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REVIEW

# *In vitro* models of the human heart

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## ABSTRACT

Cardiac congenital disabilities are the most common organ malformations, but we still do not understand how they arise in the human embryo. Moreover, although cardiovascular disease is the most common cause of death globally, the development of new therapies is lagging compared with other fields. One major bottleneck hindering progress is the lack of self-organizing human cardiac models that recapitulate key aspects of human heart development, physiology and disease. Current *in vitro* cardiac three-dimensional systems are either engineered constructs or spherical aggregates of cardiomyocytes and other cell types. Although tissue engineering enables the modeling of some electro-mechanical properties, it falls short of mimicking heart development, morphogenetic defects and many clinically relevant aspects of cardiomyopathies. Here, we review different approaches and recent efforts to overcome these challenges in the field using a new generation of self-organizing embryonic and cardiac organoids.

**KEY WORDS: Heart, Human pluripotent stem cells, Cardiac organoids, Self-organization, Tissue-engineering**

## INTRODUCTION

The heart is the first organ to form and has served as a fascinating paradigm of human organ development and physiology (Abu-Issa and Kirby, 2007; Kelly et al., 2014). In the last two decades, we have acquired crucial insights into signaling pathways and transcription factors acting at different stages of heart development, as well as into the specification of cardiac cell types and the structures they form (Meilhac and Buckingham, 2018; van Weerd and Christoffels, 2016). Despite these advances, there is still much to be discovered about how and when molecular factors instruct cardiac lineages to form specific heart structures and how these instructions fail in human congenital malformations.

Although heart and circulation disorders are being studied extensively, therapies that effectively tackle and reverse heart disease are missing (Van Norman, 2017). Today, cardiovascular disease is the dominant cause of death globally at 32% (https://www.who.int/health-topics/cardiovascular-diseases#tab=tab_1), far ahead of all cancers combined (17%). Simultaneously, the supply of potential breakthrough drugs is shockingly low (2%) compared with cancer drugs at 45% (Van Norman, 2017). The reasons are manifold and include declining druggable molecular targets and relatively low numbers of compounds reaching the clinical trial stage. The underlying issues are pre-clinical models with low predictive power, combined with the high cost of cardiovascular clinical trials (Eder et al., 2016; Van Norman, 2017).

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Studies in animal models have been essential for elucidating general principles of heart development and disease but have limited suitability to address human-specific aspects of development, disease and therapy. Animal versus human cardiac gene expression patterns differ and hence mouse disease models often do not recapitulate human disease (Bruneau, 2008; Cui et al., 2019; Lowey et al., 2018; Uosaki and Taguchi, 2016). A major bottleneck that hampers our understanding of human heart development, as well as disease and therapy, is the lack of human cardiac *in vitro* models that sufficiently recapitulate cardiogenesis and thus heart physiology and function (Brandão et al., 2017; Kreutzer et al., 2021; Stein et al., 2020). Physiological human cardiac models will be crucial for the pre-clinical development of therapies predictive of outcomes in patients (Moffat et al., 2017; Stein et al., 2020). In recent years (Table 1), we have observed an acceleration in establishing different *in vitro* systems using diverse methodological approaches to fill this need (Drakhlis et al., 2021; Giacomelli et al., 2017, 2020; Hofbauer et al., 2021; Ma et al., 2015; Mills et al., 2017, 2019; Protze et al., 2019; Ronaldson-Bouchard et al., 2018; Rossi et al., 2020; Tiburcy et al., 2017; Voges et al., 2017; Zhao et al., 2019). This Review highlights some of these efforts by pointing out their advantages, limitations, impact and future developments. For a more detailed overview and discussion of specific aspects of cardiac development, molecular regulation and disease modeling, we recommend several highly informative recent reviews (Bruneau, 2020; Devalla and Passier, 2018; Karbassi et al., 2020; Meilhac and Buckingham, 2018; Mummery, 2018; Protze et al., 2019).

### Cardiac development

Organogenesis requires the coordination of cell types and tissue morphogenesis to shape a functional unit. Importantly, specification and morphogenesis are interdependent and intricately linked, with far-reaching implications for physiology and disease. Most of our knowledge about cardiac development derives from animal models that are likely similar to humans, but important aspects may vary and are not corroborated yet.

#### Specification of cardiac cell types

Three main cardiac cell lineages integrate to build the heart: the cardiomyocyte (CM), endocardial (EC) and epicardial lineages (Abu-Issa and Kirby, 2007; Kelly et al., 2014). Cardiac specification begins during gastrulation [at embryonic day (E)6.5-E7 in mice, which would correspond to 15-16 days post fertilization (dpf) in humans] when pluripotent epiblast cells enter the mid and anterior regions of the primitive streak at different times and, as a result, give rise to distinct mesodermal populations (Fig. 1). Both time and place of primitive streak mesoderm ingression direct the subsequent differentiation into separate subtypes of cardiac mesoderm (Ivanovitch et al., 2021; Lescroart et al., 2014, 2018; Schoenwolf et al., 1993). The first population of cardiac mesoderm cells to migrate anteriorly from the primitive streak is called the first heart field (FHF), which gives rise to the left ventricle and a portion of the atria. The second population comprises the anterior and posterior second heart

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**Table 1. Comparison of cardiac-specific 3D models**

| 3D system | Shape | Generation (scaffold) | Cell source (cell types) | Stimulation (assays) | Reference |
|---|---|---|---|---|---|
| Aggregates (spheroids or microtissues) | Spherical | Aggregation in ULA well plates; no scaffold. | hPSC-CMs; hPSC-ECs; hPSC-FBs. | N.A. | Giacomelli et al., (2017); Giacomelli et al., (2020) |
| | | Aggregation in ULA well plates; no scaffold. | hPSC-CMs; primary cardiac ECs; primary cardiac FBs. | N.A. | Archer et al., (2018) |
| | | Made in agarose micro-molds. | hPSC-CMs; FBs; HUVECs; adipose-derived stem cells. | N.A. | Richards et al., (2017); Richards et al., (2020) |
| Micro-chambers | Spherical | Formed in Matrigel-coated, PEG-patterned substrates (microfabricated grooves). | hPSC differentiation. | N.A. | Ma et al., (2015); Hoang et al., (2018) |
| Engineered heart tissue (EHT) | Rod-like | Fibrin (+Matrigel)-based EHTs formed in agarose casting molds. | hPSC-CMs. | Electro-mechanical | Hansen et al., (2010); Mannhardt et al., (2016) |
| | | Fibrin hydrogel formed between two PDMS pillars. | hPSC-CMs; dermal FBs | Electro-mechanical | Ronaldson-Bouchard et al., (2018) |
| | | Polymer wire and microchannel molds used to form a collagen (+Matrigel) hydrogel. | hPSC-CMs; cardiac FBs. | Electro-mechanical | Zhao et al., (2019) |
| | | Circular and rod-like molds used to cast collagen I (+Matrigel) EHTs. | hPSC-CMs; stromal cells. | N.A. | Voges et al., (2017); Mills et al., (2017); Mills et al., (2019) |
| | | Collagen hydrogel wrapped around circular molds. | hPSC-CMs. | Passive stretch | Goldfracht et al., (2019) |
| | Chamber shape | Collagen (+Matrigel) hydrogel used to engineer a chamber shape. | hPSC-CMs; dermal FBs. | N.A. | Li et al., (2018) |
| | | ECM cell-containing bioink used to bioprint a chamber-shaped EHT. | hPSC-CMs overexpressing cyclin D2. | N.A. | Kupfer et al., (2020) |
| Embryonic organoids/ gastruloids | N.A. | Self-organization in ULA plates; no scaffold. | mESC differentiation. | N.A. | Rossi et al., (2020) |
| | Spherical | Self-organization in ULA plates; embedded in Matrigel matrix. | hPSC differentiation. | N.A. | Drakhlis et al., (2021); Silva et al., (2020) |
| Organoids /cardioids | Chamber | Self-organization in ULA plates; no scaffold. | hPSC differentiation. | N.A. | Hofbauer et al., (2021) |

CM, cardiomyocytes; EC, endothelial cells; FB, fibroblasts; hPSC, human pluripotent stem cell; HUVECs, human umbilical vein endothelial cells; mESC, mouse embryonic stem cells; N.A., not applicable; PDMS, polymethylsiloxane; PEG, polyethylene glycol; ULA, ultra-low attachment.

fields (SHF), which give rise to the right ventricle outflow tract and atria, respectively (Meilhac and Buckingham, 2018) (Fig. 1). Progenitors of both FHF and SHF further specify into CM and EC lineages, whereas the epicardial lineage has been reported to originate from a separate mesodermal compartment (Cao and Poss, 2018; Maya-Ramos et al., 2013) (Fig. 1).

The expression levels of marker genes encoding transcription factors and signaling components characterize the FHF and SHF populations (de Soysa et al., 2019; Kelly et al., 2014). However, Tyser and colleagues have recently challenged this traditional view by suggesting that heart fields are transcriptional states through which cells from different sources can progress (Tyser et al., 2021). In support of their model, they have demonstrated the presence of a transient cardiac progenitor population, which can give rise to CMs via an FHF state, as well as to epicardial cells. Thus, marker gene expression is highly dynamic and time-dependent, and therefore limits inferring cardiac progenitor origin and fate.

After the cardiac mesoderm stage, the different CM progenitor populations differentiate and mature further into specific CM subtypes (e.g. pacemaker, septal, chamber CM, etc.) within the distinct compartments of the heart. Similarly, EC precursors give rise to distinct endocardial, valvular and cardiac fibroblast cell populations (Zhang et al., 2018), whereas the epicardium differentiates into cardiac smooth muscle cells, ECs and fibroblasts (Simões and Riley, 2018). As the heart develops, additional important cell lineages, such as cardiac neural crest, innervating neurons, different subtypes of macrophages and other immune cell types (George et al., 2020; McNally, 2020; Stevens et al., 2016), contribute by migrating into the heart at specific times. These cardiac cell types interact to varying degrees and stages through signaling and extracellular matrix (ECM) contacts and mutually influence specification at the level of individual cells, tissue morphogenesis, heart function and disease.

## Cardiac morphogenesis

While cardiac progenitors progressively differentiate into distinct cardiac cell types, complex morphogenetic processes coordinately shape the future heart (Christoffels and Jensen, 2020). In addition to cardiac mesoderm, the adjacent foregut endoderm and anterior ectoderm become patterned into distinct subtypes, rendering this crucial stage of organogenesis highly complex and dynamic (de Bakker et al., 2016; Kelly et al., 2014). The process starts with cells exiting pluripotency and specifying into mesoderm within the primitive streak while undergoing an epithelial-to-mesenchymal transition (EMT). The EMT facilitates the anterior migration of these cells to the cardiac mesoderm region to form the FHF. Subsequently, precursors of the SHF migrate dorsally and medially to the FHF and proliferate in a progenitor state, while the FHF differentiates. Notably, differential signaling, ECM and tissue contacts impact the different populations within the cardiac mesoderm; for example, the proximity of the adjacent endoderm on the ventral side and the ectoderm on the dorsal side coincide with varying levels of mesodermal epithelialization along the dorsoventral axis (Linask, 1992; Sugi and Markwald, 1996). The FHF cells facing the endoderm undergo

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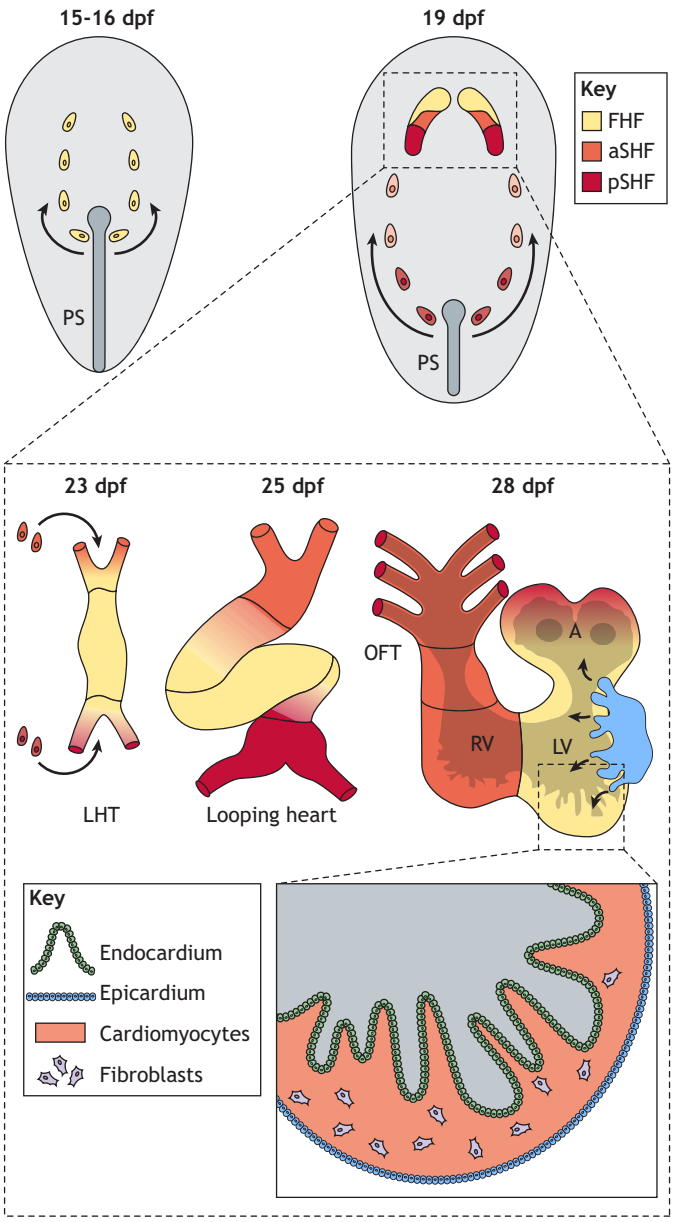


**Fig. 1. Different stages of human heart development.** Illustrations shown in days post fertilization (dpf) showing the contribution of different progenitor populations (FHF, first heart field; aSHF, anterior second heart field; pSHF, posterior second heart field) to the main cardiac developmental structures. Each compartment (A, atrium; LHT, linear heart tube; LV, left ventricle; OFT, outflow tract; RV, right ventricle) contains specific cell types derived from the three cardiac lineages (cardiomyocyte, endocardial and epicardial).

EMT, differentiate into ECs and subsequently form the bilateral endocardial tubes. Concomitant foregut constriction and the migration of FHF mesodermal cell sheets pull these endocardial tubes together, leading to their fusion at the embryo midline (Aleksandrova et al., 2015; Ivanovitch et al., 2017). During this process, the FHF mesoderm differentiates into CMs while enveloping the fusing endocardial tubes and form the first functional contractile structure, the heart tube. Thus, the heart tube consists of an outer CM layer and an inner EC layer separated by an ECM (cardiac jelly).

After heart tube formation, SHF progenitors start migrating into both poles of the tube. As they differentiate and proliferate, the tube grows and loops to form recognizable compartments: the future ventricles, atria, atrioventricular canal, inflow and outflow tracts (Sizarov et al., 2011). At this point, the pro-epicardial cell cluster starts spreading as epicardium on the entire surface of the heart. Complex crosstalk between the different lineages, together with hydromechanical cues within the heart, then lead to the formation of distinct structures, including valves, trabeculae, septa, compacted myocardium, coronary vascularization, the great vessels and the branched cardiac conduction system (Kelly et al., 2014; Lin et al., 2012; Mohun and Anderson, 2020; O'Donnell and Yutzey, 2020; van Weerd and Christoffels, 2016).

The heart tube starts contracting at 23 dpf of human embryogenesis. At this point, it quickly becomes indispensable for driving embryonic circulation, organogenesis and growth, because it sustains the rapidly growing embryo, dramatic lineage migrations, differentiation and interactions that coordinate tissue morphogenesis within the heart. At ∼60 dpf, the main functional structures of the human heart are formed, followed by further growth and structural, metabolic and functional maturation. Thus, the complex and dynamic heart development process itself links intimately to the gradual establishment of its proper physiology and function. We propose that recapitulating developmental aspects strongly influences the success of *in vitro* disease modeling and therapy.

## Modeling cardiac specification *in vitro*

Human cardiac *in vitro* models are widely used and complementary to *in vivo* animal models because they allow: (1) studying human-specific aspects; (2) rapid genetic modifications; (3) mechanistic interrogation due to limited complexity; (4) simple application of biochemistry and microscopy; (5) upscaling and automation of processes.

### Generating cardiac cell types

Historically, there are two approaches for generating cardiac cell types *in vitro* from human pluripotent stem cells (hPSCs): embryoid body formation [floating three-dimensional (3D) culture of differentiating aggregates] and directed differentiation of attached cells in 2D culture (Burridge et al., 2014; Gadue et al., 2006; Kattman et al., 2011; Kehat et al., 2001; Kwon et al., 2007; Lian et al., 2012; Lim et al., 2011; van Laake et al., 2010; Yang et al., 2008; Zhang et al., 2009) (Fig. 2A). These early studies narrowed the requirements for cardiac differentiation down to a few essential signaling molecules; generally, the activation of WNT, ACTIVIN and BMP signaling to induce mesoderm, followed by WNT-inhibition to generate cardiac mesoderm and eventually CMs. In its simplest form, bi-phasic modulation of WNT signaling (i.e. activation followed by inhibition) is sufficient for general cardiac specification. To better model the diverse developmental trajectories of cardiac differentiation, different groups have generated protocols that more closely resemble the much more complex signaling environment in the embryo (Birket et al., 2015; Devalla et al., 2015; Lee et al., 2017; Protze et al., 2017). Consequently, they could specify CMs into further subtypes, such as atrial and ventricular CMs, or CMs of the conduction system (Protze et al., 2019). Similar to CMs, in 2014, Orlova and colleagues published a study showing that the addition of a few crucial factors involved in vascular EC development were sufficient to differentiate ECs in chemically-defined conditions from hPSCs (Orlova et al., 2014). Through further improvements, Patsch and colleagues (Patsch et al., 2015) generated ECs from hPSCs at high efficiencies (>80%) in chemically-defined conditions. Finally, a series of studies demonstrated efficient differentiation of epicardium from hPSC and epicardium-derived cell types, such as fibroblasts and smooth muscle cells (Guadix et al., 2017; Iyer et al., 2015; Witty

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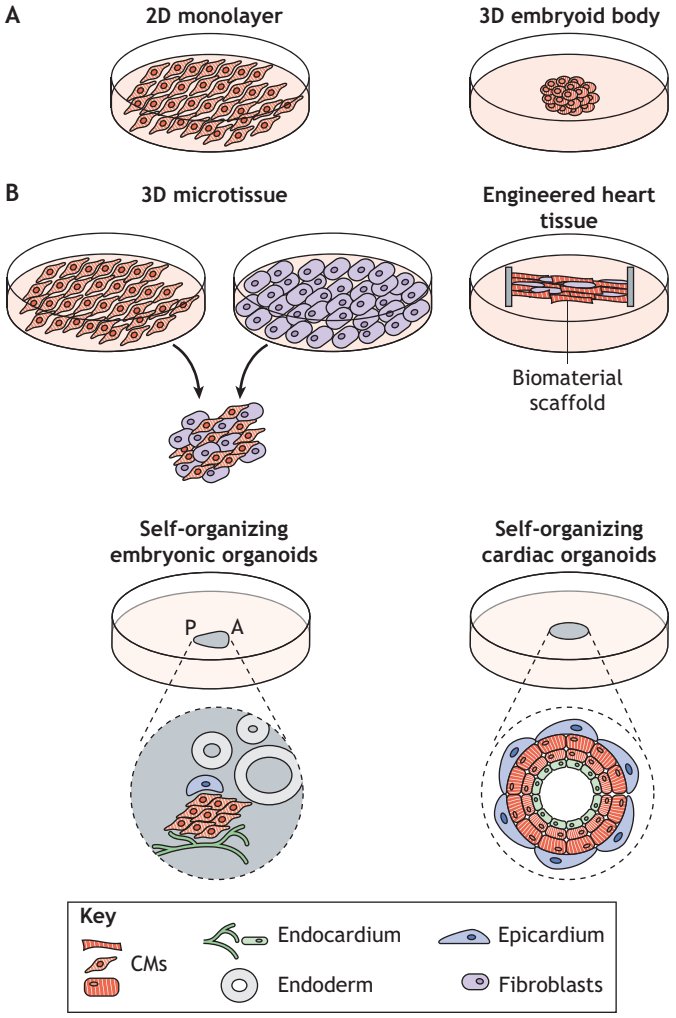


**Fig. 2. *In vitro* specification of cardiac cell types.** (A) Traditionally, cardiac cell types were generated as a 2D adherent monolayer culture (left) or as free-floating 3D embryoid bodies (right). (B) Advanced cardiac culturing techniques include generation of 3D microtissues by combining different cell types derived in 2D (top left). In engineered heart tissues, cardiac cells are combined with biomaterials that facilitate mechanical/electrophysiological manipulation and analysis (top right). Self-organizing embryonic organoids are polarized structures containing cardiac and non-cardiac tissues (e.g. endoderm and/or ectoderm) (bottom left). Self-organizing cardiac organoids (cardioids) composed of cardiac lineages contain a cavity and exhibit a physiological tissue architecture (bottom right).

et al., 2014). With the three main cardiac lineages available from hPSCs, more complex constructs to model cell-type interactions in the heart became accessible.

### Co-culture and co-specification of cardiac cell types

CMs, ECs and epicardium co-differentiate and interact at specific stages during cardiogenesis. Several studies have demonstrated simultaneous differentiation of CMs and ECs from hPSCs based primarily upon VEGF addition during the mesoderm and cardiac mesoderm specification stages (Giacomelli et al., 2017; Palpant et al., 2017). The Mummery lab further developed this approach by sorting and combining 2D differentiated or co-differentiated CMs and ECs into 3D microtissue aggregates (spheroids; Fig. 2B), which contained CMs, ECs and cardiac fibroblasts derived from the same hPSCs (Giacomelli et al., 2020). Crucially, they obtained these three cell types by providing the necessary developmental cues based on embryonic specification. The cardiac fibroblasts were generated from hPSC-derived epicardial cells and, unlike adult dermal fibroblasts, contribute to CM maturation through a mechanism mediated by connexin 43 (GJA1) and cyclic AMP. This study demonstrated how important correct developmental origin is for subsequent function. Using this approach, the group observed key hallmarks of CM maturation, such as T-tubules and a ventricular action potential notch. This microtissue platform allowed for generating a model of arrhythmogenic cardiomyopathy, highlighting the influence of non-CMs on genetic defects.

In a separate effort, Archer and colleagues combined CMs with adult primary cardiac ECs and fibroblasts to generate cardiac aggregates and assess their predictive value for drug toxicity testing (Archer et al., 2018). Similarly, Richards and colleagues employed an aggregate system by combining CMs, adult cardiac fibroblasts, human umbilical vein ECs and adipose-derived stem cells to model aspects of a hypoxic response to assess the response to cardiotoxic drugs (Richards et al., 2020). Overall, several cardiac microtissue systems have been developed to interrogate signaling between cardiac cell types as they mature, model arrhythmias and study drug cardiotoxicity. Their primary advantages are the simplicity and reproducibility of the model and defined cell type ratios in a high-throughput setting. However, aggregation models do not recapitulate *in vivo* morphogenesis, and some of these systems employ non-cardiac primary cells, limiting their potential.

## Modeling cardiac tissues *in vitro* using organoids

Up until the past decade, the term ‘organoid’ was primarily used to describe aspects of tumor morphology and in embryo explant studies attempting to understand organogenesis (Clevers, 2016; Lancaster and Knoblich, 2014; Pierce and Verney, 1961; Smith and Cochrane, 1946; Steinberg, 1964; Weiss and Taylor, 1960). More recently, its meaning has been redefined to allude to organ-like tissue structures. With a wide variety of organoid systems being developed, it is important to clarify that there are two main approaches for generating organoids: first, using tissue engineering methods, and second, using developmental mechanisms of self-organization (Box 1). The prevalent approach in the cardiac field has been the former because cardiac muscle has biomechanical properties, the output of which can be measured directly and accurately using engineered setups. These engineered tissue-like constructs have been termed heart/cardiac organoids or engineered heart tissues (EHTs) (Fig. 2B). They can partially model mechanical and electrophysiological aspects of cardiac tissues but do not rely on developmental morphogenesis.

Outside of the cardiac field, the following criteria have been introduced (Clevers, 2016; Lancaster and Huch, 2019; Lancaster and Knoblich, 2014; Sasai, 2013; Schutgens and Clevers, 2019) to distinguish self-organizing (Box 1) organoid models from engineered tissues and simpler spheroids/aggregates: (1) ‘a 3D structure containing cells that establish or retain the identity of the modeled organ; (2) the presence of multiple cell types, as in the organ itself; (3) the tissue exhibits some aspect of the specialized function of the organ; (4) self-organization (Box 1) according to the same intrinsic organizing principles as in the organ itself’ (Lancaster and Huch, 2019). In addition to these organ-specific organoids, more complex embryonic organoid models rely on self-organization principles but exhibit co-development of multiple germ layers and organ lineages instead of only one. For example, gastruloids are an embryonic organoid model composed of all three germ layer derivatives and thus many different cell types (Moris et al., 2020; Turner et al., 2017). Hence, in this Review, we distinguish between EHTs and cardiac-

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**Box 1. Self-organization**

The principles of self-organization in biological systems have been comprehensively reviewed by Yoshiki Sasai (Sasai, 2013). Based on previous work on complex systems (von Bertalanffy, 1969; Camazine and Bonabeau, 2003; Dobrescu and Purcarea, 2011; Saetzler et al., 2011), Sasai defined self-organization as the 'spontaneous formation of ordered patterns and structures from a population of elements that have no or minimal patterns'. Furthermore, he postulated that multicellular self-organization encompasses three key processes: (1) self-assembly – spatiotemporal control of relative cell positions [e.g. the re-arrangement of cells and thus their segregation (Whitesides and Grzybowski, 2002)]; (2) self-patterning – spatiotemporal control of cell status [e.g. the spontaneous appearance of tissue patterns, such as stripe formation in fish skin (Kondo and Asal, 1995)]; and (3) self-driven morphogenesis – spatiotemporal control of intrinsic tissue mechanics, [e.g. development of tissue shapes through tissue deformation (Eiraku et al., 2011)]. Thus, the crucial challenge of self-organization is the occurrence of patterns without pre-patterns or external force. An added complication in biological systems is their dynamic nature; they dramatically change in a spatiotemporal and context-dependent fashion during proliferation and differentiation, contrary to some self-organizing phenomena in physics or chemistry (e.g. formation of snowflakes from water). In both cases, however, there is a need for an initial symmetry-breaking event. A break in symmetry could be initially driven by stochastic processes and subsequently stabilized by intercellular interactions. Despite the above criteria and definitions, current self-organizing organoid systems are not 100% intrinsic and rely in part on external instructions in the form of extracellular matrix and signaling molecules. These are often essential to support the correct balance of symmetry breaking, morphogenesis and specification. Self-organizing organoids, therefore, use a combination of external signaling control and intrinsic developmental mechanisms of patterning and morphogenesis. This approach is fundamentally different from tissue engineering because the goal is not only to generate *in vivo*-like tissues, but to do so by using developmental principles of organogenesis. In brief, the route matters as much as the destination.

specific self-organized organoids, as well as more universal embryonic organoid models. Importantly, recent developments combine and synergize engineering and self-organization to overcome their respective limitations (Tables 1 and 2) (Brassard and Lutolf, 2019; Nikolaev et al., 2020).

### Modeling cardiac tissues by engineering heart tissues

In parallel to hPSC-derived cell specification into cardiac cell types, efforts started to construct more complex heart tissues *in vitro*. The main aim was to either generate structures more reminiscent of the *in vivo* architecture or a more *in vivo*-like CM maturation status. Maturation is a crucial aspect because most *in vitro* hPSC-derived cardiac cell types suffer from developmental and functional immaturity (Karbassi et al., 2020; Yang et al., 2014). However, although the goal of engineered cardiac tissues is to generate more *in vivo*-like systems, their production does not rely primarily on developmental mechanisms. Instead, the tissue engineering approach combines engineering methods and biological materials to create 3D models of tissues and organs (Langer and Vacanti, 1993). In essence, it encompasses the combined use of bioinert, bioactive or biodegradable scaffolds, cells and bioreactors to engineer 3D constructs of the desired shape for a specific cardio-physiological assay (e.g. contraction, electrophysiology, pumping, etc.). In cardiac tissue engineering, most approaches employ soft hydrogel scaffolds and decellularized ECM from *in vivo* settings (e.g. mouse or human heart ECM) (Guyette et al., 2016; Ott et al., 2008). Alternatively, they use bioink (e.g. containing ECM molecules and/or cells) to 3D print cardiac structures (Devalla and Passier, 2018; Gao et al., 2017; Kupfer et al., 2020). By far, most structures developed so far use hydrogels containing collagen and Matrigel, an undefined ECM mixture derived from the mouse Engelbreth-Holm-Swarm sarcoma (Hughes et al., 2010), or fibrin-based formulations (Goldfracht et al., 2019; Hansen et al., 2010; Ma et al., 2015; Mannhardt et al., 2016; Mills et al., 2017, 2019; Ronaldson-Bouchard et al., 2018; Tiburcy et al., 2017; Zhao et al., 2019).

Virtually all current tissue engineering approaches employ multiple cell types (e.g. CMs, ECs, fibroblasts, etc.) within hydrogels to achieve ideal contractility (Table 1). Several labs pioneered this approach 15-20 years ago (Carrier et al., 1999; Eschenhagen and Zimmermann, 2005; Eschenhagen et al., 1997; Radisic et al., 2004). More recently, some of these EHTs were used as drug screening platforms, in combination with a collagen or Matrigel hydrogel wrapped around two poles to measure contractility (Hansen et al., 2010; Mannhardt et al., 2016; Mills et al., 2017, 2019; Voges et al., 2017). In a similar approach, the Vunjak-Novakovitch lab recently used hPSC-derived CMs and dermal fibroblasts embedded in fibrin hydrogels to generate and exercise cardiac muscle-like tissues in a bioreactor (Ronaldson-Bouchard et al., 2018; erratum in Ronaldson-Bouchard et al., 2019). The continued electro-mechanical stimulation over 21 days using a specific regimen resulted in a mature 'adult-like' CM phenotype. However, the resulting high level of maturation has not been widely reproduced yet, making it difficult to assess in the context of current cardiac *in vitro* systems. A similar system developed by the Radisic lab deployed hPSC-derived CMs and adult cardiac fibroblasts in a collagen/Matrigel hydrogel suspended between two wires to electrically stimulate the generated EHTs (Zhao et al., 2019). This method also included half atrial/half ventricular EHTs and exercise-based maturation. Together, these studies have shown partial maturation of CMs and the ability to use them for disease modeling based on strength and contractility measurements, as well as for arrhythmias. Finally, large chamber-shaped EHTs showed some chamber-specific characteristics, such as liquid pumping ability (Kupfer et al., 2020; Li et al., 2018). These studies allowed measuring of *in vivo*-like parameters, such as fraction of fluid ejected with each heartbeat and pressure-volume relationship within a fluid-filled heart chamber.

In summary, several different methods for generating cardiac 3D models with varying cell type compositions have been developed, and 3D tissue engineering approaches show tremendous promise in modeling aspects of cardiac functionality based on strength/contractility, electrophysiological measurements and pumping parameters. Nonetheless, sophisticated tissue-engineered systems also come with caveats. For example, virtually all these approaches require the use, and the preparation of, biomaterials (such as hydrogels, including the addition of not fully-defined components such as Matrigel) and non-physiological/non-cardiac (primary) cell types. This complexity may hinder reproducibility and diminish the predictive validity of these models. Moreover, their elaborate process of generation limits widespread use and easy high-throughput applicability, because they are unlikely to scale to standard 96- and 384-well plate screening-relevant formats (Magdy et al., 2017). Finally, complex tissue engineering requires specialized machinery and infrastructure that can be challenging to establish in non-specialist labs.

### Modeling cardiogenesis in self-organized embryonic organoids

Self-organization comprises a sophisticated interplay of multiple symmetry-breaking events and their stabilization, combined with

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**Table 2. Evaluation of 2D and 3D human cardiac-specific models, embryonic organoid models and animal models**

| | 2D models | 3D aggregates | 3D engineered tissues | Cardioids | Embryonic organoids | Animal models |
|---|---|---|---|---|---|---|
| Recapitulation of cardiac physiology | Limited | Partial | Partial | Partial | Partial | Full |
| Recapitulation of human-specific physiology | Limited | Partial | Partial | Partial | Partial | Limited |
| High throughput applicability | High | High | Medium | High | High | Low |
| Affordability of the system | High | High | Medium | High | High | Low |
| Technical simplicity of the system | High | High | Low | High | High | Medium |
| Reproducibility of the system | High | High | Medium–high (system dependent) | High | Medium–high (system dependent) | High |
| Applicability for electrophysiological/ mechanical assays | Partial | Partial | Full | Partial | To be determined | Partial |

dynamic spatiotemporal alterations of local organizational rules (Box 1). Self-organization of cells, tissues, organs and ultimately embryos can only be modeled *in vitro* by giving the elements of a system a high degree of freedom. Cardiac self-organization can be studied either in the context of co-development with foregut endoderm and ectoderm in more complex systems, such as embryonic organoids/gastruloids (Baillie-Benson et al., 2020), or in a cardiac-specific context using cardioids (Hofbauer et. al., 2021), which are similar to self-organizing organoids representative of other organs (see below) (Lancaster and Huch, 2019; Schutgens and Clevers, 2019) (Fig. 2B).

Recent studies reported several self-organizing embryoids that include cardiac, foregut and other embryonic tissues (Drakhlis et al., 2021; Israeli et al., 2020 preprint; Rossi et al., 2020; Silva et al., 2020 preprint). Rossi and colleagues generated mouse ESC-derived self-organizing gastruloids (Turner et al., 2017) that showed an anterior-posterior polarity reminiscent of the embryonic axis. They observed a *Tnnt2*$^{+}$ (encoding cardiac troponin T) CM population in the anterior region of the gastruloids, as well as cells expressing T-box transcription factor 1 (Tbx1), which is suggestive of SHF progenitors (Meilhac and Buckingham, 2018). In addition, they noted vascular-like networks within the anterior and posterior poles of the gastruloid. The group also attempted to model a cardiac crescent-like formation of CMs, and its co-development with a gut-like tube cavity separated by endothelial cells. However, this system could still not completely recapitulate *in vivo*-like foregut constriction with heart tube and chamber formation, and it awaits translation in human gastruloids (Moris et al., 2020).

Recently, Zweigerdt and colleagues used hPSCs to derive self-organizing structures in Matrigel that consist primarily of foregut endoderm, fibroblasts, CMs and ECs (Drakhlis et al., 2021). This system is simpler than using gastruloids, because it did not contain ectoderm derivatives. Although the lineages in this model self-organized in a particular order, the arrangement did not correspond to the embryonic heart and foregut development, and major cardiac structures (such as a heart chamber) were still missing. Nevertheless, there appears to be a regularity in lineage composition and patterning typical for self-organization. The usefulness of the system was demonstrated by showing that the deletion of *NKX2-5*, a gene encoding a major cardiac transcription factor, caused a defect in the CM layer of the 3D structure. As it was unclear whether this defect stems from lacking *NKX2-5* expression in EC, foregut or CM precursors, it will be interesting to compare these defects with gastruloids and cardiac-specific organoids.

Similarly, Silva and colleagues (Silva et al., 2020 preprint) generated self-organizing embryoids from induced hPSCs that co-differentiated into gut-like and cardiac tissues, but also lacked typical cardiac structures such as a heart chamber. By modifying conventional microtissue protocols, they were able to generate multi-tissue embryoids that predominantly contained a CM-core, surrounded by epithelial cells in the surrounding looser areas. Eventually, after long-term culture, they showed that CMs were encircled by gut-like tissue, as well as epicardial-derived cells. CMs differentiated in these multilineage embryoids were more mature than in 'purer' CM microtissues, highlighting that co-development with endoderm can impact functional maturation. Overall, embryonic organoid models contain various non-cardiac cell types and are useful to start dissecting the mechanistic principles of lineage crosstalk during foregut-heart co-development. Simultaneously, the presence of non-cardiac lineages makes a cardiac-specific physiological analysis and application challenging, compared with simpler and better-defined microtissues/aggregates and 2D models.

### Self-organizing cardiac organoid models: cardioids

Our group recently developed a self-organizing cardiac-specific organoid model called cardioids (Hofbauer et al., 2021). Cardioids undergo patterning and morphogenesis to form a chamber-like cavity in the absence of non-cardiac tissues (e.g. derivatives of endoderm and ectoderm) and exogenous ECM (Fig. 2B). As cardioids do not contain foregut endoderm, they did not recapitulate the process of heart tube fusion at the midline but instead mimicked early cardiac mesoderm morphogenesis during endocardial tube formation. They patterned into separate myocardial and endothelial layers, and interacted with migrating and differentiating epicardium, which resembled aspects of early heart chamber development. Using this system, we identified signaling mechanisms that control and coordinate cardiac mesoderm self-organization and cavity formation, as well as how these processes fail in cardiac transcription factor mutants. In contrast to bioengineered organoids (Voges et al., 2017), cardioids showed accumulation of ECM proteins upon cryoinjury, which reflects an important pathophysiological response. Thus, human cardioids recapitulated some aspects of early myocardial, endothelial and epicardial morphogenesis. They therefore, resemble other organ-specific self-organizing organoid models (Lancaster and Huch, 2019). Although cardioids provide a foundation for the incorporation of additional lineages and structures of the heart, they are derived exclusively from the FHF mesoderm and, in the present form, are unlikely to recapitulate most cardiac defects that occur during SHF development. Comparing congenital mutants of transcription factor and signaling genes in cardioids and embryonic organoid models will be interesting to start dissecting lineage crosstalk in cardiac defect etiology. More advanced assays that exist for some engineered cardiac systems are still to be established for the cardioid model to assess its full potential in modeling cardiomyopathies.

## Future perspectives

This Review provides an overview of the current three main approaches for modeling heart physiology and disease *in vitro*: cardiac specification models, engineered heart tissues/organoids and self-organizing embryonic and cardiac-specific organoids. Each of these methodological approaches has advantages and disadvantages in addressing specific questions and objectives (Tables 1 and 2). Therefore, we argue that it will be necessary for the future to have easy access to most of these systems to tackle more complex questions and challenges in the field. This effort will require more open sharing of protocols, simplified engineering procedures and rigorous quality control of cell lines and reagents to improve reproducibility. Importantly, findings need to be validated *in vivo* – especially developmentally relevant aspects, requiring closer collaborations between labs working on *in vivo* and *in vitro* models. Finally, studies using self-organizing organoids that represent other organs have shown that they could be highly predictive of patient responses to therapy, unlike previous efforts using more artificial systems (Kopper et al., 2019; Ooft et al., 2019; Sachs et al., 2018; Schutgens and Clevers, 2019). This fact again underscores the importance of using developmental principles to build organ-like structures that are physiologically relevant. Simultaneously, combining tissue engineering with self-organization is a possible future avenue that might further improve the robustness and development of suitable cardio-physiological assays (Brassard and Lutolf, 2019; Nikolaev et al., 2020). We expect this combinatorial approach to eventually yield an advance in the cardiac field that is necessary to overcome the mounting challenges in understanding key developmental processes, modeling disease and recapitulating therapy response.

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### Competing interests

The Institute for Molecular Biotechnology (IMBA) filed a patent application (EP20164637.9) on different types of cardiac organoids (cardioids) with P.H., S.M.J., and S.M. named as inventors. P.H. and S.M. are co-founders of HeartBeat.bio AG, an IMBA spin-off company aiming to explore the potential of a cardioid drug discovery platform.

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