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COMPENDIUM ON BASIC MODELS OF CARDIOVASCULAR DISEASE

Cellular and Engineered Organoids for Cardiovascular Models

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ABSTRACT: An ensemble of in vitro cardiac tissue models has been developed over the past several decades to aid our understanding of complex cardiovascular disorders using a reductionist approach. These approaches often rely on recapitulating single or multiple clinically relevant end points in a dish indicative of the cardiac pathophysiology. The possibility to generate disease-relevant and patient-specific human induced pluripotent stem cells has further leveraged the utility of the cardiac models as screening tools at a large scale. To elucidate biological mechanisms in the cardiac models, it is critical to integrate physiological cues in form of biochemical, biophysical, and electromechanical stimuli to achieve desired tissue-like maturity for a robust phenotyping. Here, we review the latest advances in the directed stem cell differentiation approaches to derive a wide gamut of cardiovascular cell types, to allow customization in cardiac model systems, and to study diseased states in multiple cell types. We also highlight the recent progress in the development of several cardiovascular models, such as cardiac organoids, microtissues, engineered heart tissues, and microphysiological systems. We further expand our discussion on defining the context of use for the selection of currently available cardiac tissue models. Last, we discuss the limitations and challenges with the current state-of-the-art cardiac models and highlight future directions.

Key Words: cardiovascular disease ■ heart ■ organoids ■ phenotype ■ pluripotent stem cells ■ tissue engineering

For decades, stem cell scientists have attempted to deconvolute developmental processes using human and early embryonic tissues that are scarce. From a cardiovascular disease modeling and drug development perspective, animal models have played an important role in the validation of several common and rare diseases.[1] Both safety and efficacy parameters continue to be tested in animals before the commencement of first-in-human clinical trials. Despite the vital role of animal models in research and pharmaceutical development, the discordance between animal and human physiology and pathophysiology is evident with over 80% drug attrition rate in clinical studies.[2] Thus, animal models have not been able to mirror the complexity or pathological diversity present in human systems, thereby limiting clinical success. During embryonic development, there is a notable divergence in the pattern and timing of tissue morphogenesis between humans and animals. For instance, the differences in the stem cell surface marker repertoire between the mouse and human contribute to the key differences in the kinetics and propagation of cell populations.[3] Therefore, the use of human models is considered more reliable to uncover molecular underpinnings of human cardiac disorders and the development of targeted therapies.

The discovery of pluripotent stem cells (PSCs), such as human inner cell mass embryonic stem cells (ESCs)[4] and induced pluripotent stem cells (iPSCs),[5] has emerged as a groundbreaking tool to study human cardiomyogenesis and the basis of congenital and mature heart diseases. Particularly, the iPSC technology has solved the issues surrounding availability of primary cells from patients, resulting in the development of more accurate disease modeling platforms. Moreover, using iPSC-derived cardiomyocytes (iPSC-CMs) and genome editing approaches, a wide number of monogenic and complex cardiac pathogenesis have been studied in vitro, providing newer insights into disease mechanisms.[6] Human iPSCs-CMs has also emerged as a cornerstone in drug

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Nonstandard Abbreviations and Acronyms

2D	2-dimensional
3D	3-dimensional
ACTA2	α-smooth muscle actin
AM	atrial myocyte
APLNR	apelin receptor
BMPs	bone morphogenetic proteins
CF	cardiac fibroblast
CM	cardiomyocyte
CNN1	calponin 1
CO	cardiac organoid
Cx43	connexin 43
EB	embryoid body
EC	endothelial cells
ECM	extracellular matrix
EHT	engineering heart tissue
EMP	erythromyeloid progenitor
ESC	embryonic stem cell
FGFs	fibroblast growth factors
FLT3L	fms-like tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN-γ	interferon γ
IL	interleukin
iPSC	induced pluripotent stem cell
M-CSF	macrophage colony-stimulating factor
MPS	microphysiological system
MTF	muscular thin films
NK	natural killer cell
NM	nodal myocyte
PDGFRB	PDGF receptor B
PECAM	platelet endothelial cell adhesion molecule
PSC	pluripotent stem cell
RA	retinoic acid
RALDH-2	retinaldehyde dehydrogenase 2
SCF	stem cell factor
SHF	second heart field
SMC	smooth muscle cell
TGF	transforming growth factor
TNF-α	tumor necrosis factor α
TPO	thrombopoietin
VEGF	vascular endothelial growth factor
VM	ventricular myocyte

development and drug repurposing.[7] Two-dimensional (2D) iPSC-CM culture was built in part on in vitro system using neonatal rat myocytes to explain the biophysics of conduction block.[8] Parallel development in 3-dimensional (3D) cardiac models and generation of several supportive cell types such as iPSC-derived endothelial cells (iPSC-ECs),[9] iPSC-derived cardiac fibroblasts (iPSC-CFs),[10] and iPSC-derived smooth muscle cells (iPSC-SMCs),[11] has significantly enriched the cellular diversity in cardiac models, supporting physiological maturation and recapitulation of properties like that of an adult heart.

In this review article, we discuss the recent developments in the generation of state-of-the-art protocols for engineering cellular diversity in 3D cardiac model systems, translational utility of miniaturized cardiac models in basic research, and high-throughput screening studies. Finally, we discuss the need for standardization to fully exploit the in vitro cardiac platforms using unprecedented developments in precision genomics and artificial intelligence.

OVERVIEW OF METHODOLOGIES TO GENERATE CARDIOVASCULAR CELL TYPES IN A DISH

In vivo cardiovascular cells are obtained through cardiac morphogenesis, which occurs via modulation of carefully orchestrated pathways. The main pathway modulators include repressors of canonical Wnt/β-catenin bone morphogenetic proteins (BMPs) (bone morphogenetic proteins), nodal/Activin-A, FGFs (fibroblast growth factors), and VEGF (vascular endothelial growth factor) signaling pathways. Among these, Wnt/β-catenin is one of the key signaling pathways required to exit pluripotency and induce formation of early mesodermal intermediates expressing T-box transcription factors Brachyury (T) and eomesodermin which gives rise to multipotent progenitors, such as KDR (kinase insert domain receptor)$^{+}$CD235a/b^{+} and MesP1^{+} (mesodermal posterior 1) cells.[12,13] The manufacturing of iPSC-CMs through stage-wise specification in vitro has been one of the major milestones in cardiovascular research for understanding disease mechanisms and testing therapeutic strategies for clinical translation. The most common and efficient methods to drive cardiogenic program are based on temporal modulation of Wnt, Activin-A, BMP2/4, and TGF (transforming growth factor)-β signaling pathways (Figure 1). Among cardiomyocyte subpopulations, the available methods predominantly generate ventricular myocytes (VMs); however, small proportions of atrial, pacemaker, and nonmyocyte populations introduce heterogeneity that requires further purification or selection.[14,15]

METHODOLOGIES TO GENERATE CHAMBER-SPECIFIC CARDIOMYOCYTE PROPORTIONS FROM PLURIPOTENT STEM CELLS

Obtaining chamber-specific cardiac myocytes is important for many reasons such as understanding altered cardiac

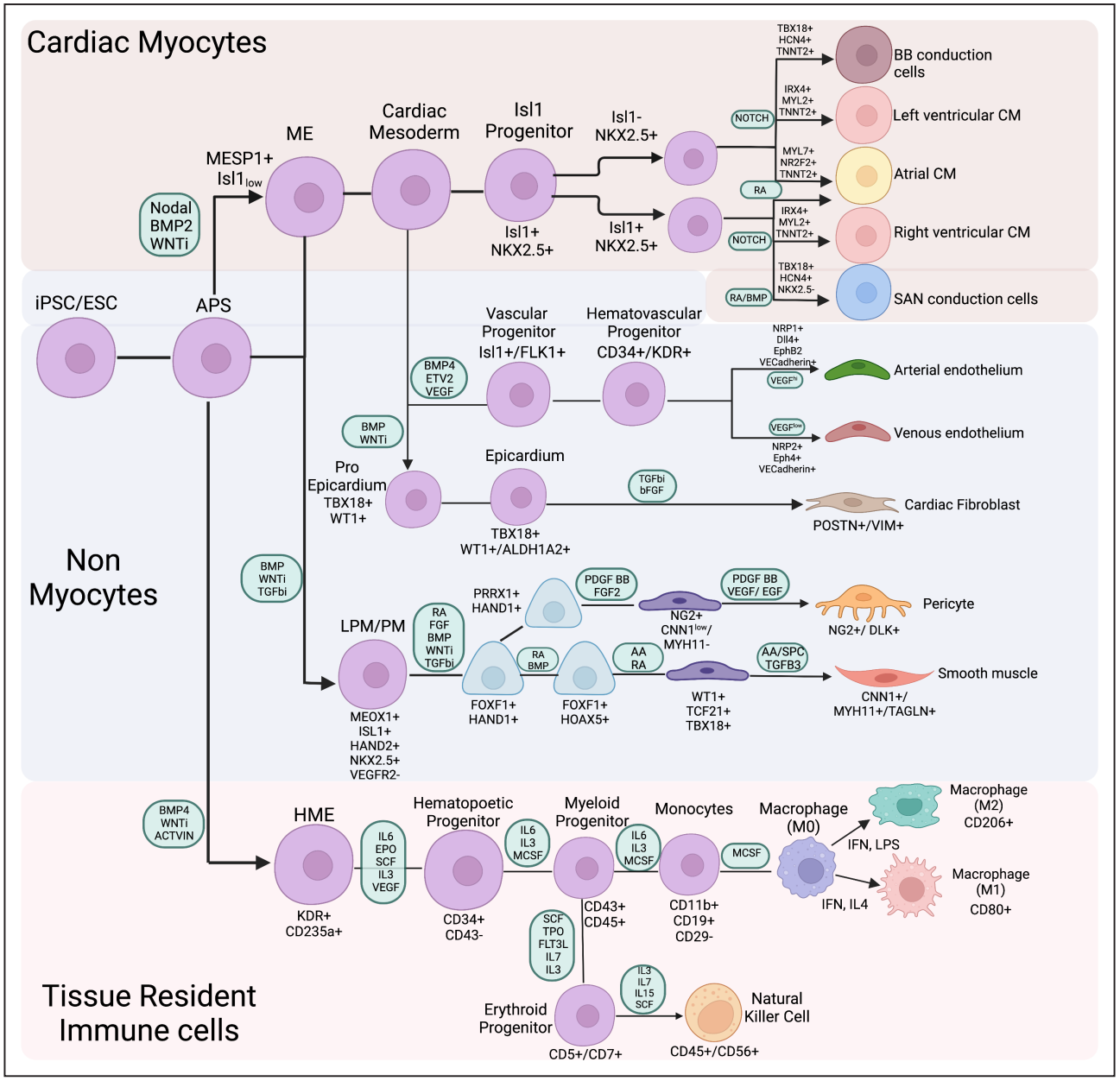


Figure 1. Deconstructing cardiogenesis using induced pluripotent stem cell (iPSC) technology.
An overview of cell lineage trajectory to derive cardiac cellular subtypes in vitro from pluripotent stem cells. Key factors that induce cell-specific morphogenesis and their identity is depicted in the development pathway. Most commonly, all cardiac cell subtypes originate from the mesendoderm progenitors (KDR [kinase insert domain receptor] and MESP1 [mesodermal posterior 1]) that arise from the primitive streak (PS). Further biphasic Wnt modulation leads to the generation of cardiomyocytes (CMs), endothelium. Chamber-specific myocyte cell types are obtained through bone morphogenetic protein (BMP) and retinoic acid (RA) mediated NOTCH signaling activation. Smooth muscles cells and pericytes develop from lateral plate mesoderm (LPM) and paraxial mesoderm (PM). Cardiac fibroblasts are derived from epicardial and mesodermal progenitors. Macrophages and natural killer (NK) cells that naturally reside in the cardiac tissue can be successfully derived from hemogenic precursors. Improving cellular diversity in the human cardiac model systems will offer higher resemblance to cardiac tissue composition. APS indicates anterior primitive streak; ESC, embryonic stem cell; ETV2, ets variant transcription factor 2; FGF, fibroblast growth factor; IFN, interferon; IL, interleukin; SAN, sinoatrial node; TPO, thrombopoietin; and VEGF, vascular endothelial growth factor.[32,33, 42,51,58,62,64,69,82,84]

physiology, pharmacology, and cell-specific toxicology responses. Particularly, a limitless supply of patient-specific iPSC sources can serve as an invaluable resource to understand inherited, acute or chronic, and even racial differences to existing or new pharmaceutics. To generate enriched myocyte population, a guided iPSC differentiation for VM, nodal myocyte (NM), and atrial myocyte (AM) is desirable. For more precise modeling of a cell-specific disease, it is essential to limit heterogeneity which may lead to confounding results. For example, atrial fibrillation is a phenotype associated with atrial cells, hence derivation of pure iPSC-AMs is desirable to model arrhythmic phenotype in familial[16] or sodium channel-linked[17] atrial fibrillation. Similarly, iPSC-VMs can be used to understand

mechanisms that drive channelopathies and cardiomyopathies caused by long-QT syndrome,[18–20] dilated cardiomyopathy,[22] hypertrophic cardiomyopathy[21] among others. In addition to inherited cardiac disorders, metabolic diseases such as diabetic cardiomyopathy,[22,23] iron-overload cardiomyopathy,[24] and ischemia-perfusion injury[25] have also been successfully modeled using iPSC-VMs. From a cell therapy perspective, obtaining pure chamber-specific cell types may prevent abnormal automaticity or arrhythmia due to differing action potentials and conduction velocities from the mixture of different cardiac myocytes. Several selection strategies have been developed for purification such as genetically coded fluorescent reporters,[26,27] modification of culture conditions,[28] metabolic selection,[29] and addition of cell-selective factors.[30] However, it is important to note that despite the enrichment techniques, residual fractions of other cell types may remain.

Ventricular Cardiomyocytes

Differentiation methods to derive PSC-CMs aim to mimic the developmental trajectory of cardiomyogenesis in vivo. In vivo, left VMs develop from first heart field (FHF) and right VMs develop from the second heart field (SHF). First heart field cardiac progenitor ($TBX5^+/NKX2\text{-}5^+$) and SHF cardiac progenitor ($TBX5^-/NKX2\text{-}5^+$) populations have been isolated from reporter lines to generate VMs.[26,27] However, due to the multipotent nature of these cardiac progenitors, it currently remains a challenge to obtain pure left or right VMs to model ventricular-specific diseases. Current protocols using both ESCs and iPSCs generate a mixture of right and left VMs that arise from the cardiac mesoderm induced by manipulating BMP and Activin-A signaling followed by Wnt inhibition.[31,32] Due to the lack of control and diffusional barrier posed by earlier embryoid body (EB) methodology, a monolayer approach was introduced that reported higher efficiency and yield.[33] Delivery of these endogenously long-range acting morphogens, such as BMP and Activin-A, was limited due to the lack of tunability of growth factor concentrations and timing to obtain reproducible results. Therefore, a small molecule-based approach was developed for exogenous activation of Wnt signaling using CHIR99021 (an inhibitor of glycogen synthase kinase 3β) that led to endogenous upregulation of BMP and nodal pathways.[34,35] Adoption of small molecule-based approach significantly improved differentiation efficiency and reduced the reliance on recombinant proteins that were expensive and less stable. This approach simplified the VM derivation in 3 defined stages: (1) induction phase, where T-Brachyury expressing mesendoderm induction is mediated by Wnt activation with small molecules such as CHIR99021, followed by (2) cardiac specification phase wherein Wnt inhibition by small molecules such as IWP-2 (chemical inhibitor of Wnt pathway) or C59 to promote upregulation of cardiac-specific markers, such as NKX2-5 (NK2 homeobox 5) and ISL-1,[34] and (3) the enrichment or metabolic selection phase by glucose starvation that drives the myocytes to shift to oxidative metabolism and simultaneous elimination of glucose-dependent nonmyocytes.[36] In a normal heart, >95% of ATP is generated in the mitochondria from oxidative phosphorylation and 2% to 5% is derived from glycolysis. Hence, supplementation with optimal oxidative substrates such as lipids in a maturation medium will significantly improve function and mitochondrial output.[29] Overall, this 3-stage protocol is shown to generate >90% cardiac troponin T (TNNT2, cardiac troponin T) expressing myocytes reproducibly in several iPSC lines.[35] iPSC-derived VMs typically express genes, such as HAND1 (heart and neural crest derivatives expressed 1), HEY2 (hes related family bHLH transcription factor with YRPW motif 2), MYL2 (myosin light chain 2), MYH7 (myosin heavy chain 7), GJA1 (gap junction alpha-1), and KCNJ2 (potassium inwardly rectifying channel subfamily J member 2) at higher levels compared with common myocyte genes such as NKX2-5, phospholamban, TNNT2, and CASQ-2 (calsequestrin 2). In addition, iPSC-derived VMs when stimulated at 0.5 Hz display a ventricle-like tracing with longer action potential duration at 50% repolarization (action potential duration 50: ≈350–400 ms) with a slower rise (time to peak: ≈300 ms) and decay (≈800 ms) in Ca^{2+} transients.[37] However, most iPSC-CMs derived using this approach are deemed immature in terms of ultrastructural features, gene expression and function. To tackle this maturity issue, several 2D and 3D techniques have been developed that significantly improve iPSC-CM maturity.[38]

Atrial Cardiomyocytes

For the generation of AMs, several in vitro[17,30,39] and in vivo studies[40,41] have demonstrated the role of retinoic acid (RA) as a key regulator in the formation of atrial and pacemaker cell lineages. RA-induced differentiation of AMs is primarily driven by the transcription factor COUP-TFII (chicken ovalbumin upstream promoter-transcription factor 2). Progenitors that express RALDH-2 (retinaldehyde dehydrogenase 2) in the lateral plate mesoderm give rise to AMs. Building on this understanding, several protocols have been developed where the first primitive streak-like induction is followed by cardiac mesoderm specification via biphasic modulation of Wnt pathway. After this stage, enrichment to obtain AMs is guided by stimulation of the cardiac mesoderm (between days 3–5) with RA to enrich the AM population up to 60%.[42,43] Lineage tracing studies have shown that differentiation efficiency can be increased to 20- to 120-fold via activation of JNK signaling pathway using BMP antagonist (GREM2, gremlin 2) which acts upstream of atrial-specific transcriptional factors COUP-TFII and HEY1.[44] However, it is important to note that expression of COUP-TFII has an inverse effect on NOTCH signaling, which is important in ventricular development. Hence, the inhibition of NOTCH pathway can stunt ventricular

development but enhance atrial specification without exogenous RA.[45,46] iPSC-derived AMs show higher gene expression of HEY1, NRF1, TBX5, ATP2A2, MLC2a, and MYH6, in addition to potassium ion channel genes, such as KCNJ3, KCNA5, and KCNK3. Electrophysiological characterization of these cells show an atrial-specific action potential tracing with a shorter plateau (action potential duration 50: ≈160–180 ms), and an overall rapid Ca^{2+} kinetics with a faster rise (time to peak: ≈150 ms) and decay (≈600 ms).[37]

NMs or Pacemaker Cells

NMs of the conduction system are responsible to initiate contractions of the heart by relaying electrical signals from the atria to the ventricles. One of the main molecular hallmarks of NMs is the absence of transcription factor NKX2-5. The earliest attempt to generate NMs from PSCs involved activation of mesoderm using BMP/Activin-A followed by supplementation with neureglin neutralizing antibody and ErbB signaling antagonist, to yield >50% pacemaker cells.[47] Recent protocols developed to generate NMs have taken 3 distinct approaches: (1) overexpression of transcription factors that control NM development, such as TBX3[48] and SHOX2,[49] (2) enrichment of NKX2-5^{-} population through overexpression of c-MYC oncogene,[50] and (3) activation of BMP-RA signaling and blocking FGF signaling after mesoderm induction.[51] Typically the differentiation efficiency of NMs using BMP-RA signaling is around 4% to 10%, but a significant improvement in enrichment up to 35% can be achieved by fine-tuning of BMP-4 concentrations in the presence of TGF-β inhibitor. Unlike strong expression of HCN4 in vivo, NMs derived from iPSCs show a low diffuse expression. Hence for marker-based selection and enrichment, it is important to characterize NMs for the presence of several additional nodal ion channel genes, such as KCNJ and HCN1. Typical NMs reveal small action potential amplitudes and duration (action potential duration 50: <100 ms) with upstroke velocities of <30 V/s.[51]

Derivation of Nonmyocytes to Engineer Cellular Diversity in Cardiovascular Models

Other than cardiomyocytes, the heart is composed of nonmyocytes, such as endothelial cells (ECs), cardiac fibroblasts (CFs), smooth muscle cells (SMCs), tissue-resident macrophages, and natural killer (NK) cells. The nonmyocytes are functionally intertwined with the cardiomyocytes and are important in physiology and pathophysiology. Development of protocols focused on derivation of nonmyocyte subtypes helps to discern mechanisms that drive both maturation and cardiac dysfunction due to cell-cell interactions in coculture studies.[52,53] Given the important role of nonmyocytes in cardiac physiology, it is pertinent to derive them reproducibly from iPSCs to understand disease processes that result from cellular crosstalk in a de novo manner.

Cardiac Fibroblasts

Both CFs and SMCs in vivo originate from the epicardium, endocardium and neural crest progenitors. Compaction of the epicardium brought about by these cell types during development further promotes proliferation of adjacent cardiomyocytes. Epicardial cells expressing markers, such as WT1 and TBX18, are the major source of CFs. These epicardial cells further undergo epithelial-to-mesenchymal transition giving rise to CFs and SMCs.[54] Following a similar trajectory, in vitro differentiation of CFs from iPSCs is guided through epicardial transition and epithelial-to-mesenchymal transition[55,56] via Wnt and FGF signaling pathways.[10] Since FGF2 is one the potent inducers of fibrogenesis, direct stimulation of mesodermal progenitors was shown to generate CFs without the intermediate epicardial cells.[57] Similar to fetal and adult ventricular CFs, iPSC-CFs derived using this method show a comparable expression of fibroblast marker, TE-7, in 80% to 90% cells during early passage. However, iPSC-CFs generated using these protocols express low levels of TBX20 which is more consistent with embryonic CF phenotype.[58]

Endothelial Cells

The majority of the EC population in the heart emerge from the endocardium forming a significant portion of both coronary and valvular endothelium.[59] Recent lineage tracing studies have also demonstrated the formation of valvular interstitial cells through VEGF and FGF stimulation of CD31 expressing precursor derived from MESP1 endocardial progenitors.[60] ECs from iPSCs or ESCs are generated from $CD34^{+}/KDR^{+}$ hematoendothelial or cardiogenic precursors.[61] With subsequent cardiomyocyte protocol development, earlier EC protocols initially relied on EBs and cardiogenic mesoderm induction followed by addition of angio-inductive factors, such as DKK1, VEGF-A, and FGF2.[31] However, this method was unable to generate high yield of ECs due to uncontrolled endothelial-to-mesenchymal transition. Taking advantage of the progress of differentiation protocols developed in EBs, several monolayer protocols were developed using CHIR99021, wherein T-Brachyury mesodermal cells primed in endothelial growth media were sufficient to drive endothelial specific fate determination.[62] Several modifications to this protocol with the addition of endothelial-inductive factors, such as VEGF, BMP-4, and forskolin, were found to enhance the yield of ECs in the presence of TGF-β inhibitor to limit endothelial-to-mesenchymal transition.[62–64] ECs have also been derived from PSCs from hemogenic precursors such as $CD34^{+}$ cells either by delivery of exogenous hematopoietic cytokines[65] or culturing $CD34^{+}$ cells[61] in endothelial growth media. A recent study explored derivation of ECs using BMP/Activin-A signaling to generate progenitors of both mid-primitive-streak cardiac mesoderm and posterior hemogenic mesoderm.[66] Such scalable approach allows for

further investigation into EC phenotypes that are cardiac tissue-specific versus the arteriovenous EC derivatives to study the cell-specific roles in a disease.

Smooth Muscle Cells

Vascular SMCs play an important role in maintaining vascular tone and blood pressure. During development, SMCs primarily emerge from lateral mesoderm, paraxial mesoderm, and neuroectoderm. Several methodologies have been introduced based on EB differentiation[67] or differentiation of selective CDH5 (cadherin 5)$^-$ cell populations[68] in smooth muscle medium containing VEGF and bFGF.[11] SMCs show specific markers such as ACTA2 (α-smooth muscle actin) and CNN1 (calponin 1). Based on these markers, >90% purity was obtained through negative sorting or metabolic selection, although the lineage origin of the SMCs was unclear. The discovery of Wnt mediated mesodermal induction made it feasible to derive SMCs of the mesodermal origin that exclusively expressed PDGFRB (PDGF [platelet-derived growth factor] receptor B).[64] Similarly, it was shown that mesodermal mesenchymal progenitors expressing APLNR (apelin receptor) and the PDGFA receptor when exogenously treated with TGF-β and sphingophospholipid led to SMCs expressing ACTA2 and CNN1.[69] To mimic generation of in vivo-like lineage-specific SMCs, Cheung et al. introduced a stepwise protocol that gave rise to mesodermal and ectodermal progenitors via exogenous delivery of FGF2, BMP-4, and PI3K inhibitor, followed by TGF-β inhibition to derive neural crest lineage through ectodermal precursors. These precursors led to the formation of SMCs when supplemented with (PDGF)-BB and TGF-β for 6 days.[70] Due to the immature phenotype of the SMCs, several attempts have been made to obtain a mature contractile SMC phenotype through overexpression of myosin heavy chain MYH11.[69,71] To date, iPSC-SMCs have been used to study pathological mechanisms in several vascular diseases, such as hypertension,[72] atherosclerosis,[73] and supravalvular aortic stenosis among others.[74,75]

Pericytes

Pericytes together with SMCs stabilize newly formed vasculature through physical and molecular interactions with the endothelium. In the heart, the absence or depletion of pericytes can result in vascular leakage and hemorrhaging in the microvasculature. The loss of pericytes due to disease or toxicity can change the shape of the blood vessels and compromise permeability.[76] Pericytes emerge mainly from the epicardium,[77] lateral mesoderm, and paraxial mesoderm.[78] Mesodermal pericytes are obtained through endothelial intermediates expressing CDH5 and PECAM (platelet endothelial cell adhesion molecule) which in the presence of FGF2 and PDGF-BB give rise to NG2 (neural/glial antigen 2)-expressing immature pericytes.[69] Cardiac tissue-specific pericytes develop through an epithelial-to-mesenchymal transition process together with vascular SMCs from the epicardial progenitors expressing PDGFR-β, whereas PDGFR-α expressing epicardial cells diverge and give rise to CFs. PDGFR-β-derived cardiac pericytes are characterized by expression of NG2 and ACTA2.[79] Recent reports also indicate that cardiac pericytes can arise from endocardial endothelial intermediates,[80] which develop as low NG2-expressing PDGFR-β cells, eventually leading to high NG2 expressing pericytes after integration into the capillaries.

Tissue-Resident Macrophages and NK Cells

Cells of the monocytic lineage originate from the hemogenic progenitors from the mesoderm. Most tissue-resident innate immunity cells such as macrophages arise from circulating blood monocytes. Monocyte differentiation cascade begins with CD34$^+$/KDR$^+$ hematopoietic progenitors to erythromyeloid progenitors (EMPs), which further give rise to cells of the erythroid and myeloid lineage. Further differentiation of EMPs to monocytes is dependent on exogenous stimulation with hematopoietic cytokines. For monocyte derivation, CD45$^+$/CD73$^+$ EMPs is induced with exogenous cytokines such as IL (interleukin)-3, IL-6, TPO (thrombopoietin), SCF (stem cell factor), and GM-CSF (granulocyte-macrophage colony-stimulating factor). Continued stimulation with IL-3, IL-6, and M-CSF (macrophage colony-stimulating factor) further gives rise to CD45$^+$/CD14$^+$ monocytes. These monocytes can be further polarized using lipopolysaccharide and IFN (interferon)-γ to derive M1 macrophages, or via IL-4 stimulation to derive M2 macrophages.[81,82] Other than the monocytic-derived macrophages from the blood, recent lineage tracing studies have shown the presence of 2 unique subsets of cardiac tissue macrophages expressing CCR2$^+$/Ly6c$^+$ and CCR2$^-$/Ly6c$^+$.[83] One of the key questions that requires further investigation is whether we can generate macrophages that resemble cardiac tissue-resident macrophages. In a similar fate determination route as the macrophages, NK cells also emerge from EMPs. To direct in vitro differentiation of NK cells, EMPs are supplemented with FLT3L (fms-like tyrosine kinase 3 ligand), IL-3, IL-15, and IL-7 over 4 weeks to generate CD45$^+$/CD56$^+$ NK cells.[84] The resulting NK cells have been shown to be functionally active, producing cytokines, such as IFN-γ and TNF (tumor necrosis factor)-α. Studies using these cells in a coculture or with cardiac organoid (CO) models will help reveal inflammatory processes that lead to monocyte recruitment in inflammatory cardiac diseases.

Identifying Biological Variability for Better Differentiation Outcomes

Despite the advances made in deriving cardiovascular cell types in a dish, there are several challenges that stem from the inherent variability among patient-derived

iPSC lines.[85] Hence, it is important to consider genetic background and the effect of a known variant within a specific genetic background for both iPSC derivation and differentiation. Some genetic changes are amplified in culture in both ESC and iPSC lines. For example, in nearly 18% to 20% of the lines screened by the International Stem Cell Initiative, a gain of prosurvival oncogenic mutations such as 20q11.21 is seen.[86] Karyotypically increased expression of such oncogenes often downregulate differentiation associated genes,[87] although the influence of epigenetic factors is mostly removed due to the reprogramming process.[88] Some residual DNA methylations could influence the differentiation efficiency based on source of the donor cells.[89] Large-scale iPSC expression quantitative trait loci studies further point to the differences in genetic variants across different individuals are higher than interindividual differences.[85,90] Such interdonor variability introduced by the variants that affect the differentiation genes[91] and by the protocols themselves, affect the activation of gene regulatory networks that define cell fate.[92,93] On a protein level, similar studies with >200 iPSC lines from 151 donors that compare protein quantitative trait loci suggest phenotypic outcomes are also influenced by protein expression levels. In cases of rare or unknown variants, such differences may confound our understanding when investigating risk factors for a specific disease causing phenotype.[94] One of the approaches to tackle the variability is to apply rigorous characterization protocols to determine genomic integrity between passages within the same line,[95] across different clones,[96] and across lines from different donors. Outliers based on variation in genes pertinent for somatic differentiation can be screened out by defining an outlier enrichment scale based on whole genome, transcriptomic, and proteomic profiles.[97] Such a system can also be established for genetic and nongenetic disorders that are classified based on disease-specific and rare variants.[98]

SEMI-HIGH THROUGHPUT STEM CELL-DERIVED 3D CARDIAC STRUCTURES

Redefining Terminologies: Distinguishing CO Models, Assembloids, and Anisotropic Cell-Based Models

COs or self-assembled microtissues or assembloids are broadly defined as 3D tissue surrogates composed of different cell types that reasonably mimic cellular heterogeneity and finite functions of the target organ. Generation of COs involves germ-layer specification through activation or nodal/activin/BMP signaling using naturally occurring morphogens or small molecule chemical analogs. Developmental COs are believed to undergo developmental trajectories as seen in vivo, whereas microtissues assembled from terminally differentiated cells undergo forced aggregation in a defined cavity or mold. The common denominator between these 2 classes of models is that they do not require exogenous extracellular matrix (ECM) as a primer to initiate cellular self-assembly, but they have major differences in their patterning or spatially organized features. However, the main difference between the resulting constructs after spontaneous cardiogenesis and cellular assemblage lies in the patterning or spatially organized features.[99]

COs are generally used as broad term for precardiac structures and feature-specific organoids, which, in part, constitute the cell or cardiac tissue-like assembly in a miniature form. Terminologies, such as gastruloids, cardiods, heart-forming organoids, and heart organoid, are confusing due to their striking differences in the features exhibited by different CO models. In addition, it is challenging to identify the model based on the in vivo morphogenetic stage of cardiac development. Due to the current limitations in the organoid models which do not resemble morphologically identifiable heart or heart chambers, there is a need for unifying classification based on the level of patterning and cavity formation. COs that do not exhibit any morphogenetic or chamber-like features can be collectively categorized as amorphic COs, whereas single or multicavity-forming organoids can be categorized as cavity-forming COs. Cardiac microtissues or assembloids are user-defined multicellular assemblies that are formed with predifferentiated cardiomyocytes with or without nonmyocytes, such as CFs, ECs, and SMCs. These models are limited in terms of cellular diversity but allow for dissecting cell-cell interactions in a controlled microenvironment. Most CO and assembloid models do not adopt a rod-shaped morphology reminiscent of an adult cardiomyocyte, which is a hallmark of maturation. Using tissue engineering techniques, the differentiated cell types can be embedded in a suitable ECM to achieve cardiac tissue-like anisotropy, and exert strain in a uniaxial direction due to passive tension offered by deformable substrates. In general, these ECM-guided constructs are broadly classified under engineered heart tissues (EHTs). However, the term EHT does not provide a reasonable commensurability to morphology or the tissue composition of a human heart. Regardless of the form factor (strip, ring, or patch), ECM-based 3D tissue preparations composed of ventricular cardiomyocytes with or without supporting nonmyocytes is a tissue-like representation of the myocardium.[100] Therefore, engineered heart muscle would be an apt terminology for better comprehension for both users and a nonspecialized audience.

COs: Mimetics for Cardiogenesis

In the past several years, there has been tremendous efforts focusing on generating developmental COs to study cardiac biology and regeneration.[101] In vivo, the chronology of heart formation begins with the appearance

of first heart field giving rise to the cardiac crescent, further merging into a linear heart tube that gives rise to left ventricle and parts of atria. The emergence of SHF drives arterial and venous polarity in the tube-like stage, further guiding the folding and chamber formation that gives rise to right ventricle, outflow tract and parts of atria.[102,103] The myocardium formed from the mesoderm is sandwiched between the endocardium and epicardium, demarcating a layered assembly.

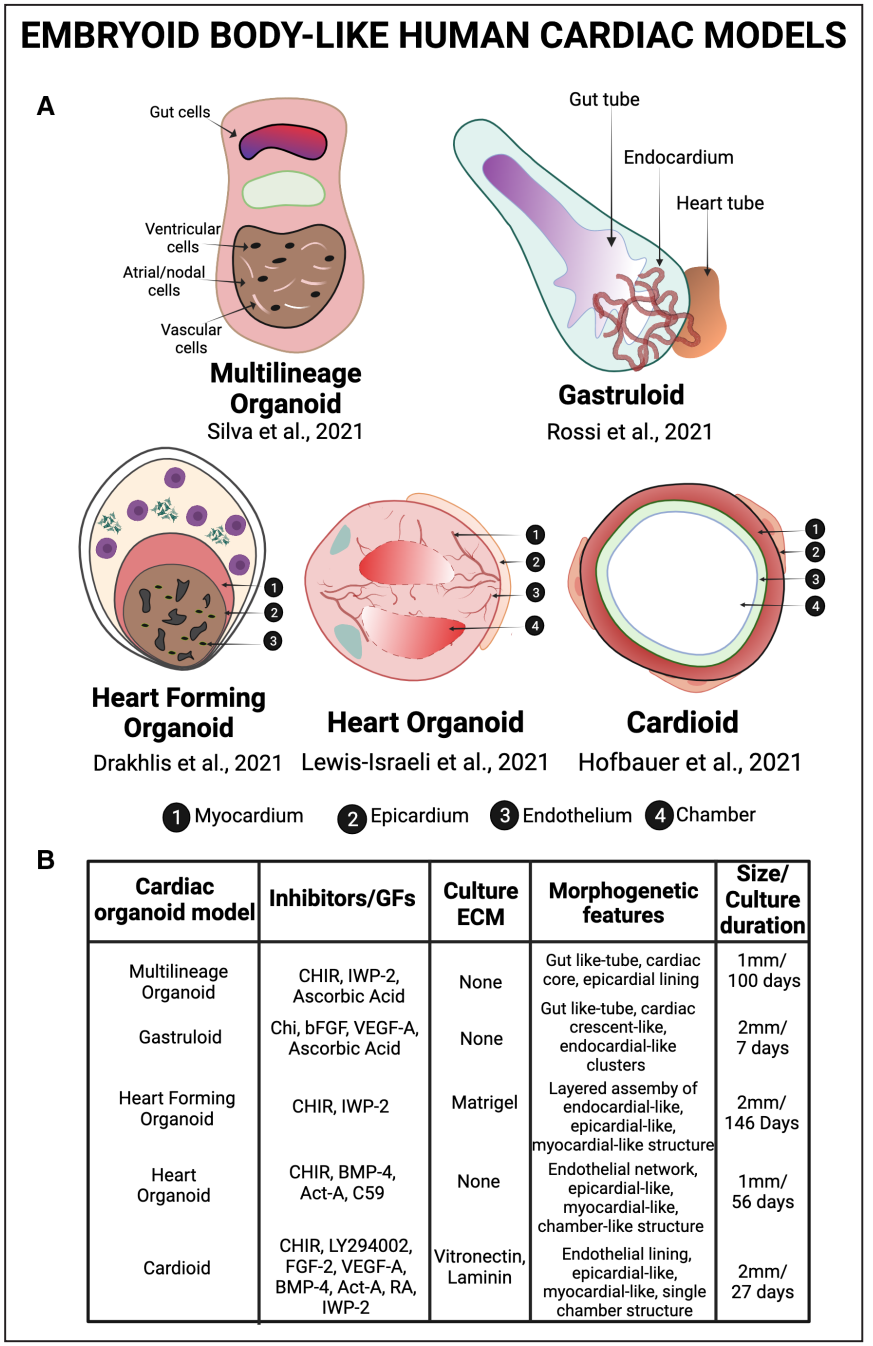
Most in vitro CO models are derivatives of previous protocols that first demonstrated the role of transcriptional regulators driving mesoderm and cardiac differentiation in vivo.[31,32] Wnt modulation using naturally occurring in vivo morphogens such as BMP and Activin-A or chemically synthesized small molecules such as CHIR/IWP-2 is used to obtain cardiomyocytes from ESCs or iPSCs. Before the adoption of 2D culture protocols for higher yield of cardiomyocytes[35,104,105], earlier methods obtained cardiomyocytes from spheroid EBs formed from aggregates of ESCs or iPSCs.[31,32,106–110] Building on the EB differentiation approach, recently developed CO models have been successful in demonstrating hallmarks of early cardiomyogenesis through the formation of cardiac mesoderm, endoderm, coemergence of gut-like structures, chamber-like cavities, and EC networks induced by vascular growth factors. In one of the first examples, EBs were subjected to Activin-A and BMP that gave rise to distinct first heart field and SHF regions but lacked polarity and structural features of a cardiac crescent or tube-like structures, for which it was aptly termed as a pre-CO. Using a small molecule-based approach, the emergence of multiple lineages during gastrulation was demonstrated with mouse ESCs that gave rise to cardiac crescent progenitors, neural, mesodermal, endodermal derivatives, and hematopoietic progenitors.[111]

Applying the same principles of gastrulation that provides a primer for multiple germ-layers and isotropic assembly, Rossi et al[112] using mouse iPSC-derived EBs were able to enrich cardiac structures within gastruloids by modulating Wnt signaling and addition of cardiogenic factors such as ascorbic acid, bFGF, and VEGF. The gastruloids supported the formation of primitive gut-like structures that codevelop with cardiomyocytes during embryogenesis. However, despite the appearance of cardiac progenitors and tube-like formation, it does not follow in vivo stages that lead to diversification of atrio-ventricular population followed by looping to form 4 chambers. In a slightly different approach, Silva et al[113] used human iPSCs to form gastruloid structures but deviated from spontaneous assembly by early dissociation during the organoid formation, followed by cellular reconstitution into spheroids. It remains unclear how disruption of self-organization may provide in vivo-like patterning in the resulting gastruloid. Heart-forming organoids introduced by Drakhlis et al[114] presented a layered self-assembly of the COs consisting of endodermal core, endocardial cell layer, a myocardial layer, and interspersed liver anlagen and septum-like cells. In this protocol, the bulk of the cardiomyocytes arose from SHF giving rise to higher ventricular and a smaller fraction of atrial-like myocytes.

One of the main reasons for the disproportionality of germ-layer assembly and maturation could be because in vivo Wnt pathway modulation occurs in a region-specific and cyclically controlled manner, which is challenging to achieve in vitro while using small molecules as they randomly diffuse in a static culture setting. There are other examples wherein cavity-forming COs have been developed using BMP and Activin-A molecules to induce microcavities within the COs (Figure 2A). These cardiac morphogens dictate the cell fate and trajectory in a dose-dependent manner as shown in one example wherein a higher BMP-4-to-Activin-A ratio doubled the atrial cell fraction compared with that of ventricular cells within the COs, leading to the enrichment of nonmyocytes from epicardial and endocardial progenitors.[115] Similarly, in an improved cavity-forming CO model called cardiods, a multitude of cardiogenic factors including both naturally occurring and chemically derived small molecules were shown to drive cavity formation. The resulting concentric assembly of epicardium and myocardium enveloped by a single central cavity was lined by a layer of ECs.[116] The cavity formation was a result of higher Wnt activation that adversely affected development of a myocardium. The addition of epicardial cells and subsequent self-assembly led to repatterning which mimic the exterior epicardial cell layer. However, it is important to note that this model lacks endodermal cells and temporal chronology of in vivo development from crescent-like stage, tube formation, and looping that precede chamber formation. The unique characteristics of each model are dissimilar to the early heart due to lack of fine-tuned control over spatially controlled signaling aspects of morphogenesis (Figure 2B). Despite the concerns of reproducibility, a thorough interrogation of these processes may allow the tracing of cellular trajectories useful to studying congenital diseases or aid in the drug development process. Therefore, it is imperative that a translational fit for purpose be defined based on the similarity between the CO model of choice and the disease being modeled compared with the relevant in vivo heart development stages.

Cardiac Assembloids and Microtissues

Cardiac assembloids or microtissues are beating clusters of cells formed by controlled aggregation and self-organization of stem cell-derived cardiovascular



Cardiac organoid model	Inhibitors/GFs	Culture ECM	Morphogenetic features	Size/ Culture duration
Multilineage Organoid	CHIR, IWP-2, Ascorbic Acid	None	Gut like-tube, cardiac core, epicardial lining	1mm/ 100 days
Gastruloid	Chi, bFGF, VEGF-A, Ascorbic Acid	None	Gut like-tube, cardiac crescent-like, endocardial-like clusters	2mm/ 7 days
Heart Forming Organoid	CHIR, IWP-2	Matrigel	Layered assemby of endocardial-like, epicardial-like, myocardial-like structure	2mm/ 146 Days
Heart Organoid	CHIR, BMP-4, Act-A, C59	None	Endothelial network, epicardial-like, myocardial-like, chamber-like structure	1mm/ 56 days
Cardioid	CHIR, LY294002, FGF-2, VEGF-A, BMP-4, Act-A, RA, IWP-2	Vitronectin, Laminin	Endothelial lining, epicardial-like, myocardial-like, single chamber structure	2mm/ 27 days

Figure 2. Embryoid body-like human cardiac models.
Summary of recent noteworthy human cardiac organoid (CO) models that mimic some features of heart development in vivo. **A**, The COs can be classified based on the presence or absence of cavity that resemble chamber-like features during early heart development. **B**, A brief summary of the morphogenetic factors or small molecules used to direct the formation of distinct precardiac structures in vitro. Act-A indicates Activin-A; BMP, bone morphogenetic protein; CHIR, an inhibitor of glycogen synthase kinase 3β; ECM, extracellular matrix; FGF, fibroblast growth factor; RA, retinoic acid; and VEGF, vascular endothelial growth factor.

cell types or from established human cell lines. In comparison to COs, the user-defined control achieved in these types of models depends on its composition, size and shape, simplicity in fabrication, and high reproducibility. Several different cell types, such as CMs, ECs, and CFs, can be coaggregated in an ECM-free form to derive beating spheroids of uniform size and structures. These resulting structures are specifically termed as cardiac microtissues or assembloids. Giacomelli et al[53,117] developed a microtissue model through codifferentiation of CMs and ECs wherein the presence of cardiac stromal cells enhanced gene expression and function by improving cardiomyocyte Ca^{2+} kinetics and electrophysiology. Owing to such benefits of nonmyocytes and their role in maturation of CMs, recently developed protocols[118,119] now combine all 3 major cell types to dissect cell-cell responses in disease and drug responses. Applications of well-defined microtissues have also been demonstrated for the evaluation of cardiotoxicity and injury-related fibrotic response or ischemia to the myocardium. For example, Richard et al[120] demonstrated that cardiomyopathy due to injury or cardiotoxicity can be recapitulated on a microtissue platform using primary human CFs and umbilical vein

ECs, together with iPSC-CMs. The microtissues thus formed showed the presence of a putative EC networks resembling a vascular network with lumen-like structures.

Cardiac microtissues or assembloids has a unique niche in disease modeling and cardiotoxicity high-throughput screening due to its facile and cost-effective fabrication methodology.[121] More importantly, the control of over cellular composition allows for ease of dissecting cellular crosstalk between cardiomyocytes and nonmyocytes in the context of in vitro maturation and disease progression in a dish. Further development of the platforms will be crucial to understand the long-term benefits or consequence of in vitro maintenance of such heterotypic microtissues.[122]

ECM FOR STRUCTURE AND FUNCTION OF THE HEART

In addition to cell-cell interaction in a confined geometry, cell-ECM interactions are essential to obtain cellular polarity and cell-cell junctions in axial direction for mechanical and electrical coupling. In 2D cultures, the cell-cell junctions are isotropic whereas in native heart tissue the CMs are highly polarized within fibrous ECM-forming intercalated discs with adjacent cells axially, while forming costamere complexes with surrounding ligands in the lateral direction.[123,124] Hence, engineering the right extracellular environment is not only significant for functional coupling but also for force transduction through load bearing contractions.

ECM in Regulation of Myofibrillogenesis

Cell-ECM binding plays an important role in myofibrillogenesis during cell development. When the cells adhere to the ECM, focal adhesions (FAs) which are multiprotein complex linking the ECM and cytoskeletal actin are assembled,[125,126] activating downstream signaling to regulate cytoskeletal and myofibril assembly along the ECM structures (Figure 3A).[127,128] When cardiomyocytes are cultured on the single-cell sized rectangular micropatterned ECM, localized FAs are observed at the corner as the adhered cells are spread to ECM pattern. From the corner, FAs initiate actin polymerization and organize myofilaments parallel to the boundary of ECM pattern,[129] resulting in anisotropic organization of cytoskeletal architectures.[127] Rectangular patterned myocytes with a 7:1 aspect ratio show anisotropic cytoskeleton structure, exhibiting maximum contractility and fast Ca^{2+} handling compared with shapes with other aspect ratios (Figure 3B).[130,131] This corresponds to cardiomyocytes found in the healthy adult heart.[132] However, ECM patterns with aspect ratios lower or higher than 7:1 and stiff ECM substrates cause misalignment of the sarcomere or exhibit a short sarcomere length, leading to reduced contractility. These results suggest that the mechanical and geometric environment of ECM affect the heart function and disease development.[133–135]

Structural, Electrophysiological, and Mechanical Connection of Cell-Cell to Cardiac Tissue

Cardiac tissue is formed as the cells are connected end-to-end (Figure 3C). Here, ECM geometry also plays a vital role in regulating cell-cell coupling.[136] In the in vitro cell-pair model, cell-ECM adhesion constrains the cell-to-cell junction morphology and regulates tissue assembly (Figure 3D).[137] As the assembly of FAs leads to ECM shape-dependent cell-migration and myofibrillogenesis at an early stage of cell culture, the FAs near the cell-cell interface are disassembled to interconnect their myofilament. The ECM geometry also organizes the laminar architecture of cardiac tissue.[138] Geometric line patterns of ECM proteins such as fibronectin allow for inter/intracellular organization of cardiac tissue (Figure 3E). This affects the alignment of cytoskeleton architecture.

Cell-to-cell junction location also affects the structure and function of the heart. The intercellular junctions provide mechanical binding, force transmission, and ionic communication among cells.[139] Adherens junctions and desmosomes form direct links to the cytoskeletal structure, thus transmitting contractile force. Gap junctions play a critical role in intercellular communication by transmitting electrical impulses. Cx43 (Connexin 43) is the most abundant gap junction protein in the myocardium. In a normal adult heart, Cx43 expression and localization mainly occurs at the longitudinal cell ends, called intercalated discs. A direct correlation between Cx43 immunosignal and electrical intercellular conductance suggests that Cx43 plays an important role in cell communication throughout the heart.[140] In in vitro studies have found an intimate correlation between mechanical adherens junctions and electrical coupling junctions, and Cx43 expression occurs after the formation of the adhesion junction protein, N-cadherin.[141] Therefore, the remodeling of cell-to-cell adhesion is occurred due to a stiff ECM environment, may influence gap junction redistribution, which can disrupt abnormal electrophysiological leading to arrhythmogenesis that is associated with many cardiomyopathies.[137]

The end-to-end connections of cardiomyocytes are arranged on cardiac tissue within a collagen ECM fibrous network in the heart (Figure 3E).[142] In in vitro cardiac tissue models, the inter/intracellular organization is also affected by geometric patterns of ECM proteins, such as fibronectin or gelatin.[143] The aligned cytoskeleton structure and junction proteins results

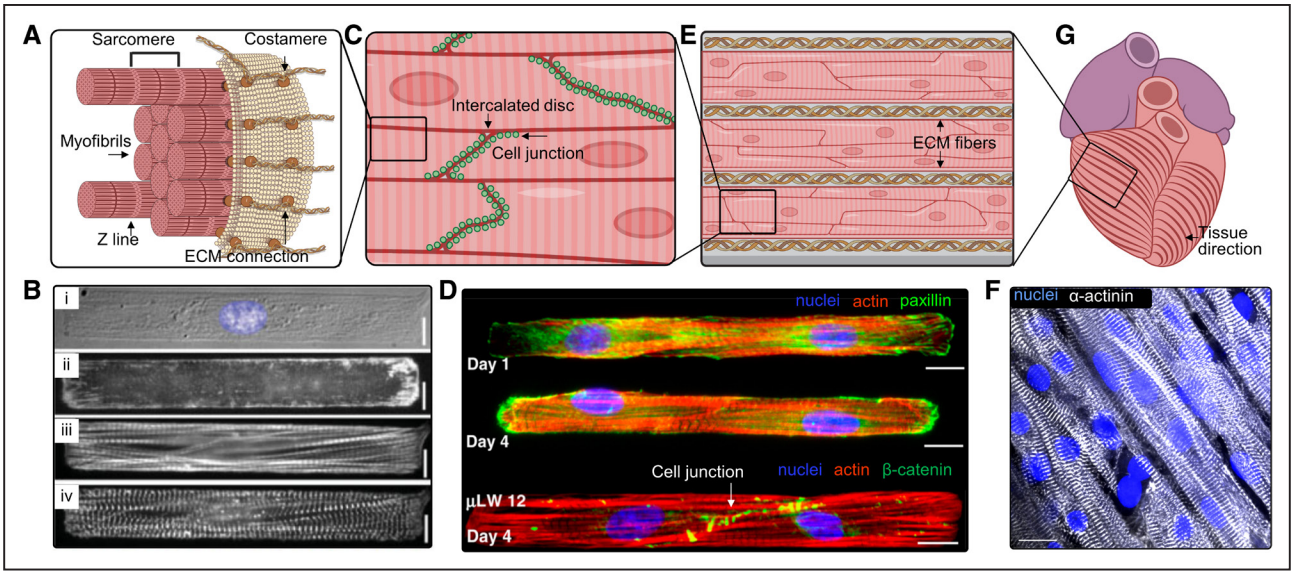


Figure 3. Hierarchical structure of cardiomyocyte and extracellular matrix (ECM) structure in the heart.
A, Cardiomyocytes shows aligned myofilament structure which is connected to ECM through costamere. Costameres contain focal adhesions (FAs) complex connecting with cytoskeletal actin filaments. **B**, A cardiomyocyte cultured on 1:7 aspect ratio of rectangular microcontact printed ECM pattern, showing intracellular organization using a DIC image and immunostaining images for vinculin, F-actin (Actin filament), and sarcomeric Alpha-actinin in (i–iv), respectively. Scale bar, 10 μm. Reprinted from Bray et al[130] with permission. Copyright ©2008, Wiley-Liss, Inc. **C**, Junction formation is occurred at the intercalated disc where the cells are connected end-to-end. **D**, Immunostained images of cardiomyocytes cell-pair images showing FAs and junction formation and intracellular organization according to culture days. Scale bar, 10 μm. Reprinted from McCain et al[137] with permission. Copyright ©2012, NAS. **E**, Fibrous ECM structures supporting cardiomyocytes with their intra/extracellular organization. **F**, In vitro cardiac tissue organization by ECM geometry patterns. Scale bar, 20 μm. Reprinted from Lee et al[143] with permission. Copyright ©2022, AAAS. **G**, Hierarchical cardiac muscle tissues are organized into the heart, inducing cyclic blood pumping with coordinated tissue contraction.

in faster action potential propagation in the longitudinal tissue direction than the lateral direction.[138,144] A monolayer of anisotropic neonatal rat VMs exhibited a longitudinal to transverse conduction velocity ratio of 1.89±0.38, similar to the ratio found in the in vivo rat heart.[145] This, in turn, activates the organized cardiac tissue that leads to synchronized contraction. Healthy cardiomyocytes with well-organized tissue structure show strong contractile stress that can be analyzed by muscular thin film (MTF) cantilever[146] or EHT platforms.[147,148] Therefore, the intra/extracellular structure of cardiac tissue is inextricably related to electrical and mechanical functions which are important factors in determining cardiac function.

In Vitro Organ Model of the Heart

The in vitro heart has been studied on multiple scales, from cellular assays to engineered tissue by recapitulating the structure and function of the heart (Figure 3F).[149] The human heart consists of hierarchical cardiac muscle tissues organized into chambers which pump blood throughout the body with coordinated tissue contraction (Figure 3G). Three dimensional organ-level in vitro models of the heart provide a direct in vivo comparison of clinically relevant functional parameters such as pressure-volume change, stroke volume, and Ca^{2+} propagation signal.

An important step in constructing the 3D organ-level in vitro heart is assembling cardiomyocytes into a 3D chamber structure that pumps fluid in and out cyclically. Collagen, the most abundant protein in cardiac ECM, is mixed with cardiomyocytes and cultured for 10 days to fabricate a balloon-shaped chamber.[150,151] The chamber also exhibited responses to drugs that induced a fast-beating frequency or weaker pumping function. Three dimensional printing is another promising manufacturing method for building organ models due to its simple design control and reproducibility. However, ECM-based hydrogel inks used in 3D printing are usually soft or fluidic, and do not retain 3D shape without additional support. To overcome this challenge, researchers have developed sacrificial baths that support 3D structure temporarily during printing.[152,153] Collagen gel inks and cardiomyocyte inks are printed in the shape of a ventricle chamber. These printed ventricle models show synchronized contraction, resulting in anisotropic Ca^{2+} wave propagation, reaching 16% wall thickening at peak systole,[153] and a 0.7% ejection fraction.[154]

Considering the main functions of ECM other than cell adhesion, tissue scaffolds provide structural integrity in cell assembly, as well as mechanical support to the tissue. Early 3D in vitro organ-level tissue models used decellularized rat heart matrix as a tissue scaffold to preserve the underlying ECM.[155] Recellularizing the heart by intramural injection of cardiac-derived cells

and perfusion of ECs allowed the building of an artificial heart after 8 days of culture. Alternatively, synthetic biocompatible fiber scaffolds also replicate the role of ECM by facilitating cell alignment and providing mechanical support to cardiac tissue.[156] Aligned microscale fibers in a ventricle-shaped scaffolds can be used to build organized tissue structure in a 3D in vitro model of the heart with electromechanically coupled cardiomyocytes, resulting in pressure-volume change by the tissue-engineered ventricle contraction.

However, in vitro ventricle models pump fluid with 50 to 200 times lower efficiency than in vivo ventricles.[156] For better performance, additional anatomic features need to be recapitulated in in vitro heart models. Tissue maturation significantly affects pumping strength.[147] Although long culture periods often prove challenging, mature cells tend to show stronger contractility. This requires advancement in better long-term cell culture techniques highly relevant to building accurate heart models.

In vitro tissue thickness is another challenge. Ventricular myocardium consists of laminar tissue that is approximately 4 cells thick.[149,157] However, cells situated deeper than 100 μm in in vitro tissue do not survive due to the lack of nutrient and oxygen diffusion. Integrating vascular structure within 2D cardiac muscle tissue is one approach to solving the thickness limitations.[158,159] Another approach is to build 3D scaffolds that allow for cell infiltration or in situ cell differentiation to recapitulate the layered laminar tissue structure of the heart.[154] In addition, studies have found that cardiac output performance is highly influenced by tissue direction, with helical direction exhibiting better performance than circumferential direction.[160,161] Developing advanced techniques for precisely controlling tissue direction will improve cardiac output in future 3D organ models, and will allow us to develop more powerful preclinical testing platforms.

INTEGRATIVE CARDIAC MICROPHYSIOLOGICAL SYSTEMS

Pharmaceutical research for drug development involves high-risk, long-term process, and substantial costs. During the drug development's preclinical stages, animal models are historically, and remain, the primary platform for drug efficacy and toxicity. However, fundamental differences between animal models and humans often cause safety issues or low efficacy. For example, the resting heartbeat in mice (≈600 bpm) is higher than human (≈60 bpm). Additionally, Ca^{2+} handling properties, myosin expression, and ion channel expression vary significantly between animal models and humans.[162,163] Two-dimensional and 3D iPSC platforms offer advantages over animal models in several respects.[164] Human iPSC-CMs can recapitulate inherited cardiomyopathies by using patient-derived iPSCs and also extend culture periods by months, allowing for the assessment of drug efficacy and toxicity with chronic drug administration or gene therapy treatments (as opposed to acute studies). However, the maturity of human iPSC-CMs represents significant obstacle when attempting to replicate complex cardiomyopathies in vitro. For that reason, rodent cell models, coupled to well understood animal models (eg, spontaneously hypertensive rat), suggest that in so far as diseases that affect adults are concerned, animal models will remain an important platform for drug discovery for the near future.

Microphysiological systems (MPSs), commonly referred to as organs-on-a chip, are great potential tools that can minimize the uncertainty of the existing preclinical animal model testing.[165,166] Cardiac MPS can be defined to replicate the cellular microenvironment of the diseased heart with well-defined geometric, topological, mechanical, and biochemical cues drawn from postmortem histological studies of explanted hearts. Compared with conventional biochemical assays, cardiac MPSs allows high throughput and real-time monitoring of electrophysiological or contractile functional changes upon electrical or biochemical intervention.[167] More recently, methods to conduct real-time immunoassays on organ chips further enhance the ability to understand the mechanisms of efficacy or toxicity.[168] Eventually, these tools will be used to screen patients for efficacy and toxicity before their enrollment in a clinical trial, lowering the risk associated with the trial and reducing the time and cost of bringing medicinal and genetic therapies to market.[169,170]

Electrophysiological Assessment of Cardiac Tissue on a Chip

Cardiomyocytes generate action potential and pass the electrical impulses in an organized tissue direction that leads to coordinated actions. Optical mapping is widely used as a noninvasive assessment tool to visualize action potential and its wave propagation to study the cardiac electrophysiology (Figure 4A). Voltage sensitive dyes (eg, Di-4-ANEPPS) or Ca^{2+} sensitive dyes (eg, X-Rhod, Fluo-4) are used to monitor transmembrane potential changes or Ca^{2+} transient propagation, respectively.[171,172] Measuring Ca^{2+} transient provides quantitative information of Ca^{2+} handling properties of the cardiac tissue, such as conduction velocity and signal intensity.[173] This allows us to investigate clinical phenotype of inherited heart diseases in vitro, such as recapitulating hallmark features of catecholaminergic polymorphic ventricular tachycardia by showing ectopic Ca^{2+} propagation from patient-derived cardiac tissues with rapid electrical pacing or adrenergic stimulation.[172]

Multielectrode arrays are another tool to record electrophysiological function for high-throughput and long-term readout platforms (Figure 4B).[174–176] High-resolution

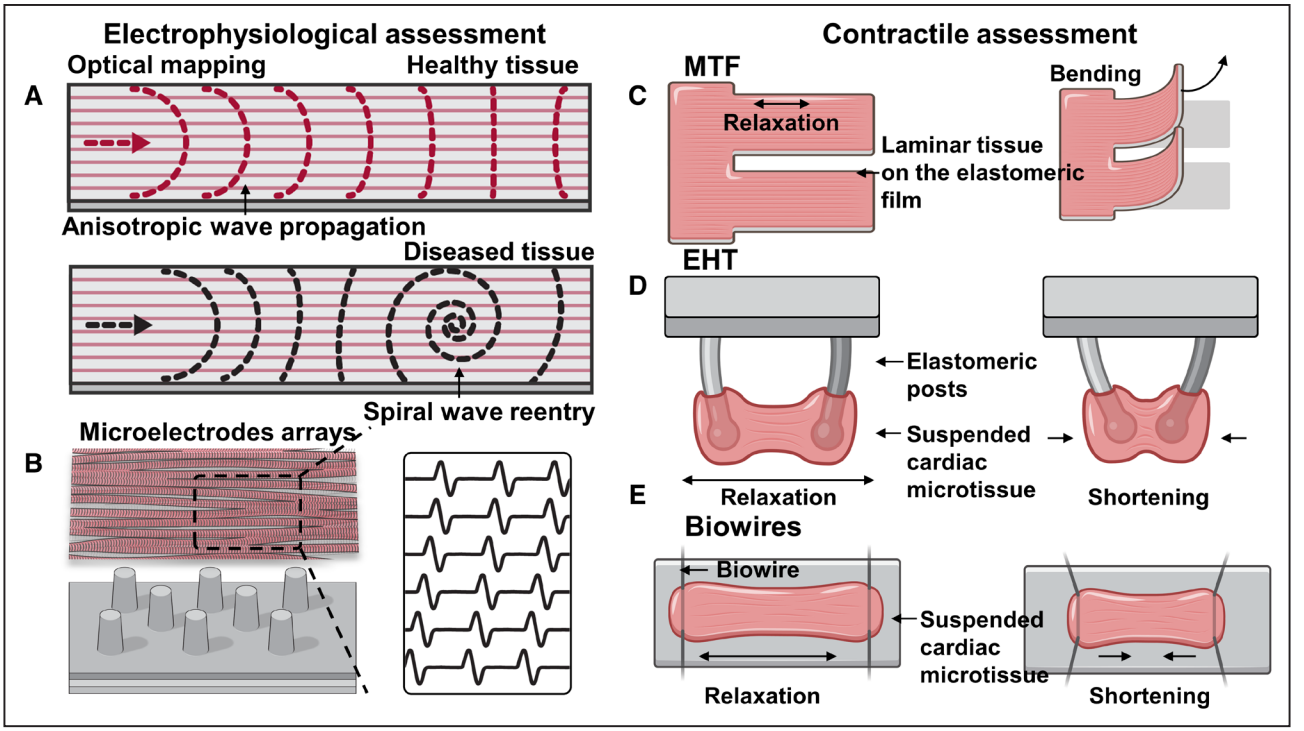


Figure 4. Cardiac microphysiological system for electrical and mechanical functional assessment.
A, Optical mapping system from in vitro cardiac tissue showing anisotropic electrical potential propagation in aligned tissue direction and arrhythmias in disease model. **B**, Microelectrodes arrays measuring the local electrical potential changes, providing high spatiotemporal bioactivity of the cardiac tissues. **C**, Muscular thin film (MTF) system to measure contractility by measuring bending force of the laminar tissues that are formed on the cantilevers. **D**, Engineering heart tissues (EHT) system allowing for monitoring contractility of the cardiac tissue that causes cyclic movement of the posts. **E**, Biowires deformation caused by contractile cardiac tissue as the tissue is sustained by wires.

electrode arrays can measure bioelectric activity with high spatiotemporal resolution at multiple length scale from cellular to tissue levels. As the electrodes are in contact with outside cells, these devices monitor extracellular electrophysiological signals, providing cardiac activity information in relative values, such as beat frequency and wavefront propagation. However, measuring the action potentials in the intracellular space is also imperative to understanding quantitative electrophysiological functions such as ion current modulation and membrane potential changes. Membrane poration techniques such as electroporation or laser optoacoustic poration overcome those limitations of the multielectrode arrays system by enabling the electrode arrays to assess intracellular space.[177,178] Integrating the advanced nano-fabrication technologies and in vitro cardiac human tissue models in cardiac MPS will enable the real-time monitoring of the intracellular action potential of cardiac tissues that are exposed to test drugs[178] or disease-inducing environments like hypoxia or ischemia.[177]

Contractile Assessment of Cardiac Tissue on a Chip

The cardiac ventricles are a 2D laminar tissues wrapped around the ventricular cavities to form the 3D form. To approximate this architecture, MTF composed of 2D laminar cardiac tissue layers on the elastomeric thin films are one of the new cardiac MPS platforms for measuring the contractility of in vitro cardiac tissue (Figure 4C). The systolic shortening and diastolic restoration of cardiac myocytes organized into a syncytium potentiate a cyclic bending motion of the laminated film allowing measurement of contractile stress.[179] These tools have been used to study complex diseases such as Barth syndrome, a genetic disease of mitochondrial function that is characterized by muscle weakness and poor myofibrillogenesis.[180] Engineered Barth syndrome iPSC-CM tissues from patients in the form of a MTF platform recapitulated the weak contractile function of the disease, showing slow and weak bending deformation. Histological studies of the engineered tissues revealed that sarcomeres were poorly formed and aligned, and that myofibrils were not laterally coupled, hence suggesting that the mitochondrial dysfunction has the collateral effect of rendering the myocytes unable to build and maintain the contractile apparatus. Further experiments went on to demonstrate the appropriate rescue of the diseased tissue by therapeutic means. Studies like these can be further enhanced by using MTF platforms integrated with strain sensors to enable real-time monitoring of contractile responses to changes in drug dose for high-throughput drug response studies.[181,182] These technical capabilities are important because they will

allow unmanned study of acute responses to medicinal therapeutics in vitro.

Another approach for measuring contractility is using EHTs that are formed and suspended around 2 elastomeric poststructures (Figure 4D).[183] In some studies, fibroblasts have been cocultured on the EHT platforms to promote cardiac tissue compaction, cell assembly, and spontaneous contraction.[147,184] The tension between the posts applies an auxotonic load to the papillary muscle-like tissues, which facilitates the longitudinal alignment of the embedded cardiomyocytes. In addition, applying electrical stimulation with gradually increasing intensity can accelerate the cardiac tissue maturation, resulting in physiologically relevant sarcomere length (2.2 μm), mitochondria density (30%), and improved Ca^{2+} handling properties.[147] The advanced maturation technique enables testing drug effects that are consistent with clinical outcomes. This property allows the EHT platform to serve as a tool for studying patient-specific pathophysiology and disease mechanisms by recapitulating electrical and mechanical pathologies reported in cardiac disease such as arrhythmogenic cardiomyopathy. Cell junctional protein mutation in the arrhythmogenic cardiomyopathy leads to disruption of sarcomere stability and organization, resulting in the impaired contractility.[185,186]

Biowires are another class of new cardiac tissue culture platforms. As the mixture of cell and ECM gel is casted, the wire structure promotes self-assembly of cardiomyocytes, forming aligned tissue along the wire roughly approximating ventricular papillary muscle.[171] Applying electrical stimulation to the tissue during culture promotes tissue maturity, resulting in increased structural myofibril organization and Ca^{2+} handling property. A more recent version of Biowire (Biowire II), which allows cardiac tissue to be generated between 2 parallel wires, enables contractility measurements (Figure 4E).[187] Deformation of the wires due to tissue contraction was optically measured and translated into contractile force. Biowire II also provides a heteropolar tissue model that combines tissues of different cell type formed on each wire, facilitating comparison of the drug effects on a specific target tissue versus the control tissue. For example, heteropolar cardiac tissues with distinct atrial and ventricular chamber models have demonstrated chamber-specific drug responses because the administered drug only affects the target tissue model.[187] With this technique, antifibrotic compounds can be tested on heteropolar cardiac tissues composites of fibrous and healthy heart tissues by the contractile functional and electrophysiological readout.

Microfluidic Integrated Cardiac Microphysiology System

Organs on chips with integrated microfluidics allow for the delivery of precise and predictable nutrients and ECM hydrogel to support cell culture environments. Bioreactors can also be used to incorporate microfluidic channels not only to support cell culture in 3D scaffolds but also providing a platform to analyze fluid movement generated by in vitro 3D chamber-shaped ventricle models.[156] While the tissue-engineered ventricle pumps fluid in and out of the chamber, the bioreactor can apply external pulsatile pressure through the fluidic channel, driving the intraventricular fluid flow. Integration of the valve structure restricts unidirectional flow, enabling functional evaluation of engineered ventricular models through measurements of physiological pressure and volume dynamics.

In addition, while drug testing in static cardiac MPS relies on diffusion-driven drug molecules, microfluidic cardiac MPS offers a spatiotemporal gradient of drugs and biochemicals that can be recapitulated through fluid flow across the cardiac tissue.[188] Microfluidic MTF platforms can evaluate cardiac function under rapid and continuous perfusion of drug solution, facilitating drug efficacy testing with real-time continuous monitoring and high throughput. A microfluidic heart-on-a chip is also amenable to control medium oxygenation, creating cell culture conditions to induce acute hyperoxia to the in vitro cardiac tissue.[177] This microfluidic MPS integrated with multielectrode arrays provides assessment of real-time disease progress and are useful for high-throughput pharmacological studies by monitoring electrical and mechanical function of cardiac tissue.

Multiorgan Assemblies to Model Pharmacodynamic Drug Interactions

Medicinal therapeutics are transported to the target organ(s) via blood circulation which takes them on a circuitous route through organs that both absorb, react to, and metabolize the drugs. Multiorgans-on-a-chip, which interconnect 2 or more organ models in a single MPS, can recapitulate those dynamic processes of absorption, distribution, metabolism, and excretion to provide accurate and efficient preclinical predictions of drug response in the human body.

The endothelium of the vasculature plays an important role in regulating the delivery of drugs or molecules to the heart muscle. Combining endothelial barrier and cardiac MTF tissue in a platform can enable the evaluation of the effect of endothelial barrier on drug transport. When Ca^{2+} channel blocker isradipine is administered through the endothelial barrier, the disruption of cardiac contractile function was significantly delayed compared with direct drug exposure to the cardiac tissues.[182] In addition, modulating permeability of the in vitro endothelial barrier can help regulate the temporal onset of cardiac drug responses.

Microfluidic systems provide platforms to simulate drug transportation from organ-to-organ in vitro,

enabling pharmacological studies in more physiologically relevant conditions by recapitulating organ crosstalk. For example, effects of an anti-cancer drug were different in a microfluidic organ-on-a-chip model that connected bone Ewing Sarcoma tumor and heart muscle tissue. The model's results demonstrated lower cardiotoxicity and tumor response in an integrated setting. Whereas direct drug exposure to each isolated tissue significantly reduced both tumor viability and cardiac function.[189] Depending on the drugs, metabolites can be also toxic to other organs or become an active form to improve drug efficacy, which is one of the main reasons for discrepancies between single organ in vitro studies and clinical trial outcomes. Integrated liver and heart models in a single MPS can help evaluate the on-target drug efficacy on integrated liver tissue and decrease off-target cardiac toxicity as the parent drug is metabolized in the liver tissue model.[190,191]

To further simulate the complexity of the human body that involves immune, nervous, and vascular systems, multiorgans-on-a-chips have been developed by integrating with key functional organs models, such as liver, kidney, heart, and brain.[192,193] As researchers seek to make accurate quantitative predictions of human pharmacological responses that are translated from accurate in vitro test results, it is important to integrate physiologically appropriate cardiovascular systems for organ-organ connections. To build a semivascularized multiorgan-on-a-chip platform, researchers installed endothelial barriers into a series of organ models and connected the organ chips via vascular perfused fluid that was delivered by an automated robot system.[194] The endothelial barriers mimic physiological systemic transportation of small molecules between organs, providing better predictions of the relevant pharmacokinetic response. Arteriovenous reservoir modules from multiorgan-on-a-chip are also used to mix drugs that pass through each organ compartments, simulating systemic circulation of the body.[195]

Both microstructural recapitulation and functional analysis of cardiac MPSs are essential to study disease phenotypes and drug treatment responses. Integrating cardiac functional readout system into large number of the in vitro cardiac tissue have a potential to overcome the limitation of low throughput in vivo animal studies that minimize the cost and times in the drug discovery process. But still more efforts are required to build multiorgan-on-a-chips that incorporate cardiac tissue models close to the human body environment, such as cardiac immune response. The inflammatory response of the peripheral and central nervous systems plays a key role in the development and persistence of cardiac pathologies, with autoimmune diseases, such as rheumatoid arthritis, being linked with increases in cardiac inflammation, such as endocarditis, myocarditis, and pericarditis.[196] Furthermore, the ILs have been shown to have maladaptive effects on cardiac tissue, with IL-4 being correlated with profibrotic remodeling[197] and IL-6 being linked to reduced Ca^{2+} transients.[193] Due to the complexity of these interactions, and their inherent multicellular nature, limited works with in vitro models have been reported for mechanistically probing how these processes evolve. In addition, further progress in obtaining more accurate quantitative information from the multiorgans-on-a-chip and modeling methods to translate the information into in vivo results will help us achieve the goal of developing human surrogate systems that can replace animal models in the future.

BREAKING THE HYPE CYCLE: CHOOSING THE RIGHT MODEL IN THE RIGHT CONTEXT

As the depth of technical knowledge and subject matter expertise grows with the precise and accurate modeling of cardiovascular diseases using 2D and 3D models, the challenge of fit to purpose design of the model remains. Lack of established quality control standards for iPSC-CMs[198,199] and limited regulatory guidance on the use of models represents both an opportunity and a hurdle for the field. Whereas toxicological studies may benefit from standardized models, drug discovery models may require that MPSs are developed in such a manner as to yield a unique readout that maps to a clinical diagnostic.[167] The primary context of use of the iPSC-based cardiac models may be based on where the model fits among the 4 categories: (1) screening and drug response, (2) mature mono or polygenic disease mechanisms, (3) developmental disorders, and (4) environmental disorders and cardiac diseases with late onset (Figure 5). Lack of consensus or diligence on the appropriateness of choosing a model is based on several such factors.[200] One of the main factors is the missing microenvironmental composition and cues (eg, cellular heterogeneity, flow, ECM, mechanical stretch) in the model that is clinically known to contribute toward a disease phenotype. For example, to accurately model interstitial tissue or aortic diseases that lead to fibrosis, in addition to the relevant cell types such as patient-specific fibroblasts, SMCs, the model must also include a tailored, structural ECM component. Second, clinical phenotypic manifestation should in some form be measurable using assays that are precise, reproducible, and devoid of bias.[201,202]

Predictive Disease Modeling for Functional and Preclinical Toxicology Responses Using High-Throughput Platforms and Multiomic Profiling

Human iPSC-CMs have proven to be instrumental in the evaluation of drug-induced toxicity for the past several years. High-throughput studies using iPSC-CMs have been performed for screening novel drug candidates,

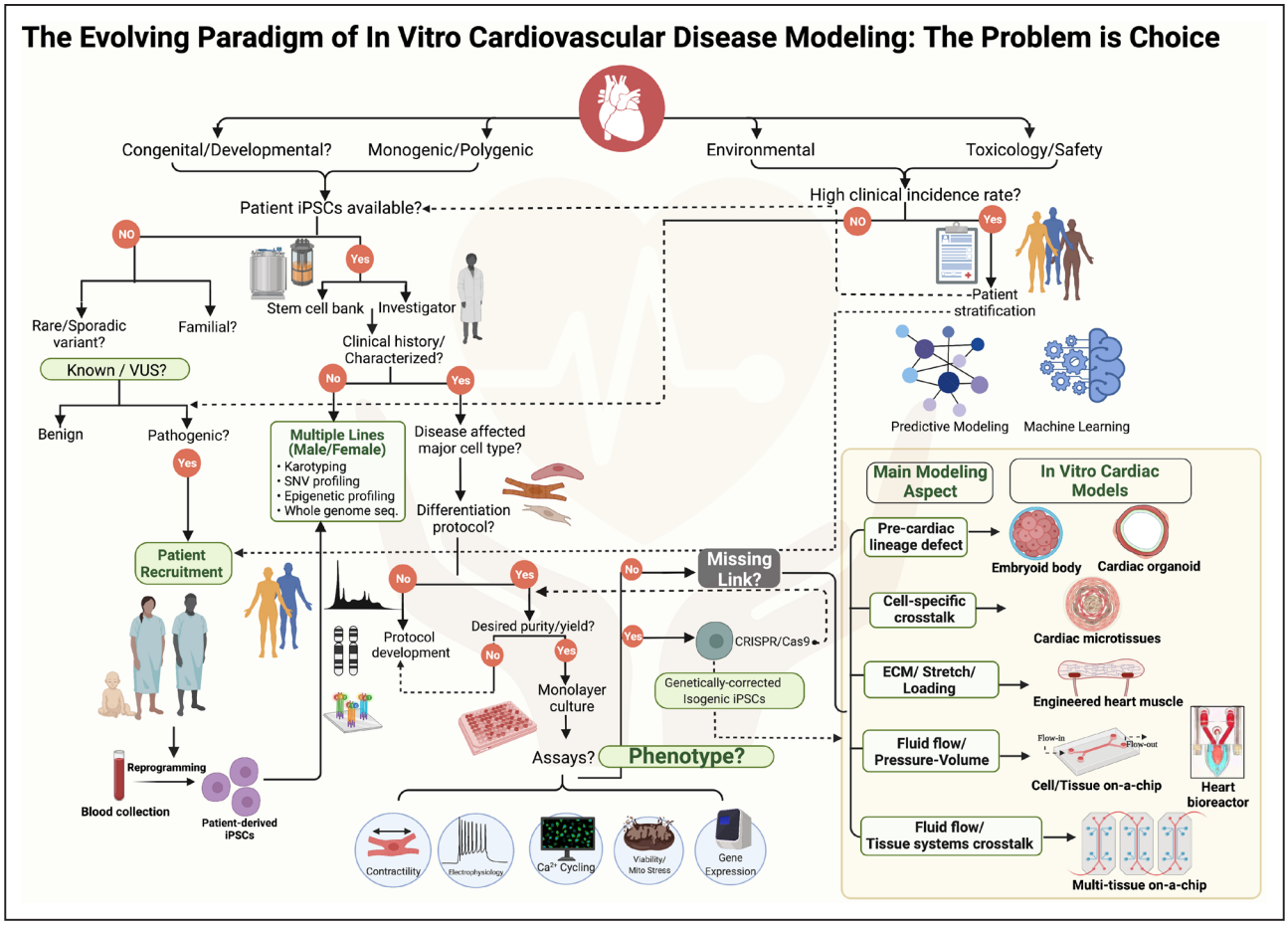


Figure 5. The evolving paradigm of in vitro cardiovascular disease modeling.
A plethora of in vitro cardiac models have been developed over several decades. Induced pluripotent stem cell (iPSC) technology has fueled the development of human cardiac models to accelerate predictability of cardiovascular disorders and drug responses to complement in vivo studies. The flowchart offers a novice guide on general principles for choosing a more relevant model in the context of the disease in question, and validity of the model based on an understanding of cellular contribution toward the disease, obtaining clinically translatable phenotype, and most importantly the reliability and reproducibility for disease modeling. ECM indicates extracellular matrix.

off-target drug effects, and drug repurposing. Several examples of chemotherapeutics used in the treatment of various cancers have been profiled using iPSC-CM models that show bystander cardiotoxicity.[171,203]

Several pathological mechanisms with dominant phenotype can be assayed on 2D such as cytoskeletal disarray caused due to truncation of proteins or ion channels that result in arrhythmic disorders. Although the success of modeling of the molecular events that lead to the disorder depends on the degree of maturation achieved in vitro using well-defined methodologies. For example, the β-adrenergic signaling is important in cAMP signaling to model chronotropic responses. In diseases such as Takotsubo syndrome where over stimulation of β-adrenergic leads to stress inotropy and lipid accumulation can be faithfully demonstrated at a cellular level given the comparable level of receptor expression to that of an adult myocyte, and sensitization as seen in patients with Takotsubo syndrome under adrenergic stress.[204] As alluded to previously, 2D iPSC-based cardiovascular derivatives can serve as excellent surrogates for testing drug sensitivities but disorders that are caused by disruption of more complex cardiac structures such as gap junctions can be well studied in 3D cardiac constructs. For example, 2D iPSC-CMs generated from LQT patients showed spontaneous and frequent cellular arrhythmias, whereas in 3D EHTs arrhythmias were only observed when challenged with a QT prolongation agent.[205] EHTs have also been used to study cardiotoxicity under loading conditions. Sunitinib, a TKI (tyrosine kinase inhibitor) at a clinically relevant concentration was shown to induce caspase-induced toxicity in EHTs under afterload conditions.[203] For high-throughput toxicity profiling, cardiac microtissues that are roughly a third in size to that of an EHT have been used for high-content imaging in multi-well formats to monitor dynamic changes in structure and metabolism. Drug or small molecule diffusion kinetics in 3D culture systems better represent in vivo diffusion barriers.[206] Cardiac microtissues comprised of iPSC-CMs, primary ECs and fibroblasts treated with sunitinib showed cytotoxicity above 10 μmol/L, whereas lower concentrations

on 2D culture format show severe toxicity due to higher drug bioavailability and uptake.[207] There is substantial evidence that complex cardiac model systems are amenable to high-throughput platforms to measure hundreds of parameters through an iterative approach. With the progress in next generation sequencing technologies and standardized approaches to perform genome-wide analysis, significant advances can be made in disease modeling and drug discovery using in vitro cardiac models for prospective clinical outcomes.

CHALLENGES AND OUTLOOK

Advancements in iPSC technology have to a greater extent eliminated the dependence on primary cells or tissue sources with the promise of large and reproducible quantities of various cardiovascular cell types for research. With several emerging precision medicine initiatives, there is a need to obtain high-quality patient-derived iPSCs at a manufacturing scale. However, the challenge of scale also comes with the need for rigorous standardization methods in obtaining iPSCs for differentiation into cardiovascular cell types. Despite these standardized methodologies, several reports have detected significant loads of single nucleotide variants due to age of donors,[208] prolonged culture,[209] clonal or somatic cell variation,[210] and genetic changes due to pluripotency induction methods.[211] Given the variability that may be introduced in iPSC-derived cardiovascular cell types, in addition to standard karyotyping assays, whole genome exome sequencing and chromosomal microarrays should be employed to detect abnormalities in iPSCs.[97] As previously discussed, since the genetic variability is often larger than the expression of a disease phenotype, it is important to evaluate multiple controls and patient lines to assess the nonoverlapping differences with high sensitivity and specificity.[95] For comparisons, both test and control lines should be as closely matched as possible for age, sex, and ethnicity.[212]

Three dimensional cardiac models are touted as more effective predictors for disease modeling and therapeutic testing due to the complexity and physiological relevance. The common denominator in these models is that they all are static systems which are limited in recapitulation of flow mediated stresses, nutrient gradients, vascular interaction, or systems-level biomolecular interactions offered by MPS systems. To develop the platforms that aid in generation of these models, standardization is essential for commercially availability as an off-the-shelf product for widespread use. Most MPS devices originate in the labs that develop them over many years, hence commercializability of such platforms depend on efforts made in standardization of the fabrication process, reproducibility of results, and transferability of the technology. One of the key aspects of consideration is the materials used in the model and their compatibility with the intended target cells or engineered tissues. For example, polydimethylsiloxane is widely used to fabricate MPS devices because of its versatility in molding and prototyping. However, polydimethylsiloxane can be unsuitable for drug-based studies due to its property of drug adsorption which may reduce the bioavailability in the device for the cells and tissues. Hence, other materials such as silicon or thermoplastics could be widely adopted to eliminate the concerns of unwanted substrate interactions within the multicellular/tissue chips. Standardization of these parameters will also reduce the cost of obtaining bespoke materials and shift the focus on quality control and end-user reliability measures for mass production.

One of the major hurdles using multicellular cardiac models is to find the right balance of nutrient medium to meet cell-specific demands. Currently, specialized medias are developed for individual cell types. Combination of multiple cells in organoids, engineered tissues, and MPS systems require a universal or customized media formulations. In advance of the availability of a tailored or universal medium, an approach that can be pursued in the interim is to cater each cell population with its specialized medium on an MPS platform in multiple single-pass circuits, integrated with an external loop for exchange of media metabolites.[213] In the meanwhile, to develop a design criterion for optimal universal medium a thorough characterization of the cell secretomes must be made independently for each cell type using proteomic analyses for the assessment of metabolite concentration and waste products. This will help trace cell-specific responses in coculture setup and reveal potential negative influence in cell behavior due to altered culture environment.

CONCLUSIONS

The heart is a complex organ and has greater influence on other organ systems due to its key role in providing nourishment. Hence, studying tissue and organ-level dynamics using in vitro cardiac models offers an unprecedent opportunity in heart regeneration, disease screening, and drug development. Prima facie the goal of the emerging engineered cardiac models is to build greater physiological relevance, and confidence in functional end points for the development of prospective clinical strategies. Along the same lines, long-term monitoring of the models will not only model acute effects but also assist in longitudinal studies through the course of patient care in the clinic. One of the major breakthroughs in the translation of cardiovascular disease has been our ability to model functions and responses to biological perturbations, stressors,

or compounds. For wider adoption of these advanced cardiac models, it is important to build physiological complexity using facile methodologies, and in doing so increase efficiency and cost-effectiveness. Hashing out these key considerations would help in exploiting the cardiovascular models more purposefully to understand the diseases with greater precision. Ultimately, adoption of such personalized cardiac models in drug development and early preclinical proof-of-concept studies would draw us one step closer toward the clinical trials-in-a dish frontier.

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Disclosures

J.C. Wu is a cofounder of Greenstone Biosciences. The other authors report no conflicts.

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