., 2010), and the murine equivalent of hESCs are mEpiSCs, which are representative of the postimplantation epiblast (Brons et al., 2007; Tesar et al., 2007). It is important to note that hESCs, mESCs, and mEpiSCs are all pluripotent but represent different developmental stages with different signaling requirements (Warmflash et al, 2012a).

Cell Fate Determination

Directed by external signals, a single stem cell can give rise to an array of different cell types. Signals are also required to maintain a progenitor cell in its state of stemness, and a number of different factors are required to maintain the pluripotent state in both mouse (Ying et al., 2003; Ying et al., 2008) and human (James, 2005; Vallier et al., 2005). Differentiation is traditionally divided into three stages: competence, when a cell is able to respond to a signal; specification, when the signal can be removed without changing the resultant fate; and determination, when other signals can no longer influence the fate. Signals are provided by other cells but can also consist of physical or chemical environmental factors such as substrate stiffness and oxygenation. Ideally, differentiation would be probed experimentally by measuring the response of a single cell or homogeneous population to a well-defined signaling environment, but this is difficult in vivo. For example, traditional experiments involving transplantation of tissue across the embryo (such as the pioneering experiments of Spemann and Nieuwkoop in amphibians) (Kinder et al., 2001) leave much uncertain about both the identity of the transplanted cells and the environment they are moved into. Stem cells, however, have a defined initial state and can be placed in a specified environment, allowing for exact determination of the differentiation trajectory as a function of the external stimuli.

Burning the French Flag

The paradigm for spatial differentiation in the early embryo is the French flag model of concentration-dependent response to a morphogen gradient, whereby cells closer to a localized source of diffusible ligands differentiate into different types than cells farther away (Wolpert, 1969). However, we will argue that there is essentially no direct evidence for patterning by level-dependent interpretation of long-range morphogens in vertebrate systems. The issue can be broken into two parts: gradient formation and interpretation. We will focus on the latter here and postpone discussion of the former to the next section. In what follows, we will use the word "morphogen" in a broad sense [as its originator intended (Turing, 1952)], meaning any factor that determines a spectrum of cell fates. As discussed in detail in Green & Sharpe, 2015, Turing's model focused on how morphogen gradients are established and not how they are interpreted, and is potentially consistent with any mechanism of gradient interpretation, including, but not limited to, the French flag.

A number of experiments in *Xenopus* animal cap cells provide evidence for level-dependent response to vertebrate morphogens. The animal cap cells are pluripotent and will default to a neural fate if dissociated and cultured in isolation, but exposure of dissociated animal cap cells to exogenous Activin or FGF (Green et al., 1992) induces the cells to differentiate to mesodermal fates whose identity along the dorsal-ventral axis is determined by the concentration of inducer. Similar treatment with BMP ligands does not switch the germ layer but produces progressively more ventral fates within the ectoderm so that intermediate doses induce neural crest, whereas higher doses induce neural fates (Wilson et al., 1997). Of note, in all these experiments, each concentration of ligand induced multiple fates within a shifting window of available fates; there was not a one-to-one mapping between ligand concentration and fate. Experiments with artificial Activin gradients created by mRNA injection or Activin-

soaked beads subsequently showed that the response is direct (not due to an induced secondary morphogen) and that an Activin gradient established through free diffusion can control the spatial organization of cell fates (Gurdon et al., 1994). One problem with the dissociation experiments is that the heterogeneity in obtained cell types at each concentration could not be accounted for, and could have been the result of heterogeneity in competence before treatment, noisy signal reception, and transduction, or subsequent spatial organization due to endogenous signaling. The experiments with artificial gradients suggest the latter, as the artificial gradients produced coherent domains of expression, rather than random differentiation with a proportion of different fates that depends on the distance from the source. Importantly, all experiments left undetermined how the dynamics of ligand presentation are integrated into a cell fate decision. Mammalian morphogen gradients, to the extent that they have been observed or inferred, are highly dynamic (Balaskas et al., 2012; Ferrer-Vaquer et al., 2010; Monteiro et al., 2008). Moreover, patterning of mammalian embryos takes place during a phase of rapid growth, and in all vertebrates the large-scale tissue rearrangements that occur during gastrulation result in the sources and receivers of signals constantly moving relative to one another. How patterning takes place in this highly dynamic environment is therefore left obscure by these early experiments. For the dynamic interpretation of Nodal, both the time integral of ligand levels (Ben-Haim et al., 2006) and the maximal level (Bourillot et al., 2002; Dyson and Gurdon, 1998) have been proposed to determine cell fate. Subsequently, both were ruled out by response of target genes to decreases in level, but the actual relation to fate was left undetermined (Dubrulle et al., 2015).

A More Refined Picture of Cell Fate Determination

The difficulty in understanding the link between ligand dynamics and cell fate may be due to the complexity of the relationship; there is likely no simple rule such as the proposed integral or ratchet model. To obtain a more refined understanding of morphogen interpretation, it is necessary to separate ligand presentation, signal transduction, transcription, and cell fate. Vertebrate morphogens are diffusible factors that generally signal by binding receptors, leading to the translocation of signal transducers to the nucleus. These signal transducers can then complex with other nuclear factors to induce gene expression. Finally, mutually exclusive cell fates defined by the stable expression of a particular set of genes are generally not a direct response to signal transducers but a consequence of the regulatory logic between targets. In the following paragraphs, we review what is known about each of these steps and the opportunities for progress in stem cell models.

The Importance of Signaling Dynamics

Figure 1 shows a theoretical example of how interpretation of signaling dynamics can play a fundamental role in pattern formation. Cells move across a morphogen gradient at different speeds. Three scenarios for the relation between pathway signaling and morphogen dynamics are explored: direct response to concentration, temporal integration of ligand concentration, and response to rate of increase (adaptation). For simplicity, cell fate is assumed to be determined by thresholding the maximal signaling level in each case. The result differs from the French flag model in two important ways: First, although pattern formation requires a morphogen gradient, cell fates are patterned orthogonal to the gradient. Second, level-dependent response does not lead to spatial patterning, whereas the other two scenarios lead to patterns that are inverted relative to each other, with ligand integration leading to the highest level of signaling in slow-moving cells, and response to ligand rate of increase leading to the highest signaling in fast-moving cells. The example illustrates the effect of cells moving relative to static morphogen levels, which may be relevant to the movement of mesendodermal precursors past the organizer in various organisms. However, it is an extreme case chosen for its simplicity, and examples with static cells and changing morphogen levels, or combinations of various scenarios, can be constructed equally well. The general point is that dynamics has the potential to uncouple both the axis of patterning and the arrangement of fates from the naïve expectation based on the instantaneous morphogen gradient.

The importance of information processing at the level of the signaling response to ligand was demonstrated for a number of pathways in a handful of experimental systems. Although not directly relevant to early development, signaling dynamics are perhaps best understood for the NFkB pathway, where cell culture experiments have shown a complex relationship between ligand presentation, signaling response, and target gene activation. A fast transcriptional feedback on the nuclear localization of the signal transducer leads to an initial burst of signaling followed by a slower, possibly oscillatory phase (Hoffmann, 2002; Nelson et al., 2004; Tay et al., 2010; Turner et al., 2010), and each of these responses may be important for the induction of particular genes. Some transcriptional targets depend on sustained signaling at high doses, whereas others are transcribed with similar amplitude and finite duration regardless of the concentration and temporal profile of stimulation (Hoffmann, 2002; Tay et al., 2010).

For the TGFβ pathway, it was shown in C2C12 myoblasts (murine muscle progenitor cells) that the nuclear localization of the signal transducer Smad2 was sustained, while the nuclear localization of the co-transducer Smad4 was adaptive (Sorre et al., 2014; Warmflash et al., 2012b). There, too, adaptation depends on a transcriptional feedback. Transcriptional dynamics of many target genes follow those of Smad4 localization, and differentiation into myotubes was more effective with pulsed rather than sustained stimulation, consistent with adaptive but not sustained dynamics controlling differentiation. Adaptation in TGFβ signaling is also potentially consistent with the model that growth in the *Drosophila* wing disc depends on the rate of increase of Dpp signaling (the *Drosophila* homologue of the TGFβ superfamily member BMP4) (Wartlick et al., 2011); however, this suggestion remains controversial (Harmansa et al., 2015). In vivo, an adaptive response, i.e., gradual desensitization, to Sonic Hedgehog (Shh) was observed in patterning the chick neural tube (Dessaud et al., 2007) through a negative feedback that induces transcription of the Shh inhibitor Patched-1.

Signaling Dynamics in Early Development

In Zebrafish, patterns of activation of both the TGFβ transducer Smad2 (Dubrulle et al., 2015; Harvey and Smith, 2009) and an Shh signaling reporter (Xiong et al., 2013) have been followed in individual cells. However, in vivo, ligand levels cannot be controlled precisely or dynamically, and direct interpretation of an

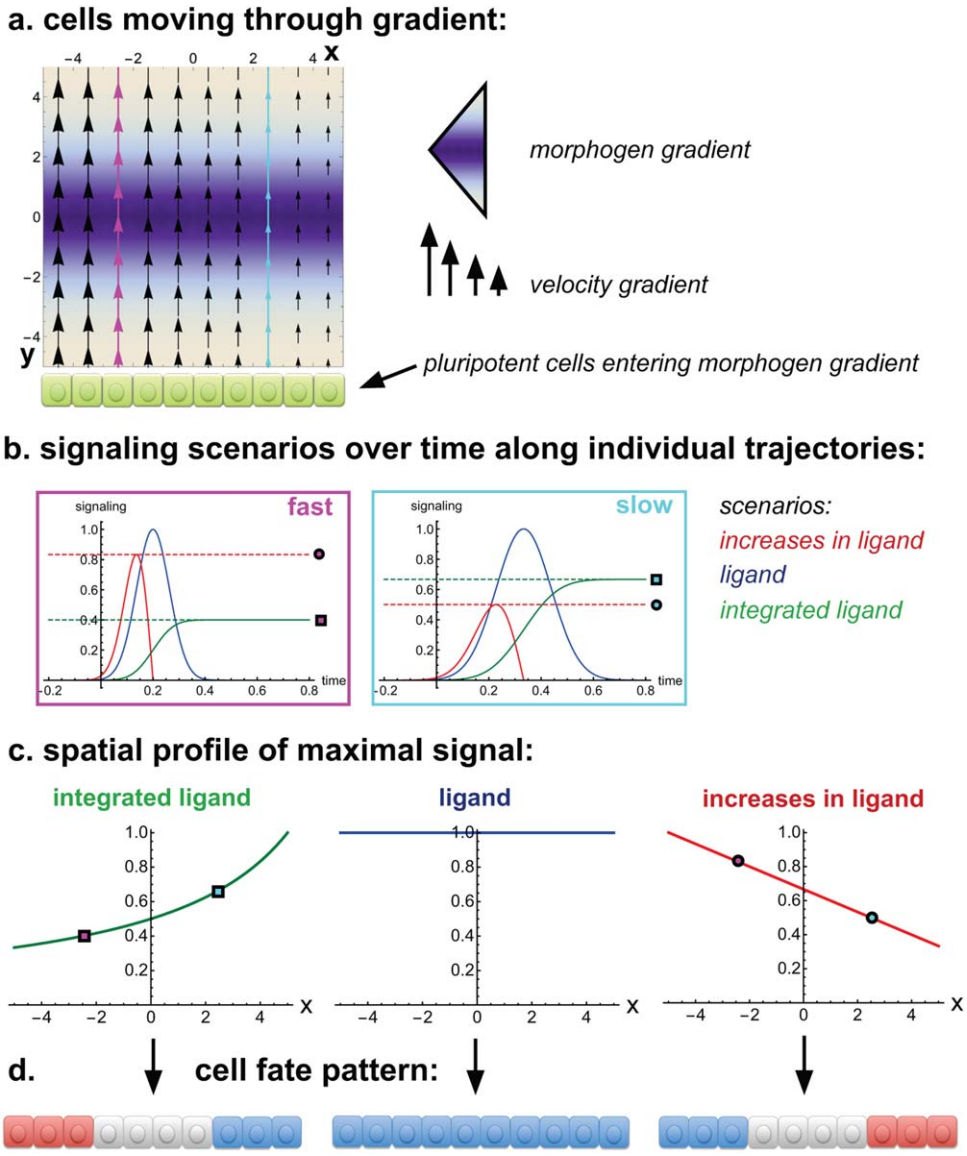

Fig. 1. Importance of signaling dynamics for spatial patterning. Toy model demonstrating the dramatic effect signaling dynamics can have on pattern formation. For simplicity, cells move relative to a static morphogen gradient, and cell fate is assumed to be determined by the signaling maximum in one of three possibilities for the relation between ligand dynamics and signaling response. **A**: Morphogen gradient from high (purple) to low (white) along Y direction, velocity field of cell trajectories (black) with two trajectories singled out (magenta and cyan). **B**: Signaling response along the cyan and magenta trajectories for three scenarios: response to ligand level (blue), response to increases in ligand (red), and response to time integral of ligand (green). Dashed lines indicate signaling maximum; note reversed positions of integral and increase response for the two trajectories. **C**: Maximal signaling response along X direction at late times for different scenarios. The maximal level for the trajectories in A and B are indicated by cyan and magenta squares (ligand integration) and circles (ligand increase). **D**: Resultant pattern for all three scenarios requires morphogen gradient but is orthogonal to it, instead following the velocity gradient. Integral and increased response lead to opposite patterns, while concentration-dependent response produces no pattern at all.

applied morphogen cannot be separated from response to endogenous signaling. In mammalian systems, observing the dynamics of signal production and transduction is much more challenging, and little work has been done quantifying signaling, morphogen distributions, and cell fate. Stem cells not only simplify understanding differentiation, they may ultimately be the only system that allows the degree of control required to relate dynamics of ligand presentation to cell fate. For example, given that both BMP and Nodal are TGFβ family members, it is entirely possible that germ layer specification, like myoblast differentiation, depends on adaptive dynamics. It is now feasible to evaluate this possibility using embryonic stem cells exposed to changing ligand levels controlled by microfluidics.

Of course, it then remains to be determined which ligand dynamics cells are actually exposed to during embryonic patterning, and whether, for example, pulsed input has any in vivo relevance. We will return to this topic in the next section. However, regardless of in vivo relevance, it is of great value to know the most effective way to differentiate cells to a particular fate, either for studying more advanced stages of development or for therapeutic purposes. Current protocols for obtaining particular cell fates coarsely recapitulate the signaling environment known to

achieve those cell fates in vivo (Chambers et al., 2009; McLean et al., 2007; Mendjan et al., 2014). Lack of in vivo understanding challenges the ability to obtain differentiated cell types at higher resolution, e.g., to consistently obtain lateral plate mesoderm. For further progress, differentiation protocols cannot rely only on in vivo studies. Instead, systematic study of stem cell responses in different environments should shed light on in vivo patterning and simultaneously allow for rational protocol design.

Gene Regulation and Cell Fate

Recent quantitative work in Zebrafish showed differential response of target genes to signaling. In particular, transcription rates of target genes were shown to correlate with the ligand level required to induce them stably (Dubrulle et al., 2015). In this study, the complexity of the embryonic system was reduced by knocking down the genes for endogenous Nodal ligands and injecting recombinant Nodal protein into the extraembryonic space of the embryo, resulting in uniform exposure of the cells to the supplied morphogen. In mammals, studies such as these would be much easier to perform in vitro with stem cells and would provide similar information about pattern formation. Moreover, in cell culture, the activity of signaling reporters of various pathways (Ferrer-Vaquer et al., 2010; Sorre et al., 2014), as well as reporters of target genes, can be followed in individual cells as the dynamics of morphogen stimulus are varied, allowing a more complete picture of the link between signaling dynamics and the regulation of direct targets.

In vivo, the relation between signaling and cell fate is best understood for Shh in the vertebrate neural tube (Cohen et al., 2013). In this system, it was shown that regulatory interactions between Shh targets are essential for "thresholding" the signal and establishing coherent gene expression domains (Balaskas et al., 2012). Similar mechanisms were suggested for interpreting Activin levels in the *Xenopus* embryo (Saka and Smith, 2007) and are almost certainly involved in interpreting other morphogens.

A great deal of progress has been made in understanding cell fate decisions at the level of the gene regulatory networks downstream of signaling using stem cells. Small molecule interference with signaling, RNAi of particular genes, and, with the advent of CRISPR technology, gene knockouts are more rapid and straightforward to perform in stem cells than in developmental model systems. Furthermore, while interpreting whole body phenotypes is an advantage of animal systems, assessing signaling activity, gene expression, and cell fate proportions is performed much more easily and quantitatively using stem cells.

As one example, the gene regulatory network controlling human primitive streak (mesodermal and endodermal) fates is being unraveled using embryonic stem cells. Teo et al. established the hierarchy of transcription factors in endoderm differentiation (Teo et al., 2011): The pluripotency-associated gene Nanog is essential for inducing Eomes early during differentiation, and Eomes subsequently combines with Activin/Nodal signaling to induce a network governing endoderm differentiation. Mendjan et al. (Mendjan et al., 2014) revealed how anterior and posterior streak fates depend on mutually repressing transcription factors Cdx2 and Nanog. Both studies were able to establish links between the networks of transcription factors and key upstream developmental signals Activin/Nodal, BMP, and Wnt. Furthermore, both studies checked consistency of their findings with expression patterns in mouse, demonstrating the power of this approach for understanding development.

Differentiation vs. Pluripotency

In non-mammalian species, it is not known if a stable pluripotent state exists or if differentiation is an unavoidable consequence of the onset of zygotic transcription. However, in mammals, differentiation requires destabilizing the pluripotent state. As such, understanding pluripotency is complementary to, and perhaps required for, understanding differentiation. Paradoxically, many of the same factors that control differentiation also maintain pluripotency. Pluripotency in hESCs can be maintained by FGF and Activin (James, 2005; Vallier et al., 2005). In mESCs, BMP, LIF, and Wnt all play a role in maintaining the pluripotent state (ten Berge et al., 2011; Smith et al., 1988; Ying et al., 2003). These differences between mouse and human ESCs most likely result from the fact that mESCs model the ICM, whereas hESCs model the epiblast.

It has now become clear that the transcription factors essential to maintain pluripotency are also involved in differentiation. Until recently, pluripotency was considered a stable state maintained by a network of dedicated transcription factors in the absence of destabilizing external signals. This was supported by the maintenance of mESCs in conditions named 2i or 3i (Ying et al., 2008), ostensibly blocking all differentiation-promoting signals. However, the core transcription factors Sox2, Oct4, and Nanog are, respectively, required for differentiation toward ectoderm, mesoderm, and endoderm (Malleshaiah et al., 2016; Teo et al., 2011; Thomson et al., 2011; Wang et al., 2012). Moreover, their overexpression can induce differentiation, as opposed to what would be expected from factors simply blocking differentiation toward particular lineages. Pluripotency therefore seems to be a delicate equilibrium between mutually inhibitory lineage specifiers (Loh and Lim, 2011). This perhaps explains why the same signaling pathways can both stabilize pluripotency and promote differentiation. Further supporting this picture, lineage specifiers can substitute for pluripotency-associated transcription factors in reprograming differentiated cells to the pluripotent state (Shu et al., 2013). This picture is complicated, however, by the demonstration that combinatorial interactions between pathways are important for influencing the balance between pluripotency and differentiation. It is not only the levels of Activin/Nodal signaling, but the state of the PI3K pathway that determines whether Nodal signaling supports pluripotency or differentiation. When PI3K is activated, Nodal maintains pluripotency; but when it is inhibited, Nodal directs differentiation, and these effects are mediated by interactions between PI3K and the Wnt and MAPK pathways (McLean et al., 2007; Singh et al., 2012). Consequently, nearly all modern protocols for endoderm differentiation include modulation of a second pathway in addition to Activin/Nodal (Pagliuca et al., 2014; Rezania et al., 2014; Touboul et al., 2010).

These in vitro studies elucidating the relationship between pluripotency and differentiation can also shed light on how this transition occurs in the embryo; they would have been extremely difficult to perform in vivo because, in that case, pluripotency is a transient state. Recently, the ability to stabilize cellular states has also been used to uncover the heterogeneity in those states (Hough et al., 2014; Singer et al., 2014) (earlier work reviewed in Torres-Padilla and Chambers, 2014). Finally, using stem cells

with cell cycle reporters, multiple groups have reported a preference for cells to differentiate during the G1 phase of the cell cycle, and have linked this both to patterns of TGFβ signaling and to epigenetic modifications (Pauklin and Vallier, 2013; Singh et al., 2013; Singh et al., 2015). The relevance of cellular state heterogeneity and cell cycle modulation of differentiation in vivo will be an interesting subject of future investigation, requiring tight integration between stem cell work that generates hypotheses and embryonic assays to test their in vivo relevance.

Other Environmental Factors Controlling Differentiation

Cell fate is determined not only by signaling molecules but also by other environmental factors. For example, the low oxygen levels that the early embryo naturally experiences before vascularization have been shown to inhibit differentiation in hESC (Ezashi et al., 2005), and hypoxia has also been shown to play a role in maintenance of adult stem cells (Mohyeldin et al., 2010) and in trophoblast differentiation (Choi et al., 2013).

Mechanical forces could also control differentiation and are more likely to play a role in early patterning than oxygenation, as the latter is likely uniform across the epiblast. Lineage specification in mesenchymal stem cells is dependent on matrix stiffness (Engler et al., 2006), while in mESCs, cyclic stress has been shown to down-regulate Oct4 and induce differentiation (Chowdhury et al., 2010). An interesting open question is whether there is also a role for mechanical forces between cells in cell fate determination.

Spatial Patterning

Knowledge of how extracellular signals determine cell fate is necessary but not sufficient to understand spatial patterning, which also requires knowing which signals are supplied in vivo, and how these signals are organized in space and time. This begins with understanding the dynamics of morphogen gradients, which has turned out to be of much greater complexity than anticipated in nearly all systems where it has been studied. Moreover, it is an aspect of early development in which mammals differ substantially from non-mammals, because axis formation and gastrulation do not depend on maternal factors but are completely self-organized. Stem cells hold much potential as a model to both elucidate mammalian self-organization and understand principles of gradient formation in general.

Gradient Formation in Vertebrates

There is evidence that transport—and consequently gradient formation—of diffusible signaling molecules is generally controlled by nontrivial mechanisms rather than free diffusion. These mechanisms may serve a variety of purposes such as restricting signaling to the right tissue and enhancing robustness against perturbation (Lander, 2007). Morphogen transport has been studied in most detail in *Drosophila*, where an unexpected level of complexity was revealed, but it is becoming clear that vertebrate gradient formation is no simpler, and that some of the same mechanisms play a role. The role of BMP in patterning the dorsoventral axis of the early embryo is conserved across bilaterians, and it was shown in *Xenopus* that the establishment of a BMP gradient in this process depends on shuttling of BMP by its inhibitor Chordin (Lee et al., 2006; Piccolo et al., 1997), just like it does in *Drosophila* (Bier and De Robertis, 2015; Shimmi and O'Connor, 2003). Cell surface molecules may restrict diffusion of FGF (Duchesne et al., 2012; Müller et al., 2013) and BMP (Hu et al., 2004). The Nodal gradient is shaped by proteolytic cleavage (Beck et al., 2002). Long cellular protrusions that deliver morphogen signals over distances of several cell diameters, termed cytonemes, have been shown to be important for Shh patterning of the chick limb bud (Sanders et al., 2013). Given that all nontrivial mechanisms of gradient formation rely on the presence of additional molecules, it is likely that artificial morphogen sources created with ligand-soaked beads or mRNA injection saturate these molecules and overwhelm physiological gradient-forming processes. Therefore, while it has been convincingly demonstrated that a gradient *can* be formed by free diffusion in vertebrates, it is not clear that it *does* in vivo, or that the dynamics are similar. Caution is warranted in accepting claims regarding diffusion constants and the mechanism of diffusion from overexpression studies (Gurdon et al., 1994; Müller et al., 2012; Williams et al., 2004; Yu et al., 2009). In agreement with this, both Wnt signals in intestinal crypts (Farin et al., 2016) and Nodal signals in the early Zebrafish embryo (van Boxtel et al., 2015) have been shown to be short range, only directly affecting cells in contact with the source. Much could be learned from observing the dynamics of endogenous morphogens, but progress on this front is hampered by the difficulty of tagging endogenous proteins in Zebrafish, and the difficulty of imaging morphogens at endogenous concentrations over long timescales in mouse.

Spatial Patterning in Stem Cells

Stem cells show spatial patterning (Poh et al., 2014; van den Brink et al., 2014; Warmflash et al., 2014), which is likely due to self-organized morphogen gradients. Recent advances in CRISPR gene-editing technology (Cong et al., 2013; González et al., 2014; Mali et al., 2013) allow for creation of fusions of fluorescent proteins to endogenous signaling molecules via homologous recombination with much greater ease than previously possible. Stem cells can be grown to allow for optimal imaging conditions so that the dynamics of self-organized gradient formation could in principle be observed in real time. As such, they hold great potential for revealing the mechanisms of gradient formation in mammalian embryos. In the following paragraphs, we describe systems in which patterning is beginning to be studied using ESCs.

Patterning in Embryoid Bodies

Three-dimensional aggregates of mESCs or hESCs, termed embryoid bodies, will differentiate spontaneously into tissues from all three germ layers and, under the right conditions, form an epithelium with a central lumen (Coucouvanis and Martin, 1995) and an outer endodermal layer (Martin, 1981). Embryoid bodies formed from mESCs were shown to recapitulate aspects of axis formation and showed polarized expression of mesendodermal genes that correlated with markers of the epithelial-mesenchymal transition (ten Berge et al., 2008). This was demonstrated to depend on localized Wnt signaling, and larger embryoid bodies were found to have more than one Wnt signaling region, suggesting a gradient with a fixed-length scale. It was shown that Wnt expression in the embryoid body can be activated by Activin A in the presence of BMP4 inhibitors, challenging the model suggested

by in vivo data, according to which Wnt depends strictly on BMP (Ben-Haim et al., 2006), and suggesting the possibility that the in vitro Wnt gradient is formed by interaction with a Nodal gradient. A recent study made further advances by introducing a defined protocol to consistently produce patterned aggregates of mESCs. This study also demonstrated that aggregates of a particular size will both pattern and change shape to elongate along the axis of polarization (van den Brink et al., 2014). Similar self-organization and patterning have also been observed in P19 embryonic carcinoma cells (Li and Marikawa, 2015; Marikawa et al., 2009).

The facts that embryoid bodies show features of patterning and morphogenesis associated with gastrulation, and have already refined in vivo findings, show their potential as a model system. However, they also suffer from a number of limitations. Their features are not sufficiently reproducible to allow for quantitative comparison between gene expression patterns in different embryoid bodies, which makes it difficult to construct a detailed model for spatial pattern formation. Moreover, they are too large and opaque for in toto live imaging, and high-quality data require fixation followed by optical clearing or cryosectioning.

Biologically, there are both pros and cons to using embryoid bodies as a model for gastrulation. The epiblast is a well-defined epithelial monolayer that is topologically a disc (as opposed to geometrically, i.e., it is a monolayer with a single connected boundary) and receives signals from extraembryonic tissues at its boundary and on its basal side. Embryoid bodies are able to form structures that resemble the double-layered epithelium found in vivo, but they do so slowly and incompletely, through a mechanism different from that of the embryo in vivo (Bedzhov and Zernicka-Goetz, 2014), and result in a spherical topology. Under many culture conditions, they simply remain solid 3-D aggregates. Therefore, two-dimensional (2-D) colonies can be argued to more faithfully model the epiblast, as they form epithelial monolayers and are topological discs.

Spatial Heterogeneity in 2-D hESC Colonies

When grown in two dimensions, removal of pluripotency factors or treatment with BMP4 triggers differentiation to both embryonic and extraembryonic tissues in an apparently random fashion. This random differentiation is a consequence of endogenous gradients of morphogens and inhibitors that depend on random colony shape and density variations. One of the first studies to investigate endogenous signaling gradients in stem cell colonies examined differentiation in hESCs upon removal of pluripotency-maintaining factors from the media (Peerani et al., 2007). Under these conditions, the cells differentiated to XEN inhomogeneously with a local resistance to differentiation that depended on cell density. This resistance resulted from the secretion of the BMP inhibitor GDF3 by pluripotent cells, counteracting the differentiation signal from BMP2 secreted by all cells and, to a larger degree, the differentiated ones. By directly manipulating colony size, and therefore average density, using micropatterning, the authors demonstrated an inverse relationship between density and likelihood of differentiation.

This study elegantly showed how quantitative analysis of even disorganized stem cell colonies can provide information about the mechanisms behind spatial organization, and how the level of environmental control that is only possible in vitro can more rigorously test relationships between variables that are hard to manipulate or separate in vivo. Quantitative analysis and reproducibility are key in understanding self-organization, because it is a highly nonlinear process with feedback both between signaling pathways and between signaling, cell density, and colony geometry. Only by systematically varying one variable at a time and evaluating the effects on gradient formation and patterning can we hope to understand the underlying mechanisms.

Reproducible Spatial Differentiation on Micropatterned Surfaces

While the colonies in Peerani et al., 2007 showed a variety of cell fates, no reproducible pattern was formed. Recently it was found by one of the authors that hESCs grown in circular colonies using micropatterned surfaces show reproducible spatial differentiation upon treatment with BMP4 (Warmflash et al., 2014). Cells positive for markers of all three germ layers as well as extraembryonic tissue form concentric rings at particular radii in the colony (Fig. 2). Micropatterned surfaces contain defined regions of extracellular matrix proteins such as laminin or matrigel typically surrounded by a coating that prevents cell and protein adhesion, such as PLL-PEG. A number of techniques are available to produce the surfaces, including photolithography (Azioune et al., 2009; Ma et al., 2015; Warmflash et al., 2014), microcontact printing (Peerani et al., 2007; Théry and Piel, 2009), and stencil micropatterning (Toh et al., 2011; Xing et al., 2015). These methods were originally applied to study the biophysics of cell shape and cell division (Théry et al., 2006; Théry et al. 2007) and were subsequently adapted to study multicellular, colony-level events.

Although BMP4 is administered homogeneously in the experiments of Warmflash et al., 2014, the layout of cell fates appears to be controlled by spontaneously formed gradients of BMP inhibitors and Nodal. Modulation of pathway activity with RNAi and small molecule inhibitors, as well as immunofluorescence analysis for activated signal transducers, revealed both a graded response to the initial BMP stimulation and a Nodal activity gradient. These two signals are responsible for differentiation to extraembryonic and mesendodermal cell fates, respectively. Similar to the embryo, this signaling gradient relied on secreted inhibitors of Nodal and BMP, as patterning was lost upon knockdown of these inhibitors.

The length scale of the germ layer rings is independent of colony size and the pattern forms from the boundary inward, as smaller colonies lose inner cell fates (Fig. 2). Such a fixed-length scale cannot be obtained by uniform production and degradation combined with free diffusion, and implies a nontrivial mechanism for gradient formation, reaffirming the need for studying endogenous morphogen gradients. Although the mechanism responsible for this length scale is not yet known, its existence provides a hypothesis regarding the results of earlier work relating colony size to cell fate. In Lee et al., 2009, micropatterning was used to relate colony size to mesendoderm differentiation, and it was found that smaller colonies end up with higher fractions of endoderm relative to mesoderm. Evaluating the spatial patterns shows that this effect was likely due to the relative positions of these fates in the colony, as the more central-lying mesoderm is lost in smaller colonies.

The organization of cell fates into rings, rather than layers, differs from the in vivo situation, and the cause of this remains to be identified. However, all evidence indicates that the same regulatory logic as in the embryo controls spatial organization in

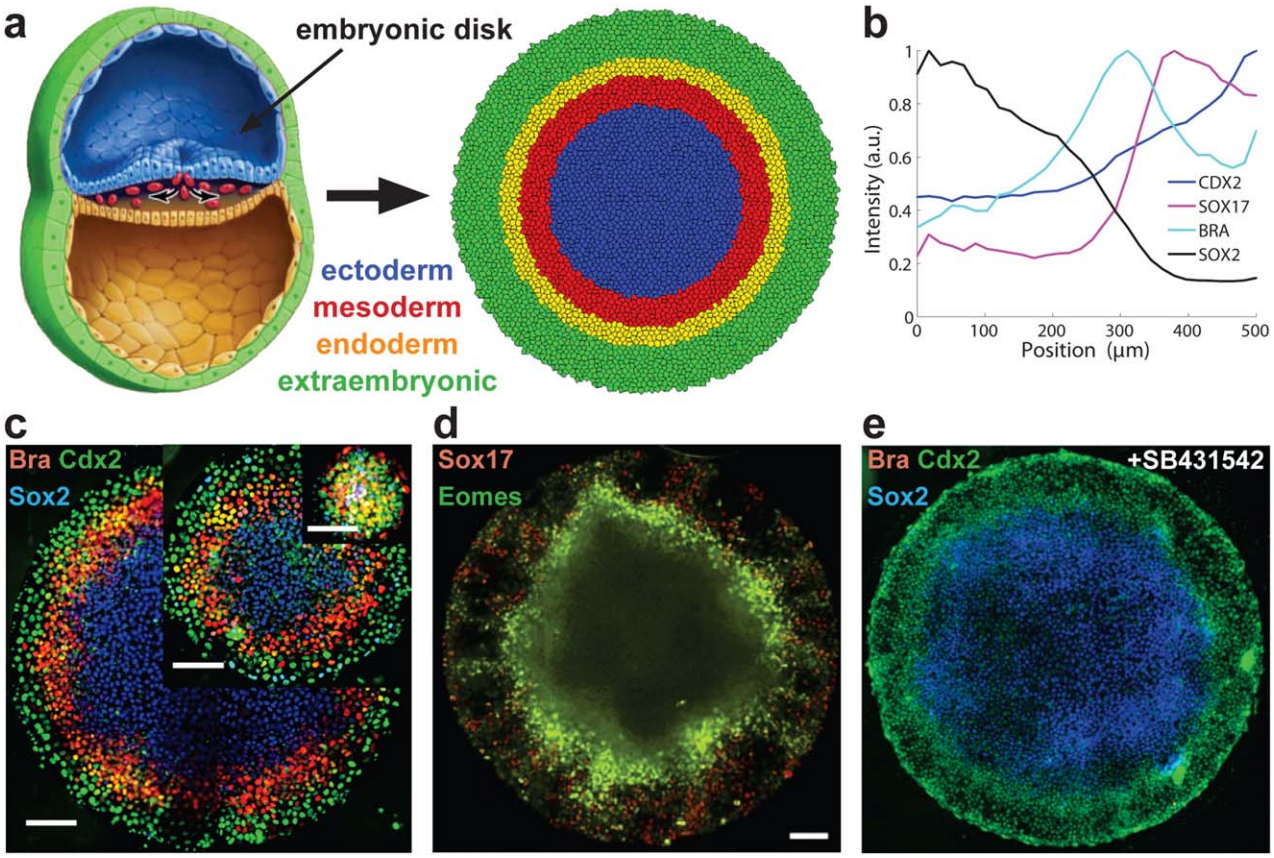

Fig. 2. Recapitulating early embryonic spatial patterning with embryonic stem cells. **A**: Schematic of correspondence between the gastrulating human embryo and micropatterned hESC colony 42 hours after BMP4 treatment. **B**: Radial averages of immunofluorescence quantification for markers for each lineage on colony of 500-micron radius. Extraembryonic: Cdx2+, Sox17-, Bra-, Sox2-; Endoderm: Sox17+, Sox2-; Mesoderm: Bra+, Sox17-; Ectoderm: Sox2+(Nanog-, not shown). **C,D**: Immunofluorescence stainings for markers of the lineages. Pattern forms from the boundary inward, and smaller colonies contain less ectoderm. **E**: Colony treated with the Nodal inhibitor SB431542; mesendoderm is absent and ectoderm has expanded into the mesendodermal territory.

vitro, and systematic manipulation of pattern formation with reproducible outcomes makes it possible to reveal this logic and understand the differences with the embryo. Rather than being a drawback, the dependence of the pattern on conditions such as ligand concentrations and colony geometries provides additional information that can be used to unravel patterning mechanisms.

Patterning depends on tissue geometry, but also controls it through its effects on differential growth, cellular mechanics, and cell migration. Micropatterning provides a simplified model for patterning by breaking this feedback between pattern and shape, and controlling geometry and cell density independently. Even if the precise geometry and cell density of the embryo are not accessible this way, the fact that these parameters can be varied allows determination of their effect on pattern formation. An additional advantage of this method is the large volume of high-quality data it produces. A single chip yields hundreds of embryoid colonies of the same shape, density, and developmental stage, under ideal imaging conditions, allowing for high-throughput quantitative analysis of perturbations.

In Vitro Models for the Embryo Involving Multiple Cell Lines

In micropatterned culture, BMP4 is added to the media homogeneously, whereas in the mouse embryo, it is supplied at the edge of the epiblast by the extraembryonic ectoderm (ExE). In addition to providing BMP, extraembryonic tissues play an essential role in patterning the mouse embryo by secreting convertases required for the proteolytic cleavage that produces functional Nodal (Ben-Haim et al., 2006; Mesnard et al., 2011). Moreover, the distal visceral endoderm secretes Nodal inhibitors that position the primitive streak. To some extent, it is therefore surprising that pattern formation happens without these tissues, and future studies will reveal whether the outer ring of induced extraembryonic cells plays an essential role. However, it is also possible to more closely approximate the in vivo situation before gastrulation and evaluate the interaction between stable extraembryonic and embryonic cell lines. The first steps in this direction were taken in Toh et al., 2011, where micropatterning techniques were combined to create a layer of mESC on top of an interface between TS cells (trophoblast) and XEN cells, and it was shown that this organization was sufficient to polarize the ESC colonies along an induced proximal-distal axis. Such techniques also hold potential for genetic mosaic experiments, which are commonly used in vivo tools that have revealed much about morphogen gradient formation, in particular in the *Drosophila* wing disc (Blair, 2003). Whereas the position, shape, and density of clones are hard to control in vivo, micropatterning would allow very precise control of these parameters.

Interspecies Differences

So far we have focused on the experimental strengths of stem cells as a model for early development and compared stem cells to vertebrate in vivo systems including fish and frog. However, gastrulation differs substantially both between mammalian and non-mammalian systems and between different mammals. As such, even the mouse may not be an accurate model for human gastrulation. Stem cells provide an opportunity to study human development and determine interspecies differences. We briefly review some of the known interspecies differences in gastrulation and, in particular, differences in the roles of conserved morphogens. This discussion is not intended to be complete, but serves to illustrate the extent of the dissimilarities. We then discuss how hESCs may help in better understanding uniquely human aspects of embryogenesis.

Mouse vs. Frog

BMP has a conserved function in dorso-ventral and neural patterning across bilaterians, although the axis is inverted in vertebrates relative to arthropods (Bier and De Robertis, 2015; Holley and Ferguson, 1997). Even though parts of the mechanism of dorso-ventral gradient formation are conserved, both the function and formation of the BMP gradient differ significantly between mouse and frog. In frog, BMPs are initially expressed throughout the ectodermal and mesodermal parts of the embryo (Gilbert, 2014). In contrast, mouse BMP is not produced in embryonic cells: BMP2 is secreted from the VE and BMP4 from the ExE (Arnold and Robertson, 2009; Madabhushi and Lacy, 2011; Winnier et al., 1995). Nodal, on the other hand, is broadly expressed in the mammalian embryo but restricted to a particular domain in frog. In mouse, it is initially expressed throughout the epiblast and is required to maintain pluripotency, but in frog it is restricted to the dorsal-vegetal side. Moreover, BMP is required in mouse to establish a Nodal gradient and initiate gastrulation (Mishina et al., 1995; Winnier et al., 1995) through a feedback loop that also involves Wnt, whereas in frog, Nodal is activated by VegT, a maternal gene with no mammalian ortholog. Overexpression of BMPs in frog will cause cells to adopt progressively more ventral fates but will not convert cells between germ layers (Wilson et al., 1997), whereas in mammalian stem cells, BMP treatment leads to conversion from pluripotency to both extraembryonic and mesodermal lineages, although this is likely through feedback loops with other pathways (Bernardo et al., 2011; Xu et al., 2002; Yu et al., 2011). Nodal signaling appears to induce mesendodermal fates in both species; however, the dose-dependent patterning of mesendoderm by Nodal (Green et al., 1992) has not been directly demonstrated in mammals.

Differences in Mammalian Development and Stem Cells

Many of the differences between mouse and frog involve the extraembryonic tissues that are absent in anamniotes [although equivalencies between the frog embryo and particular extraembryonic tissues may still exist (Beddington and Robertson, 1998)]. It is precisely in the development of the extraembryonic tissues that mammalian species differ considerably. During the blastocyst stage, differences in the timing of lineage specification have been found: For example, a delay in expression of the trophectodermal marker Cdx2 (Niakan and Eggan, 2013) and the absence of Eomes expression in human trophectoderm (Blakeley et al., 2015). The fact that it has been possible to derive stable cell lines for all three lineages of the blastocyst in mouse but not in human is another indication that the very first cell fate decisions may be different. Immediately after the blastocyst stage, not only molecular but anatomical differences arise. It appears that the human amnion and extraembryonic mesoderm both form well before gastrulation (Dobreva et al., 2010; Enders et al., 1986), in contrast to mouse, where these derive from the primitive streak. As a consequence, the human trophectoderm is separated from the epiblast by several tissue layers and a substantial distance by the onset of gastrulation. Considering the importance of ExE-derived signals for early pattering in mouse, this observation raises the question of what takes the place of the feedback loops between the ExE and epiblast in human.

In the context of these findings, it is particularly interesting to determine which extraembryonic cell types hESCs can differentiate into, and what role they play in in vitro self-organization. If, as current data suggest, hESCs can differentiate into both trophectoderm and primitive endoderm, the genetic network involved in these cell fate decisions can be quantitatively dissected using the tools we discussed in previous sections. Moreover, the behavior of hESCs can be compared to that of mESCs or mEpiSCs under identical conditions to determine species differences in molecular processes that are independent of embryonic geometry or the relationship to extraembryonic lineages. The variety of readouts available in the stem cell platform can be used to straightforwardly determine whether different behavior is due to signal input or response, e.g., whether a delay in Cdx2 expression reflects a delay in cell signaling or in competence to respond. More generally, this opens up the possibility of a new kind of in vitro comparative embryology in which behavior of embryonic cells from different species can be rigorously and quantitatively compared.

One of the most striking differences between human and mouse embryos is the geometry (but not topology) of the epiblast (Behringer et al., 2000). Whereas the mouse embryo is cup-shaped, the human embryo, like most mammalian embryos, is disc-shaped, and it is not known how the shape of the pregastrulation embryo is controlled or how it affects later events. Using micropatterning technology, one can grow human and mouse stem cells in a variety of geometries to determine how shape affects pattern formation and how the self-organization of morphogen gradients in each species has adapted to a particular geometry.

Discussion and Conclusion

Differentiation Outlook

Substantial evidence shows the importance of ligand and signaling dynamics in determining cell fate. Unraveling this relationship requires precise control of the dynamic environment, real-time imaging of signaling reporters and fate outcomes, and quantitative analysis of the resulting data. In contrast to in vivo systems, ESCs provide unparalleled control to probe the signaling dynamics and gene regulatory networks controlling early cell fate decisions. The largest conceptual challenge with this approach is to relate the lessons learned back to embryo. It is often not clear which aspects of the embryonic environment need

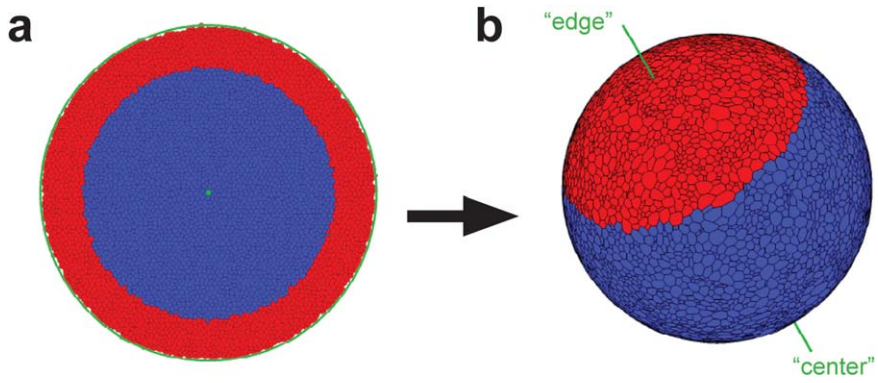

Fig. 3. The symmetry of gastruloids is the same as that of disc-shaped colonies.**A**: Disc-shaped colony with edge and center fate. **B**: Spherical colony with polarized gene expression possesses symmetry around the axis that is the same as that of the disc around its center (both green); one pole can be thought of as the disc center while the other can be thought of as the disc edge.

to be reproduced in vitro, and conversely, the physiological relevance of in vitro states can be difficult to discern. What is the relevance of the method of growing mESCs in a so-called "ground state" (Ying et al., 2008)? Can the conversion of hESCs to trophectoderm by BMP4 treatment (Li and Parast, 2014; Xu et al., 2002) shed light on human extraembryonic development in vivo? The important differences in the role of BMP and Wnt signaling between mESC and mEpiScs and their in vivo correlates highlight the need for caution when applying in vitro findings to the embryo (Biechele et al., 2013; Tang et al., 2010). Nonetheless, stem cells may offer the only opportunity to quantitatively map the relationship between external stimulus and fate. A combination of careful evaluation of these relationships and confirmation of resulting hypotheses in vivo should ultimately allow us to understand how cell fate decisions in the embryo are shaped.

Spatial Patterning Outlook

Although still an emerging model, spatially controlled stem cell colonies show great promise in revealing the mechanisms of embryonic patterning and, more generally, in understanding the formation of self-organized morphogen gradients. The in vitro environment in current models differs substantially from the in vivo environment and likely is the cause of the differences in spatial organization, but uncovering the underlying logic still provides essential information about the in vivo mechanism. A gap in the current approach is that results with hESCs cannot be compared directly to human embryos due to ethical and practical limitations. Differences between hESC colonies and other mammalian embryos could be due to culture artifacts or interspecies differences. Comparing in vivo and in vitro patterning in mouse could clarify these issues by revealing the extent and nature of culture artifacts. More generally, comparison to embryos to verify in vitro findings will remain necessary, but regardless of the connection to in vivo development, improved understanding of endogenous signaling and spatial heterogeneity in stem cells will directly impact regenerative medicine.

A substantial challenge for stem cell models of early development is to more faithfully reproduce aspects of morphogenesis. The recent demonstrations that optic cups (Eiraku et al., 2011), "mini-guts" (Sato and Clevers, 2013), patterned neural tubes (Meinhardt et al., 2014), and cerebral organoids (Lancaster et al., 2013) can be grown in vitro shows the potential of cells to undergo morphogenetic movements outside of the embryo. As 3-D culture moves forward, it will be of particular importance to create models that undergo patterning and morphogenesis in three dimensions but retain the reproducibility of the 2-D micropatterning models. The extensive progress made to date in organoid cultures and the bioengineering approaches for generating and manipulating them are beyond the scope of this commentary, and the reader is referred to recent reviews of these topics (Gjorevski et al., 2014; Turner et al., 2016).

For the early embryo, study of morphogenesis must start with the formation of a trilaminar disc. Although in the current micropatterned model, cells undergo an epithelial-mesenchymal transition as they differentiate into mesendodermal fates (Warmflash et al., 2014), they do not properly form a separate cell layer between the epiblast and visceral endoderm as in the embryo. It is possible that growing cells on a substrate that allows cells to invade and remodel their local environment, as in organoid models, will allow for forming properly polarized multilayered structures. It is also possible that other features of the in vivo environment must be recapitulated before morphogenesis can take place. Interaction with the underlying visceral endoderm layer or with gradients of signaling molecules guiding cell migrations may be required. If this migration could be reproduced, much could be learned about the relation between morphogenesis and differentiation. In vivo this process may depend on the establishment of an anterior-posterior (AP) axis, which is absent in micropatterned differentiation. Understanding AP symmetry breaking therefore constitutes another major challenge for in vitro models of gastrulation.

The discussion of symmetry breaking is at times complicated by inconsistent language referring to the dynamics of axis formation in mouse, as well as possible confusion about how to compare symmetry of 2-D and 3-D colonies. In the work on gastruloids, polarized expression of mesendodermal genes is referred to as anterio-posterior axis formation. This is because in the mouse embryo, AP patterning depends on the anterior migration of distal visceral endoderm cells that secrete Nodal and Wnt inhibitors that restrict expression of mesendodermal genes to the posterior side. However, in mutants where distal visceral endoderm (DVE) migration fails, these genes are still expressed, but now in a ring that is symmetric around the axis of the egg cylinder. This is the rotational symmetry that is also present in micropatterned colonies, as well as in polarized gastruloids, which spontaneously break the symmetry of a sphere to the symmetry

of a disc, as illustrated in Figure 3. When we refer to in vitro AP axis formation, we have in mind breaking rotational symmetry of micropatterned colonies to bilateral symmetry. Comparison of micropattern colonies to mouse mutants with failed DVE migration could shed light on whether the defects in these models are similar (Migeotte et al., 2010; Nowotschin et al., 2013). Conversely, localized external supply of Nodal and Wnt inhibitors may be sufficient to initiate AP patterning in stem cells. Current technologies for patterning multiple cell types on surfaces (Toh et al., 2011) and delivering ligands with microfluidics (Cosson and Lutolf, 2014; Wu et al., 2006) allow us to start testing these hypotheses, and time will tell whether in vitro development can be engineered to mimic the embryo. Of course, success in this program raises ethical questions, and decisions must be reached about what experiments should be permitted with stem cell colonies that accurately mimic gastrulation and later stages (Pera et al., 2015).

Conclusion

Modern techniques in bioengineering, gene editing, and imaging have now opened the door to using stem cells to address long intractable questions in developmental biology. Combining imaging of live cell reporters with exquisite control over the microenvironment has the potential to reveal the relationship between the cues cells receive and the fates they adopt. The ability of cells to self-organize into patterns and morphologies in vitro will allow us to manipulate and study these processes with a resolution that cannot be accessed in vivo. At all stages of this process, it is essential to use what is known about the embryo in vivo to guide the design of in vitro experiments, and to test the relevance of the in vitro outcomes back in the embryo. By closing this loop, artificial stem cell systems have the potential to revolutionize our understanding of patterning and morphogenesis in the embryo.

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References

Arnold SJ, Robertson EJ. 2009. Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. Nat Rev Mol Cell Biol 10(2):91–103.

Azioune A, Storch M, Bornens M, Théry M, Piel M. 2009. Simple and rapid process for single cell micro-patterning. Lab Chip 9(11):1640–1642.

Balaskas N, Ribeiro A, Panovska J, Dessaud E, Sasai N, Page KM, et al. 2012. Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. Cell 148(1-2):273–284.

Beck S, Le Good JA, Guzman M, Ben-Haim N, Roy K, Beermann F, Constam DB. 2002. Extraembryonic proteases regulate Nodal signalling during gastrulation. Nat Cell Bio 4(12):981–985.

Beddington RS, Robertson EJ. 1998. Anterior patterning in mouse. Trends Genet 14(7):277–284.

Bedzhov I, Zernicka-Goetz M. 2014. Self-organizing properties of mouse pluripotent cells initiate morphogenesis upon implantation. Cell 156(5):1032–1044.

Bedzhov I, Bialecka M, Zielinska A, Kosalka J, Antonica F, Thompson AJ, et al. 2015. Development of the anterior-posterior axis is a self-organizing process in the absence of maternal cues in the mouse embryo. Cell Res 25(12):1368–1371.

Behringer RR, Wakamiya M, Tsang TE, Tam PPL. 2000. A flattened mouse embryo: Leveling the playing field. Genesis 28(1):23–30.

Ben-Haim N, Lu C, Guzman-Ayala M, Pescatore L, Mesnard D, Bischofberger M, et al. 2006. The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev Cell 11(3):313–323.

Bernardo AS, Faial T, Gardner L, Niakan KK, Ortmann D, Senner CE, et al. 2011. BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. Cell Stem Cell 9(2):144–155.

Bianco P. 2014. "Mesenchymal" stem cells. Annu Rev Cell and Dev Biol 30:677–704.

Biechele S, Cockburn K, Lanner F, Cox BJ, Rossant J. 2013. Porcn-dependent Wnt signaling is not required prior to mouse gastrulation. Development 140(14):2961–2971.

Bier E, De Robertis EM. 2015. BMP gradients: A paradigm for morphogen-mediated developmental patterning. Science 348(6242):aaa5838.

Blair SS. 2003. Genetic mosaic techniques for studying Drosophila development. Development 130(21):5065–5072.

Blakeley P, Fogarty NME, del Valle I, Wamaitha SE, Hu TX, Elder K, et al. 2015. Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. Development 142(18):3151–3165.

Bourillot P-Y, Garrett N, Gurdon JB. 2002. A changing morphogen gradient is interpreted by continuous transduction flow. Development 129(9):2167–2180.

Brons IGM, Smithers LE, Trotter MWB, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, et al. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448(7150):191–195.

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27(3):275–280.

Chen LT, Hsu YC. 1982. Development of mouse embryos in vitro: preimplantation to the limb bud stage. Science 218(4567):66–68.

Chen Y, Kim JK, Hirning AJ, Josić K, Bennett MR. 2015. Emergent genetic oscillations in a synthetic microbial consortium. Science 349(6251):986–989.

Choi HJ, Sanders TA, Tormos KV, Ameri K, Tsai JD, Park AM, et al. 2013. ECM-dependent HIF induction directs trophoblast stem cell fate via LIMK1-mediated cytoskeletal rearrangement. PLoS One 8(2):e56949.

Choi J, Lee S, Mallard W, Clement K, Tagliazucchi GM, Lim H, et al. 2015. A comparison of genetically matched cell lines reveals the equivalence of human iPSCs and ESCs. Nat Biotechnol 33(11):1173–1181.

Chowdhury F, Na S, Li D, Poh Y-C, Tanaka TS, Wang F, Wang N. 2010. Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. Nat Mater 9(1):82–88.

Cohen M, Briscoe J, Blassberg R. 2013. Morphogen interpretation: the transcriptional logic of neural tube patterning. Curr Opin Genet Dev 23(4):423–428.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823.

Corson F, Siggia ED. 2012. Geometry, epistasis, and developmental patterning. Proc Nat Acad Sci U S A 109(15):5568–5575.

Cosson S, Lutolf MP. 2014. Microfluidic patterning of protein gradients on biomimetic hydrogel substrates. Methods Cell Biol 121, 91–102.

Coucouvanis E, Martin GR. 1995. Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. Cell 83(2):279–287.

Deglincerti A, Croft GF, Pietila LN, Zernicka-Goetz M, Siggia ED, Brivanlou AH. 2016. Self-organization of the in vitro attached human embryo. Nature 533(7602):251–254.

Dessaud E, Yang LL, Hill K, Cox B, Ulloa F, Ribeiro A, et al. 2007. Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. Nature 450(7170):717–720.

Dobreva MP, Pereira PNG, Deprest J, Zwijsen A. 2010. On the origin of amniotic stem cells: of mice and men. Int J Dev Biol 54(5):761–777.

Dubrulle J, Jordan BM, Akhmetova L, Farrell JA, Kim S-H, Solnica-Krezel L, Schier A. F. 2015. Response to Nodal morphogen gradient is determined by the kinetics of target gene induction. eLife 4.

Duchesne L, Octeau V, Bearon RN, Beckett A, Prior IA, Lounis B, Fernig DG. 2012. Transport of fibroblast growth factor 2 in the pericellular matrix is controlled by the spatial distribution of its binding sites in heparan sulfate. PLoS Biol 10(7):e1001361.

Dyson S, Gurdon JB. 1998. The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. Cell 93(4):557–568.

Eakin GS, Behringer RR. 2004. Diversity of germ layer and axis formation among mammals. Semin Cell Dev Biol 15(5):619–629.

Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, et al. 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472(7341):51–56.

Enders AC, Schlafke S, Hendrickx AG. 1986. Differentiation of the embryonic disc, amnion, and yolk sac in the rhesus monkey. Am J Anat 177(2):161–185.

Engler AJ, Sen S, Sweeney HL, Discher DE. 2006. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell 126(4):677–689.

Ezashi T, Das P, Roberts RM. 2005. Low O2 tensions and the prevention of differentiation of hES cells. Proc Nat Acad Sci U S A 102(13):4783–4788.

Farin HF, Jordens I, Mosa MH, Basak O, Korving J, Tauriello DVF, et al. 2016. Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. Nature 530(7590):340–343.

Ferrer-Vaquer A, Piliszek A, Tian G, Aho RJ, Dufort D, Hadjantonakis A-K. 2010. A sensitive and bright single-cell resolution live imaging reporter of Wnt/ß-catenin signaling in the mouse. BMC Dev Biol 10:121.

Gafni O, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, et al. 2013. Derivation of novel human ground state naive pluripotent stem cells. Nature 504(7479):282–286.

Gilbert SF. 2014. Developmental biology (10 ed.). Sunderland, MA: Sinauer Associates Incorporated.

Gjorevski N, Ranga A, Lutolf MP. 2014. Bioengineering approaches to guide stem cell-based organogenesis. Development 141(9): 1794–1804.

González F, Zhu Z, Shi Z-D, Lelli K, Verma N, Li QV, Huangfu D. 2014. An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. Cell Stem Cell 15(2):215–226.

Green JB, New HV, Smith JC. 1992. Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. Cell 71(5):731–739.

Green JB, Sharpe J. 2015. Positional information and reaction-diffusion: two big ideas in developmental biology combine. Development 142(7):1203–1211.

Guo G, von Meyenn F, Santos F, Chen Y, Reik W, Bertone P, et al. 2016. Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass. Stem Cell Reports 6(4):437–446.

Gurdon JB, Harger P, Mitchell A, Lemaire P. 1994. Activin signalling and response to a morphogen gradient. Nature 371(6497):487–492.

Hanna JH, Saha K, Jaenisch R. 2010. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 143(4):508–525.

Harmansa S, Hamaratoglu F, Affolter M, Caussinus E. 2015. Dpp spreading is required for medial but not for lateral wing disc growth. Nature 527(7578):317–322.

Harvey SA, Smith JC. 2009. Visualisation and quantification of morphogen gradient formation in the zebrafish. PLoS Biol 7(5): e1000101.

Hatten ME, Roussel F. 2011. Development and cancer of the cerebellum. Trends Neurosci 34(3):134–142.

Hochedlinger K, Jaenisch R. 2015. Induced Pluripotency and Epigenetic Reprogramming. Cold Spring Harb Perspect Biol 7(12).

Hoffmann A. 2002. The Ikappa B-NF-kappa B Signaling Module: Temporal Control and Selective Gene Activation. Science 298(5596):1241–1245.

Holley SA, Ferguson EL. 1997. Fish are like flies are like frogs: conservation of dorsal-ventral patterning mechanisms. BioEssays 19(4):281–284.

Hough SR, Thornton M, Mason E, Mar JC, Wells CA, Pera MF. 2014. Single-cell gene expression profiles define self-renewing, pluripotent, and lineage primed states of human pluripotent stem cells. Stem Cell Reports 2(6):881–895.

Hsu YC. 1979. In vitro development of individually cultured whole mouse embryos from blastocyst to early somite stage. Dev Biol 68(2):453–461.

Hu Q, Ueno N, Behringer RR. 2004. Restriction of BMP4 activity domains in the developing neural tube of the mouse embryo. EMBO Rep 5(7):734–739.

James D. 2005. TGF/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 132(6):1273–1282.

Keenan TM, Folch A. 2008. Biomolecular gradients in cell culture systems. Lab Chip 8(1):34–57.

Kinder SJ, Tsang TE, Wakamiya M, Sasaki H, Behringer RR, Nagy A, Tam PP. 2001. The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. Development 128(18):3623–3634.

Kwon GS, Viotti M, Hadjantonakis A-K. 2008. The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev Cell 15(4):509–520.

Lancaster MA, Renner M, Martin C.A, Wenzel D, Bicknell LS, Hurles ME, et al. 2013. Cerebral organoids model human brain development and microcephaly. Nature 501(7467):373–379.

Lander AD. 2007. Morpheus unbound: reimagining the morphogen gradient. Cell 128(2):245–256.

Lee HX, Ambrosio AL, Reversade B, De Robertis, EM. 2006. Embryonic dorsal-ventral signaling: secreted frizzled-related proteins as inhibitors of tolloid proteinases. Cell 124(1):147–159.

Lee LH, Peerani R, Ungrin M, Joshi C, Kumacheva E, Zandstra P. 2009. Micropatterning of human embryonic stem cells dissects the mesoderm and endoderm lineages. Stem Cell Res 2(2):155–162.

Li AS. W, Marikawa Y. 2015. An in vitro gastrulation model recapitulates the morphogenetic impact of pharmacological inhibitors of developmental signaling pathways. Mol Reprod Dev 82(12): 1015–1036.

Li Y, Parast MM. 2014. BMP4 regulation of human trophoblast development. Int J Dev Biol 58(2-4):239–246.

Li Y, Moretto-Zita M, Soncin F, Wakeland A, Wolfe L, Leon-Garcia S, et al. 2013. BMP4-directed trophoblast differentiation of human embryonic stem cells is mediated through a ΔNp63 + cytotrophoblast stem cell state. Development 140(19): 3965–3976.

Loh KM, Lim B. 2011. A precarious balance: pluripotency factors as lineage specifiers. Cell Stem Cell 8(4):363–369.

Ma Z, Wang J, Loskill P, Huebsch N, Koo S, Svedlund FL, et al. 2015. Self-organizing human cardiac microchambers mediated by geometric confinement. Nat Commun 6, 7413.

Madabhushi M, Lacy E. 2011. Anterior visceral endoderm directs ventral morphogenesis and placement of head and heart via BMP2 expression. Dev Cell 21(5):907–919.

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. 2013. RNA-guided human genome engineering via Cas9. Science 339(6121):823–826.

Malleshaiah M, Padi M, Rué P, Quackenbush J, Martinez-Arias A, Gunawardena, J. 2016. Nac1 Coordinates a Sub-network of Pluripotency Factors to Regulate Embryonic Stem Cell Differentiation. Cell Rep 14(5):1181–1194.

Marikawa Y, Tamashiro DAA, Fujita TC, Alarcón VB. 2009. Aggregated P19 mouse embryonal carcinoma cells as a simple in vitro model to study the molecular regulations of mesoderm formation and axial elongation morphogenesis. Genesis 47(2):93–106.

Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Nat Acad Sci U S A 78(12):7634–7638.

McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, et al. 2007. Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when

phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells 25(1):29–38.
Meinhardt A, Eberle D, Tazaki A, Ranga A, Niesche M, Wilsch-Bräuninger M, et al. 2014. 3D Reconstitution of the Patterned Neural Tube from Embryonic Stem Cells. Stem Cell Reports 3(6): 987–999.
Mendjan S, Mascetti VL, Ortmann D, Ortiz M, Karjosukarso DW, Ng Y, et al. 2014. NANOG and CDX2 pattern distinct subtypes of human mesoderm during exit from pluripotency. Cell Stem Cell 15(3):310–325.
Mesnard D, Donnison M, Fuerer C, Pfeffer, PL, Constam DB. 2011. The microenvironment patterns the pluripotent mouse epiblast through paracrine Furin and Pace4 proteolytic activities. Genes Dev 25(17):1871–1880.
Migeotte I, Omelchenko T, Hall A, Anderson KV. 2010. Rac1-dependent collective cell migration is required for specification of the anterior-posterior body axis of the mouse. PLoS Biol 8(8): e1000442.
Mishina Y, Suzuki A, Ueno N, Behringer RR. 1995. Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev 9(24): 3027–3037.
Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A. 2010. Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7(2):150–161.
Moledina F, Clarke G, Oskooei A, Onishi K, Günther A, Zandstra PW. 2012. Predictive microfluidic control of regulatory ligand trajectories in individual pluripotent cells. Proc Nat Acad Sci U S A 109(9):3264–3269.
Monteiro RM, de Sousa Lopes SMC, Bialecka M, de Boer S, Zwijsen A, Mummery CL. 2008. Real time monitoring of BMP Smads transcriptional activity during mouse development. Genesis 46(7):335–346.
Morris SA, Grewal S, Barrios F, Patankar SN, Straus B, Buttery L, et al. 2012. Dynamics of anterior-posterior axis formation in the developing mouse embryo. Nat Commun 3:673.
Morsut L, Roybal KT, Xiong X, Gordley RM, Coyle SM, Thomson M, Lim WA. 2016. Engineering Customized Cell Sensing and Response Behaviors Using Synthetic Notch Receptors. Cell 164(4):780–791.
Müller P, Rogers KW, Jordan BM, Lee JS, Robson D, Ramanathan S, Schier AF. 2012. Differential diffusivity of Nodal and Lefty underlies a reaction-diffusion patterning system. Science 336(6082):721–724.
Müller P, Rogers KW, Yu SR, Brand M, Schier AF. 2013. Morphogen transport. Development 140(8):1621–1638.
Nakano T, Ando S, Takata N, Kawada M, Muguruma K, Sekiguchi K, et al. 2012. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10(6):771–785.
Nelson DE, Ihekwaba AEC, Elliott M, Johnson JR, Gibney CA, Foreman BE, et al. 2004. Oscillations in NF-kappaB signaling control the dynamics of gene expression. Science 306(5696): 704–708.
Niakan KK, Eggan K. 2013. Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. Dev Biol 375(1):54–64.
Nichols J, Chambers I, Taga T, Smith A. 2001. Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. Development 128(12):2333–2339.
Nowotschin S, Costello I, Piliszek A, Kwon GS, Mao C-A, Klein WH, et al. 2013. The T-box transcription factor Eomesodermin is essential for AVE induction in the mouse embryo. Genes Dev 27(9):997–1002.
Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, et al. 2014. Generation of functional human pancreatic β cells in vitro. Cell 159(2):428–439.
Pauklin S, Vallier L. 2013. The cell-cycle state of stem cells determines cell fate propensity. Cell 155(1):135–147.
Peerani R, Rao BM, Bauwens C, Yin T, Wood GA, Nagy A, et al. 2007. Niche-mediated control of human embryonic stem cell self-renewal and differentiation. EMBO J 26(22):4744–4755.
Pera MF, de Wert G, Dondorp W, Lovell-Badge R, Mummery CL, Munsie M, Tam PP. 2015. What if stem cells turn into embryos in a dish? Nat Methods 12(10):917–919.
Perea-Gomez A, Vella FDJ, Shawlot W, Oulad-Abdelghani M, Chazaud C, Meno C, et al. 2002. Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. Dev Cell 3(5):745–756.
Piccolo S, Agius E, Lu B, Goodman S, Dale L, De Robertis EM. 1997. Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. Cell 91(3):407–416.
Piliszek A, Kwon GS, Hadjantonakis A-K. 2011. Ex utero culture and live imaging of mouse embryos. Methods Mol Biol 770:243–257.
Poh Y-C, Chen J, Hong Y, Yi H, Zhang S, Chen J, et al. 2014. Generation of organized germ layers from a single mouse embryonic stem cell. Nat Commun 5:4000.
Przybyla L, Voldman J. 2012. Probing embryonic stem cell autocrine and paracrine signaling using microfluidics. Annu Rev Anal Chem (Palo Alto, Calif.), 5:293–315.
Raj A, Rifkin SA, Andersen E, van Oudenaarden A. 2010. Variability in gene expression underlies incomplete penetrance. Nature 463(7283):913–918.
Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. 2014. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 32(11):1121–1133.
Robinton DA, Daley, GQ. 2012. The promise of induced pluripotent stem cells in research and therapy. Nature 481(7381):295–305.
Rossant J. 2015. Mouse and human blastocyst-derived stem cells: vive les differences. Development 142(1):9–12.
Roybal KT, Rupp LJ, Morsut L, Walker WJ, McNally KA, Park JS, Lim WA. 2016. Precision Tumor Recognition by T Cells With Combinatorial Antigen-Sensing Circuits. Cell 164(4):770–779.
Saka Y, Smith JC. 2007. A mechanism for the sharp transition of morphogen gradient interpretation in Xenopus. BMC Dev Biol 7: 47.
Sanders TA, Llagostera E, Barna M. 2013. Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. Nature 497(7451):628–632.
Sato T, Clevers H. 2013. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. Science 340(6137):1190–1194.
Scognamiglio R, Cabezas-Wallscheid N, Thier MC, Altamura S, Reyes A, Prendergast ÁM, et al. 2016. Myc Depletion Induces a Pluripotent Dormant State Mimicking Diapause. Cell 164(4):668–680.
Shahbazi MN, Jedrusik A, Vuoristo S, Recher G, Hupalowska A, Bolton V, et al. 2016. Self-organization of the human embryo in the absence of maternal tissues. Nat Cell Bio.
Shimmi O, O'Connor MB. 2003. Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. Development 130(19):4673–4682.
Shu J, Wu C, Wu Y, Li Z, Shao S, Zhao W, et al. 2013. Induction of pluripotency in mouse somatic cells with lineage specifiers. Cell 153(5): 963–975.
Singer ZS, Yong J, Tischler J, Hackett JA, Altinok A, Surani MA, et al. 2014. Dynamic heterogeneity and DNA methylation in embryonic stem cells. Mol Cell 55(2):319–331.
Singh AM, Chappell J, Trost R, Lin L, Wang T, Tang J, et al. 2013. Cell-cycle control of developmentally regulated transcription factors accounts for heterogeneity in human pluripotent cells. Stem Cell Reports 1(6):532–544.
Singh AM, Reynolds D, Cliff T, Ohtsuka S, Mattheyses AL, Sun Y, et al. 2012. Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. Cell Stem Cell 10(3): 312–326.
Singh AM, Sun Y, Li L, Zhang W, Wu T, Zhao S, et al. 2015. Cell-Cycle Control of Bivalent Epigenetic Domains Regulates the Exit from Pluripotency. Stem Cell Reports 5(3):323–336.
Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336(6200): 688–690.

Sorre B, Warmflas, A, Brivanlou AH, Siggia ED. 2014. Encoding of temporal signals by the TGF-β pathway and implications for embryonic patterning. Dev Cell 30(3):334–342.

Sprinzak D, Elowitz MB. 2005. Reconstruction of genetic circuits. Nature 438(7067):443–448.

Sudheer S, Bhushan R, Fauler B, Lehrach H, Adjaye J. 2012. FGF inhibition directs BMP4-mediated differentiation of human embryonic stem cells to syncytiotrophoblast. Stem Cells Dev, 21(16):2987–3000.

Takahashi K, Yamanaka S. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126(4):663–676.

Takashima Y, Guo G, Loos R, Nichols J, Ficz G, Krueger F, et al. 2014. Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell 158(6):1254–1269.

Tam PP. 1998. Postimplantation mouse development: whole embryo culture and micro-manipulation. Int J Dev Biol 42(7):895–902.

Tang F, Barbacioru C, Bao S, Lee C, Nordman E, Wang X, et al. 2010. Tracing the derivation of embryonic stem cells from the inner cell mass by single-cell RNA-Seq analysis. Cell Stem Cell 6(5):468–478.

Tay S, Hughey JJ, Lee TK, Lipniacki T, Quake SR, Covert MW. 2010. Single-cell NF-kappaB dynamics reveal digital activation and analogue information processing. Nature 466(7303):267–271.

ten Berge D, Koole W, Fuerer C, Fish M, Eroglu E, Nuss R. 2008. Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell 3(5):508–518.

ten Berge D, Kurek D, Blauwkamp T, Koole W, Maas A, Eroglu E, et al. 2011. Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. Nat Cell Bio, 13(9):1070–1075.

Teo AKK, Arnold SJ, Trotter MWB, Brown S, Ang LT, Chng Z, et al. 2011. Pluripotency factors regulate definitive endoderm specification through eomesodermin. Genes Dev 25(3):238–250.

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, et al. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448(7150):196–199.

Theunissen TW, Powell BE, Wang H, Mitalipova M, Faddah DA, Reddy J, et al. 2014. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell 15(4):471–487.

Théry M, Piel M. 2009. Adhesive micropatterns for cells: a microcontact printing protocol. Cold Spring Harb Protoc 2009(7):pdb.prot5255.

Théry M, Jiménez-Dalmaroni A, Racine V, Bornens M, Jülicher F. 2007. Experimental and theoretical study of mitotic spindle orientation. Nature 447(7143):493–496.

Théry M, Racine V, Piel M, Pépin A, Dimitrov A, Chen Y, et al. 2006. Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity. Proc Nat Acad Sci U S A 103(52):19771–19776.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147.

Thomson M, Liu SJ, Zou L-N, Smith Z, Meissner A, Ramanathan S. 2011. Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 145(6):875–889.

Toh Y-C, Blagovic K, Yu H, Voldman J. 2011. Spatially organized in vitro models instruct asymmetric stem cell differentiation. Integr Biol (Camb) 3(12):1179–1187.

Torres-Padilla M-E, Chambers I. 2014. Transcription factor heterogeneity in pluripotent stem cells: a stochastic advantage. Development 141(11):2173–2181.

Touboul T, Hannan NRF, Corbineau S, Martinez A, Martinet C, Branchereau S, et al. 2010. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology 51(5):1754–1765.

Trounson A, McDonald C. 2015. Stem Cell Therapies in Clinical Trials: Progress and Challenges. Cell Stem Cell 17(1):11–22.

Turing AM. 1952. The chemical basis of morphogenesis. Philos Trans R Soc Lond B Biol Sci 237(641):37–72.

Turner DA, Baillie-Johnson P, Martinez-Arias A. 2016. Organoids and the genetically encoded self-assembly of embryonic stem cells. BioEssays 38(2):181–191.

Turner DA, Paszek P, Woodcock DJ, Nelson DE, Horton CA, Wang Y, et al. 2010. Physiological levels of TNFalpha stimulation induce stochastic dynamics of NF-kappaB responses in single living cells. J Cell Sci 123(Pt 16):2834–2843.

Vallier L, Alexander M, Pederse RA. 2005. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118(Pt 19):4495–4509.

van Boxtel AL, Chesebro JE, Heliot C, Ramel M-C, Stone RK, Hill CS. 2015. A Temporal Window for Signal Activation Dictates the Dimensions of a Nodal Signaling Domain. Dev Cell 35(2):175–185.

van den Brink SC, Baillie-Johnson P, Balayo T, Hadjantonakis A-K, Nowotschin S, Turner DA, Martinez-Arias A. 2014. Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. Development 141(22):4231–4242.

Wang Z, Oron E, Nelson B, Razis S, Ivanova N. 2012. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell 10(4):440–454.

Warmflash A, Arduini BL, Brivanlou AH. 2012a. The molecular circuitry underlying pluripotency in embryonic stem cells. Wiley Interdiscip Rev Syst Biol Med, 4(5):443–456.

Warmflash A, Sorre B, Etoc F, Siggia ED, Brivanlou AH. 2014. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. Nat Methods 11(8):847–854.

Warmflash A, Zhang Q, Sorre B, Vonica, A, Siggia ED, Brivanlou AH. 2012b. Dynamics of TGF-β signaling reveal adaptive and pulsatile behaviors reflected in the nuclear localization of transcription factor Smad4. Proc Nat Acad Sci U S A 109(28):E1947–56.

Wartlick O, Mumcu P, Kicheva A, Bittig T, Seum C, Jülicher F, González-Gaitán M. 2011. Dynamics of Dpp signaling and proliferation control. Science 331(6021):1154–1159.

Williams PH, Hagemann A, González-Gaitán M, Smith JC. 2004. Visualizing long-range movement of the morphogen Xnr2 in the Xenopus embryo. Curr Biol 14(21):1916–1923.

Wilson PA, Lagna G, Suzuki A, Hemmati-Brivanlou A. 1997. Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. Development 124(16):3177–3184.

Winnier G, Blessing M, Labosky PA, Hogan BL. 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9(17):2105–2116.

Wolpert L. 1969. Positional information and the spatial pattern of cellular differentiation. J Theor Biol 25(1):1–47.

Wu H, Huang B, Zare RN. 2006. Generation of complex, static solution gradients in microfluidic channels. J Am Chem Soc 128(13):4194–4195.

Xing J, Toh Y-C, Xu S, Yu H. 2015. A method for human teratogen detection by geometrically confined cell differentiation and migration. Sci Rep 5:10038.

Xiong F, Tentner AR, Huang P, Gelas A, Mosaliganti KR, Souhait L, et al. 2013. Specified neural progenitors sort to form sharp domains after noisy Shh signaling. Cell 153(3):550–561.

Xu R-H, Chen X, Li DS, Li R, Addicks GC, Glennon C, et al. 2002. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol, 20(12):1261–1264.

Ying Q-L, Nichols J, Chambers I, Smith A. 2003. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115(3):281–292.

Ying Q-L, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, et al. 2008. The ground state of embryonic stem cell self-renewal. Nature 453(7194):519–523.

Yu P, Pan G, Yu J, Thomson JA. 2011. FGF2 sustains NANOG and switches the outcome of BMP4-induced human embryonic stem cell differentiation. Cell Stem Cell 8(3):326–334.

Yu SR, Burkhardt M, Nowak M, Ries J, Petrásek Z, Scholpp S, et al. 2009. Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules. Nature 461(7263):533–536.