# Alliance of Heart and Endoderm: Multilineage Organoids to Model Co-development

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## Abstract

Studies in animal models tracing organogenesis of the mesoderm-derived heart have emphasized the importance of signals coming from adjacent endodermal tissues in coordinating proper cardiac morphogenesis. Although *in vitro* models such as cardiac organoids have shown great potential to recapitulate the physiology of the human heart, they are unable to capture the complex crosstalk that takes place between the co-developing heart and endodermal organs, partly due to their distinct germ layer origins. In an effort to address this long-sought challenge, recent reports of multilineage organoids comprising both cardiac and endodermal derivatives have energized the efforts to understand how inter-organ, cross-lineage communications influence their respective morphogenesis. These co-differentiation systems have produced intriguing findings of shared signaling requirements for inducing cardiac specification together with primitive foregut, pulmonary, or intestinal lineages. Overall, these multilineage cardiac organoids offer an unprecedented window into human development that can reveal how the endoderm and heart cooperate to direct morphogenesis, patterning, and maturation. Further, through spatiotemporal reorganization, the co-emerged multilineage cells self-assemble into distinct compartments as seen in the cardiac-foregut, cardiac-intestine, and cardio-pulmonary organoids and undergo cell migration and tissue re-organization to establish tissue boundaries. Looking into the future, these cardiac incorporated, multilineage organoids will inspire future strategies for improved cell sourcing for regenerative interventions and provide more effective models for disease investigation and drug testing. In this review, we will introduce the developmental context of coordinated heart and endoderm morphogenesis, discuss strategies for *in vitro* co-induction of cardiac and endodermal derivatives, and finally comment on the challenges and exciting new research directions enabled by this breakthrough.

## Non-standard Abbreviations and Acronyms

Shh – Sonic hedgehog
Wnt2 – Wingless-type MMTV integration site family, member 2
Wnt2b – Wingless-type MMTV integration site family, member 2b
Tbx5 – T-box transcription factor 5
hPSC – Human pluripotent stem cell
hiPSC – Human induced pluripotent stem cell

hESC – Human embryonic stem cell
WNT – Wingless-related integration site family member
CHIR99021 – 6-((2-((4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl)amino)ethyl)amino)nicotinonitrile
IWP2 – N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d]pyrimidin-2-yl)thio]-acetamide
SOX2 – SRY (Sex Determining Region Y)-Box 2
ECM – Extracellular matrix
cTnT – Cardiac Troponin T
TBX18 – T-Box Transcription Factor 18
MLC2V – Myosin Light Chain 2V
NKX2.1 – NK2 Homeobox 1
NKX2.5 – NK2 homeobox 5
SFTPC – Surfactant Protein-C
COVID-19 – Coronavirus Disease 2019
SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2
ACE2 – Angiotensin-Converting Enzyme 2

**Short Title**

Engineering cardiac-endodermal organoids

**Keywords**

Multilineage organoid, cardiac organoid, intestine, lung, foregut, endoderm, co-development

## 1. Developmental relationship between cardiac mesoderm and endoderm

Extensive investigations of embryonic organogenesis have revealed comprehensive mechanisms regarding how tissue morphogenesis is regulated by interactions among cells within each individual organ, including the mesoderm-derived heart and the endoderm-derived lung or intestine. However, accumulating evidence suggests the importance of the inter-organ, cross-lineage communications taking place between the co-developing heart and the surrounding endoderm in coordinating their individual developmental programs (Figure 1) [1-4]. During heart tube formation, differential growth of the cardiac mesoderm and adjacent endoderm collectively drive their diagonal folding and elongation through a process called convergent extension [2,5,6]. As the endoderm folds to form the foregut tube along the body's midline, the bilateral heart primordia also folds towards the midline and fuses to give rise to the heart tube located ventrally to the newly emerged foregut (Figure 1A, B) [6]. The crucial role of the neighboring endoderm for cardiac mesoderm migration is well demonstrated by the removal of foregut endoderm which results in cardiac bifida [7,8], the failure of the bilateral cardiac cells to coalesce into a single heart tube. Following their arrival at the midline, the foregut and the heart tube undergo coordinated elongation [5], driven by active contraction of the actomyosin cytoskeleton that exerts forces within and across cells [9]. It has been suggested that the accompanying endoderm may provide mechanical forces to guide cardiac cells to undergo directional rearrangement and thereby heart tube extension [5,6].

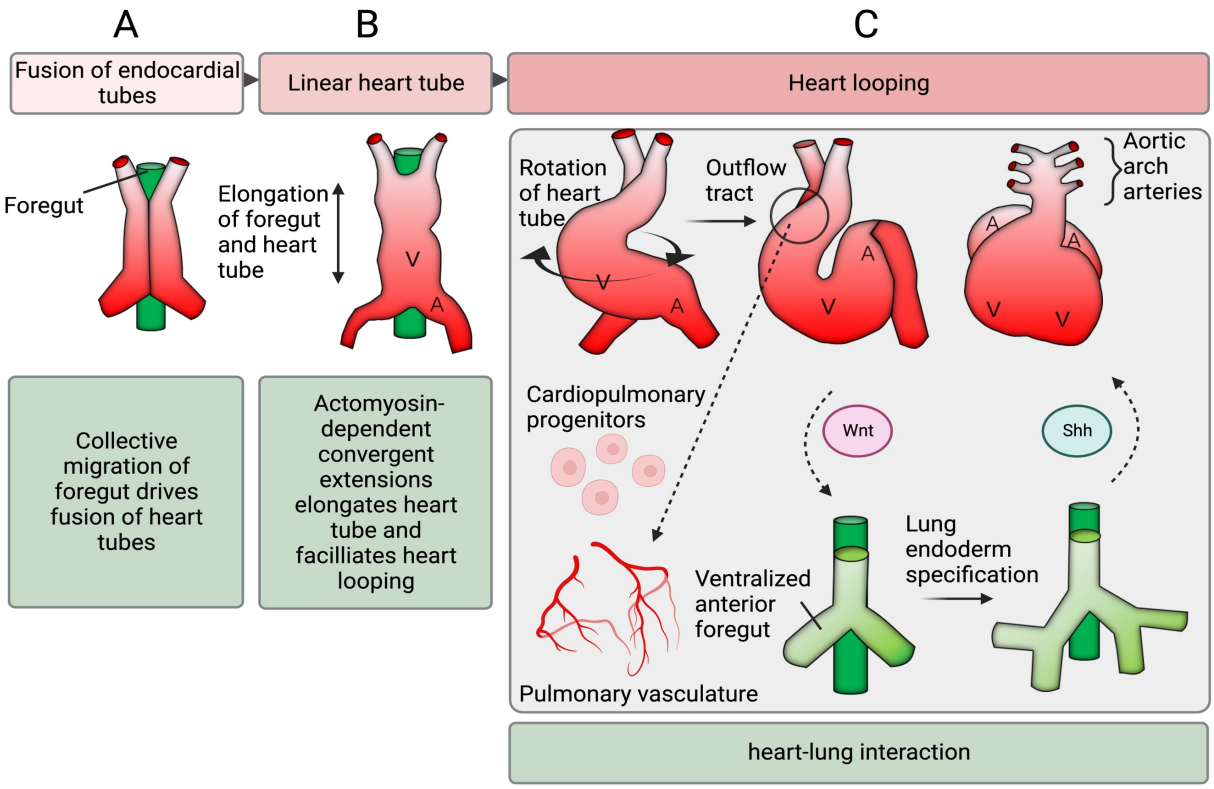


**Figure 1. *In vivo* co-development of heart and endoderm with an emphasis on the co-emergence with the lung. Figure created with BioRender.com.**

One of the well-established examples of developmental cardiac-endoderm crosstalk is the coordinated morphogenesis of the heart and the lung that are located in close vicinity since their emergence during embryogenesis. An investigation tracing mesodermal lineage progression during mouse heart and lung co-development revealed a group of mesoderm-derived, multipotent cardiopulmonary progenitors originating from the second heart field that provide extensive lineage-specific differentiation cues to the lung, including specifying the pulmonary vasculature and airway smooth muscle, proximal vascular endothelium, and perivascular cells (Figure 1C) [10]. The specification of these mesodermal cardiopulmonary progenitors is regulated by sonic hedgehog (Shh) signaling through ligands expressed by the nearby foregut endoderm [10]. Furthermore, as one of the descendants of foregut endoderm, the pulmonary endoderm forms a mutual-interacting, Wnt2/Wnt2b-Shh paracrine signaling loop with the adjacent cardiac mesoderm to coordinate their co-development (Figure 1C) [3,4]. Tbx5-expressing cardiac progenitors from the second heart field supply Wnt2/Wnt2b signals that activate pulmonary specification, and subsequently in return, the pulmonary endoderm secretes Shh ligands that signal back to the developing heart to regulate atrial septation [4]. In summary, findings at the morphological, molecular, and cellular levels during embryogenesis all underscore the importance of the extensive mutual influences between the co-developing heart and endodermal derivatives.

## 2. Engineering multilineage organoids to model the co-emergence of cardiac and endodermal derivatives

Although human development has been challenging to investigate in the past, advances in organoid engineering have created new opportunities for *in vitro* exploration and modeling of human cardiogenesis [11-16]. Cardiac organoids derived from human pluripotent stem cells (hPSCs), including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) have the ability to undergo a series of differentiation events that parallel *in vivo* development, resulting in the formation of three-dimensional tissues that mimic certain aspects of cardiac function and physiology. Studies investigating early heart development in animal models have recognized the neighboring endodermal tissues as an important influence in guiding cardiac morphogenesis [2-4]. Accordingly, there has been a growing interest in introducing additional cell lineages into cardiac organoids, especially those derived from adjacent endodermal tissues to recapitulate and investigate the inter-organ, cross-lineage interactions leading up to cardiogenesis. Discussed below are some recent examples of multilineage cardiac organoids that model cardiac and endoderm co-development.

**2.1 Heart and foregut:** Drakhlis et al., 2021 reported an organoid model for tracing the early stages of human heart development (Figure 2A) [12]. Here, hPSC aggregates embedded in Matrigel, a mouse sarcoma derived matrix that is rich in laminin and collagen IV, were differentiated towards cardiac fate in a stepwise manner. This includes an initial exposure to the WNT agonist CHIR99021 for mesoderm induction, followed by WNT inhibition using IWP2 for further specification towards cardiac lineage. Histological analysis of the resulting organoid revealed a multilineage tissue architecture that was composed of an inner core of SOX2+ anterior foregut endodermal cells, encapsulated by a middle layer of mostly cardiomyocytes and epicardial cells, and finally an outer layer of posterior foregut endoderm-derived liver cells and mesenchymal cells. Additionally, a small population of endocardial cells were observed at the interface between the inner endoderm and the middle cardiac mesoderm layers. This finding is in line with prior studies indicating that signals from the developing endoderm are needed for the specification of precardiac mesoderm to endocardium [17,18].

In this organoid model, while there is a clear separation between foregut tissues of the anterior versus posterior identities, spatial patterning of these tissues along an established tissue axis as is seen in the native gut tube remains to be demonstrated. Further, during embryogenesis, the endodermal gut tube expands in parallel to the adjacent, co-developing heart tube, a unique tissue arrangement that remains distinct from the concentric tissue organization observed in the cardiac-foregut organoid discussed here. Further, of note, organoids differentiated in the ECM-free condition or in matrices alternative to Matrigel, such as Geltrex and Collagen, failed to reproduce the cardiac-foregut co-differentiation. Thus, setting an important precedent in modeling cardiac-foregut co-development, this study is expected to inspire future investigations to pinpoint the specific biochemical and biomechanical cues within the ECM microenvironment that trigger or permit this unique morphogenic process.

**2.2 Heart and intestine:** In the same vein, Silva et al., 2021 generated a highly sophisticated organoid model of heart and gut co-development (Figure 2B) [11]. In this study, hiPSC-derived mesendodermal aggregates were directed towards cardiac fate in an ascorbic acid-supplemented medium. The addition of ascorbic acid induced two major waves of growth in the multilineage organoid. During the first 30 days in culture, the initial growth was largely attributed to the cardiac region within the organoid, comprising cTnT+ cardiomyocytes at the center, an intermediate layer of stromal cells, and finally an outer layer of TBX18+ epicardial cells. The organization of the cardiomyocyte center was further characterized by a MLC2V+ ventricular core surrounded by a layer of MLC2V- atrial/nodal cells. The self-organization of these cardiac cell lineages into distinct compartments parallels the chamber-specific distribution of cardiomyocytes in the native heart [19,20]. The next wave of growth of the multilineage organoid was marked by the expansion of the

intestinal epithelium, where the endodermal cysts that had emerged around the cardiac region by day 30 eventually matured into intestinal tissues by day 100. This was accompanied by further maturation of the cardiac elements, where the majority of the cardiomyocytes exhibited an atrial/nodal phenotype, while the population of ventricular cardiomyocytes gradually declined. Such atrial-dominant cardiac induction is absent in other multilineage cardiac organoids as well as conventional, cardiac-only organoids that usually exhibit a more prominent ventricular phenotype [12,13,21]. For instance, although Drakhlis et al., 2021 reported a similar combination of cardiac and endoderm-derived cells, further specification into atrial/nodal fate was absent in their model. Additionally, while it was indicated that the addition of ascorbic acid to the existing cardiac differentiation protocol could promote specification into epicardial lineage, the medium composition is insufficient to specify atrial/nodal phenotype in the absence of retinoic acid signaling. Altogether, this implies that the inter-organ interaction between the cardiac and intestinal cells in this model was crucial for atrial/ nodal specification. Further comparison of cardiomyocytes dissociated from the cardiac-intestinal versus cardiac-only organoids revealed that cardiomyocytes in the multilineage organoids possessed a more elongated morphology and sarcomere structure, indicative of maturity. The authors concluded that co-emergence with the intestinal tissue was essential for the

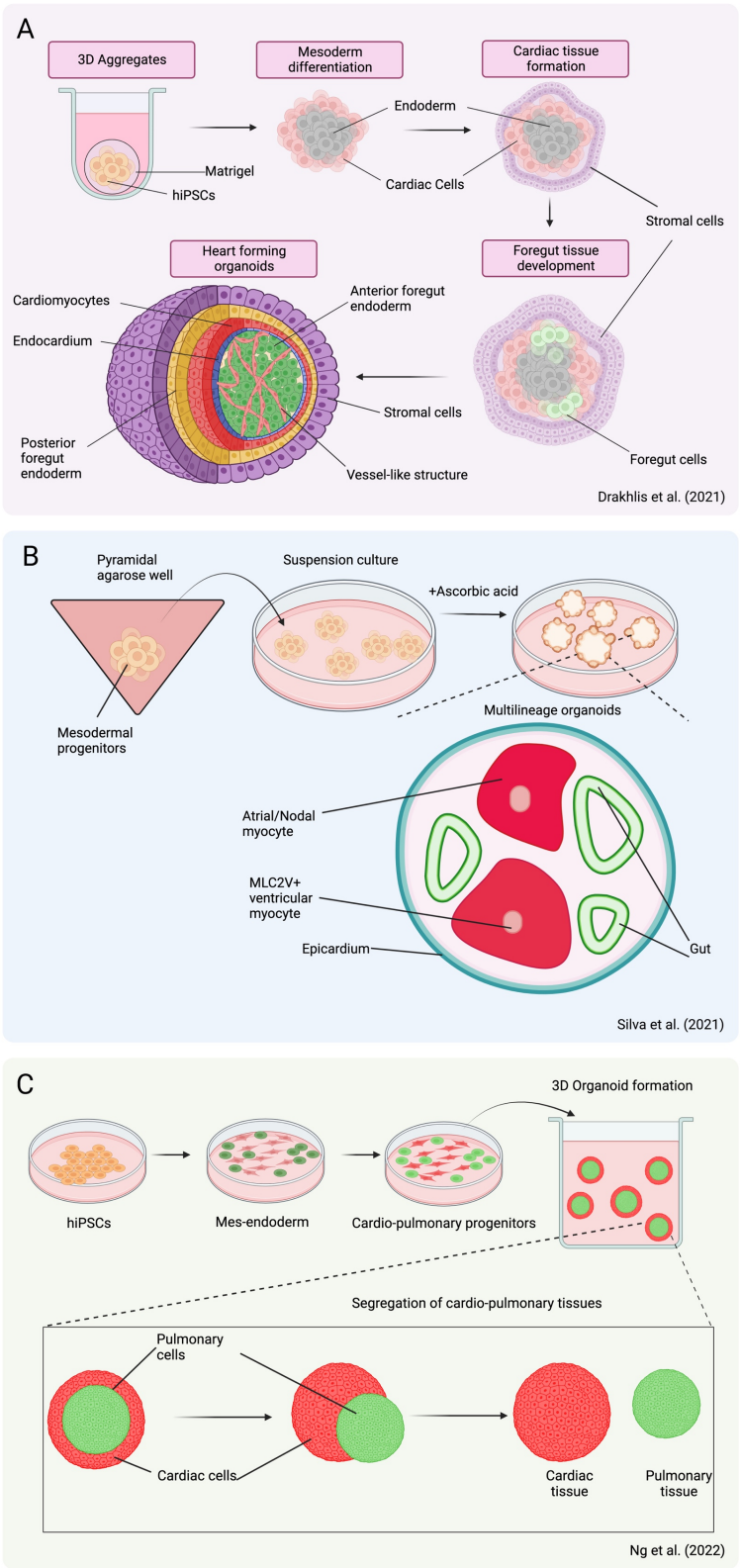


**Figure 2. Existing organoid models of multilineage co-development, including (A) heart and foregut, (B) heart and intestine, and (C) heart and lung. Figure created with BioRender.com.**

functional and phenotypic maturation of the induced cardiomyocytes, as well as their specification into atrial/nodal fate. In contrast to the tissue organization seen in Drakhlis et al., 2021 where the cardiac and foregut tissues encapsulate one another, the co-emerging intestinal and cardiac tissues in this organoid model gradually moved towards the opposite ends of the organoid into distinct compartments, separated by mesenchymal tissue and a layer of smooth muscle that displayed peristalsis-like contractile movements.

This study elegantly recapitulates the embryonic co-emergence of heart and intestinal tissues. For instance, the heart is the first organ to develop during embryogenesis, and fittingly, it is also the first to emerge in this organoid model of co-development. Further, although the heart and intestinal tissues exhibit an initial concentric organization, they eventually migrate away from each other to form parallel tissue domains, a unique feature that closely mimics the dorsal-ventral patterning of the native gut and heart tubes.

**2.3 Heart and lung:** While the above studies were primarily focused on investigating cardiac morphogenesis, Ng et al, 2022 reported a study that investigated the role of cardiac mesoderm in regulating the maturation of the co-developing pulmonary epithelium (Figure 2C) [22]. There, hiPSCs were simultaneously differentiated into NKX2.1+ lung progenitors and NKX2.5+ cardiac progenitors within the same culture through stepwise modulation of WNT and Nodal signaling. At first, the concentration of CHIR99021 (WNT agonist) was titrated to facilitate a balanced induction of mesoderm and endoderm [23,24]. This was followed by the dual inhibition of both WNT and Nodal signaling to direct the specification of germ layer progenitors towards cardiac mesoderm and anterior foregut endoderm fate. A similar approach was taken in both reports from Drakhlis et al., 2021 and Silva et al., 2021, where WNT signaling was activated and then inhibited to enable effective induction of both cardiac and endodermal derivatives [11,12]. Given the close spatial proximity of the co-developing cardiac and endodermal lineages within the embryonic body patterning, it makes sense that both of their fates are influenced by the exposure to a similar set of paracrine factors.

The co-induced cardiac and pulmonary progenitors by day 15 of differentiation were then aggregated in an ECM-free suspension culture to induce the formation of 3D dual-lineage organoids and tissue maturation. Initially, a dense layer of cardiac tissue formed at the outer layer to encapsulate the centrally located pulmonary component. The presence of the cardiac tissue expedited the maturation of the pulmonary progenitors into SFTPC+ alveolar type 2 cells within as short as 3 days in 3D suspension culture. This finding echoes the cardiac-assisted pulmonary specification observed in mouse embryogenesis [4]. Further, similar to the lineage-specific tissue re-organization observed in the cardiac-intestinal organoid model [11], the cardiac and pulmonary compartments gradually migrated away from each other and eventually split into separate cardiac and pulmonary tissues. This provides further experimental support implying the presence of intrinsic mechanisms within cells originating from distinct lineages to self-assemble based on lineage similarity and segregate from cells with distant lineage relationship, a process that mimics inter-organ boundary formation.

Although analysis of the cardiac component within the dual-lineage, cardio-pulmonary organoid confirmed both contractile function and structural maturation, there was a general lack of cellular diversity in the cardiac population, which could be due to the relatively short culture time (less than 3 weeks) as compared to the more than 100 days of culture in the cardiac-foregut and cardiac-intestinal organoid models. Ultimately, while this study provides a long-sought *in vitro* platform for understanding the developmental co-emergence of heart and lung, the two major organs within the chest cavity, there was a greater emphasis on pulmonary maturation in the

study. Accordingly, future efforts are needed to further investigate cardiac morphogenesis and how the co-emergence with pulmonary cells may modulate this process *in vitro*.

## 3. Challenges and Future Directions

Current organoid models of cardiac and endoderm co-emergence capture many trends that are reminiscent of *in vivo* heart development and its reciprocative influence on the developmental programs of adjacent endodermal organs [11,12,22]. The multilineage cardiac organoids support the differentiation of a diverse population of cardiac cells, including cardiomyocytes, cardiac fibroblasts, endocardial cells, endothelial cells, and epicardial cells [11,12]. The specification of cardiomyocytes into atrial and ventricular subtypes typically relies on the use of separate differentiation protocols involving retinoic acid and NOTCH signaling, respectively [25-27]. Comparatively, simultaneous specification of both atrial and ventricular cardiomyocytes into chamber-specific compartments can be readily observed in the cardiac-intestinal organoid [11], where the cardiomyocytes also possessed a more mature phenotype and sarcomere structure in comparison to traditional cardiac organoids [11,28]. Although the multilineage cardiac organoids approach an impressive degree of complexity and cellular heterogeneity, they remain unable to fully recreate the trajectory of events leading up to *in vivo* cardiogenesis [29]. These events include critical developmental processes such as heart tube formation, and chamber morphogenesis via cardiac looping [29]. Although we are yet to uncover the exact biochemical and biomechanical cues that mediate these essential cross-lineage, cardiac-endoderm interactions, technologies, such as single-cell transcriptomics coupled with cellular lineage barcoding and spatial transcriptomics, can offer potential insights regarding coordinated lineage specification and the involved paracrine signaling [30-33].

Besides understanding orchestrated organogenesis, multilineage cardiac organoids would also be a valuable tool for investigating multi-organ interactions during disease pathogenesis and drug discovery. For example, in the recent Coronavirus Disease 2019 (COVID-19) pandemic, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been well recognized to compromise the respiratory system severely, due to its nature as an airborne pathogen. However, due to the shared expression of Angiotensin-Converting Enzyme 2 (ACE2), the cognate receptor for the viral spike protein needed for viral entry into cells, other organs including the heart, kidneys, and liver can also be affected [34-36]. To understand SARS-CoV-2 infection mechanisms, multiple animal models have been explored but were unable to fully represent the pathogenesis observed in human patients [37-39]. Traditional platforms for studying coronavirus infection also include 2D culture of cell lines such as VeroE6 [40,41], nonetheless, anti-viral drugs discovered through 2D culture models have been found to be challenging for clinical translation [42]. This warrants the development of models that can better recapitulate human pathogenesis to facilitate therapeutic development for diseases such as COVID-19. Organoids, due to their ability to closely mimic the cellular composition and internal architecture of native organs and their ability to be derived from patient-specific cells, offer the promise to overcome the major limitations in using animal and 2D cell culture models as mentioned above to investigate viral infection in human. Using respective organoid models, studies have demonstrated that COVID-19 affects not only the lung [43,44], but also the heart [45,46], gastrointestinal tract [47], blood vessels and kidney [48]. These echo the multi-organ dysfunction observed in human patents infected by SARS-CoV-2 [49,50]. Accordingly, the newly emerged multilineage organoid models are uniquely positions to enable expedited investigation of diseases like COVID-19 that affect the heart and other organ systems and to reveal how inter-organ interactions shape the pathological progression.

The multilineage cardiac organoids show a tendency to self-organize into distinct, lineage-specific tissue compartments that find counterparts in native embryogenesis. However, the lack of

established tissue axes, similar to those seen in the anterior-posterior and dorsal-ventral patterning during early development limits the extent of these organoid models to recapitulate the native-like spatial organization of the co-induced tissues [11,12,22]. In an effort to introduce spatial axes into organoid models of embryogenesis, the recent development of gastruloids, axially patterned embryonic organoids [13] hold the potential to investigate cardiogenesis in the context of "whole-embryo" patterning. For instance, Rossi et al., 2021 reported the self-organization of mouse ESCs into gastruloid that demonstrated the capacity to model cardiac tissue emergence at the anterior portion of the gastruloid that was accompanied by an adjacent gut-tube-like epithelial structure extending along the anterior-posterior axis [13].

Another technical limitation in the use of existing multilineage organoids thus far is the invariability in cellular composition and organoids morphology upon maturation. This is partly due to the reliance of current models on the intrinsic ability of multilineage cells to self-aggregate followed by self-reorganization with minimal external guidance. Emerging engineering approaches, such as scaffold-guided tissue morphogenesis and hydrogel-based 3D-printing [51-54], could be used to provide further instruction and improve the reproducibility in producing multilineage organoids, and thereby enhance their utility in development and disease modeling, drug development, and personal medicine industry.

In their current form, the multilineage cardiac organoids summarized here are more representative of fetal organs than their adult counterparts. The cellular composition and organization of multilineage organoids also appear to be in constant flux, and extensive steps need to be taken towards improving the cellular maturity and overall tissue complexity of multilineage organoids so they can be used to model the development, physiology, and pathology of the organs involved.


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## Competing of Interests

None.

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