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From embryos to embryoids: How external signals and self-organization drive embryonic development

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SUMMARY

Embryonic development has been traditionally seen as an inductive process directed by exogenous maternal inputs and extra-embryonic signals. Increasing evidence, however, is showing that, in addition to exogenous signals, the development of the embryo involves endogenous self-organization. Recently, this self-organizing potential has been highlighted by a number of stem cell models known as embryoids that can recapitulate different aspects of embryogenesis *in vitro*. Here, we review the self-organizing behaviors observed in different embryoid models and seek to reconcile this new evidence with classical knowledge of developmental biology. This analysis leads to reexamine embryonic development as a guided self-organizing process, where patterning and morphogenesis are controlled by a combination of exogenous signals and endogenous self-organization. Finally, we discuss the multidisciplinary approach required to investigate the genetic and cellular basis of self-organization.

Reducing the generation of patterns and forms in the embryo to exogenous signals does not explain how multicellular organization emerges, but rather shifts the focus to external pre-patterns. This reductionist stance entails a hierarchical view of development that ultimately reduces all structures in the embryo to external causes, in a way that resembles preformationism. As an alternative, embryonic development can be studied by putting emphasis on developmental processes rather than their causes—i.e., by studying the autonomous capacity of the embryo to regulate itself and generate order. This has been done in embryology, where the attention has often focused on the self-regulatory capacity of the embryo to adapt to size (Tarkowski, 1961) and to perturbations (Snow and Tam, 1979). At the forefront of this self-regulatory capacity, we can find the concept of self-organization, which explains the emergence of order as a complete autonomous process without invoking external organizational causes. For a long time, self-organization was looked upon with skepticism in developmental biology, but increasing evidence has shown that, during embryonic development, tissues can organize independently of exogenous signals (Marcon and Sharpe, 2012; Schweisguth and Corson, 2019). In recent years, it has been shown that three-dimensional cultures of stem cells can spontaneously form complex biological structures that resemble organs (organoids) and embryos (embryoids). These discoveries have prompted a renaissance of the concept of self-organization. We are currently faced with the challenge of reconciling new evidence for embryonic self-organization with classical knowledge of developmental biology. Here, we take a step in this direction.

We begin by presenting the different exogenous inputs and endogenous processes that control embryonic development. We continue with an analysis of the endogenous self-organizing processes that have been observed in different *in vitro* models of the epiblast. This analysis leads to propose the idea of "guided self-organization" as an appropriate framework to describe the development of the epiblast in the embryo, where endogenous self-organizing processes are modulated by exogenous instructive inputs. In light of this framework, we discuss the processes of self-assembly and patterning observed in embryonic explants and in co-cultures of embryonic and extra-embryonic stem cells. Finally, we present a multidisciplinary approach to study embryonic self-organization, which combines experiments on embryoids, quantitative biology, and computational modeling. We conclude by proposing that a deeper knowledge on self-organization will be key to devise novel bioengineering strategies to control and improve embryoid development.

EXOGENOUS INPUTS AND ENDOGENOUS DEVELOPMENTAL PROCESSES

Embryonic development can be described as a combination of exogenous inputs and endogenous developmental processes (Figure 1A). On one side, exogenous inputs can be of two types: permissive, which are typically homogeneous and are required for the progression of a developmental trajectory that would otherwise fail to continue; and instructive, which are typically localized or heterogeneous and can steer the development toward distinct trajectories depending on the nature and strength of the input (Slack, 1993). On the other side, endogenous developmental processes can fall under two categories: hierarchical processes, which typically involve open loop or feedforward regulatory logics and are completely under the control of external instructive inputs, such as the interpretation of positional information; and self-organizing processes, which involve feedback regulatory logics and can

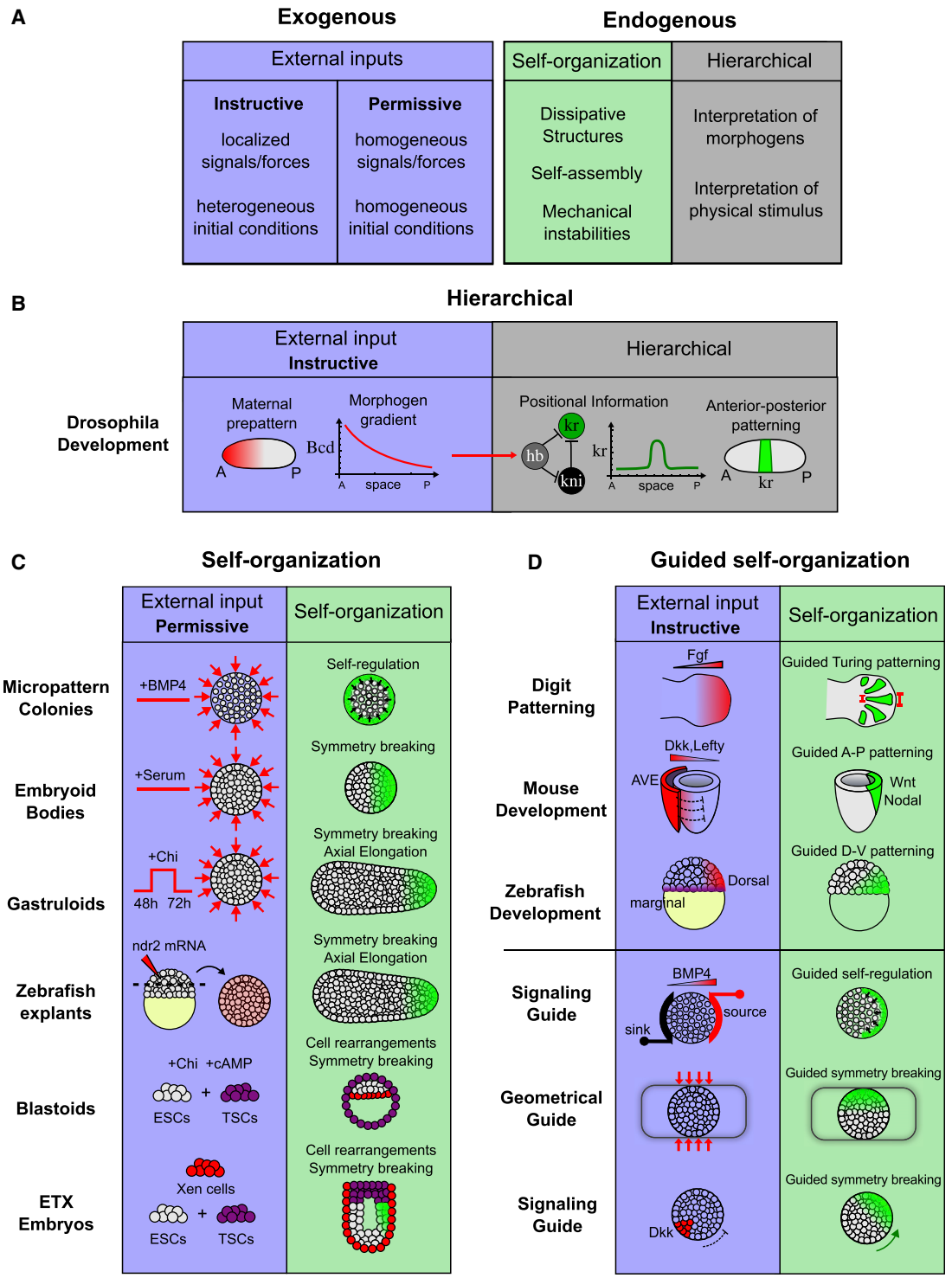

Figure 1. Embryonic development as guided self-organization

(A) Exogenous inputs and endogenous processes that drive the emergence of organization during embryonic development.

(B) Early *Drosophila* development is a classical example of a hierarchical developmental system. The emergence of order in this case is controlled completely by external instructive signals, like the morphogen gradient Bicoid that activates GAP genes, such as Hunchback (hb), anteriorly, which in turn promotes Kruppel (kr) and Knirps (kr) to create a band of Kruppel expression in the middle of the embryo (green).

(legend continued on next page)

create order independently of exogenous instructive signals (Figure 1A).

Examples of self-organizing processes during embryonic development include the formation of appendages, such as hair follicles and feathers (Jung et al., 1998; Painter et al., 2012), pigmentation patterns on the fish and lizard skin (Kondo and Asal, 1995; Manukyan et al., 2017), digits in the mouse limb (Raspopovic et al., 2014), fin rays in dogfish (Onimaru et al., 2016), ridges on the mouse palate (Economou et al., 2012), branching patterns during lung and kidney morphogenesis (Menshykau et al., 2012, 2019), and the arrangement of leaf primordium in the plant stem (Turing, 1952). In all these examples, the emergence of order relies on endogenous regulatory feedbacks that generate a series of repetitive dissipative structures (Goldbeter, 2018). An influential hypothesis to explain the formation of these self-organizing repetitive patterns is diffusion-driven instability, proposed by Alan Turing in 1952 (Turing, 1952). This theoretical model describes how a system of chemical substances, called morphogens, can react together and diffuse between cells to generate emergent periodic patterns of dissipative structures. Aside from these chemical changes, subsequent studies have shown that self-organizing patterning can also occur in mechanochemical models that consider changes in the physical properties and morphology of the tissue (Mercker et al., 2016; Shyer et al., 2017). The patterning capabilities of these mechanochemical models go beyond classical Turing systems and include the ability to scale patterns with tissue size (Recho et al., 2019). Finally, there are alternative self-organizing mechanisms that can explain the emergence of repetitive structures that include mechanical instabilities (Tallinen et al., 2016) or coordination of autonomous oscillations (Venzin and Oates, 2020).

While Turing's theory has been quite successful in explaining the formation of periodic structures during late developmental processes, it was originally proposed to explain the first steps of embryonic development, and in particular the breaking of the initial symmetry of the embryo to form the main body axis (Turing, 1952). This process, however, has been considered for a long time a "gold standard" example of a hierarchical process, with strong genetic evidence in *Drosophila* that the maternal gradient Bicoid acts as an instructive morphogen (Driever and Nüsslein-Volhard, 1988), which is interpreted by a hierarchical GAP gene network to specify the anterior-posterior axis (Jaeger, 2011) (Figure 1B). Similarly, the formation of the anterior-posterior axis in the mouse epiblast has been described as an inductive process controlled by inhibitory signals from an anteriorly localized population of cells in the primitive endoderm (PrE), known as the anterior visceral endoderm (AVE) (Tam and Loebel, 2007). Nevertheless, other studies have proposed that earlier steps of mouse development, which involve the segregation between PrE and epiblast lineages in the blastocyst, could be instead driven by self-organization (Zhang and Hiiragi, 2018). Experimental evidence suggested that this self-organizing process depends on the gradual sorting of an initially heterogeneous population of cells that could be facilitated by differential cell adhesion. This has often been referred to as an example of self-assembly, which can be distinguished from the formation of dissipative

(C) Examples of self-organizing systems where endogenous self-organizing processes are triggered by external permissive signals. From top to bottom: stimulated by permissive medium that contains BMP4 (red arrows), micropattern colonies generate a radial self-regulatory wave of mesendoderm markers (green) that propagates from the edge of the colony to its inner core; embryoid bodies (EBs) stimulated by permissive medium that contains serum (red arrows) spontaneously break their radial symmetry expressing primitive streak markers at one EB pole (green); when EBs are stimulated with permissive pulse of Chiron (Chi) between 48 and 72 h of development (red arrows and red line), they generate axially elongated embryo-like structures known as gastruloids; upon homogeneous expression of ndr2, Nodal (injection needle, red), naive animal poles of zebrafish embryos generate axially elongated structures; in medium with permissive conditions (+Chi and +cAMP), co-cultures of mouse trophoectoderm stem cells (TSCs) (purple) and mouse embryonic stem cells (ESCs) (white) form blastoids showing asymmetrical structure and the emergence of primitive endoderm cells (red); ETX post-implantation embryo-like structures can be formed under minimal permissive conditions by mixing TSCs (purple), ESCs (white), and primitive endoderm-like cells (XEN, red). These structures break their symmetry and express primitive streak markers only on one side (green).
(D) Example of guided self-organization where instructive external signals influence endogenous self-organizing processes. Upper half, embryonic development, from top to bottom: digit patterning is controlled by a self-organizing Turing mechanism that creates a series of periodic stripes (green) and an external gradient of Fgf (red) that aligns stripes and avoids digit bifurcation by promoting larger wavelength (red marks); anterior-posterior (A-P) patterning in the mouse embryo is controlled by a self-organizing symmetry-breaking process in the epiblast (white) marked by the formation of the primitive streak (green) that is influenced by inhibitors (Dkk, Lefty) from the anterior visceral endoderm (AVE) (red); cells in the developing zebrafish embryo, which have the ability to self-organize in explants, are modulated by signals coming from the marginal and dorsal organizer (red) to generate a dorsal-ventral axis (D-V) (green). Lower half, multicellular engineering, from top to bottom: self-regulatory waves in micropattern colonies (green) can be established only one side of the colony under the control of an external BMP4 gradient provided by microfluidics (red); last two bottom rows: the symmetry breaking of EBs can be biased by physical inputs or external localized signals (red) provided by micropools and by grafting cells that constitutively express Dkk (red), respectively.

Box 1. Self-assembly and dissipative structures

Different types of self-organization have been distinguished according to three criteria: the initial configuration of the self-organizing system, the plasticity of the elements that compose the system and the energetic requirements of self-organization. At one side of the spectrum, we find the classical definition of self-assembly, that considers systems with heterogeneous initial configurations, whose elements are immutable (such as atoms or molecules) and where a new spatial order is achieved by minimizing the energy of the system (Halley and Winkler, 2008). At the opposite side of the spectrum, we find the generation of dissipative structures (e.g., diffusion-driven instability) that does not require a heterogeneous initial configuration, it gives rise to dynamically changing elements and where self-organization requires a continuous supply of energy (Goldbeter, 2018). Multicellular self-organizing processes fall into the second category, because cell identities and the properties that drive the assembly of cells are often maintained through the continuous supply of energy and can change dynamically over time (Kirschner et al., 2000). It is important to notice that self-organizing dissipative structures can also be generated from heterogeneous initial conditions and regenerated from disrupted patterns and therefore do not require the components of the system to be equal. In summary, multicellular self-organization is a process that combines the generation of dissipative structures with cell rearrangements to form patterns on a scale larger than the typical length of cell interaction. The concept of guided self-organization proposed in this study refers to cases where this broader type of self-organization is influenced by pre-existing asymmetries.

structures because it does not consume energy to create structures far from equilibrium but, rather, it minimizes the energy of the system to converge to a new order (Halley and Winkler, 2008; Turner et al., 2016). This distinction is easy to draw in physics where immutable atoms self-assemble into ordered structures, such as crystals, that have minimal energy. However, in multicellular organisms, the identity of cells and the properties that determine cell assembly (e.g., adhesion) can change over time and are often dynamically maintained using energy as in dissipative systems (Kirschner et al., 2000; Yanagida et al., 2020), see the discussion in Box 1.

Therefore, as a whole, embryonic self-organization should be viewed as a combination of different processes (Figure 1A) that can happen simultaneously and include: the formation of dissipative structures, which can be temporal as in self-regulatory oscillating systems, or spatial as in periodic patterns driven by diffusion-driven instability; self-assembly, which can form structures at lower molecular levels and mechanical instabilities that can drive spatially heterogeneous changes in conformation of soft matter under ubiquitous force.

EMBRYONIC STEM CELL MODELS TO STUDY EPIBLAST SELF-ORGANIZATION

Recently, the reexamination and the establishment of new stem cell models has allowed to recapitulate distinct aspects of embryonic self-organization *in vitro*. The first observations that *in vitro* systems could mimic embryonic development can be traced back to the 1970s, with the establishment of three-dimensional suspension cultures of teratocarcinomas and teratomas cells called embryoid bodies (EBs) (Martin and Evans, 1975). These cultures were known to differentiate into various cell types observed during embryonic development (Martin and Evans, 1975) and, if generated with mouse embryonic stem cells (mESCs), they could differentiate into most descendants of the three germ layers, but this was assumed to happen in a disorganized fashion (Murry and Keller, 2008). However, more recently it was shown that EBs generated with mESCs were able to form stereotypical localized patterns of *Brachyury* expression and Wnt signaling that were reminiscent of the primitive streak in the embryo (ten Berge et al., 2008). This provided the first concrete evidence that an initially homogeneous population of mESCs could self-organize to form an embryonic axis when exposed to permissive signals (Figure 1C). This spontaneous symmetry-breaking process happened in the absence of instructive inputs, since the differentiation medium that surrounded EBs provided only ubiquitous signals that could not instruct specific parts of the EB to become posteriorly fated. Another study showed that, when mouse EBs were cultured in minimal basal medium, they could self-organize into polarized cortical tissues (Eiraku et al., 2008), demonstrating that EB self-organization could be directed by exogenous permissive signals toward more anterior fates.

A more extensive characterization of the permissive signals that could direct EB self-organization, revealed that under different conditions, basal medium (N2B27) could also stimulate the formation of a main embryonic axis (Turner et al., 2017; van den Brink et al., 2014). This study showed that EBs exposed to basal medium formed a polarized expression of posterior markers (Turner et al., 2017), but that the majority of them failed to continue axis development and remained morphologically rounded. However, it was found that EBs could undergo robust elongation, when a pulse of WNT signaling agonist (Chiron) was provided between the second and the third day after EB

Box 2. Symmetry breaking

Symmetry breaking is the ability of a system to autonomously generate a new spatial symmetry that is not manifest in the initial state of the system. This implies that symmetry breaking always gives rise to an emergent spatial order that cannot be reduced to the initial configuration of the systems or to external stimuli. For this reason, the self-organizing patterns formed in micropatterned colonies of hESC are not symmetry-breaking events, because they maintain the initial radial symmetry of the colony and they are directly correlated with permissive factor in the differentiation medium (Figure 1C). These *in vitro* systems, however, show self-regulatory dynamics that are characterized by the formation of signaling waves in response to the external stimuli (Chhabra et al., 2019). It is also important to highlight that symmetry breaking cannot be associated to cases where asymmetries are a direct reflection of an external localized signal. In this case, the patterns that form are just an interpretation of the initial asymmetry imposed to the system (e.g., external localized signals) similar to the case of micropatterned colonies stimulated by external gradients (Manfrin et al., 2019) (Figure 1D). It is nevertheless possible to use external stimuli or localized signals to bias a symmetry-breaking process, as it has been done in EBs with geometric constrictions (Sagy et al., 2019) (Figure 1D). In this case, the symmetry-breaking process can be interpreted as an example of guided self-organization.

formation (Turner et al., 2017; van den Brink et al., 2014) (Figure 1C). Due to the resemblance between this morphogenetic event and some of the tissue movements observed during gastrulation, this *in vitro* system was named gastruloid. Subsequent studies showed that, in addition to the formation of an anterior-posterior axis, gastruloids can also recapitulate the formation of a dorsal-ventral axis, the establishment of the midline and the expression of body segment markers (Beccari et al., 2018; van den Brink et al., 2020). More recently, two studies showed that, when gastruloids are embedded in Matrigel, they can also recapitulate the formation of somites and the neural tube (van den Brink et al., 2020; Veenvliet et al., 2020), showing that mechanical permissive inputs can also play a central role in directing EB self-organization.

Another stem cell model that has been developed to investigate embryonic self-organization are micropatterned colonies (Warmflash et al., 2014). This quasi two-dimensional culture system was developed to control the initial geometry of colonies by growing human ESCs (hESCs) on micropatterned adhesive surfaces. When these colonies were exposed to medium containing BMP4, they formed an outer ring of primitive streak markers that propagated from the periphery of the colony to its inner core (Heemskerk et al., 2019; Martyn et al., 2019). It has been proposed that this self-organizing process is controlled by a Turing system implemented by BMP signaling and its antagonist Noggin (Tewary et al., 2017) and more recently also by Wnt signaling and its antagonist Dkk1 (Etoc et al., 2016). However, in contrast to EBs, this system does not break its initial radial symmetry, since all the cells along the outer edge of the colony show an identical behavior under the influence of BMP4. Therefore, while micropattern colonies give rise to self-regulated radial waves of WNT and NODAL signaling that are limited by their own inhibitors (Chhabra et al., 2019) (Figure 1C), these dynamics do not invoke a Turing model that can break the symmetry of the system by generating periodic patterns (see discussion in Box 2). On the other hand, it has been shown that, when hESCs are cultured as three-dimensional aggregates, they can indeed undergo symmetry breaking (Simunovic et al., 2019) end elongate (Moris et al., 2020). Intriguingly, similar differences between the self-organization of micropattern colonies (Morgani et al., 2018) and three-dimensional cultures of ESCs (ten Berge et al., 2008) have been observed in mouse, suggesting that the three-dimensional arrangement of ESCs could be a general requirement for symmetry breaking. The molecular basis of these differences are unknown, but they can be related to the different distribution of transforming growth factor β receptors that have been observed along the edge of micropatterned colonies of hESCs (Etoc et al., 2016; Sozen et al., 2020).

In summary, stem cell models offer a unique opportunity to investigate embryonic self-organization, but different *in vitro* systems highlight different self-organizing capabilities of ESCs. On one side, micropattern colonies highlight the self-regulatory capacity of the signaling pathways that control the formation of the primitive streak. On the other side, three-dimensional aggregates, such as EBs and gastruloids, reveal the additional ability of ESCs to break their initial symmetry and undergo axial elongation. How these different self-organizing behaviors relate with normal embryonic development remains unclear.

EPIBLAST DEVELOPMENT AS GUIDED SELF-ORGANIZATION

Altogether different embryoid models of the epiblast can be interpreted as different trajectories of a common ground state of the self-organizing potential of ESCs, which in the embryo is canalized and modulated by specific extra-embryonic signals, initial conditions and tissue geometry. These exogenous inputs can be permissive as well as instructive, such as the inhibitory signals secreted from the AVE in the mouse gastrula or signals released from the marginal zone

and the dorsal-ventral organizer in zebrafish (Figure 1D). When exogenous instructive signals and endogenous self-organization are combined, they give rise to a process that cannot be interpreted as the result of self-organization alone, but that evolves also according to an external pre-pattern. These types of processes are examples of guided self-organization (Hartmann et al., 2020; Prokopenko, 2009). Therefore, the development of the epiblast in the embryo is an example of guided self-organization, where interactions between maternal inputs, extra-embryonic tissues, and endogenous self-organizing processes control patterning and morphogenesis (Figure 1D).

There are two main ways to guide a self-organizing system: by using specific initial conditions to exploit the tendency of the system to behave according to its history, a property usually referred as stigmergy (Sasai, 2013); or by modulating the rate or extent of the internal self-organizing dynamics with external signals (Prokopenko, 2009). A prime example of the second type of guided self-organization is the combination of positional information and Turing patterning (Miura, 2013), which has been proposed to explain digit formation during limb development (Raspopovic et al., 2014; Sheth et al., 2012). These studies interpreted digit patterning as the emergence of a series of repetitive dissipative structures generated by a two-dimensional Turing model. The model alone generated a random stripy pattern but, when it was modulated by a gradient coming from the tip of the limb, it reliably generated a pattern similar to the experimental expression of digit markers (Raspopovic et al., 2014; Sheth et al., 2012) (Figure 1D). This pattern could not be explained by external pre-patterns or by the Turing system alone, but only as a combination of both patterning processes.

Despite their simplicity, these models capture the important idea that complex patterns, such as the one observed during digit patterning, can emerge from the interaction between exogenous instructive inputs and endogenous self-organizing processes. The self-organizing potential of the limb was revealed by depleting exogenous instructive inputs, such as Shh, Gli3, and distal Hox genes, and by observing that, in the absence of these signals, the limb could form up to 14 digits (Sheth et al., 2012). This example demonstrates that one of the best approaches to study guided self-organization is by minimizing the influence of external inputs to uncover the endogenous self-organizing potential of the system.

EMBRYONIC EXPLANTS: PATTERNING AND SELF-ASSEMBLY

In the context of early embryonic development, the influence of external inputs can be minimized by isolating embryonic explants to study their self-organizing capabilities. These are classical experiments in embryology that have been performed in avians and in amphibians, and have often revealed that, in explants, embryonic cells can undergo directed movements and can sort according to their original identity in the embryo (Townes and Holtfreter, 1955). A more recent study has generated explants by mixing cells dissociated from the anterior and the posterior region of the forming mesoderm in the *Xenopus* gastrula (Ninomiya et al., 2004). In agreement with previous findings, this study showed that cells in the explant have the ability to self-organize and to sort into two distinct populations according to their original anterior-posterior identity. Moreover, the presence of these two populations could stimulate axial elongation, while explants obtained with cells dissociated only from the anterior or the posterior mesoderm remained rounded. This showed that *Xenopus* explants could self-organize and break their symmetry only in the presence of initial heterogeneous cell populations, supporting the idea that this process was mediated by cell sorting. Recently, two studies have drawn similar conclusions by suggesting that zebrafish explants require the self-assembly of an initially heterogeneous cell population to break their symmetry and elongate (Fulton et al., 2020; Schauer et al., 2020). However, another recent study showed that zebrafish explants can break their symmetry starting from a more homogeneous cell population (Williams and Solnica-Krezel, 2020). This was achieved by generating explants made of identical cells from the animal pole that were stimulated with permissive signals provided by ubiquitous Nodal expression (Williams and Solnica-Krezel, 2020) (Figure 1C). Although, the presence of small asymmetries in the explant and in the injection of Nodal mRNA cannot be completely excluded, this experiment demonstrates that naive explants derived from the animal pole are able to self-organize by regulating cell fates, similarly to the symmetry breaking observed in EBs. Altogether, experiments on explants show that embryonic development can involve both self-organizing patterning and cell rearrangements (see Box 1). In line with this idea, exciting advances in synthetic biology have recently showed that robust multicellular self-organization can be achieved by combining contact-mediated patterning and changes in cell adhesion that drive cell sorting (Toda et al., 2018).

SELF-ORGANIZATION OF EMBRYONIC AND EXTRA-EMBRYONIC STEM CELLS

The ability of ESCs to spontaneously assemble together has also been highlighted by co-culturing embryonic and extra-embryonic cell lineages *in vitro*. These systems can generate structures that resemble the morphology of the

early embryo by self-organizing through crosstalk between different tissues. While EBs, gastruloids, and embryonic explants can be considered as an attempt to deconstruct the embryo to reveal its self-organizing potential; co-culture systems can be considered an attempt to reconstitute the embryo from different cell lineages.

One of the first studies that used this approach combined small clumps of mouse trophoblast stem cells (TSCs) and mESCs to form embryo-like structures, named ETS, similar to the mouse gastrula (Harrison et al., 2018). The same group extended this system by co-culturing extra-embryonic endoderm-like cells, known as XEN cells, in addition to TSCs and mESCs (Sozen et al., 2018), this time allowing a cell mixture to spontaneously self-organize into embryo-like structures named ETX embryos (Figure 1C). The cells in these aggregates self-organized into a complex morphology that resembled the inner core of the gastrula, characterized by an external layer of PrE that surrounded two clusters of TSCs and mESCs with a common central cavity and a basal membrane. The majority of aggregates that underwent proper self-organization showed a remarkable resemblance with post-implantation mouse embryos, which included the formation of localized anterior-posterior markers. More recently, ETX embryos were generated using a population of PrE cells induced from mESCs rather than XEN cells (Amadei et al., 2021). These aggregates had an improved developmental potential showing markers of gastrulation and a migrating subpopulation of cells in the distal visceral endoderm that resembled the AVE.

Another study used co-cultures of TSCs and mESCs to show that they can self-organize into structures named blastoids (Rivron et al., 2018). This system was able to undergo symmetry breaking forming the embryonic-abembryonic axis characteristic of the blastocyst, with the formation of a cavity on one side of the blastoid, and a group of mESCs reminiscent of the inner cell mass (ICM) on the opposite side. In addition, blastoids could also recapitulate the formation of the PrE lineage that emerged as a group of cells positioned at the interface between the ICM and the cavity (Figure 1C). Blastoids could not develop further into the post-implantation stages of the mouse embryo, but it was shown that they could recapitulate key aspects of uterine implantation (Rivron et al., 2018). More recently, the same experiments were repeated by forming blastoids with a mixture of mESCs exposed to extended pluripotent conditions and TSCs (Sozen et al., 2019). These blastoids could progress into a structure similar to the embryo at the peri-implantation stage characterized by an external layer of PrE surrounding a cylindrical core of mESCs and TSCs. Finally, another recent work performed a systematic study to identify chemical conditions that can stimulate the formation of PrE cells and applied these conditions to blastoids to stimulate a transition toward post-implantation embryo-like structures (Vrij et al., 2019).

In summary, three-dimensional co-cultures of extra-embryonic and ESC lineages have the remarkable potential to self-organize into structures that resemble the early embryo. Nevertheless, these structures fail to progress to later developmental stages. Surprisingly, simpler three-dimensional cultures generated only with ESC, such as human and mouse gastruloids, can form structures that are observed at later developmental stages, such as progenitors of the neural tube and anterior-posterior segment markers. A possible interpretation for these differences is that gastruloids might represent a more unconstrained self-organizing potential of the epiblast that can explore different developmental trajectories to find its own way toward later differentiation programs. In contrast, co-cultures of extra-embryonic stem cells and ESCs could give rise to a more constrained system, where the self-organizing process is canalized by interactions between the different tissues. This canalization may push the system toward the specific developmental trajectory of the mouse blastocyst and the peri-implantation embryo, which can fail to progress if the epiblast and extra-embryonic tissues that emerge from self-organization are not in same state as the corresponding tissues in the embryo.

STUDYING EMBRYONIC SELF-ORGANIZATION TO IMPROVE MULTICELLULAR BIOENGINEERING

Co-culture systems that mimic the early human embryo could have a profound impact in the study of human disease and in tissue engineering. Establishing these systems, however, has been more challenging because human extra-embryonic stem cells have emerged only recently (Fu et al., 2021). To overcome this limitation a recent study has controlled the development of an epiblast-like cyst of human pluripotent stem cells (hPSCs) by using two microfluidics compartments that stimulated the formation of amniotic ectoderm on one side of the cyst and epiblast fates on the opposite side (Zheng et al., 2019). This promoted the efficient generation of cysts with a dorsal-ventral axis that mimicked the post-implantation human embryo. However, while other studies have shown that hPSC cysts can break their symmetry spontaneously to form a dorsal-ventral axis (Shao et al., 2017), in this microfluidics setup the emergence of asymmetries was a direct consequence of the signals localized in the two compartments.

The idea of using microfluidics to control PSCs development has also been applied in micropattern colonies, where an engineered BMP4 gradient was used to modulate the self-regulatory signaling waves generated in the colonies (Manfrin et al., 2019) (Figure 1D). This modulation

promoted a polarized expression of germ layer markers that mimicked the anterior-posterior axis of the embryo. However, similarly to the case of human cysts, the formation of the axis was a direct consequence of the external gradient and did not involve an endogenous symmetry-breaking process (see the discussion in Box 2). Asymmetric distributions of germ layer markers have also been obtained culturing micropattern colonies by using different geometries (Blin et al., 2018). This system promoted cell rearrangements that formed cell density patterns that reflected the geometry of the colony and promoted localized expression of Brachyury. These experiments provide further evidence that multicellular self-organization can often involve feedbacks between cell rearrangements and cell signaling (see Box 1). However, it remains unclear if these localized patterns involve a reliable endogenous symmetry-breaking process or are just a reflection of the external geometry. Another study, nevertheless, showed that geometric confinement provided by microwells could bias the endogenous symmetry breaking observed in EBs (Sagy et al., 2019). In this case, although the confinement stimulated EBs on two opposite sides, primitive streak markers often formed only on one side due to the symmetry-breaking process (Figure 1D). In addition, EB symmetry breaking could also be influenced by clumps of cells that constitutively expressed Wnt or Dkk1, which were able to shift the localization of primitive streak markers (Figure 1D). These experiments are examples of guided self-organization, where the intrinsic symmetry-breaking ability of EBs is modulated by external localized inputs (Figure 1D). Moreover, they suggest that the formation of the primitive streak in the mouse embryo could be described as a guided self-organizing process, where endogenous symmetry breaking in the epiblast is modulated by external localized signals and constrictions provided by the PrE and trophoectoderm.

The use of engineering tools to drive self-organization has been incrementing at a faster pace in three-dimensional organ-like cultures, called organoids, due to the strong interest in recreating more reproducible organ structures for modeling human disease and to improve tissue engineering (Brassard and Lutolf, 2019; Garreta et al., 2021). Perhaps the vanguard of an engineering device that controls the growth and microenvironment during organoid self-organization is the "organ on a chip" system: a microfluidic chip capable of combining physiological cues, such as nutrient and growth factor flow, mechanical forces, electrical stimulation, and microbiota interactions (Park et al., 2019). With these incredible advances in tissue and organoid engineering in mind, should we expect that embryoid research would go toward a "embryo on a chip" system?

Indeed the use of more sophisticated culture methods for embryoids is at our doorstep, but there is an important difference between embryoids and organoids that can make it more difficult to develop an embryos on a chip. Organoids are generally made from adult stem cells or PSCs that have been restricted toward a specific set of fates (Garreta et al., 2021). On the contrary, ESCs can give rise to cells of all three germ layers and have the potential of a wide range of distinct self-organizing states, where small changes in timing and strength of inputs can lead to drastically different outputs. For example, mESCs aggregates can generate gastruloids with posterior fates (van den Brink et al., 2014) or cortical organoids with anterior fates (Eiraku et al., 2008) with minimal differences in permissive inputs. On the other side, there has been a struggle to achieve the full set of anterior and posterior fates in the same embryoid. Altogether, these difficulties suggest that, if embryoids are guided as a black box by adding instructive cues, eventually the outcome and reproducibility of the results could be unpredictable. Moreover, too many external inputs can extinguish the self-organizing potential of the system. Therefore, we propose that, to devise proper strategies to guide the self-organization of embryoids, a deeper knowledge of the basis of embryonic self-organization is required. The question that remains is how can we identify the underlying self-organizing process that drives embryonic development?

(1) First, the aim should be to reconcile evidence from different *in vitro* systems. A deeper understanding of the self-organizing dynamical system that drives the development of ESCs will come from comparisons between different *in vitro* systems, similarly to what has been done for decades with genetic screening to compare mutant phenotypes.
(2) The best way to study self-organization is to choose systems with the most homogeneous initial conditions as possible to avoid pre-existing asymmetries that could cover important self-organization dynamics. In this direction, choosing the right maintenance medium for the pluripotency of stem cells is key, trying to avoid media based on serum that could drag cells to a more heterogeneous and primed state (Guo et al., 2016). Pre-existing asymmetries can also be driven by uneven initial aggregation of cells in three-dimensional cultures, therefore the microwells and micropatterns used to generate aggregates should be chosen carefully to minimize initial geometrical asymmetries.
(3) Self-organizing systems are variable per se, and can reach different states despite minor differences in the initial conditions. Improving the reproducibility of *in vitro* systems with instructive cues is ideal to boost robustness (Brassard and Lutolf, 2019), but it can be counterproductive for the study of the

self-organization itself. Therefore, new techniques to cope with the intrinsic variability of self-organization should be developed. In this direction, it will be crucial to analyze data from as many specimens as possible by the use of high-throughput imaging methods and automated computational analysis. A recent study, for example, has performed a systematic screen to analyze nearly half a million intestinal organoids exposed to thousands of different chemical compounds, and used the data to derive a phenotypic landscape of intestinal organoid self-organization (Lukonin et al., 2020).

(4) Different self-organizing processes have distinct spatiotemporal dynamics. e.g., Turing patterns emerge as static dissipative structures throughout the whole tissue, while traveling waves and excitable models are characterized by temporally unstable patterns (Goldbeter, 2018). To distinguish between different models it is imperative to capture spatiotemporal dynamics of the early stage of self-organization. These dynamics are likely to involve feedbacks between gene regulation and cellular behaviors, therefore quantification of cell fates and cell movements should be performed simultaneously. Moreover, since each set of feedbacks in a self-organizing model predicts a certain distribution of activity patterns (Marcon et al., 2016), several genes should be monitored simultaneously to evaluate their relative spatial distribution. This can be done by monitoring embryoids generated with multiple reporter cell lines with lightsheet microscopy (McDole et al., 2018) and by integrating these data with single-cell or spatial transcriptomics (van den Brink et al., 2020) to infer genome-wide spatial distribution of expression patterns.

(5) Self-organizing systems have unintuitive dynamics that cannot be understood without computational simulations and mathematical analysis. The ideal theoretical tool required to study biological self-organization should include the possibility to simulate gene-regulatory networks, cellular movements, and mechanics. This can be achieve by combining partial differential equations with agent-based models to simulate patterning and morphogenesis simultaneously.

(6) Finally, the main way to evaluate if a specific model underlies a self-organizing process is by comparing the dynamics predicted by computational simulations and experimental self-organizing dynamics. This should be done both in the normal situation and upon perturbations. For this, it will be crucial to design perturbations that do not abolish self-organization completely, but rather that promote a rearrangement of the patterns (Raspopovic et al., 2014). Perturbation should also include changes in size, since different self-organizing models predict different scaling behaviors (Ishihara and Tanaka, 2018).

CONCLUSIONS

Frequently in science, anti-reductionist awakenings have been followed by a quick fall back into reductionism. This process has helped scientists to dissect reality in all its diversity, but it has often left us surrounded by data and lacking theoretical explanations. Our ability to distinguish entities must be accompanied with the awareness that reducing a system into its parts is just an approximation and that, in parallel, we must aim to put the parts back together. Three-dimensional cultures of ESCs allow us to separate the epiblast from the signals that influence its development. These new isolated wholes have the surprising capacity to form complex biological structures that resemble the developing embryo under minimal permissive conditions. This leads to re-interpret the development of the epiblast in the embryo as a guided self-organizing process, where ESCs spontaneously generate patterns that are influenced by external localized inputs (Figure 1D). This perspective represents an advance with respect to a classical hierarchical view of development, since it recognizes a previously undervalued autonomous capacity of the epiblast and reconsiders external signals as guiding cues. However, it is yet another reductionist approximation that can be applied only to systems where there is a clear distinction between instructive pre-patterns and autonomous self-organizing processes.

For instance, the development of ETX embryos and blastoids is a remarkable example of self-organization that cannot be regarded as a guided self-organizing process, because it does not involve instructive pre-patterns (Figure 1C). Indeed, these co-culture systems begin from a mixed population of embryonic and extra-embryonic stem cells that are stimulated with homogeneous permissive factors (Figure 1C). Nevertheless, during their self-organizing trajectory, these systems generate asymmetries that can act as pre-patterns to guide other endogenous self-organizing processes, similarly to what happens in the embryo. An example of these phenomena is the emergence of the AVE during early mouse development, which guides the symmetry breaking of the epiblast to form the PSCs (Sozen et al., 2020). This patterning event is an example of guided self-organization because it involves a self-organizing symmetry-breaking process in the epiblast that is guided by an exogenous pre-pattern provided by

the AVE (Figure 1D). In summary, the concept of guided self-organization is a reductionist approximation that is suitable to describe self-organizing processes that are under the influence of external asymmetries, which, within a specific developmental time frame, can be considered as prepatterns.

A fundamental question that remains to be addressed is how self-organization is implemented at the molecular and cellular levels. Evidence suggests that embryonic self-organization couples regulatory feedbacks between signaling pathways and changes in cellular behaviors, such as cell adhesions or directed movements. A marvelous array of powerful technologies is available to investigate these processes. Experimental data combined with theoretical studies and work from synthetic biology will help us to identify the principles that underlie embryonic self-organization. This highly multidisciplinary effort will require collaborations between stem cell biology, developmental biology, and theoreticians and engineers, proving that also in the scientific community the whole is more than the sum of its parts.

AUTHOR CONTRIBUTIONS

J.S.M. and J.R. wrote the manuscript. L.M. wrote the manuscript and designed the figure.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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