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Modifying gastruloids to dissect mechanisms of tissue-specific induction

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How functional organisms arise from a single cell is a fundamental question in biology with direct relevance to understanding developmental defects and diseases. Dissecting developmental processes provides the basic, critical framework for understanding disease progression and treatment. Bottom-up approaches to recapitulate formation of various components of the embryo have been effective to probe symmetry-breaking, self-organisation, tissue patterning and morphogenesis. However, these studies have been mostly concerned with axial patterning, which is essentially longitudinal. Can these models generate the appendicular axes? If so, how far can self-organisation take these? Will experimentally induced organisers be required? This short review explores these questions, highlighting how minimal models are essential for understanding patterning and morphogenetic processes.

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Introduction

A key event in early embryogenesis is gastrulation, which transforms the embryo into a tri-layered structure, progressively allocating and positioning cells to the ectodermal, mesodermal and endodermal germ layers. This process defines the body-plan of the embryo, and once established, genetic, physical and chemical inputs gradually convert these cell types into defined tissues and organs over the timescale prescribed for the species, all precisely placed and interconnected. The mechanisms governing these events are progressively being revealed, partially through advances in obtaining and culturing mammalian embryos, but also by exploiting the rapidly advancing *in vitro* embryonic stem cell (ESC) systems, including 'stembryos' as well as organoids for specific tissues [1]. Such stembryo models for mammalian development include 'gastruloids' that use mouse or human ESCs [2,3], and the related trunk-like structures (TLSs) [4]. Both of these mimic aspects of early gastrulation such as symmetry-breaking, polarisation of gene expression, axial elongation, the emergence of three orthogonal axes and (if cultured in Matrigel), the development of somites (these and other systems will be discussed later). A significant advantage of using *in vitro* technologies such as these, in parallel with studies on embryos, allows us to dissect the mechanisms involved in mammalian embryonic patterning (as well as the general patterning principles for other species), lineage segregation and organ development from two complementary directions: 'bottom-up' (stembryo/organoid/stem cell) and 'top-down' (embryos/genetics/transgenics/knockouts). However, to appreciate fully the extent to which bottom-up models may be employed, it is necessary to define and understand the limits of these systems and how far they can be manipulated before their biological relevance is lost. In the context of early mammalian development, 'embryoid bodies (EBs)' were among the first *in vitro* tools used to investigate mechanisms underlying changes in epiblast morphology [5]. Most other models for early mammalian development are concerned with axial patterning and the emergence of tissues and cell types along the trunk of the embryo, essentially generating a patterned tube. It is not yet clear if these self-organised stembryo models permit the development of appendicular axes. Furthermore, is it possible to model innervation of mammalian organoids and appendages, or migration of key cell types such as the neural crest? In this short review, we will consider the current model systems that allow us to study early development, as well as their limitations. We propose some strategies in light of recent gastruloid experiments that could be employed to allow stembryo models to develop appendages. In the context of this review, which will focus on mammalian development, we propose that the aim of these *in vitro* organoid approaches is not to generate an entire *in vitro* embryo, but to model specific aspects of embryonic development incrementally. Taking this approach, we believe we can discover more about the inherent properties of the system by homing in on specific tissue types and their interactions.

Tissue-specific organoids: the embryo is more than the sum of its parts

The isolation and culture of ESCs, and the subsequent understanding of the precise factors regulating their pluripotency and differentiation, were essential steps for *in vitro* models of development. Grown as a monolayer, and without the influence of feeder cells, ESCs provide an attractive alternative to dissecting the signalling conditions required to maintain pluripotency in the early embryo [6–9], or to direct their differentiation to defined lineages and tissues [10,11]. As a monolayer, they are amenable to live time-lapse imaging as well as fixed, single-cell analysis (e.g. immunofluorescence, *in situ* hybridisation chain reaction, RNA-Fluorescent In Situ Hybridisation), which has permitted a more nuanced understanding of their heterogeneous properties, linking cell fate to reporter gene expression, morphology and cell movements [12–15]. As useful as this highly reduced and simplified method of studying cell-fate decisions during early development is, a necessary trade-off for this simplicity is that the 3D nature of the embryo cannot be reflected. As such, this motivated many groups to utilise something more akin to the 3D nature of organs and embryos, such as EBs ([16] and other 3D culture techniques, such as mechanically supported cultures [17–19] and the development of organoids (see [20,21] for comprehensive reviews). EBs constitute a relatively simple 3D model system, where ESCs are aggregated, mostly, using hanging-drops. However, these tend to be disorganised, variable in their phenotype and are not very tractable in terms of changing the signalling environment. Combining EBs with microfluidic approaches has the potential to improve this [22–25]. The 'organoid' field evolved from EB models, changing the initial aggregation phase from hanging-drops to U-bottom plates (with different low- or no-adhesion properties), where the conditions of their growth could be tightly controlled. By changing the chemical and physical (e.g. Matrigel, Geltrex) conditions, these aggregates of ESCs could self-organise into 3D structures resembling the architecture and patterning profile of various organs, depending on the culture conditions. Examples include optic cup, retina, kidney, limb-bud and pancreas [26–29], and have the potential for disease modelling, as has been shown with the Zika virus [30,31], and drug screening [32].

Although these organoid approaches permit experimentally tractable model systems to study tissue-specific patterning and morphogenesis, they omit a range of physiological constraints for simplicity (which in some cases can be advantageous). Indeed, the tissues/organs that are being mimicked *in vitro* are, by necessity, experimentally isolated, whereas *in vivo*, organs and tissues develop in concert with one another, and signals from developing tissues help shape and pattern both themselves and tissues nearby. For example, the embryonic heart and liver, whilst originating from distinct germ layers (mesoderm and endoderm, respectively), require reciprocal signalling between the two tissues for proper development [33–35] and the notochord (axial mesoderm) is essential for secreting signals that establish a morphogen gradient, such as Sonic Hedgehog (Shh) for proper dorsoventral patterning of the neural tube (neuroectoderm) [36]. Such reciprocal interactions are not present in isolated organoid models, and although these signalling inputs may be supplemented experimentally, the subtle signalling dynamics that occur between adjacent tissues are more difficult to mimic experimentally.

Post-implantation organoid models: stembryos

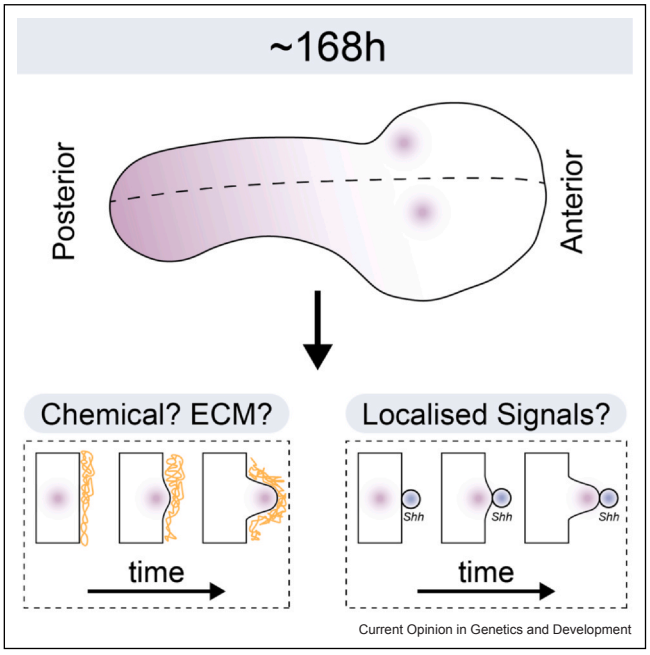
To allow a more inclusive model system that permits the development of multiple tissues simultaneously, several approaches have been established that span the developmental period encompassing pre-, peri- and post implantation [37]. Pre-implantation culture techniques for mammalian development encompass 'blastoids' that combine trophoblast stem cells and ESCs [38–42] and Embryonic-Trophoblast-Extra-embryonic endoderm (ETX) embryos that develop structures very similar to the mouse embryo between E5.5 and E7.5, by combining extraembryonic endoderm stem cells, trophoblast stem cells and ESCs [43] We direct the reader to other articles within this Issue that tackle such models directly.

Most of the recent models for post-implantation development can trace their origin back to seminal work [44]. Using hanging-drop culture, similar to traditional EBs, aggregation of embryonal carcinoma cells in medium containing dimethyl sulphoxide resulted in morphological and gene expression changes that mimicked the early stages of embryonic axial development: convergent extension/elongation of the axis, polarisation of gene expression and up-regulation of markers associated with early lineage commitment during gastrulation [44]. Interestingly, markers associated with the primitive streak were localised to one end of the elongating organoid. The critical difference between this approach and other EB studies was in the initial number of cells that were plated: ~200–300 cells per hanging drop [44]. Building on this but using U-bottom 96-well plates, the lab of Alfonso Martinez Arias aggregated small numbers of mouse ESCs and applied a pulse of a Wnt/β-catenin agonist between 48 and 72 h of culture [45,46]. The structures that formed broke symmetry, polarised the expression of primitive-streak markers to one side of the aggregate and also underwent axial elongation. Interestingly, when these structures, called '*gastruloids*' were analysed in detail, a remarkable degree of self-organisation was revealed, reminiscent of post-occipital development, with the gradual patterning of the three orthogonal axes (anteroposterior, dorsoventral and mediolateral), collinear *Hox* gene expression and organised derivatives of the three germ layers, including neural crest [47]. Furthermore,

gastruloids have a significant advantage in that they are highly reproducible between replicates [47]. However, despite the organisation of gene expression patterns, these aggregates were not able to develop proper structures such as somites, or neural/gut tubes, showing a disconnect between gene expression and structure, which indicated that additional signalling inputs may be required [2]. Indeed, Matrigel (or Geltrex), an ECM-rich medium, provided this additional input, and embedding gastruloids at ~96 h in low concentrations of Matrigel resulted in multiple features of somitogenesis being recapitulated [48], and the development of a midline neural tube flanked by pairs of somites (referred to as 'TLSs') [4]. For ethical reasons, gastrulation cannot be studied in human embryos. However, ESCs derived from blastocysts left over from assisted conception programmes, or induced pluripotent stem cells reprogrammed from human tissue, have been utilised to make gastruloids resembling those of the mouse, providing a means to model this largely inaccessible stage of development [3] and thereby identify human-specific mechanisms.

Gastruloids and their derivatives are rapidly becoming recognised as complementary models for early development, that are minimal in nature so as to remain tractable and understandable, yet close enough in mimicking the processes seen in embryonic development to remain directly relevant. To this end, modifications of the gastruloid culture conditions have permitted the refinement of model systems for cardiac development [49], haematopoiesis [50], anterior neural development [51] and elongating multi-lineage organoids (EMLOs), which generate neurons, neural crest cells and gut structures bearing molecular and functional resemblance to those derived *in vivo* [52].

Figure 1



Possible strategies for encouraging the development of gastruloid appendicular axes. HoxD9 gene expression in small, bilateral spots towards the anterior of the gastruloid may indicate the initiation of transcriptional programs that could give rise to the appendicular axes, providing the correct signalling environment is present (top). Global application of signalling molecules such as chemical modulators and specific Extra-Cellular Matrix components (possibly provided by Matrigel) could be read by cells receptive to these signals and over time, develop outgrowths (bottom left). Additionally, localised signalling molecules, applied in beads soaked with signalling factors that mimic the AER (e.g. Shh or other combinations of signals), may also be an additional strategy (bottom right). Combinations of these strategies may also be required.

From tubes to appendages: what signalling cues are missing?

Currently, the gastruloid is essentially a polarised, patterned cylinder, attributable to its extraordinary ability to self-organise within a simple signalling environment. Remarkably, *in situ* hybridisation for *Hoxd9*, a marker for limb-bud primordia in mouse, identified small spots on the flanks of gastruloids [2]. It is tempting to speculate that these may indicate regions in the gastruloids that may have the potential to respond to signals promoting outgrowth (Fig. 1). *In vivo*, such signals come from an *overlying* ectodermal layer that is recognised to be important for the formation of an apical ectodermal ridge (AER) essential for limb outgrowth. As reconstruction of an 'organiser region' in gastruloids has been used to enhance dorsoventral patterning [53], a similar strategy may therefore enable some development of lateral structures. To date, limb organoids have been generally derived from mouse embryos [54], however, attempts have been made to generate these directly from pluripotent stem cells [28]. In the case of the latter, this required a combination of chemical signalling and Matrigel embedding, which (providing the correct mechanochemical signals are added) could be a mechanism to facilitate outgrowth from gastruloids. The exciting prospect of attempting to induce limb outgrowth on gastruloids will lead to greater understanding of how chemical, mechanical and temporal mechanisms are orchestrated to create these essential appendages.

Conclusions

One argument for advancing the 'bottom-up' approach, which might appear attractive, is to be able to generate a whole-functioning embryo from its constituent components, satisfying Richard Feynman's conjecture: '*what I cannot create, I do not understand*'. However, reproduction of embryonic development *in toto* jettisons the potential to dissect the minimal requirements for interactions between adjacent-developing organs. Studying organs and tissues individually or in neighbourhood groups will allow us to examine the *minimal* requirements for

Box 1 Definition of terms commonly used to describe organoids.

Term	Definition	References
Gastruloid	3D aggregates of pluripotent stem cells that mimic embryonic axial development	Van den Brink et al. (2014) [46] Turner et al. (2017)[47] Beccari et al. (2018)[2] Moris et al. (2020) [3]
TLSs	Trunk-like structures: addition of Matrigel at late stages of development allows formation of somites flanking neural tube	Veenvliet et al. (2020) [4] Van den Brink et al. (2020) [48]
Embryonic-Trophoblast-Extra-embryonic endoderm(ETX) structures	Generation of structures that mimic some aspects of E5.5–E7.5 mouse embryos	Sozen et al (2018)[43]
EMLO	Elongating multi-lineage organoids	Olmsted and Paluh (2021) [52]
Stembryo	Portmanteau of stemcells and embryos, a term coined to bring together the *in vitro* models of early development	Veenvliet et al. (2021) [1]
Blastoid	3D aggregates combining trophoblast and ESCs to mimic blastocysts	Rivron et al. (2018)[38] Kagawa et al. (2021)[39] Liu, et al. (2021) [40] Yanagida et al. (2021) [41] Yu et al. (2021) [42]
Embryoid body (EB)	A technique that uses gravity-induced aggregation of ESCs, generally disorganised	Coucouvanis and Martin (1995) [5] Veenvliet et al. (2021) [1]
Feeder cells	A layer of mitotically inactivated cells (typically immortalised mouse embryonic fibroblasts)	Martin and Evans (1975) [58]
Directed differentiation	The process of controlling differentiation by the addition of specific signalling factors to guide pluripotent cells towards defined lineages	Multiple examples exist, but see: Neural: Ying and Smith (2003) [59] Primitive sreak: Gadue et al. (2006)[60] Turner et al. (2014)[13] Endoderm: Hashmi et al. (2022) [61] Mesoderm: Thomson et al. (2011) [62]

developmental processes, defining what is truly necessary for specific processes to be initiated, sustained and terminated. Once defined, we can incrementally add further complexity to examine whether our hypotheses generated through minimal models are upheld, or whether different strategies are employed by the embryo at key stages to integrate information from additional complex signalling environments (e.g. chemical, physical).

Finally, as developmental biologists, we are comfortable in comparing embryogenesis across different species to understand common strategies for patterning and morphogenesis. This is why we should also take into account bottom-up approaches from different species where possible, for example, comparing stembryo models from mouse, human and other vertebrates such as the zebrafish [55,56]. Indeed, zebrafish 'pescoids' show a remarkable similarity in patterning and morphogenesis to their mouse and human counterparts: when boundary conditions are removed, aggregating cells revert to a 'morphogenetic ground state' [57], providing a blank slate upon which we can examine what is required for morphogenesis of key organs and tissues when incrementally applied.

CRediT authorship contribution statement

Both authors contributed to Conceptualization, Funding acquisition and writing. Figures were designed and executed by DAT.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

We have no conflict of interest to declare.

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