Towards Improved Understanding of Cardiac Development and Congenital Heart Disease: The Advent of Cardiac Organoids

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Towards Improved Understanding of Cardiac Development and Congenital Heart Disease:

The Advent of Cardiac Organoids

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Abbreviations

bFGF: Basic fibroblast growth factor
BMP: Bone morphogenic protein
CO: Cardiac Organoid
CRISPR: Clustered regularly interspaced palindromic repeats
EC: Endothelial cell
FGF4: Fibroblast growth factor 4
GATA4: GATA binding protein 4
GSK-3: Glycogen synthase kinase-3
HAND1: Heart and neural crest derivatives expressed 1
iPSCs: induced pluripotent stem cells
MEF2: Myocyte enhancer factor 2
NKK2.5: NK2 Homeobox 5, a key regulator in cardiac morphogenesis
PSCs: Pluripotent stem cells
RA: Retinoic acid
SRF: Serum response factor
TBX5: T-box transcription factor 5
VEGF: Vascular endothelial growth factor
Wnt: Wingless and Int-1
Central Message

Human cardiac organoid systems hold significant promise for mechanistic studies of early heart morphogenesis and an improved understanding of congenital cardiac disease.

Central Illustration Abbreviated Legend

Progress and Promise of Cardioids

Central Illustration Full Legend

Icons represent major applications of cardiac organoids. Solid lines represent applications for which cardiac organoid technology is sufficiently progressed for immediate utility, such as variant screening. The dashed lines represent areas of promise, where the current complexity of cardiac organoids has not yet matured to meaningfully probe relevant research questions, such as cardiac structural disease.

Perspective Statement

Studies in self-assembled, 3-dimensional cell culture models, termed organoids, can improve the mechanistic understanding of early human development and disease. Human cardiac organoids, in particular, permit modeling of early cardiac differentiation, specification, and morphogenesis, including chamber formation, thus offering a platform to advance new therapies for congenital heart defects.
Abstract

Structural congenital heart disease remains a significant burden, affecting up to 1 in 50 births. While surgical advances have substantially improved outcomes for these patients, a deeper understanding of the underlying cellular and molecular mechanisms of cardiac birth defects may offer future opportunities for non-surgical interventions. Organoids are cell culture models in which three-dimensional self-assembly, differentiation, and rearrangement of cells mimic the earliest events of embryonic development and organogenesis, creating a versatile platform to study human congenital diseases. Here, we review recent advances in cardiac organoid technology to illustrate the investigative power of these models and invite discussion of how they might be best used as a platform for translational discovery. With continued advances in this evolving field, human cardiac organoids hold significant promise to improve our knowledge of cardiac developmental biology and stimulate the development of new therapies for congenital heart defects.
Feature Editor’s Introduction – During the last decade we have witnessed a remarkable progress in genome editing technology, stem cell research and bioengineering. The fundamental basic research discoveries accelerate rapidly into clinical translation, paving the ways for myocardial regeneration, better understanding of the structural heart disease, bioengineering of heart structures and even entire heart. The new horizon is vast and diverse, ranging from creating universal stem cell biobanking to genome edited heart xenotransplantation. Herein a group of experts from Duke University discuss the state of art and possible impact of cardiac organoids on our understanding of structural heart disease. It may not be immediately clear now in what practical ways this technology will be translated into our daily work, yet the current progress in bioengineering will likely have a very significant impact on our surgical practice.

- Igor E. Konstantinov, MD, PhD, FRACS
Introduction

Cardiac birth defects remain a significant health burden and cause of death in the United States and worldwide, with congenital heart malformations affecting 1-2% of live births\(^1\). Surgical advances have promoted longer, healthier lives in those suffering from severe genetic anomalies, but a more complete mechanistic understanding of early cardiac development and congenital structural disease could open doors to minimally invasive or non-surgical interventions. To achieve such a lofty goal, the fields of developmental cardiology and congenital heart surgery require new models to provide critical insights into biological processes underlying early human cardiac development and disease. The *in vitro* self-organizing human cardiac organoid (CO) systems, such as that recently reported by Mendjan and colleagues\(^2\), represent powerful platforms to study early cardiac morphogenic events and related abnormalities, offering the promise to eventually improve the current standard of care for patients with congenital heart disease via numerous applications (Figure 1).

Methods for cardiac organoid generation

Organoids are three-dimensional (3D), self-assembled, cellular structures that can recapitulate important spatial and functional relationships in developing and adult mouse and human tissues\(^3\). As such, organoids have been increasingly used to model early organ development, physiology, disease, and drug responses *in vitro*\(^4\). Compared to traditional animal models, organoids have the primary advantage of using human cells and having improved ability to interrogate cellular processes, higher experimental throughput, and control over 3D cellular and matrix composition and structural organization. On the other hand, they have a small size due to the absence of perfusable vasculature, possess simplified architecture, and lack complex
chemical and physical cues present in vivo. The typical protocols for organoid formation (Figure 2), involve culturing stem cells on a plate, reconstituting them into a single cell suspension, and embedding them in an extracellular matrix analog (e.g., Matrigel, a solubilized basement membrane matrix secreted by mouse sarcoma cells). Over several days, the embedded cells self-aggregate into 3D spheroid structures, followed by differentiation, migration, and formation of solid or luminal structures, depending on the cell types used and modulation of specific signaling pathways. Organoids may be constructed out of terminally differentiated cells; however, the use of pluripotent stem cells (PSCs) or their multipotent derivatives uniquely allows studies of early lineage specification and tissue morphogenesis in a controlled, in vitro setting. Recent advances in organoid development have resulted in the successful generation of in vitro models of non-cardiac tissues such as the intestines and brain. Self-organized cardiac organoids mimicking early heart development and chamberogenesis are some of the latest additions to this rapidly evolving field.

In the original and increasingly used formulation of COs, multiple terminally differentiated cardiac cell types are coaxed together to form spheroid-shaped microtissues. For example, the COs made of human induced PSC (iPSC)-derived cardiomyocytes, cardiac fibroblasts, and endothelial cells (ECs) can permit relatively high-throughput studies of drug responses, hypoxic insults, or heterocellular interactions underlying heart diseases. More complex structures made from multiple spheroid COs have been generated using 3D bioprinting to position and fuse organoids in pre-defined spatial patterns and study cardiac scarring. Although these methods do not replicate developmental organogenesis, they can recreate the realistic macroscopic architecture of an adult heart and its components, including ventricles, atria, valves, and coronary vasculature. Overall, while 2D micropatterning and 3D
bioprinting\textsuperscript{17} technologies can be used to pre-form complex organoid structures, they do not recreate dynamic spatiotemporal signals and self-organizing processes that drive early cardiac development and morphogenesis.

**Self-organizing COs for modeling early cardiac development**

Recently, several studies have demonstrated generation of sub-mm to mm-sized COs where cells are initially mixed with Matrigel, followed by a staged addition of small molecules and growth factors to guide early cardiac differentiation, lineage specification, and formation of stratified ventricular walls and chamber-like lumens. While the initial efforts to mimic early cardiac development in organoids have been limited to specification and rudimentary patterning of first and second heart fields with mouse PSCs\textsuperscript{18}, recent studies that utilize GSK-3 inhibition to activate Wnt signaling have demonstrated the formation of multi-lineage COs containing spatially distinct cardiac mesoderm and endodermal, gut-like regions\textsuperscript{5, 7, 9}. Under exogenously supplied growth factors (ascorbic acid, bFGF, VEGF) and endogenous heterocellular cues, cardiac lineage cells within these COs undergo early differentiation and morphogenic events, eventually forming linear heart tube-like structures, but not proceeding to generate cardiac chambers\textsuperscript{19}. A more accurate recapitulation of the interactions between human cardiac mesoderm and foregut endoderm\textsuperscript{8} resulted in multilineage organoids containing an outer layer of epicardial cells, cardiomyocytes, smooth muscle cells, and gut epithelial cells. While this system could allow studies of early cardiac patterning events, including those involving aberrant NKX2.5 signaling, it did not result in cardiac chamberogenesis.

**Self-organizing COs for modeling early chamberogenesis**
Only recently have two studies with human\textsuperscript{2, 11} and one with mouse\textsuperscript{10} cells been able to create \textit{in vitro} conditions for the spontaneous generation of chamber-like cavities within COs (Table 1). The cavity formation was induced by manipulating Wnt and/or retinoic acid (RA) signaling and did not require recreation of all developmental stages such as formation of the cardiac crescent, linear heart tube, or cardiac looping. Similar to other CO studies, the use of mouse PSCs yielded more advanced cardiac morphogenesis compared to use of human PSCs, with manipulation of extracellular matrix proteins and FGF4 signaling resulting in the formation of primitive atrial and ventricular chambers\textsuperscript{10}. A more rigorous structural and functional characterization of these COs and improved methods to decrease their variability in shape and cellular composition will be highly instructive for the future generation of developmentally mimetic COs made from human PSCs.

The most advanced method for forming a cavity resembling a primitive left ventricle in human COs has been reported by Hofbauer \textit{et al}\textsuperscript{2}. The authors named these COs “cardioids”, using the term Dennis and colleagues originally coined to describe self-assembled aligned cylindrical cardiac tissues made from neonatal rat heart cells\textsuperscript{19}. Approximately 1-2 mm in size, each human cardioid was made starting from 5000-7500 PSCs which, for improved reproducibility, were cultured within individual wells of a commercially available AggreWell\textsuperscript{TM} dish. Upon induction of cardiac mesoderm, chamber-like cavities rapidly formed in the presence of Wnt, Activin, and RA signals and were maintained stably during subsequent cardiomyocyte specification and maturation for up to 3 months. While exogenously induced and endogenous changes in VEGF signaling led to partial lining of cavities with endothelial cells, neither endoderm nor endothelium was required for chamber formation. Instead, chambers were formed solely by self-organization of cardiac mesoderm, through a process regulated via the Wnt-BMP-
HAND1 signaling axis. Specifically, and similar to cardiac development in vivo, chamber formation was critically dependent on the activity of HAND1, the genetic deletion of which resulted in small, poorly formed COs with rudimentary chambers. These experiments further revealed that cavity formation and cardiomyocyte specification in cardioids occur in parallel but are distinctly regulated. Interestingly, addition of PSC-derived epicardial cell aggregates to pre-formed cardioids generated stable structures in which epicardial cells both spread on the CO surface and, upon migration into the organoid, upregulated EC and fibroblast markers, thus yielding a more in vivo-like ventricular cell composition.

Use of cardioids to study injury response

The critical difference between fetal and adult mammalian hearts is the lack of proliferative capacity in adult cardiomyocytes, representing the primary barrier to robust regenerative response following myocardial injury. In contrast, developing fetal hearts consist of cardiomyocytes with significant proliferative and regenerative ability. Interestingly, the epicardium-supplemented cardioids by Hofbauer and colleagues did not regenerate after a localized cryoinjury and instead mounted a fibrotic response characterized by fibroblast, fibronectin, and collagen I accumulation at the injury site and no cardiomyocyte proliferation. While, as in mature hearts, this fibrogenic outcome in cardioids was aided by epicardial cells, the lack of injury-induced proliferation of evidently immature cardiomyocytes is puzzling and suggests either a non-physiological nature of cryoinjury or requisite roles for additional resident nonmyocytes or systemic influences (e.g., immune system) in mounting a robust regenerative response in the fetal heart. The modular nature of cardioids can be used to gain further mechanistic insights into these clinically important phenomena.
Use of cardioids for modeling developmental defects

While essential roles of various transcription factors and their interactions have been extensively studied in cardiac specification, morphogenesis, function, and disease\(^\text{23}\), genetic manipulations in cardioid systems have revealed new, stage-specific changes in transcriptional hierarchy during early cardiogenesis. Specifically, by generating organoids from human PSCs and iPSCs with HAND1 or NKX2.5 knockout, the authors determined that, in the cardiac mesoderm stage, HAND1 functions upstream of NKX2.5, while in the cardiomyocyte stage it functions downstream of NKX2.5. As HAND1 and NKX2.5 mutations are known pathogenic drivers of ventricular anomalies including hypoplastic left heart syndrome, it is conceivable that additional genetic manipulations of relevant transcription factors in cardioids (e.g., GATA4, TBX5, MEF2, SRF) could enhance our understanding of early cardiac morphogenetic defects\(^\text{8, 11}\).

On the other hand, cardioids are primarily made of first heart field derivatives and do not include second heart field or neural crest cells, which play important roles in outflow tract septation, valvulogenesis, and development of the cardiac conduction system\(^\text{24}\). As such, cardioids do not model co-emergence of a variety of intra and extracardiac cell lineages that contribute the formation of linear heart tube, cardiac looping, and patterning of later, more complex cardiac structures (right ventricle, atria, cardiac cushions, valves, Purkinje fibers, inflow/outflow tract, vasculature, lymphatics, etc.\(^\text{25}\)). Since most congenital malformations arise during these later stages of heart development, cardioids will require significant improvements to allow modeling of the common cardiac birth defects. Specifically, improving methods for human PSC differentiation and cardiomyocyte maturation\(^\text{26, 27}\) and integration of advanced
bioengineering methods for 3D cell culture will be important first steps towards realizing this goal. Nevertheless, in their state-of-the-art form, COs can currently permit systematic studies of early cardiac self-patterning, including dissecting and simultaneously investigating cardiac morphogenetic and specification events.

Conclusion

Self-organizing cardiac organoids derived from human PSCs represent important emerging platforms for studies of early heart development. Despite their structural simplicity, these in vitro systems may hold the key to improved understanding of early cardiac morphogenesis and mechanistic underpinnings of congenital heart disorders, eventually leading to improved patient care.

References


### Table 1: Overview of Cardiac Organoid Systems

<table>
<thead>
<tr>
<th>Species</th>
<th>Architecture</th>
<th>Modulated pathway</th>
<th>Findings</th>
<th>Advantages; Disadvantages</th>
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</tr>
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<tbody>
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<td></td>
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<td>Spheroid with a microchamber</td>
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<td></td>
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### Table 1 Legend: Overview of Cardiac Organoid Systems

Classification of COs based on cell type and species, structural organization, signaling pathways modulated, major findings, advantages and drawbacks.
Figure Legends

Figure 1: Progress and Promise of Cardiac Organoids
Icons represent major cardiac organoid applications. Solid lines represent applications for which cardiac organoid technology is sufficiently progressed for immediate utility, such as variant screening. The dashed lines represent areas of promise, where the current complexity of cardiac organoids has not yet matured to meaningfully probe relevant research questions, such as cardiac structural disease.

Figure 2: Schematics of generalized protocol for *in vitro* organoid formation
Pluripotent stem cells are cultured in standard tissue-culture dishes coated with laminin-based matrix. The cells are dissociated from a dish and reconstituted as a single cell suspension before being embedded at a desired density within an extracellular matrix analog. In the matrix, cells self-aggregate into a spherical organoid structure, while soluble factors are used to guide their differentiation and organization.
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