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Perspective

Establishment of the vertebrate body plan: Rethinking gastrulation through stem cell models of early embryogenesis

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SUMMARY

A striking property of vertebrate embryos is the emergence of a conserved body plan across a wide range of organisms through the process of gastrulation. As the body plan unfolds, gene regulatory networks (GRNs) and multicellular interactions (cell regulatory networks, CRNs) combine to generate a conserved set of morphogenetic events that lead to the phylotypic stage. Interrogation of these multilevel interactions requires manipulation of the mechanical environment, which is difficult *in vivo*. We review recent studies of stem cell models of early embryogenesis from different species showing that, independent of species origin, cells in culture form similar structures. The main difference between embryos and *in vitro* models is the boundary conditions of the multicellular ensembles. We discuss these observations and suggest that the mechanical and geometric boundary conditions of different embryos before gastrulation hide a morphogenetic ground state that is revealed in the stem-cell-based models of embryo development.

The emergence of an organism from a fertilized egg is the result of a coordination of cellular multiplication, diversification, and spatial organization. Each of these events is driven by schedules of gene expression within individual cells that are in turn regulated by the spatial rearrangement of cells and tissues via intercellular signaling. Early in this process, a mass of seemingly identical cells resulting from the proliferation of the zygote engages in a choreography of morphogenetic movements that shapes the recognizable outline of an organism. This is a feature of all metazoan organisms and the ensemble of movements is called "gastrulation," a term introduced by Ernst Haeckel to account for the formation of the gut in sponges and generally understood as a stage between the "blastula" and the "neurula," during which germ layers are specified (Haeckel, 1872, 1874; Stern, 2004b). The multicellular choreographies associated with gastrulation are species specific, determined by the geometry and organization of the egg of origin. In vertebrates, each of these variant gastrula morphologies nevertheless leads to a conserved phylotypic process, represented by the emergence of a segmentally organized anteroposterior (AP) axis with the pharyngeal arches at the front, an orthogonal axis demarcating the dorsoventral (DV) positions of the nervous system, and the gut and a midline (ML) that defines a bilateral symmetry. How such a conserved body plan emerges through a process that is associated with extensive differences in global morphology is a fundamental problem in biology.

Gastrulation and the emergence of the vertebrate body plan have traditionally been studied in model organisms either by experimental embryology, amphibia and birds, or genetics in mammals, with zebrafish providing a useful combination of the two (Solnica-Krezel and Sepich, 2012; Stern, 2004b). Over the last 20 years, a convergence of these approaches to the notions of embryonic induction and morphogen activity has created a framework to think about the emergence of tissues and organs during embryogenesis focused on gene regulatory networks (GRNs). In what often appears like a parallel universe stands morphogenesis, the way cells coordinate their activities to generate shapes. This is the realm of processes such as cell division, adhesion, polarization, and deformation, which, in cell ensembles, are translated into tissue flows, cell migration, cavitation, extension, and branching. These are excluded from the GRN framework, but may be elements of a set of what we suggest to calling cell regulatory networks (CRNs); GRNs specify cell fates, CRNs specify the organization of multicellular ensembles (Gorfinkiel and Martinez Arias, 2021). The omission of CRNs from the GRN framework is not deliberate, but rather reflects a new casting of an old problem: we do not yet know how to deal with the higher order activities of cells, i.e., the outputs of the integrated activity of the cytoskeleton and adhesion systems as in individual and collective cell movements, nor how to relate them to gene activity (Shook and Keller, 2008).

The notion that development results from an interaction between two different operative systems was foreshadowed by Alan Turing in his seminal paper on "the chemical basis of morphogenesis" (Turing, 1952), in which he states that "the description of the state [of a biological system] consists of two parts, the mechanical and the chemical. The mechanical part of the state describes the positions, masses, velocities and elastic properties of the cells, and the forces between them… The chemical part…is given as the chemical composition of each separate cell…" The statement has a corollary, namely, "The interdependence of the chemical and mechanical data

adds enormously to the difficulty [of understanding the system] and attention will therefore be confined, so far as is possible, to cases where these can be separated.'' We shall refer to this statement as the ''Turing conjecture'' and will use it to emphasize the importance of understanding interactions between GRNs (chemical) and CRNs (mechanical) to gain a complete picture of how the vertebrate body plan emerges. Turing began to address the problem with a theoretical analysis of the chemical component, isolated from the mechanical one, and the recent work on GRNs represents an extension of this. However, as we have pointed out, GRNs do not explain morphogenesis, a process that is the realm of cells rather than that of genes. Efforts are being made to bridge the two (Collinet and Lecuit, 2021; Shook and Keller, 2008), but our understanding of CRNs is very preliminary, largely because while we know their components, we have not yet thought of how to assemble them into networks and circuits.

The study of morphogenesis has traditionally been based on experimental embryology, an approach that, by explanting tissues and culturing them *ex vivo*, or through heterotopic transplantation of defined regions of the embryo, elucidates specific tissue interactions to determine the relationship between intercellular communication and pattern formation (Hamburger, 1988). This work is currently undergoing a renaissance through the development of novel imaging and computational techniques, but still requires the use of embryos of appropriate size and accessibility to perform microsurgical manipulations. A new experimental paradigm provided by mammalian pluripotent stem cells (PSCs) allows for the emergence of multicellular interactions to be observed as cells undergo differentiation from a common pluripotent state and is particularly useful for asking questions about these events in embryos for which direct experimental manipulation has been challenging (Moris et al., 2020b). Taking advantage of the potential of these cells to generate most, if not all, cell types of an organism *ex vivo*, it is possible to ask how much of an organism, understood as a collective of cell types, tissues, and organs, can be recapitulated without having undergone any prior instruction from signals in the embryo.

Here, we focus on gastrulation and the emergence of the vertebrate body to survey these studies in the context of experimental embryology. We suggest that experiments with PSCs uncover principles of multicellular organizations, which are hidden in embryos owing to the interdependence of chemical and mechanical signals in morphogenesis. We shall discuss how these new experimental systems begin to untangle the two components and will argue that the organization of PSCs over time reveals how conserved gastrulation processes can lead to a common morphology when released from the geometrical constraints imposed on whole embryos *in vivo*. We suggest that species-specific gastrulation movements can be considered as a conserved set of gene regulatory interactions molded by a more flexible morphogenetic driver based on multicellular interactions and is dependent on geometry and mechanics. The importance of considering the role of mechanics in gastrulation has been known for some time (Keller et al., 2003), and has recently come to the fore through the discovery of multiple mechanisms of mechanotransduction (Collinet and Lecuit, 2021; Gross et al., 2017). Here, we focus on the need to understand how feedback between GRNs and CRNs operates at the whole embryo level and emphasize its central importance in laying down the body plan.

GASTRULATION: THE EPIBLAST AS A HARMONIOUS EQUIPOTENTIAL SYSTEM

Gastrulation provides a prime example of the integration of chemical and mechanical components of living systems in that the patterning events associated with this process require a complex interplay of cell movements and cell-fate decision making. However, the common view of gastrulation primarily as a stage of development at which the germ layers are established, does not highlight this important feature. This notion of gastrulation is challenged by the observation that, in some organisms, e.g., in amphibia and fish, germ layer specification precedes the morphogenetic movements associated with gastrulation. Furthermore, the notion of germ layer is a generalization from the XIX century, which does not stand the test of detailed observation (de Beer, 1951; Oppenheimer, 1940). One example that has drawn some attention recently is the origin of the spinal cord from neuromesodermal progenitors (NMPs), a shared primordium with the paraxial mesoderm rather than the ectoderm (Cambray and Wilson, 2002, 2007) (Catala et al., 1995), but there are others, in particular the extreme examples of developmental homoplasy, convergence onto a differentiated cell type from different germ layer origin, e.g., PNS and bone/cartilage from neural crest, ectoderm, and mesoderm (Graham, 2010). In addition, hallmarks of gastrulation persist beyond the ''gastrulation stage,'' for example, during axial extension in the form of the chordoneural hinge (Cambray and Wilson, 2002; Gont et al., 1993) and, particularly during the primitive streak in amniotes, which creates some confusion in the literature as to when gastrulation ends. For these reasons, we use the term gastrulation here to describe how multiaxial structures are formed and patterned concomitantly with the onset of embryo elongation along an anteroposterior axis.

These arguments lead us to suggest that rather than a *stage* associated with the emergence of germ layers, gastrulation should be viewed as a *process* whereby the embryo acquires a system of coordinates to organize and position the primordia for the different tissues and organs. In vertebrates, this process starts from a broad range of conditions, in terms of egg size, composition, and the range of extraembryonic tissues that must also develop alongside the embryos to achieve successful development of a complete organism. Nevertheless, there exists common morphogenetic events associated with gastrulation that can be observed in multiple model organisms, albeit played out over divergent topologies (Figure 1). There is a tendency of cells to converge toward the embryonic midline, and extend along the anterior-posterior body axis—a transition from an epithelial-to-mesenchymal state and the concomitant organization of germ layers and their derivatives. There are also tissue-level variations associated with these events as to whether the cells involute or ingress. Furthermore, the output of gastrulation in each major group of vertebrates leads to the generation of a common body plan at the phylotypic stage (Slack et al., 1993) (Figure 1). This view of gastrulation raises a number of questions, in particular the question of how GRNs and CRNs act together to

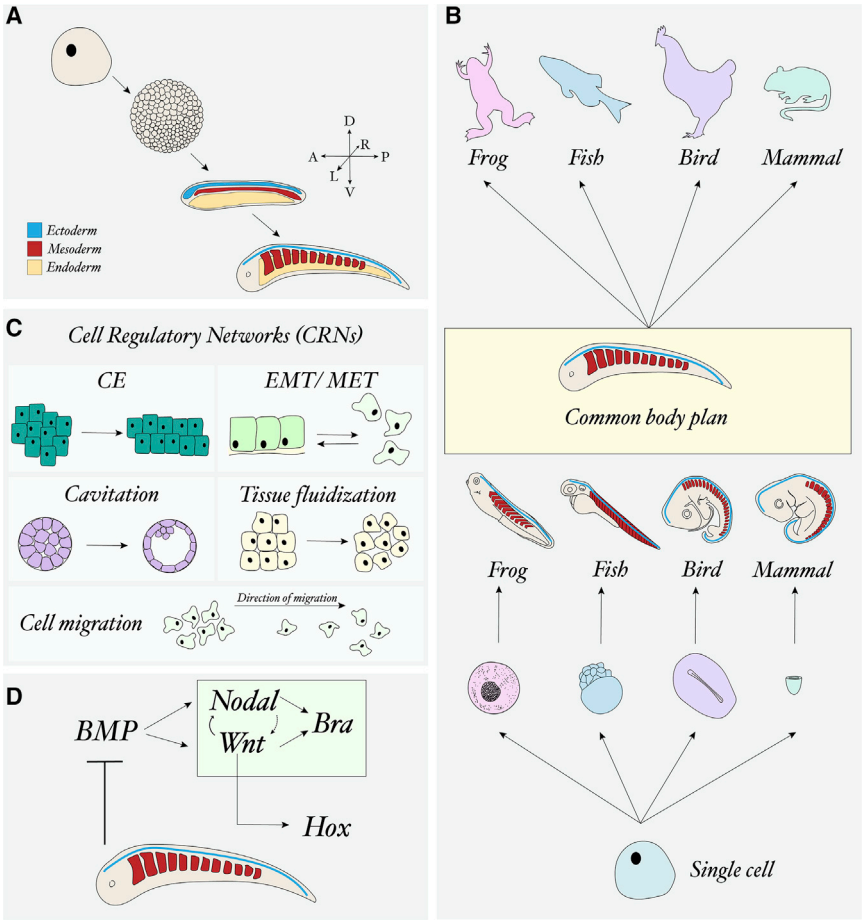

Figure 1. Elements of gastrulation

(A) Fundamental operation that transforms a cellular aggregate into an elongated structure with a multiaxial organization that leads to an archetypal body plan.

(B) Early development and morphogenesis of model organisms. Although all have different organizations as the output of the proliferation of the zygote that leads to different modes of gastrulation, this process reveals a conserved body plan that corresponds to the archetypal one.

(C) Elements of cell regulatory networks (CRNs).

(D) Fundamental GRNs driving the laying down of the body plan in vertebrate embryos.

drive pattern emergence during the morphogenetic movements that lead to the generation of a conserved body plan.

As a process, gastrulation illustrates the relationship between GRNs and CRNs (Shook and Keller, 2008). For example, the organization of the germ layers in anamniotes before gastrulation and the precise choreography that ensues suggest that transcription factors play a role in laying down a basis for the wholesale cell movements, such as coordinated cell intercalation, involution, and ingression. However, as a consequence of these movements, cells update their gene expression states as they move into alternate signaling domains, thus creating a constructive feedback between the activity of GRNs and morphogenetic processes (Busby and Steventon, 2020). This is clearly seen in amniote embryos, where gastrulation is fueled by the primitive streak, a sequence of EMT movements from the epiblast that progressively lays down the AP axis of the embryo. At the onset of gastrulation, the epiblast has signaling centers that act as references for cell fate assignments and, as the streak progresses, ingressing cells express genes associated with specific lineages in a position dependent manner, creating patterns that are elaborated as cells move through the streak in a ventral to dorsal pattern (Stern, 2004a; Tam and Gad, 2004). These patterns of fate assignment are thought to result from cells reading relative concentrations of the signaling molecules Nodal, BMP, and Wnt (Arnold and Robertson, 2009; Tam and Gad, 2004). Thus, the activity of CRNs feeds back to GRNs, as morphogenetic movements change the positioning of cells relative to these signals. Concomitant with these dynamics, there are alterations in mechanochemical signaling that are likely to also transmit additional information to the activity of GRNs as cells make their move through the early embryo (Collinet and Lecuit, 2021).

The manner in which GRNs and CRNs interact to guide the emergence of the body plan during early development underpins the regulative nature of multicellular development first revealed by H. Driesch in his work with sea urchin embryos. In these experiments, individual blastomeres were isolated at two or four cell stages and shown to give rise to one embryo each that was about half the size of the original organism (Sander, 1992). The same observation was made later in other animals, including mice (although in this case, there was size regulation), and might underlie the generation of some twins in humans (Casser et al., 2019; Martinez Arias et al., 2013). On the basis of this, Driesch referred to the early embryo as a "harmonious equipotential system," implicating the existence of regulative totipotency early in development as well as its ability to produce a size-independent proportionate outcome even after injury (Driesch, 1908; Hamburger, 1988). The regulative ability of the embryo continues through to the gastrulation stages of development, as demonstrated in experiments involving experimentally removing or bisecting the "organizer," a group of cells that, early in development, act as a source of signals to pattern both the anterior-posterior and dorsal-ventral body axis (Martinez Arias and Steventon, 2018) (Figure 2). Experiments on chicken embryos show that when the gastrula organizer is eliminated, it reforms completely and provides function (Joubin and Stern, 1999; Psychoyos and Stern, 1996). More dramatically, bisection of *Xenopus* embryos through the organizer region leads to the disappearance of the organizer from the original position and its re-appearance in a new position in each of the halves, where it will participate in the development of complete embryos, albeit of smaller size (Moriyama and De Robertis, 2018) (Figure 2). The regulative ability of the gastrula organizer is taken to the extreme in killifish embryos

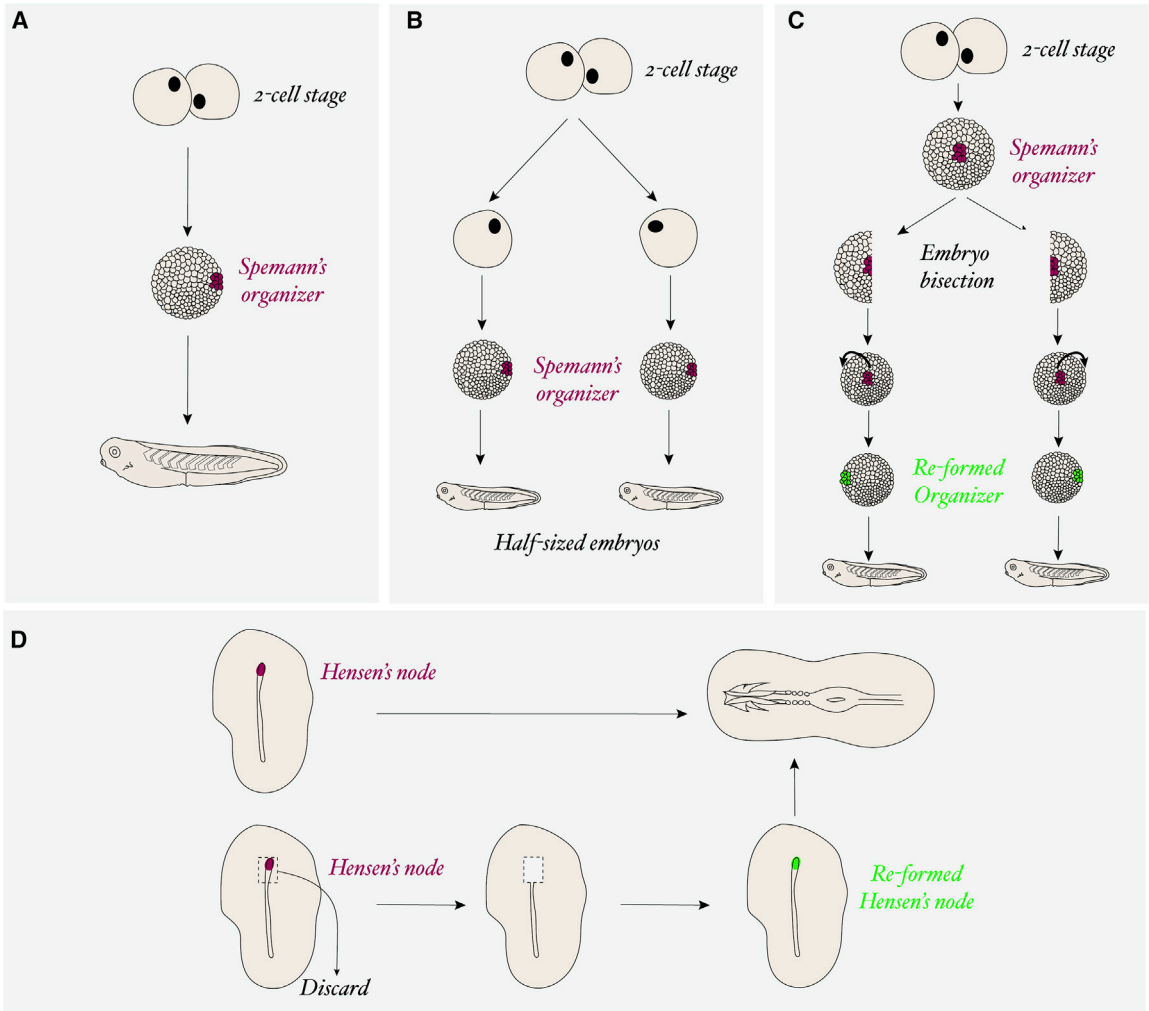

Figure 2. Regulative processes during gastrulation

(A) In *Xenopus*, gastrulation is led by the activity of the organizer (indicated), eventually leading to a tadpole.

(B) Separation of blastomeres at the two blastomere stage leads to two embryos, each with its own organizer, that will give rise to two tadpoles, half the size of the normal ones.

(C) Bisection of the blastula before organizer formation along the midline, i.e., along the region where the organizer should form, leads to a relocalization of the organizer to a new position, 90° from the original one, in each of the halves and to two smaller tadpoles as indicated (Moriyama and De Robertis, 2018).

(D) In chicken blastocysts, axial organization is led by the primitive streak that will form the node at its anterior tip. Ablation of the node leads to its regeneration and to a normal chick (Joubin and Stern, 1999).

that naturally undergo a dissociation-reaggregation event at gastrula stages within their life cycle (Naumann and Englert, 2018). Furthermore, in these embryos, the body plan emerges without evidence of a blastopore or a primitive streak, though involving cell rearrangements. In all these cases, patterns occur from a coordination of cell rearrangements (Abitua et al., 2021; Pereiro et al., 2017). These observations show that Driesch's "harmonious equipotential system" lasts until early gastrulation and that, as predicted by the Turing conjecture, the interactions between chemical and mechanical are the essence of pattern regulation.

In addition to regulation upon removal of cells, the degree to which tissues can self-assemble complex structures has been explored in explants of specific tissue regions. In a series of classical experiments, patterned structures have been shown to emerge from animal cap explants of *Xenopus* embryos when allowed to develop in an unconstrained manner in culture. This includes the subdivision of neural territories within neuralized animal caps (Lamb and Harland, 1995) and the derivation of multiple well-patterned mesodermal derivatives upon exposure to activin (Thomsen et al., 1990). Dramatically, a series of experiments from the Asashima group has shown how a multitude of organ rudiments can be generated from activin-treated animal caps with a minimal alteration in the starting conditions of culture, e.g., pronephric duct, pancreas, and cardiac tissue (Asashima et al., 2008). A part of this structural complexity is likely due to pre-existing patterns within animal cells prior to explanting, with different regions responding differently to activin treatment because they are "prepatterned" to do so (Sokol and Melton, 1991). However, experiments where animal caps are dissociated and reaggregated to disrupt the influence of these initial prepatterning events show that patterned cell differentiation can still occur (Green et al., 2004; Grunz and Tacke, 1989;

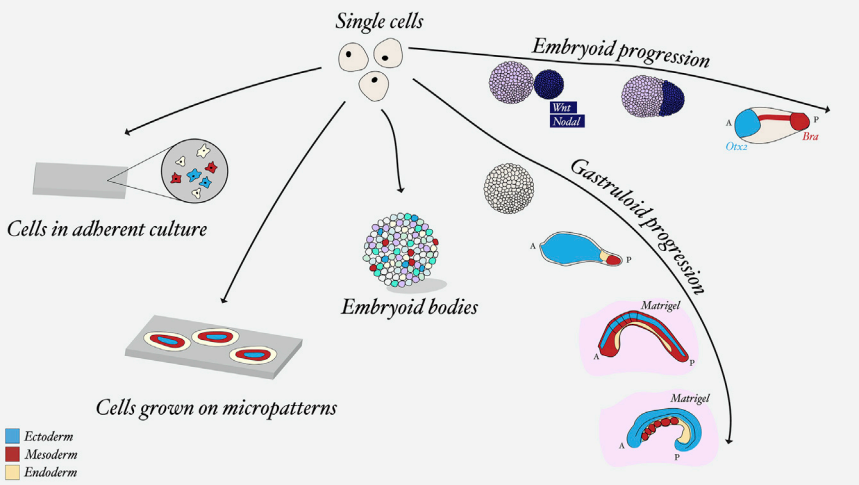

Figure 3. Examples of pluripotent stem cell (PSC) models of mammalian embryogenesis
From left to right: adherent culture differentiation, micropatterns, embryoid bodies, gastruloids, and patterned gastruloids. For details see text.

Kuroda et al., 1999; Smith et al., 1989; Wilson and Melton, 1994). In all cases, however, the initial inducing signal is added to the explant in a uniform manner, meaning that subsequent differentiation and patterning will rely to a large extent on the individual interactions between cells as a downstream event of the initial inductive response and not as a direct response to the signal. Understanding the interplay between cell movements and cell fate decisions in such scenarios remains an important question for fully understanding pattern formation in multicellular systems.

Embryonic stem cells and GRNs

PSCs are clonal derivatives of mammalian blastocysts (embryonic stem cells, ESCs), postimplantation epiblasts (epiblast stem cells, EpiSCs), or reprogrammed somatic cells (induced pluripotent stem cells, iPSCs) that have the potential to give rise to all cell types of an organism in culture; when placed back into a blastocyst, they integrate with and contribute to the developing organism, including its germ line (Martello and Smith, 2014; Nichols and Smith, 2009; Weinberger et al., 2016). These cells have been used to generate many different cell types *in vitro*, e.g., cardiomyocytes, hematopoietic progenitors and derivatives, pancreatic insulin-producing cells, or neurons (Keller, 2005; Murry and Keller, 2008). In all cases, the differentiation processes are steered by nonspecific signals and follow sequences of cell states that recapitulate events in the embryo (Gadue et al., 2005), often with similar timescales and involving complex behaviors, such as oscillatory gene expression associated with somitogenesis (Chal et al., 2018), motorneuron differentiation (Rayon et al., 2020), or the sequential onset of *Hox* gene expression (Lippmann et al., 2015).

Studies in which PSCs have been used to investigate the specification of early embryonic lineages have revealed two major initial paths that cells can follow *in vitro* (Figure 3). One, usually associated with the suppression of Wnt, BMP, and Nodal signaling, leads to anterior neural cell fates (brain), and the second one, promoted by these signals, specifies the derivatives of the endoderm, the mesoderm, and the spinal cord. This reflects the situation in the embryo where the signaling landscape in the epiblast allows both processes to run simultaneously and coordinately (see Figure 1) (Rivera-Pérez and Hadjantonakis, 2014). In the case of cell types posterior to the hindbrain, an immediate and general response to the induction of differentiation *in vitro* is the expression of Brachyury (Bra), a transcription factor universally associated with gastrulation (Marcellini et al., 2003; Technau, 2001), and other genes associated with the primitive streak (Gadue et al., 2006; Tsakiridis et al., 2014), including cell types associated with the node (Edri et al., 2018b; Sharon et al., 2011). A close observation of PSC differentiation shows that cells expressing *Bra* undergo EMT and directed migratory activity, as they do in the embryo (Turner et al., 2014b). Furthermore, a subpopulation of these cells also express *Sox2* and are able to give rise to both neural and mesodermal, specifically paraxial mesoderm, derivatives (Gouti et al., 2014; Tsakiridis et al., 2014; Turner et al., 2014a) that are likely to correspond to neuromesodermal progenitors (Wilson et al., 2009). Thus, if we were to view gastrulation as a stage associated with *Bra* expression and EMT, we could construe the adherent culture differentiation of PSCs as a model for gastrulation. However, gastrulation is not only a sequence of cell states but, importantly, a choreography of cell movements in three dimensions that organizes proportionate cell populations to generate the primordia of tissues and organs. In adherent culture, differentiating cells are not only spatially disorganized but also do not yield the proportionate subpopulations observed *in vivo*. An example of this is the mesendoderm, a population of cells that can give rise to both endoderm and mesoderm and which can be easily found *in vitro* (Tada et al., 2005), but represents only a very small proportion of the gastrulating cells in the embryo (Probst et al., 2021).

There are additional significant differences between differentiation and cell fate assignments in adherent cultures and in embryos. An intriguing one concerns the path taken by PSCs to develop into particular fates. In contrast with the process in the embryo, where cells follow unique stereotyped paths, a similar fate can be reached *in vitro* through different paths, in a protocol-dependent manner, often with varying efficiencies. This has been highlighted in the paraxial mesoderm (Edri et al., 2018a) and in the spinal cord motorneurons (Briggs et al., 2017). In both cases, it appears that it is the extracellular signaling environment that modulates this path and even the timing of the process. For example, depending on the degree of Wnt/β-catenin signaling, the timing of motorneuron differentiation from PSCs can be reduced by half (Maury et al., 2015), and in a series of recent experiments, chimeric spinal cords between human PSC-derived neural progenitors and chicken embryos have demonstrated the role that interactions between extrinsic and intrinsic factors play in defining the timing of events

(Dady et al., 2021). Nonetheless, there is some evidence for a contribution to the timing from cell autonomous mechanisms associated with protein turnover and GRN activity (Rayon et al., 2020). In a dramatic example of this feature of *in vitro* development, functional motorneurons can be obtained from mouse ESCs in just 3 days, as opposed to the normal 7 or 8, after overexpression of three transcription factors, Ngn2, Isl1, and Lhx3 in the starting population (Mazzoni et al., 2013). The resulting motorneurons exhibit physiological characteristics of the embryonic ones, emphasizing the existence of intrinsic programs of gene expression driven by GRNS that are plastic. However, by and large, the outcome of the *in vitro* differentiation is either generic neurons or, as in the case of NMPs, variable mixtures of neurons with heterogeneous and heterochronic identities (Gouti et al., 2014; Lippmann et al., 2015). In an embryo, the development of motorneurons needs to be coordinated with axial elongation so that specific motorneurons arise for specific muscles and arise in the numbers that are needed. It is clear that adherent culture cannot reproduce this essential patterning process, which is also associated with the proportionate production of the different cell types.

When human PSCs were seeded on micropatterned adhesive substrates and induced to differentiate by exposure to BMP, which triggers gastrulation in mammalian embryos, different cell fates emerged in an organized and proportionate radially symmetric pattern, reproducibly (Figure 3) (Warmflash et al., 2014). The cell types have been interpreted as samples of the classical three germ layers—ectoderm, endoderm, and mesoderm—with the extraembryonic tissue outside (Warmflash et al., 2014) (Minn et al., 2020). Changing the input signals alters the proportion and identity of the cell types in a manner that is consistent with the genetics of the embryo (Martyn et al., 2017; Morgani et al., 2018). The patterns have been interpreted as reflecting gastrulation (Martyn et al., 2019), particularly as cells that express *Bra* and attempt to move directionally within the patterned structure. However, the physical constraints limit the ability of the cells to move and to organize themselves as they do in the embryo, and the system does not evolve nor undergo any morphogenesis. It is likely that the movement simply reflects that cells with Bra expression promote cell movement (Turner et al., 2014b; Wilson et al., 1995). Nonetheless, these studies have allowed a start to dissect the role that the interface of cell biology, geometry, and mechanics play in cell fate assignments (Muncie et al., 2020; Xue et al., 2018) (Etoc et al., 2016; Knight et al., 2018; Nemashkalo et al., 2017; Xue et al., 2018).

Notwithstanding the differences between these systems in culture and the embryo, a surprising observation from these studies is that programs of gene expression are executed rather faithfully without associated morphogenetic events, e.g., a primitive streak or a neural tube. In one instance, for example, the sequence of gene expression that leads to human somitogenesis has been recapitulated from iPSCs and follows the same developmental sequence that it does *in vivo*, including the periodic oscillations that are central to the generation of somites, but without any spatial organization or morphogenesis, (Diaz-Cuadros et al., 2020; Matsuda et al., 2020). Even the emergence of cells with the signature and even the function of the organizer does not appear to require any morphogenesis in these systems (Edri et al., 2018b; Martyn et al., 2017; Sharon et al., 2011).

These experiments reflect several aspects of gastrulation, but importantly reveal that, as suggested by Turing, it is possible to separate the activity of the GRNs (chemical) from the morphogenesis (mechanical). The appearance of some order and proportionality in the micropatterns begins to suggest that the physical constraints of the system play an important role in its organization. However, the central element of gastrulation, the pivotal element that organizes the body plan—the multiaxial coordinate system, is missing in these systems.

GASTRULOIDS AND GASTRULATION

When indeterminate numbers of PSCs are cultured in nonadherent conditions, they aggregate into three-dimensional structures that, in the presence of serum, differentiate into randomly distributed collections of cell types in the form of embryoid bodies (EBs) (Brickman and Serup, 2017). Exposure of EBs to Wnt signaling as they differentiate, elicits a symmetry breaking event reflected in a variable pattern of Bra expression at one end of the aggregate (Boxman et al., 2016; ten Berge et al., 2008). Although these structures do not develop any defined patterns, they reveal a potential for organization, which is realized when they are differentiated in the presence of inhibitors of Nodal and BMP signaling, within the confines of a mechanochemical environment such as Matrigel. Under these conditions, over a time course of several days or weeks in the case of human PSCs, they differentiate into highly organized structures resembling regions of the brain, in particular the forebrain and associated structures such as neocortex and optic cups (Eiraku et al., 2011; Lancaster et al., 2013). The occurrence of these events is haphazard, particularly in the case of the cortex, but specimens can be found in cultures that reproduce much of the organization of the embryonic cortex and can be used to model physiological situations (Chiaradia and Lancaster, 2020; Qian et al., 2016; Velasco et al., 2020). Culturing single cells under more defined conditions and exposing them to Retinoic Acid, leads to cysts containing neurons with spinal cord identity (Meinhardt et al., 2014; Ranga et al., 2016). Surprisingly, in a small fraction of the cases, the cysts become patterned in a manner that resembles the DV organization of the neural tube, despite all signals being homogeneously distributed (Duval et al., 2019; Zheng et al., 2019).

When small, defined, numbers of PSCs are placed in suspension under defined culture conditions, they aggregate and, after being pulsed with Chiron, an agonist of Wnt signaling, they break symmetry, grow, and develop an elongated structure with a pole of Bra expression. After a few days in culture, the structure exhibits localized patterns of gene expression that outline the mammalian body plan, although they lack a brain and extraembryonic tissues (Figure 3) (Beccari et al., 2018; Turner et al., 2017a; van den Brink et al., 2014). These structures, called "gastruloids," have been studied in some detail with mouse PSCs (Veenvliet and Herrmann, 2021), but have also been developed from human PSCs (Marikawa et al., 2020; Moris et al., 2020a).

After 7 days in culture, mouse gastruloids exhibit a multiaxial organization, which resembles that of an embryo at E9.0. A pole of *Bra*-expressing cells fuels elongation and differentiation

into a mesoderm and spinal cord, mimicking the tail bud. At the opposite end, there is a cardiac primordium and cells expressing markers of ectodermal placodes, branchial arches, and a hemogenic tissue (van den Brink et al., 2020). Most remarkably, the *Hox* gene clusters are expressed on a schedule and with the characteristic nested organization of the embryo (Beccari et al., 2018). The analysis of the gene expression profile during gastruloid development reveals that, upon application of Wnt signaling, the aggregate expresses, genes associated with gastrulation in order, including the transition of *Cdh1* to *Cdh2* in mesodermal genes, the maintenance of Cdh1 in the endoderm, and even the appearance of primordial germ cells (Beccari et al., 2018; Rossi et al., 2021; Turner et al., 2017b; van den Brink et al., 2020; Veenvliet et al., 2020). Furthermore, there is a dynamic spatial organization of gene expression concomitant with the elongation of the cell ensembles (Beccari et al., 2018; Hashmi et al., 2020; Rossi et al., 2021; Turner et al., 2014a; Vianello and Lutolf, 2020). This degree of organization is surprising, as gastruloids lack extraembryonic tissues that have been shown to drive the axial organization of the embryo (Rossant and Tam, 2009; Takaoka and Hamada, 2012), and are also devoid of a primitive streak or a blastopore, which are structures closely associated with gastrulation. Thus, gastruloids reveal an intrinsic self-organizing activity that must be active during early embryogenesis.

The lack of anterior neural structures in gastruloids is likely due to the high levels of Wnt signaling applied for their development, which, in the embryo, is known to suppress the brain and head development (Arkell et al., 2013; Arkell and Tam, 2012). However, culture of PSCs into gastruloids with extraembryonic elements (primitive endoderm or trophectoderm) recovers these fates, even with exposure to Wnt signaling (Bérenger-Currias et al., 2020; Girgin et al., 2021). An examination of these structures reveals that, in contrast with PSC-only gastruloids, the aggregates develop an epithelial organization and that this appears to alter the interpretation of the signals by the cells (Girgin et al., 2021).

These observations highlight the importance of the interactions between the activity of the GRNs and the mechanochemical environment of the cells and, more significantly, of the groups of cells. Not only can the mechanical environment have an impact on cell biology and the programs of gene expression but the cellular organization can also alter the way cells perceive signals and, in that manner, influence the programs of gene expression. The results of these interactions also raise the question of how changing boundary conditions alter the implementation of differentiation programs in cell populations. For example, the difference between a collection of neurons and a brain organoid is the initial boundary conditions.

THE TURING CONJECTURE *IN VITRO*

Gastruloids exhibit a high degree of cell-type organization, as represented by gene expression, but have little or no morphogenesis beyond the initial elongation and subsequent extension of this pole. Cell types, particularly in the anterior part, are intermingled and disorganized. For example, in the spinal cord region, it is possible to identify several motorneuron progenitors, which are not organized in a dorsoventral pattern (Beccari et al., 2018). Strikingly, the mesoderm in the elongating domain reveals a partitioning of the paraxial mesoderm into proportionate gene expression territories that copy the comparable region in the embryo. At the posterior end, a group of cells express *Bra* and give rise to a sequence of gene expression domains. From posterior to anterior there exists a series of expression domians marked in turn by *Tbx6* (paraxial mesoderm), a narrow region expressing *Ripply2* and *Mesp2* and finally, a growing domain expressing genes associated with somitogenesis, e.g., *uncx4.1*, *Tcf15*, and *Tbx16*. However, there are neither different cellular morphologies nor multicellular organizations, and, what is more obvious, the epithelial blocks that characterize somites are absent (Beccari et al., 2018; van den Brink et al., 2020; Veenvliet et al., 2020). This observation confirms the results from differentiation in adherent cultures that GRNs can operate in the absence of morphogenesis but also extends it and shows that, under free boundary conditions, GRNs cannot only organize territories, as they do in micropatterns, but they can also organize and evolve with regard to an emergent coordinate system.

The organization of gene expression domains in gastruloids reveals a partial disconnect between the genetic (GRNs) and morphogenetic (CRNs) blueprints that are hinted at in adherent culture differentiation but are made obvious because of their three-dimensional organization. This result is unexpected as it reveals the autonomy of genetic programs that can organize cell populations in space without concomitant morphogenesis, thus opening avenues for the experimental exploration of the interaction between GRNs and CRNs. The observation provides an experimental vindication of the Turing conjecture that the integration of the mechanical and chemical elements of biological systems is central to the emergence of an embryo. It is interesting that the separation of the two components, which he used for theoretical tractability, might be an element of biological systems. Gastruloids reflect the potential of the chemical component and provide an experimental system to explore the link between the chemical and the mechanical components. This possibility is supported by the observation that embedding gastruloids in Matrigel at a precise time of their development elicits segmentation and epithelialized somite-like structures within (van den Brink et al., 2020; Veenvliet et al., 2020). This is associated with a dramatic change in the organization of gene expression—whereas in the absence of Matrigel, there is a continuous growing domain, a periodic pattern of somite specific genes emerges within the domain of somitic gene expression (van den Brink et al., 2020) that, in the presence of a neural tube, is transformed into epithelial structures with many of the features of somites, including schlerotome and dermomyotome (Veenvliet et al., 2020). The analysis of differences in gene expression between embedded and nonembedded gastruloids reveals that Matrigel changes the expression of ECM-related molecules, in particular integrins, and that this appears to be crucial in triggering morphogenesis.

This observation provides evidence for the notion that morphogenesis is latent in, but independent of, the activity of the GRNs and that it can be elicited and should allow the investigation of this phenomenon. Interactions between mechanical and chemical components can also be observed in the events that take place at the anterior end of the gastruloids, where modifications of the protocols trigger the formation and morphogenesis of a cardiac primordium (Rossi et al., 2021). Furthermore,

the single-cell analysis reveals the presence of cells with placodal and branchial arch identities in the anterior region. However, there is no structural organization suggesting that these cells might lack mechanical input at the right time. In some instances, morphogenesis is intrinsic to the genetic program. For example, gastruloids are not epithelial but the activation of the program of endodermal gene expression leads to the formation of an epithelium that can assemble into a tube (Vianello and Lutolf, 2019).

Conservation in the regulative processes of gastrulation has allowed for the emergence of common morphologies *in vitro*

The observation that gastruloids can self-assemble embryonic axes and pattern primordia for different tissues and organs in a reproducible manner is even more striking when comparing the output of similar experiments from different species. Gastruloids from mouse and human ESCs produce a similar overall structure, despite their epiblasts having different geometrical features—mouse a cylinder and human a disc (Moris et al., 2020a). In the elongating region, markers of the primitive streak are expressed together with high signal reporter activity for the canonical Wnt signaling pathway that opposes a region of high BMP activity within which cardiac mesoderm markers are expressed alongside multiple markers of ectodermal derivatives (Moris et al., 2020a). Whether this is a special feature of mammalian ESCs or a more general feature of PSCs in other organisms is difficult to test, as there are no equivalent embryonic stem cells—for example from anamniote embryos—to test with. However, using zebrafish embryos, it has been possible to explant embryonic cells away from the yolk at very early stages, prior to the onset of zygotic gene expression. When these cells are cultured as 3D aggregates, they go on to converge and extend along a single primary axis, forming gastruloid-like structures dubbed as "pescoids"(Fulton et al., 2020; Schauer et al., 2020) and exhibit a polarized arrangement of signals similar to gastruloids. Similar elongated structures emerge when animal cap explants are taken from zebrafish embryos previously injected with Nodal (Williams and Solnica-Krezel, 2020). These studies built on previous observations where similar explants were taken from the killifish embryo and were allowed to develop in culture to generate well-patterned structures (Oppenheimer, 1936).

Gastruloids and pescoids have very different starting points in terms of how the cells are derived, with cells obtained from embryonic stem cells in the former and primary cell culture in the latter. For pescoids, this means that cells have already been regionalized through the uptake of maternally derived patterning cues that can be observed as early as the 4-cell stage (Gore et al., 2005; Kelly et al., 2000; Schneider et al., 1996). However, prior to breaking morphological symmetry, cells rearrange extensively, yet still manage to polarize Nodal activity in a similar manner to what has been observed in gastruloids. Therefore, both display a similar set of starting conditions prior to elongation and axial patterning that include a single pole of (1) Nodal activity, (2) polarized canonical Wnt activity, and (3) high expression levels of the pan-mesodermal marker Brachyury. More recently, pescoids have also been generated from both surface and cavefish populations of *Astynax mexicanus* (Torres-Paz and Retaux, 2020) and similar structures emerge when animal caps of *Xenopus* embryos are exposed to a dose of activin sufficient to induce the specification of early mesoderm (Symes and Smith, 1987), even when the cells are disaggregated and mixed (Green et al., 2004). Thus, there seems to be a convergence of multicellular ensembles toward a similar elongated morphology, as long as a similar set of starting conditions are met.

The observation that such similar morphogenetic outcomes emerge from cells derived from different organisms is striking, particularly when considering the morphological differences that each species displays during gastrulation in intact organisms, and begs the question of what the underlying basis for morphological symmetry breaking is. One of the earliest events in the axial organization of vertebrate embryos is the restriction of Wnt signaling to one pole of a cellular aggregate and the ensuing elongation driven by the Wnt responsive cells (Petersen and Reddien, 2009). This results in a basic organization similar to that observed in gastruloids and pescoids, where Wnt signaling localized to the posterior pole of the aggregate, maybe through the self-organizing potential of the Wnt signaling network (Stückemann et al., 2017). Usually, Wnt signaling is associated with Brachyury expression, creating a blueprint associated with the vertebrate body plan (Martin and Kimelman, 2009; Martindale, 2005; Technau, 2001). The transient localization of BMP to the opposite pole, associated with the specification of the cardiac primordium, is another feature common to vertebrate embryos. Thus, we would suggest that the structure and signaling arrangement of gastruloids and pescoids reflect the behavior of the conserved signal and transcriptional regulatory networks of early vertebrate embryos and the tendency of Wnt signaling to promote elongation.

A second question relates to why similar structures emerge from aggregates of embryonic cells that normally display highly divergent gastrula morphologies *in vivo*. Similarities in organizational features between different species at comparable stages of development are often used to infer the description of an ancestral state (Martindale, 2005). This inference is based on the assumption that observed homologies between organisms reflect a common origin and are a product of a particular state being continually conserved through evolution of the multiple species in question. The fact that the gastruloid morphology can only be observed *in vitro* makes an argument about homology unfeasible, as it is inconceivable that selection could act on a developmental process that is only seen upon experimental manipulation. Instead, we propose that gastruloid experiments reveal how a conserved set of processes, when combined with a similar set of starting conditions, can lead to similar morphogenetic outcomes at the multicellular level when allowed to develop in an unconstrained manner.

We have previously outlined a number of conserved morphogenetic processes that are driven by multicellular interactions (CRNs) and are often associated with gastrulation across multiple species (Figure 1). These include the tendency of cells to converge toward the embryonic midline, and concomitantly extend along the anterior-posterior body axis. A second conserved process involves the transition from an epithelial-to-mesenchymal state at the onset of mesoderm formation. A third involves the progressive regionalization of cell types that is closely linked to cell movements, in that they result in the spatial separation of signal sources for known patterning molecules, as

discussed earlier. Comparative developmental biology has taught us that these elements are intricately linked and deeply conserved in evolution and are often associated with conserved GRNs. However, the relative timing at which these events take place, and their precise spatial configuration can vary greatly in a species-specific manner (Martinez Arias and Steventon, 2018), therefore revealing an inherent flexibility in gastrulation. Thus, as long as a pole of Nodal activity emerges alongside Brachyury and Wnt/beta-catenin signaling within a gastruloid, cells will begin to undertake similar cell behaviors, as observed *in vivo* but in an altered mechanical environment. The output of this is likely a set of morphogenetic movements that are altered compared to *in vivo* settings due to altered boundary conditions of the system.

Gastruloids self-organize a body plan, however, while they develop the main derivatives of the different germ layers, there is a variability in the organization and representation of the different primordia. In a recent variation of the gastruloid theme, inspired by earlier work in zebrafish (Xu et al., 2014), an aggregate was engineered with a group of ESCs where BMP has induced the expression of Wnt3 and Nodal, as opposed to another aggregate made up of naive ESCs (Figure 3) (Xu et al., 2021). This situation mimics the early gastrula, where, at the posterior pole, gastrulation is initiated by the emergence of a group of cells expressing Wnt3 and Nodal, and, anterior to it, there is a population of, still, pluripotent cells. Cells in the engineered gastruloids develop a series of polarized movements and, importantly, structures that are more organized and faithful to the embryo than in self-organized gastruloids. This observation underscores the role that the localization of signaling centers plays in the patterning of cell ensembles within embryos.

A further example of the influence of cell organization and boundary conditions on the initiation of symmetry breaking can be observed in the behavior of aggregates of mouse PSCs. In the case of gastruloids, the body plan emerges from nonepithelial cells, in the absence of extraembryonic tissues, without a need for BMP signaling and without a primitive streak (Beccari et al., 2018; Turner et al., 2017b). However, if the starting condition is an epithelium, conditions change, and now the initiation of symmetry breaking requires BMP, which is associated with a local EMT and with the initiation of a primitive streak-like structure (Girgin et al., 2021; Sozen et al., 2018). It is of interest that, in these structures, the primitive streak does not progress and a very truncated body plan emerges with a prevalence of anterior structures (Girgin et al., 2021). Recently, the reconstruction of the epiblast with elements of the three lineages generated a structure that approximated the primitive in embryo, although it did not go beyond the mid-stages of gastrulation (Amadei et al., 2021). This suggests that the organization of individual cells and their interactions play a very important role in the response to signals (Etoc et al., 2016). Another example of the influence of boundary conditions in morphogenesis is in somitogenesis in gastruloids, where boundary conditions are minimal as they are allowed to develop in free-floating media and are therefore unconstrained in any mechanical sense. The addition of such a constraint in the form of Matrigel then imposes a degree of such constraint, with dramatic consequences to the resulting tissue types and their patterning (Girgin et al., 2021; Veenvliet et al., 2020). However, when left to develop in an unconstrained manner, gastruloids and pescoids converge to a similar morphology, likely due to the self-organizing activity of Wnt signaling, which might drive the elongation of the aggregates. It is clear that Matrigel not only provides mechanical feedback, but it is likely that this feature plays a very important influence on its effects (Simian and Bissell, 2017).

MATERNAL-FETAL TRADE-OFF IMPACTS THE BOUNDARY CONDITIONS OF GASTRULATION *IN VIVO*

A conclusion from our proposal is that when considering embryos, a major component of the evolution of gastrulation must relate to variation in boundary conditions. This may be in the form of alterations in the extraembryonic tissues that surround and develop alongside the embryo. In addition, there is a large variation in starting conditions, as eggs come in many different sizes, largely due to the degree of yolk given to the early embryo, and the nature in which this is taken up by embryonic cells. In both cases, the evolution of alternate strategies for imparting nutritional supply to gastrula-stage embryos will greatly alter the mechanical constraints within which conserved gastrulation processes are acting out (Figure 4). Many of these differences are related to selective pressures acting elsewhere in the reproductive process. For example, anamniote embryos display a large variation in egg size as a trade-off exists between producing many eggs with a small amount of yolk given to each, or much fewer eggs for which a larger energy commitment is made to each individual. Another example lies in the transition toward viviparity—something that has occurred over 120 times and has been seen in reptiles, amphibians, sharks, rays, teleost fish, and mammals. At each alteration in reproductive strategy, the morphology of the gastrula and the corresponding set of cell behaviors driving gastrulation must also compensate, presumably leading to a large selective pressure on a system of pattern formation that is in itself able to adapt rapidly to such alterations.

The observed morphological variation observed during early developmental stages through to the completion of gastrulation underpins a pattern of species diversification that has been described as the developmental hourglass (Duboule, 1994) (Figure 4). In this view, the morphological diversity of these early stages is followed by a short period of increased similarity between embryos of different species as they develop a common body plan. This "phylotypic process" represents the thin constriction of an hourglass structure and occurs prior to the development of species-specific characteristics that mark an increasing divergence of embryonic forms through to the generation of adult forms (Duboule, 1994). The generation of more species-specific characteristics of development was anticipated early on by Karl Ernst von Baer in his "Laws of Embryology" (Abzhanov, 2013). In simple terms, this supposes that embryos of different species are likely to first develop general characteristics, and then increase in complexity as development continues. This is in part due to an increase in developmental constraints that act against the development of novel characteristics, as any change early on in development would have large consequences at later stages in the process. In this light, it is perhaps not all that surprising that a similar body plan emerges at those stages of development when the extraembryonic

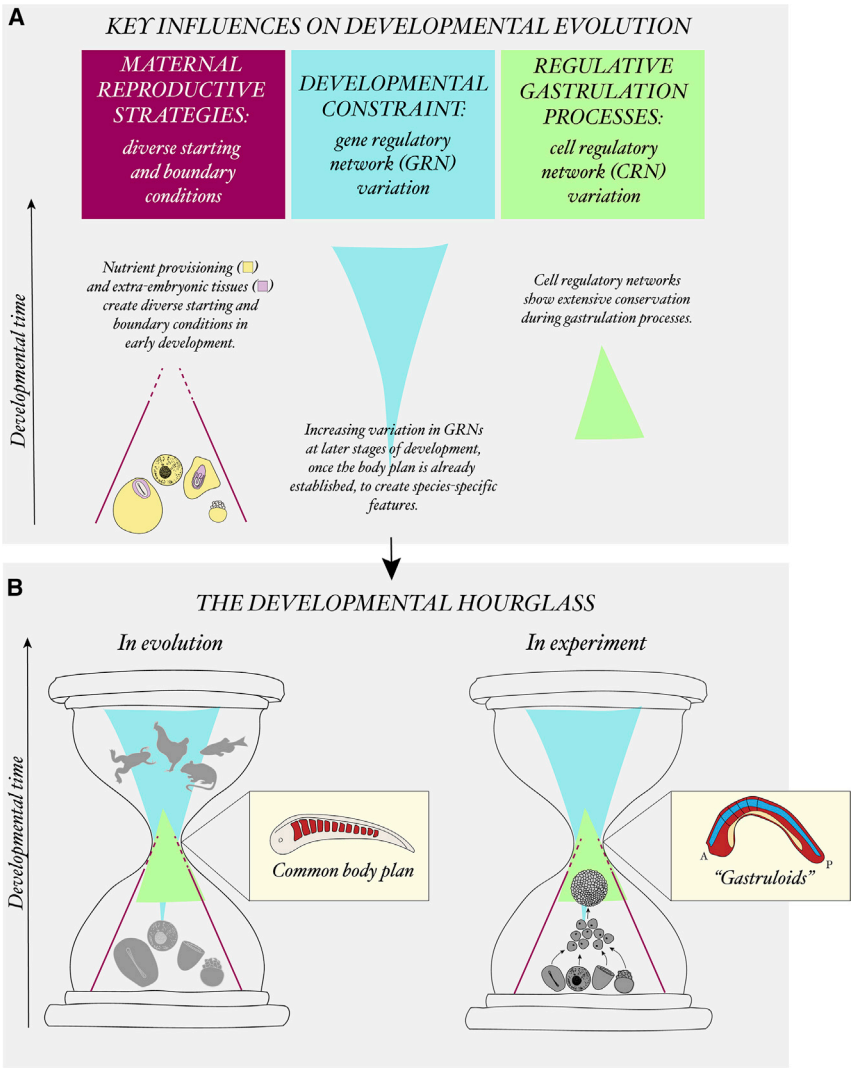

Figure 4. Key evolutionary developmental influences on the emergence of the developmental hourglass

The developmental hourglass describes a pattern of comparative morphologies that exhibit high divergence at the beginning of development, converge to a common body plan as a consequence of the phylotypic process, and then display increasing divergence again as species-specific adult characteristics emerge.

(A) We propose that this can be explained by a combination of three interlinked aspects of development that are underpinned by different selective pressures. In the first (purple), maternal reproductive strategies create diverse starting conditions in terms of egg size and nutrient content. This, together with a variation in the mechanical boundary conditions to gastrulation, generates a diversity in early embryo morphologies up until the point of the phylotypic process. In the second (cyan), developmental constraints are acting to conserve the function of gene regulatory networks (GRNs). As these interactions are used repeatedly through development, conservation is maintained at a maximum through early stages of development, before diverging to generate species-specific characteristics. Third (green), a set of conserved cell regulatory networks of gastrulation act to drive morphogenesis.

(B) In the evolution of gastrulation (left), the external boundary conditions to the embryo become released through the tailbud stages of development. Conserved gene and cell regulatory networks then allow for a common body plan to be generated prior to the generation of species-specific characteristics. Experiments in which aggregates of embryonic cells are allowed to develop *in vitro* from multiple species (right) can also lead to the emergence of a common morphology. Provided that opposing BMP and Nodal/Wnt signaling poles can be established, elongation allows for the spatial-temporal patterning of multiple axes through the deployment of conserved gene and cell regulatory networks.

structures imposing radically different gastrula morphologies have been fully externalized from the embryo proper.

We propose that the phylotypic process itself has not been developmentally constrained in an evo-devo sense, i.e., via the repeated conservation of developmental mechanisms at this stage above that of earlier stages. Instead, such a conservation has been acting throughout the proceeding gastrulation stages. It is merely a mechano-geometrical constraint that has been relaxed to allow for the observed hourglass structure upon reaching a conserved body plan (Figure 4). Therefore, von Baer might have been correct in supposing that there are developmental constraints happening to drive conservation during essential stages of development. However, this is happening in the context of conserving a highly regulative mechanism of multiaxial patterning that can allow for a concomitant variation in morphology. The main difference between embryos are the boundary conditions imposed to their pluripotent cells, and likely contributed significantly to the feedback between CRNs and GRNs that shapes the embryo (Figure 4). If boundary conditions are removed, as in the case of pescoids and gastruloids, the multicellular system reverts to a morphogenetic ground state, which appears to be associated with an elongated structure patterned by GRNs. Therefore, we suggest that what is unveiled in gastruloid and pescoid experiments is an underlying mechanism that is able to respond to alterations in boundary conditions and self-assemble multiaxial patterning through a wide range of morphologies. When all such boundary conditions are released in culture, a morphology emerges that is a product of such conserved gastrulation processes coupled with similar starting conditions.

Several questions remain as to how exactly pattern regulation is achieved, how is it that biological systems exhibit the size invariant proportionality of tissues and organs, and how is it that such a critical process as gastrulation is so robust to certain manipulations. This might be in an experimental context upon manipulation of the early embryo, in the generation of embryo-like structures *in vitro*, and through the evolution of gastrulation. The answers will most likely lie in the fact that gastrulation is a highly integrated process that relies on continued information exchange across many levels of biological organization. Within

each cell, GRNs drive cells to differentiate and adopt specific behaviors. Their coordination at the level of cell populations then results in the progressive deformation of tissues during morphogenesis. Tissues then interact via inductive interactions to build multitissue structures, which, in turn, form interacting organs of the animal body. Concomitant with the causal relationships outlined above is a reversal of this information flow to confer changes to GRN activity in response to alterations in the size and shape of tissues. In other words, downward causation must exist from higher levels of biological organization (i.e., at the whole organism or multitissue level) to lower levels (at the level of gene expression within single cells). Such a mechanism for downward causation in development has been termed "tissue tectonics" in reference to the multitissue repositioning that lies at the heart of patterning processes, such as body plan establishment during gastrulation (Blanchard et al., 2009; Busby and Steventon, 2020).

Understanding the mechanisms of multiscale information flow during development is at the heart of what we have termed here as "Turing's conjecture" and represents one of the most important questions facing modern developmental biology. It has a huge potential impact in terms of influencing the bioengineering of embryo-like structures and organoids *in vitro*. In selecting the appropriate control parameters with which to alter boundary conditions and engineer multicellular systems and embryo-like structures *in vitro*, it makes sense to take inspiration from comparative developmental biology to define which elements of development evolution has selected upon to generate diversity in form. We define such an approach as "evo-engineering," in reference to observations of how alterations in properties such as progenitor growth rates have generated diversity in the developmental mechanisms of axial elongation (Crespi and Semeniuk, 2004; Steventon and Martinez Arias, 2017). Where the limits of robustness are reached, it is likely the development and patterning of multicellular systems that can be manipulated and guided in a controllable manner. In many ways, this could not come at a better time, as the ability to obtain quantitative information from multiple levels of biological organization offers a real possibility for the development of predictive models for multicellular development.

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