3D gastruloids: a novel frontier in stem cell-based in vitro modeling of mammalian gastrulation

Susanne C. van den Brink1,*,@ and Alexander van Oudenaarden1,@

3D gastruloids, aggregates of embryonic stem cells that recapitulate key aspects of gastrula-stage embryos, have emerged as a powerful tool to study the early stages of mammalian post-implantation development in vitro. Owing to their tractable nature and the relative ease by which they can be generated in large numbers, 3D gastruloids provide an unparalleled opportunity to study normal and pathological embryogenesis from a bottom-up perspective and in a high-throughput manner. Here, we review how gastruloid models can be exploited to deepen our understanding of mammalian development. In addition, we discuss current limitations, potential clinical applications, and ethical implications of this emerging model system.

Stem cell-based embryo models: new tools to study mammalian gastrulation in vitro

The body plan of the developing embryo is laid down during an essential process, named ‘gastrulation’ (see Glossary), which specifies the three germ layers (endoderm, ectoderm, and mesoderm) (Box 1) and organizes those along the anterior–posterior, left–right, and dorsal–ventral body axes (Box 2) [1]. In mammals, gastrulation starts with the formation of a primitive streak; a ‘groove’ in the single-layered epiblast that marks the midline of the developing embryo. During gastrulation, mesodermal and endodermal progenitors in the epiblast ingress through the primitive streak via an epithelial-to-mesenchymal transition (EMT) that allows them to leave the epithelial epiblast and that results in the formation of the mesodermal and endodermal germ layers (see Figure I in Box 1).

The coordination between germ layer formation and body axis establishment that occurs during gastrulation ensures that all embryonic tissues and organs end up in the correct spatial location within the developing embryo. Defects in this coordination can result in severe congenital malformations, such as heart laterality defects [2] or vertebral abnormalities [3], highlighting the clinical relevance of reinforcing our understanding of these processes. However, due to the technical, ethical, and legal difficulties associated with experimentation on gastrula-stage mammalian embryos, our knowledge about body axis establishment and gastrulation, particularly in a human context, is currently limited.

The discovery that pluripotent stem cells can be coaxed into embryo-like structures (Box 3) resulted in the development of model systems, such as: (i) ETS/X embryos [4–6]; post-implantation amniotic sac embryoid (PASE) models [7,8]; human epiblast models [9]; micropatterns (which were recently relabeled as ‘2D gastruloids’) [10–15]; and gastruloids (which were recently relabeled as ‘3D gastruloids’ to avoid confusion with 2D gastruloids) [16–25], that allow in vitro studies into the processes that occur during mammalian gastrulation. Similar to other stem

Highlights

Gastruloids, 3D aggregates of embryonic stem cells that recapitulate the axial organization of post-implantation embryos, have emerged as a powerful tool to study embryonic development in vitro.

Recent improvements have resulted in more complex mouse 3D gastruloid models that generate brain, somite, neural tube, gut tube, and beating heart-like structures in vitro and led to the first human versions of the 3D gastruloid system.

Gastruloids provide fundamental new insights into embryonic development and may have applications for high-throughput studies into normal and pathological development in the near future.

Given the rapid expansion of the gastruloid field, there is an increasing need for debates about the ethical aspects of human gastruloid models and for methods that improve the reproducibility of this emerging model system.

© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
cell-based embryo models that recapitulate earlier developmental stages, these models have several key advantages over mammalian embryos: they can be generated in large numbers, allowing screens; they are easier to genetically modify than embryos since they bypass the need to create genetically modified animals; they allow studies into stages of human development that are difficult to access for research; and their tractable nature allows experimental approaches that cannot readily be applied to embryos [26–31].

In this review, we specifically zoom in on mammalian (mouse and human) 3D gastruloids, which we here define as: ‘Three dimensional aggregates of pluripotent stem cells that, under appropriate culture conditions, develop an embryo-like organization with three orthogonal axes and a precise distribution of the multiple derivatives of the three germ layers’. The 3D gastruloids generate most embryonic tissues with spatial and temporal precision in vitro [16,20,21,23,25,30,32], however, in contrast to mammalian embryos, they do not start from a single-layered epithelial state, they do not undergo any classical EMT-related mechanisms [22,33,34], and they do not form a primitive streak. These observations suggest that mammalian cells might be able to undergo a gastrulation-like process in an EMT- and primitive streak-independent manner and illustrate how observations made with gastruloids can challenge classical textbook paradigms of gastrulation. Illustrated by key examples, we review how 3D gastruloid models can be exploited to obtain fundamental new insights into the processes that occur during gastrulation. In addition, we discuss potential clinical applications, current limitations that should be considered, and ethical considerations associated with this model system.

3D gastruloids: origin and basic concepts

The first protocol to generate 3D gastruloids was established in the Martinez Arias laboratory in 2014 [16,17]. With this protocol, small numbers of mouse embryonic stem (ES) cells could be coaxed to robustly form elongating aggregates that form derivatives of all three germ layers with reference to clearly defined anterior–posterior, left–right, and dorsal–ventral body axes (Figure 1, Key figure) [16,25,32]. The innovations that led to the first gastruloids protocol were based on decades of in vitro stem cell work. In particular, the discovery that elongating mesodermal embryoid bodies (EBs) can be derived from mouse embryocarcinoma cells [35] and advances that resulted in protocols with which 2D mouse ES cell cultures could be induced to differentiate towards a neuromesodermal progenitor (NMP)-like fate in vitro [36,37].

In the years following their discovery, more advanced mouse 3D gastruloid models were developed that are able to generate brain [18,19], somite [20,21], neural tube [21], gut tube [21,22], and beating heart [23]-like structures (Figure 1A) [36]. In addition, recent advances resulted in human ES cell-based elongating mesodermal EBs [38], in human ES cell-based 3D gastruloids (Figure 1B) [24], in human ES and human induced pluripotent stem (iPS) cell-based neural tube-like structures [39], and in human iPS cell-based structures (‘elongating multi-lineage organized (EMLO) gastruloids’) that develop embryonic central and peripheral neurons that interact with co-developing trunk mesodermal tissues [40].

Recent single-cell RNA-sequencing (scRNA-seq)-based characterizations revealed that mouse 3D gastruloids generate most embryonic cell types, including NMP, endothelial, head mesenchymal, and primordial germ cell (PGC)-like cells in vitro [20,21,23,32]. Further spatial transcriptomics and imaging-based characterizations revealed that these cell types are formed in the correct location along their anterior–posterior axis [20,21,32] and showed that the spatial and temporal patterns of Hox gene expression that determine the anterior–posterior organization of embryos are recapitulated in mouse gastruloids [32]. Finally, live imaging experiments revealed that the periodic Notch-signaling oscillations that are thought to regulate the timing of somite

Glossary

Anterior–posterior axis: head–tail axis.
Anterior visceral endoderm (AVE): extraembryonic cells located near the future anterior side of the epiblast.
Chiron: CHIR-99021; Wnt agonist that inhibits GSK-3 (a component of the β-catenin destruction complex).
Conceptus: the ensemble of the embryo with its extraembryonic tissues, not to be confused with the term ‘embryo’ (which refers to the embryo proper only, without extraembryonic tissues).
Dorsal–ventral axis: back–belly axis.
Ectoderm: germ layer that gives rise to the nervous system and epidermis, along with other tissues.
Embryocarcinoma cells: pluripotent cells derived from an embryo-derived teratocarcinoma.
Embryoid bodies (EBs): 3D aggregates of ES cells that generate all three germ layers [78,79]. EBs are disorganized and lack the axial organization of mammalian embryos, although some degree of anterior–posterior organization was achieved in EBs in a small number of studies [80,81].
Embryonic stem (ES) cells: cells derived from the inner cell mass of blastocyst-stage embryos. These cells can be propagated in a pluripotent state and can be differentiated into all three embryonic germ layers in vitro.
Endoderm: germ layer that gives rise to the gut tube and its derivatives.
Endothelial cells: cells that line the inner surface of blood and lymphatic vessels.
Extraembryonic endoderm stem (XEN) cells: cells that recapitulate the primitive endoderm of blastocyst-stage embryos.
Gastrulation: the process that reorganizes the embryo into an axially organized structure in which the three germ layers appear and are patterned with reference to the three body axes.
Head mesenchyme: cells that populate the embryonic branchial arches, which give rise to the connective tissues, muscles, and skeletal elements of the head.
Induced pluripotent stem (iPS) cells: pluripotent cells that are obtained through reprogramming adult cells towards an embryo-like state.
Lateral plate mesoderm: sheets of mesodermal cells that are located laterally of the paraxial mesoderm and
formation in embryos [41] are present in gastruloids with a periodicity that is very similar to that in embryos [20]. Similar to mouse 3D gastruloids, human 3D gastruloids also generate derivatives of all three germ layers in the correct spatial location [24] and comparisons with human embryo samples from the Carnegie collection revealed that they recapitulate events associated with the post-occipital region of ~18–21 days post fertilization (dpf) human embryos. However, both human and mouse 3D gastruloids generated with the first protocols [16,17,24,42] do not generate anterior neural (brain) or extraembryonic tissues and lack embryonic architecture (i.e., they are not able to form somite or neural tube-like structures) (Figure 1).

3D gastruloids challenge long-standing paradigms of body axis establishment and provide new insights into gastrulation

Anterior–posterior axis formation in absence of extraembryonic tissues

The observation that gastruloids are able to form axially organized embryo-like structures in the absence of any extraembryonic tissues (Figure 2A) [16,25] challenged the long-standing theory that the **anterior visceral endoderm (AVE)** is essential for anterior–posterior axis formation in embryos (see Figure IA in Box 2) [43]. This raised the possibility that the role of the AVE might not necessarily be to induce symmetry breaking, but rather to ensure that an event that can occur spontaneously happens reproducibly and in the correct spatial position relative to the site of implantation [16]. Alternatively, gastruloids may break their symmetry via a mechanism that does not occur in vivo. In EBs, the location of anterior–posterior symmetry breaking was recently shown to be influenced by physical contact between the EB and the surface of the microwells in which they were cultured [44], raising the possibility that a similar mechanism might be responsible for the observed AVE-independent symmetry breaking process in gastruloids. Future follow-up experiments, during which gastruloids are, for instance, cultured in the presence of extraembryonic tissues or a local source of Wnt antagonists, may further increase our understanding of the mechanisms that drive anterior–posterior axis formation during mammalian embryogenesis.

Brain formation can be induced by adding extraembryonic tissues or through Wnt inhibition

Gastruloids generated with the first protocols [16,17,24] do not generate brain-like structures [18]. In agreement with the prevailing view that extraembryonic tissues are essential for brain development in mice (Box 2) [45], two studies recently showed that the formation of brain-like structures can be induced in gastruloids by adding mouse *trophoblast stem (TS) cells* [19] or *extraembryonic endoderm stem (XEN) cells* [46]. A third study showed that anterior brain-like structures can also be induced in the absence of any extraembryonic cell types through early homogeneous Wnt inhibition in gastruloids grown in an alternative, Chiron-free protocol (Figure 2B) [18]. While the observations are in accordance with the well-known role of Wnt inhibition in brain formation [45] (Box 2), they challenge the prevailing notion that an external, localized source of Wnt inhibition is essential for anterior brain development [45].

Node-like structure, but randomized left-right asymmetry

Microscopy-based observations revealed that both mouse [32] and human [24] gastruloids are able to generate a *node*-like structure, and preliminary observations suggest that left–right asymmetry (which in embryos is set up by the node [1,47,48]; see Figure IB in Box 2) is present but randomized in mouse gastruloids that are generated with the initial protocol [32]. This randomization could result from the absence of extraembryonic tissues in these gastruloids, which might
Box 1. Embryogenesis: from fertilization to gastrulation and organogenesis

During the first 4.5 days of mouse and the first ~5–6 days of human development, regular mitotic divisions of the fertilized egg cell give rise to a multicellular structure referred to as the “blastocyst” (Figure I). The outer layer of the blastocyst contains trophectodermal cells; these cells are responsible for implantation of the blastocyst in the uterus and later contribute to the chorion and placenta of the developing embryo. Below the trophectoderm of the early blastocyst resides a cluster of ‘inner cell mass’ cells. During the last stages of blastocyst formation these inner cell mass cells further specialize towards either the primitive endoderm lineage (which predominantly contributes to the yolk sac), or the epiblast lineage from which the actual embryo develops [1].

At day 6.5 in mice and day ~16 in humans, a process named ‘gastrulation’, which lays down the body plan of the embryo, starts [1]. During this process, cells from the epiblast differentiate towards either the mesodermal, endodermal, or ectodermal lineage. These differentiation processes are coordinated with extensive morphological rearrangements that transform the epiblast into a three-layered (ectoderm, endoderm, and mesoderm) structure with clearly defined anterior–posterior, dorsal–ventral, and left–right body axes. The coordination between the differentiation and morphological processes that happen during gastrulation ensures that all embryonic tissues end up in their correct spatial location within the axially organized embryo.

During the second phase of gastrulation, rapid divisions in a posteriorly localized population of neuromesodermal progenitor (NMP) cells drive posterior growth and elongation of the embryo [1]. These NMPs give rise to both the elongating neural tube and the paraxial mesoderm in the post-occipital region of the embryo. The NMP-derived part of the neural tube is connected to the developing embryonic brain, which develops from the anterior neural plate in an NMP-independent manner. At E7.5 in the mouse, and ~20 dpf in humans, the presomitic mesoderm starts to condense into two longitudinal strings of blocks (the ‘somites’). These somites appear in anterior-to-posterior direction over time and give rise to the vertebrae, ribs, skeletal muscles, tendons, cartilage, and dermis of the embryo. During somitogenesis, organogenesis starts with the formation of a heart, which starts to beat at E8.0–E8.5 in mice and at ~22–23 dpf in humans [1].

Figure I. Schematic explaining mouse and human embryonic development. Abbreviations: A, anterior; dpf, days post fertilization; E, embryonic day; NMPs, neuromesodermal progenitors; P, posterior.

expose the fluid in their node-like structure to the culture medium, interfering with directional fluid flow (Figure 2C). In the near future, a detailed characterization of the node-like structure (e.g., are cilia present and functional) and comparisons between gastruloids generated with and without extraembryonic cell types could shed light on the regulation of left–right asymmetry establishment in gastruloids.
Matrigel-embedding or culture in presence of extraembryonic cell types induces the formation of somite and neural tube-like structures

Two independent studies recently discovered that the formation of somite \[20,21\] and neural tube-like \[21\] structures can be induced in mouse gastruloids by embedding them in a low percentage (5–10%) of Matrigel at 96 h (Figure 1A). Similar to the situation in embryos, the somite-like structures in Matrigel-embedded mouse gastruloids display clearly defined rostral–caudal polarity and they appear rhythmically one by one in anterior-to-posterior direction with an in vivo-like (~2 h) periodicity. A possible explanation for these observations is that Matrigel may recapitulate aspects of the extracellular matrix (ECM) that is produced by the extraembryonic endoderm cells that directly surround embryos in vivo (Box 1) and that might be essential for somite and neural tube formation (Figure 2D) \[21\]. In accordance with this hypothesis, a third study showed that the formation of neural tube-like structures can also be induced by adding a layer of XEN cells instead of Matrigel to mouse gastruloids \[46\]. It is currently unknown whether the effect induced by the addition of Matrigel/XEN-cells is the result of a chemical component, or instead results from mechanical forces that the Matrigel/XEN cells may exert on gastruloids. The tractable nature of the gastruloid system allows scientists to probe into such questions, for example, by testing the effect of engineered synthetic hydrogels with variable stiffness \[49,50\], or by exploring the effect of individual components of Matrigel/XEN cells on the architecture of gastruloids.

Partial dorsal-ventral polarity in the absence of a notochord

A remarkable feature of Matrigel-embedded gastruloids is that the somite and neural tube-like structures that they generate display some degree of dorsal-ventral polarity \[21\], while a
notochord-like structure (which plays an important role in dorsal–ventral patterning of somites and the neural tube in embryos [1]; see Figure IC in Box 2) has so far never been observed in mammalian gastruloids [16,32]. The somite and neural tube-like structures present in Matrigel-embedded mouse gastruloids are, probably due to the absence of the notochord, localized in close proximity to an underlying gut tube-like structure [21], raising the possibility that signals from the gut tube can partially substitute the role of the notochord in patterning the neural tube and somites (Figure 2E).

Alternative route of endoderm formation
In mouse embryos, the endoderm is thought to be derived from progenitors in the epithelial epiblast that undergo an EMT [51]. These cells subsequently migrate towards the visceral endoderm as individual cells, after which they integrate into the visceral endoderm through re-epithelialization. Two independent studies recently suggested that endoderm development follows a different route in gastruloids: the endoderm progenitors in gastruloids migrate in clusters with an epithelial-like configuration, suggesting that endoderm formation in gastruloids does not depend on any EMT-related mechanisms (Figure 2F) [22,33,34]. These observations raise questions about the long-standing paradigm of endoderm formation in mouse embryos and future studies may reveal whether a similar mechanism also plays a role during endoderm development in embryos [33].
Endoderm epithelialization in gastruloids can occur in the presence or absence of Matrigel. Endodermal gut tube-like structures have been observed in Matrigel-embedded gastruloids [21]. However, epithelial gut tube-like structures were recently also reported in a study in which gastruloids were cultured in freely floating conditions [22], indicating that, in contrast to what...
seems to be the case for mesoderm [20,21], the gastruloid endoderm can epithelialize without an external supply of ECM-like components.

Extraembryonic tissues: essential role in evolution?
Comparisons between gastruloid models generated from mouse, human, and other (non-mammalian) cells recently provided unexpected new insights into the role of the extraembryonic environment in evolution: while the morphology of gastrula-stage embryos differs vastly across species, removing embryonic cells from their extraembryonic environment and culturing them in a gastruloid protocol induces them to adopt strikingly similar morphologies across species [52]. These observations indicate that differences between species might largely be attributed to differences in their extraembryonic environment and suggest that the embryonic environment plays a pivotal role in speciation events during evolution.

Taken together, the findings discussed here demonstrate that gastruloid models can provide fundamental new insights into the processes that direct embryogenesis. The tractable nature of the system, in combination with the accuracy with which the model captures germ layer differentiation and body axis specification, makes the system particularly well suited to study the role of the extraembryonic environment in body axis formation and morphogenesis.

Potential clinical applications of 3D gastruloid models
Over recent years, various proof of principle experiments demonstrated that 3D gastruloid models can be used to study developmental defects resulting from genetic or environmental alterations. For instance, mouse gastruloids were shown to be useful for studying the role of Nodal signaling [25] and Cripto mutations [53] in anterior–posterior axis establishment;

Figure 2. Examples of new insights provided by 3D gastruloid models. (A) Mouse gastruloids grown in suspension spontaneously break symmetry in the absence of any extraembryonic cells [16,25], suggesting that the AVE might not be as essential for anterior–posterior axis formation as believed previously (Box 2). (B) The formation of anterior neural rosettes can be induced in mouse gastruloids through uniform exposure to Wnt inhibition [18], suggesting that localized Wnt antagonists might not be essential for brain formation (Box 2). (C) Even though gastruloids generate a node-like structure [24,32], left–right asymmetry in mouse gastruloids (cultured without Matrigel) is randomized [32], perhaps because they do not generate extraembryonic parietal endoderm (Box 2). (D) The discovery that Matrigel addition can induce somite formation in mouse gastruloids [20,21] indicates that the extraembryonic environment plays an essential role in somitogenesis. (E) The somite and neural tube-like structures that appear in Matrigel-embedded mouse gastruloids display partial dorsal–ventral polarity in the absence of a notochord [21] (which is thought to be essential for dorsal–ventral patterning; Box 2). These observations suggest that the gut tube might secrete ventralizing signals (green arrows). (F) In mouse gastruloids, endodermal cells migrate towards the future gut tube in clusters that remain in an epithelium-like configuration [22,33,34]. This raises the possibility that alternative, EMT-independent mechanisms of endoderm formation may exist. Grey arrows show randomized nodal flow. Abbreviations: A, anterior; AVE, anterior visceral endoderm; D, dorsal; EMT, epithelial-to-mesenchymal transition; h, hours after aggregation; P, posterior; V, ventral.
Matrigel-embedded gastruloids generated from a Tbx6 knockout mouse ES cell line were shown to form ectopic neural tubes at the expense of somitic tissues [21] and hence display a phenotype similar to that of Tbx6 knockout mouse embryos [54]; FGF inhibition was shown to result in shorter gastruloids [20] matching previous observations in embryos [55]; addition of the known teratogen retinoic acid was shown to result in defective human gastruloid formation [24]; and knockdown of the BMP inhibitor Noggin resulted in increased extension of human stem cell-based neural tube-like structures [39], matching previous observations in mouse knockout models [56]. In some studies, new phenotypes were discovered through experimentation with gastruloids. For instance, Wnt activation at 96–120 h in Matrigel-embedded mouse gastruloids resulted in an excess of somite-like structures in the anterior region of gastruloids [21]. These promising results suggest that 3D gastruloids can be used to study (human) congenital malformations that arise during gastrulation, such as spinal cord defects and vertebral abnormalities, in a patient-specific manner [Figure 3] [57].

The relative ease with which gastruloids can be generated in high numbers makes the system compatible with large-scale toxicological, genetic, or drug screens (Figure 3). The 96-well plate format of gastruloid cultures (Figure 1) makes the system compatible with automated (live-) imaging, which may allow the automated collection of changes in morphology, body axis formation, elongation speed, and gene expression patterns (when reporter lines are used) during such screens [58]. Encouragingly, pioneering work using elongating mouse carcinoma [59,60] and elongating human ES cell aggregates [38], and a small proof-of-principle study that used 3D mouse and 3D human gastruloids [58], revealed a good correspondence between results obtained with gastruloids and known in vivo teratogenicity of a selection of compounds. Furthermore, it was recently shown that thalidomide, which has a known teratogenic effect in human but not in mouse embryos [61], has a stronger effect on 3D mouse than on 3D human gastruloids [58], suggesting that gastruloids can be used to study species-specific responses to external perturbations. An important challenge of in vitro toxicological screenings is that some drugs are in vivo first metabolized in the maternal liver and that toxicity may in some cases come from

Figure 3. Potential clinical applications for mouse and human 3D gastruloid models. The recently published human 3D gastruloid models open up the possibility to study congenital malformations in a human context. Disease models built from human patient-derived induced pluripotent stem (iPS) cell lines may allow the identification of the genetic alterations or environmental factors that cause such congenital malformations. Since gastruloids can relatively easily be generated in large numbers, the system should be amenable to high-throughput screening procedures that can be used to identify genetic or environmental causes of congenital malformations. Finally, it might be possible to derive transplantable tissues or cells with relevant clinical applications from (iPS cell-based) gastruloids.
drug metabolites rather than from the substance itself [59]. It will therefore be important to keep in mind that a gastruloid-based screening might not be conclusive about the toxicity of new substances. Yet, the system could be used to identify promising compounds before these are submitted to further studies in animals [58].

Since the cell types and tissues present in gastruloids develop within an environment that accurately resembles the spatial-temporal organization of natural embryos [20,21,23,32], the tissues that develop within gastruloids may more accurately resemble their in vivo counterparts than tissues derived with traditional cell or organoid culture protocols. Therefore, gastruloids could perhaps also be used as a source of organoids or tissues for transplantation purposes in clinical settings. In line with this idea, recent work suggested that it might be possible to derive brain organoids [46], to derive endodermal cell types that are difficult to obtain with 2D cultures [22], or to derive transplantable blood progenitors [62] from mouse gastruloids.

In summary, recent observations indicate that gastruloid models can be used to study the genetic and environmental causes of congenital malformations and suggest that it might be possible to use the system to obtain transplantable tissues with clinical relevance from stem cells. Since it is now starting to become possible to derive gastruloids from human iPS cells [39,40] it might ultimately be possible to use 3D gastruloids to model congenital malformations in a patient-specific manner or to produce transplantable tissues from patient-derived iPS cells.

**Concluding remarks**

The results described in this review demonstrate that gastruloid models have many applications for both fundamental and translational studies. We anticipate that the model system can be used to reduce and complement animal studies, to identify new genetic and environmental causes of congenital malformations, to search for new drugs that prevent congenital malformations, to develop new contraceptives, or to identify previously unknown teratogens (Figure 3).

Since the gastruloid field is still young, the currently available versions of this system still display several limitations that will have to be addressed in the near future (see Outstanding questions). For example, while mouse gastruloid models with brain [18,19], somite [20,21] neural tube [21,46], gut tube [21,22], and beating heart [23] -like structures have been generated, an integrated model that develops all these tissues simultaneously is not yet available. In addition, there are currently no reports of human gastruloid models that generate somite and beating heart-like structures and notochord, primitive streak, and limb bud-like structures have not yet been observed in mouse or human 3D gastruloid models. Furthermore, while some mouse and human iPS cell-based gastruloid models were recently reported [32,39,40], these models are not yet as advanced as the currently available mouse and human ES cell-based versions of the system. We anticipate that recent advances in high-throughput microscopy [63,64], and in the field of single-cell and spatial transcriptomics, will speed up the search for improved gastruloid protocols. In particular, advances that allow multiplexed scRNA-seq analysis, such as cell hashing [65], may allow a vast number of gastruloids cultured in various conditions to be analyzed at the single cell level simultaneously, allowing a systematic search for improved gastruloid culture protocols. These innovations will make it possible to, for example, screen for culture conditions that induce notochord formation (which could be achieved by applying high-throughput microscopy-based screenings to gastruloids generated from a notochord reporter line), or to identify components that reduce variability in gastruloid formation. In the longer term, such screens could be performed using robots that generate, culture, screen, and image gastruloids in an automated, reproducible, and high-throughput manner.

---

**Outstanding questions**

Can we develop a more advanced version of the mouse gastruloids model that displays the integrated development of all tissues that are present in mouse embryos?

Will it be possible to extrapolate improvements recently made to mouse gastruloid models to the human version of this system and as such to create human gastruloids that are able to form morphologically correct gut tube, somite, and beating heart-like structures?

How can we efficiently derive gastruloids from both mouse and human iPS cells? And can such iPS cell-based gastruloids model congenital malformations in a patient-specific manner?

How can we improve the reproducibility of gastruloid models (within experiments, across experiments, and across laboratories)?

Can we develop alternative gastruloid protocols that are free of any animal-derived products, thereby omitting batch effects that are associated with the use of animal-derived products? That is, can we find animal-free alternatives of the fetal bovine serum that is used in 2D cultures prior to gastruloid formation and can we engineer synthetic matrices that can replace Matrigel?

To what extent do human 3D gastruloids resemble human embryos in their cell type composition and in their response to chemical or genetic perturbations?

Can we indeed use gastruloids for high-throughput toxicological, drug, and genetic screenings? If so, to what extent do results obtained with such screenings match with those obtained with embryos?

Can gastruloids be used as a source for transplantable cells or tissues with clinical applications?

When do human gastruloid models become so advanced that their moral and regulatory status should be considered equivalent to that of the human embryo?
An additional challenge of gastruloid models is that their reproducibility is not yet optimal. While ~80–90% reproducibility can by now be achieved with older [16] versions of the mouse gastruloids protocol, which have been optimized over the years following their discovery [17], results with more recent protocols are currently less reproducible. In Matrigel-embedded mouse gastruloids, for example, somite-like structures are only observed in 50% of the gastruloids [20,21]. In addition, the percentage of gastruloids that successfully form somite-like structures after Matrigel embedding varies between experiments and the morphology of gastruloids with somites varies significantly across individual samples, even within one experiment [20]. Although such variability can sometimes be scientifically interesting, it is problematic in many experimental settings and optimization strategies that ensure higher robustness will therefore have to be developed in the coming years. Preliminary observations have suggested that the status of the 2D culture prior to gastruloid formation (such as density, passage number, and presence/absence of feeder cells) may play an essential role in this variability [20] and optimization efforts could therefore start with attempts to improve the 2D culture conditions for the cells from which gastruloids are generated.

Another limitation associated specifically with human gastruloid models is that it is difficult to validate results obtained with these models. Although it will be difficult to address this issue, some level of validation might be achieved in future experiments, for instance, through comparisons between non-human primate embryos and non-human primate ES cell-based gastruloids, through detailed comparisons between human gastruloids and non-human primate embryos, or through comparisons with gastrula stage human embryos that can be obtained on rare occasions [66].

Finally, work with human gastruloid models is associated with new ethical, societal, and regulatory questions [29,57,67–71] that need to be addressed before the avenues described here can be pursued with human cells. While currently available versions of the human gastruloid model do not generate anterior neural or extraembryonic tissues and are therefore considered a safe alternative for human embryo research [24], it is not unlikely that more advanced versions of such models may appear in the future and it will not be straightforward to determine when these models become so advanced that their moral and regulatory status should be considered equivalent to that of the human embryo [69]. Furthermore, while work with human iPSC cells does not require human embryos and therefore avoids some of the ethical concerns associated with human ES cell research, the possibility to generate human iPSC cell-based gastruloids also raises several new questions. For instance, should informed consent for such experiments be obtained from donors before their cells can be used to generate gastruloids? And, if so, should donors be informed that the human iPSC cell-based gastruloids generated from their cells might generate functional (early) PGC-like cells? To address the ethical considerations associated with human embryo models, scientists should consult with ethicists, philosophers, and policy-makers to explain their work and the potential implications thereof, with as much clarity and as early as possible, so that well-informed decisions can be made on the guidelines surrounding such work [72,73].

In conclusion, 3D gastruloids are promising tools that can be used to obtain fundamental new insights into the processes that drive mammalian embryogenesis. Since it is now starting to become possible to derive 3D gastruloids from human ES and human iPSC cells, the system may have many applications for modeling both genetic and environmental causes of human congenital malformations in vitro in a patient-specific manner.

Note added in proof

Our review article was in the proof process when this interesting study [82] was published describing ‘embryoids’, which are generated by merging a small, BMP4-treated mouse ES cell
aggregate that functions as a morphogen signaling center with a larger untreated ES cell aggregate. Similar to mouse 3D gastruloids, embryoids model neurula-stage mouse embryos and generate the three germ layers in an axially organized context. Remarkably, embryoids generate a notochord-like structure, together with beating heart, blood vessel, gut tube and neural tube-like structures. Embryoids display more accurate axial patterning than gastruloids, and detailed comparisons between embryoids and gastruloids could shed light on the role of morphogen signaling centers and on the role of the notochord in these patterning events. The embryoid system could perhaps be used to generate tissues that resemble their in vivo embryonic counterparts with even higher accuracy, and might therefore be particularly useful for studies that aim to in vitro generate tissues or cell types for transplantation purposes in clinical settings.

Acknowledgments
We thank Anna Alemany and other members of the van Oudenaarden lab for their contributions to this review. This work is part of the Oncode Institute, which is partly financed by The Dutch Cancer Society.

Declaration of interests
The authors declare no conflicts of interest related to this work.

Author contributions
S.C.B. wrote the manuscript, with supervision from A.O.

References
1. Wolpert, L. et al. (2015) Principles of Development, Oxford University Press
2. Ramsdell, F. (2005) Left-right asymmetry and congenital cardiac defects: getting to the heart of the matter in vertebrate left-right axis determination. Dev. Biol. 288, 1-20
3. Sadler, T.W. (2019) The Notch signaling pathway. Annu. Rev. Cell Dev. Biol. 35, 131-164
4. Harrison, S.E. et al. (2017) Assembly of embryonic and extra-embryonic stem cells to mimic embryogenesis in vitro. Science 356, eaal1810
5. Sozen, B., T., and E. (2018) Autonomous control of left-right axis in mouse embryonic stem cells. Nat. Cell Biol. 20, 979-988
6. Amadei, G., T., S., and A. (2021) Inducible stem-cell-derived embryos capture mouse morphogenetic events in vitro. Dev. Cell 56, 395-399
7. Shao, Y., T., Y., and J. (2017) A pluripotent stem cell-based model for post-implantation human amniotic sac development. Nat. Commun. 8, 208
8. Zheng, Y., T., Y., and J. (2019) Controlled modelling of human epiblast and amnion development using stem cells. Nature 573, 421-425
9. Simonovs, M., T., M., and A. (2019) A 3D model of a human epiblast reveals BMP4-driven symmetry breaking. Nat. Cell Biol. 21, 900-910
10. Warmflash, A., T., and A. (2014) A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. Nat. Methods 11, 847-854
11. Elia, F., T., F., and A. (2016) A balance between secreted inhibitors and edge sensing controls gastruloid self-organization. Dev. Cell 39, 302-315
12. Martyn, I., T., I., and A. (2019) Mapping cell migrations and fates in a gastruloid model to the human primitive streak. Development 146, dev175644
13. Martyn, I., T., I., and A. (2018) Stereotypic left-right patterning in mouse and human early embryos. Nat. Cell Biol. 20, 878-887
14. Blin, G., T., C., and A. (2016) Geometrical confinement controls the asymmetric patterning of brachyury in cultures of pluripotent cells. Development 145, dev160023
15. Munic, J.M., T., J., and A. (2020) Mechanical tension promotes formation of gastrulation-like nodules and patterns mesoderm specification in human embryonic stem cells. Dev. Cell 55, 679-694
16. van den Brink, S.C., T., S., and A. (2014) Symmetry breaking, germ layer specification and axial organisation in mouse embryonic stem cells. Development 141, 4251-4262
17. Balas, J.C., T., C., A., and A. (2015) Generation of aggregates of mouse embryonic stem cells that show symmetry breaking, polarization and emergent collective behaviour in vitro. J. Vis. Exp. 105, e53252
18. Girgin, M.U., T., M.U., and A. (2021) Gastruloids generated without exogenous Wnt activation develop anterior neural tissues. Stem Cell Rep. 16, 1143-1155
19. Girgin, M.U., T., M.U., and A. (2021) Bioengineered embryos mimic post-implantation development in vitro. bioRxiv Published online January 10, 2021. https://doi.org/10.1101/2021.01.10.426096v1
20. van den Brink, S.C., T., S., and A. (2020) Single-cell and spatial transcriptomics reveal somitogenesis in gastruloids. Nature 582, 405-409
21. Vianello, S., J.V., and A. (2020) Mouse embryonic stem cells self-organize into trunk-like structures with neural tube and somites. Science 370, eaaz9376
22. Vianello, S., T., J.V., and A. (2021) In vitro endoderm emergence and self-organisation in the absence of extraembryonic tissues and embryonic architecture. bioRxiv Published online June 10, 2021, http://doi.org/10.1101/2021.06.07.138803v0
23. Rossi, G., J.T., and A. (2021) Capturing cardiogenesis in gastruloids. Cell Stem Cell 28, 230-240
24. Vianello, S., J.V., and A. (2020) An in vitro model of early anteroposterior organization during human development. Nature 582, 410-415
25. Tournier, D.A., T., D.A., and A. (2017) Anteroposterior polarity and elongation in the absence of extra-embryonic tissues and of spatially localised signalling in gastruloids: mammalian embryonic organizers. Development 144, 3894-3906
26. Shababi, N., T., N., and M. and A. (2018) Defining and reconstructing the mouse and human early embryo. Nat. Cell Biol. 20, 878-887
27. Schauer, A. and Hensenberg, C-P. (2021) Reassembling gastrulation. Dev. Biol. 474, 71-81
28. Rosado-Olivieri, E.A. and Brivanlou, A.H. (2021) Synthetic blastogen: exploiting tissue self-organization to explore early human embryology. Dev. Biol. 474, 16-21
29. Ghimire, S., et al. (2021) Human gastrulation: the embryo and its models. Dev. Biol. 474, 100-108
