Generating 3D human cardiac constructs from pluripotent stem cells

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Summary

Human pluripotent stem cell (hPSC) technology has offered nearly infinite opportunities to model all kinds of human diseases *in vitro*. Cardiomyocytes derived from hPSCs have proved to be efficient tools for cardiac disease modeling, drug screening and pathological mechanism studies. In this review, we discuss the advantages and limitations of 2D hPSC-cardiomyocyte (hPSC-CM) system, and introduce the recent development of three-dimensional (3D) culture platforms derived from hPSCs. Although the development of bioengineering technologies has greatly improved 3D platform construction, there are certainly challenges and room for development for further in-depth research.

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Introduction

Cardiovascular diseases are the leading causes of death worldwide,[1-3] and therefore understanding the mechanism of human cardiac pathologies is not only of great importance but will have a tremendous impact on majority of the population. The prevailing physiological and disease models in the past few decades were based on rodents due to their advantage in acting as disease models, in genetic engineering, breeding as well as ease of tissue accessibility.[4-7] However, since primates and rodents have huge interspecies differences,[8,9] it is difficult for rodent model to fully recapitulate human cardiac homeostasis and pathogenesis. For example, the physiological characteristics of the mouse and human hearts are significantly different, with repolarization of the mouse myocardium mediated by ultra-rapid delayed rectifier potassium current (I_{Kur}), whereas the repolarization of human myocardium is mediated by rapid and slow delayed rectifier potassium current (I_{Kr} and I_{Ks}). The composition of myofibrillary filaments of mouse and human cardiomyocytes is also very different. α-Myosin heavy chain isoform is mainly expressed in adult mouse hearts, while β-Myosin heavy chain isoform is mainly expressed in adult human hearts. Moreover, with regards to PDE regulation, PDE4 is the most important regulator in cardiomyocytes regulating L-type calcium current ($I_{Ca,L}$) and contractility in rodents, while PDE3 is the dominant regulator in adult human cardiomyocytes.[10] In addition, 2D and 3D culture models constructed by patient-derived iPSC can reflect variations in phenotypes caused by disease-related mutations and human genetic background diversity, which cannot be reproduced in mouse models. In this context, the rapid evolution of human pluripotent stem cell (hPSC) technology, that includes embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), provides an opportunity to generate various biologically relevant human disease models *in vitro*.[11-13] This approach has been largely favored due to its advantage in high-throughput drug screening, and its ability to faithfully recapitulate non-genetic and genetic human diseases in pathology phenotypes.[13]

The advantages and limitations of 2D human cardiac disease models

hPSC derived cardiomyocytes (hPSC-CMs) have shown their potential as *in vitro* model of human cardiac diseases.[11] Nowadays, hPSC cardiac-differentiation

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technology has been greatly improved and hPSC-CMs are now prevailing tools for studying pathological mechanisms[14,15] and drug screening.[16] hiPSC-derived cardiomyocytes (hiPSC-CMs) have served as a limitless resource of patient-specific CMs, and played an important role in advancing the field of precision medicine.[17] Until now, by generating cardiomyocytes from hiPSC-CM, various genetic and non-genetic cardiac pathological models have been established in two-dimensional (2D) culture system. For example, genetic cardiac disease models have been constructed using iPSCs derived from patients suffering from hereditary long QT syndrome, hypertrophic cardiomyopathy, and dilated cardiomyopathy.[18-21] Non genetic diseases, such as ischemia injury,[22-24] hormone induced hypertrophy,[14,15,25] drug induced cytotoxicity[26-28] and metabolic dysfunction[29-31] have also been successfully modeled using hPSC-CMs *in vitro*. 2D PSC-CM model has several advantages: (1) Compared with 3D model, 2D cell culture system is relatively simpler and easier to conduct molecular biological experiments such as gene editing. (2) Under 2D culture condition, the number of available cells is larger compared with 3D culture system, which makes it easier to carry out molecular biochemical, electro-physiological and metabolic detection. (3) At the 2D level, the research object is usually fixed, which makes it applicable to depict cell-specific molecular events in a certain type of cells.

On the contrary, limitations of 2D PSC-CM model apply as well: (1) The monolayer culture lacks complex structure and multi-cellular organization present in the living organ which supports cellular growth *in vivo*. Pure hPSC-CMs population lack crosstalk between multiple cell types, which is essential to understand cardiac pathogenesis triggered by hormonal stimulation and paracrine signaling. (2) The cell maturity is relatively low. For example, compared with hPSC-CMs in 3D model, hPSC-CMs in 2D culture have smaller cell size, shorter sarcomere length, lower mitochondrial density, and lower α-adrenaline response. (3) Due to the heterogeneity of differentiated cells in 2D model, the contractile force records of individual cells vary greatly. However, in the 3D model, because the contraction force of the entire microtissue is recorded, the heterogeneity is relatively lower. (4) Traditional 2D PSC-CM model can only be used to detect the direct effects of drugs on cardiomyocytes, the systemic effects of drug metabolism through the liver cannot be evaluated in this system. Advanced bioengineering is required to construct 3D culture system for cardio-genesis and pathogenesis study. Therefore, a growing number of studies focus on hPSC 3D constructs, which can recapitulate tissue and organ level pathophysiology more accurately.[32-34] In this review, we summarize the progress of cardiac 3D constructs from hPSCs by analyzing several contemporary research studies.

The types of 3D cardiac constructs

The development of biomaterials and microfluidic technologies has largely increased the diversity of hPSCs derived 3D cardiac constructs, including strip-format engineered heart tissue (EHT), cardiac organoids (cardioids), spherical cardiac microtissues and micro-bio-fabricated heart-on-chip (Figure 1). EHT is formed by mixing hydrogels and multiple types of hPSCs-derived cardiac cells in casting molds. Human cardiac organoids are sphere shaped mini-organs formed by cell aggregation and self-organization of hPSCs due to their self-renewal and differentiation capabilities (Figure 2). Scaffold-free cardiac spherical microtissues are cardiac spheroids formed by cardiomyocytes (monoculture spheroid)[35] or mixing various cell types together (multicellular microtissue)[36,37] in a 3D culture system. Heart-on-chip usually refers to miniature bioreactor culture system using a microfluidic technology to build a network simulating real blood circulation in the body.[38] Heart-on-chip is also known as biosensor constructed with Muscular Thin Film (MTF) technology.[39] In this MTF system, tissue contraction can be real-time monitored with sensors embedded in hiPSC-cardiac thin-film tissue.

EHTs are ideal models for analyzing cardiac contractile force and electrophysiology, and enable accurate disease phenotype simulation and drug testing. The hydrogel-based EHT was initially conceptualized by forming coherently beating constructs between 2 glass tubes using chicken embryonic heart cells.[40] The system was then improved such that it could be adapted to standard 24 well plates using neonatal rat heart cells.[41] Other researchers also significantly contributed to the human EHT system by miniaturization and multiplexing[42] and developing EHT with chamber-specific drug responses[43] or increased maturity.[44] Nowadays, EHTs from hPSCs derived cardiac cells have been successfully synthesized to model cardiac diseases caused by genetic mutation, such as hypertrophic cardiomyopathy caused by BRAF mutation,[45] ACTN2 mutation[46] or MYBPC3 mutation,[47] left ventricular hypertrophy caused by PRKAG2 mutation,[48] dilated cardiomyopathy caused by phospholamban (PLN) mutation,[49] and diverse muscle dystrophy caused by X-linked dystrophin gene mutation.[50] Miniaturized and multiplexed EHTs are ideal for *in vitro* drug testing, while EHTs with clinically relevant sizes could also be generated for implantations.[51] Taken together, engineered cardiac tissues have great potential in drug screening, cardiac pathogenesis studies and therapeutic applications.

As mentioned above, EHTs are formed by engineering pre-differentiated cardiac cell types. These engineered microtissues could mimic some aspects of adult heart tissue, but do not reproduce the patterning of early heart development. Studies focused on heart development based on 3D constructs are relatively rare. Pioneering studies in this front have initiated the development

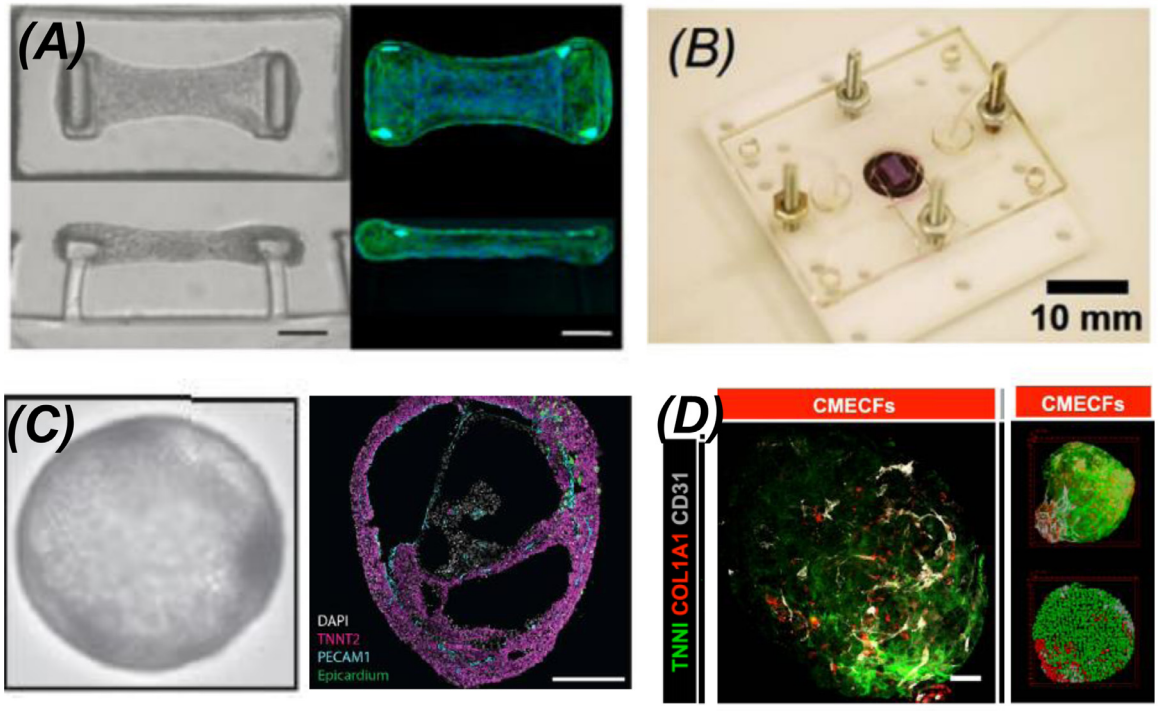

Figure 1. 3D Cardiac constructs.

(A) hiPSC-EHT from top-down (upper) and side (lower) views. Scale bar, 50 μm; Images (bright field (left) and fluorescent (right); green, Phalloidin; blue, DAPI). (B) Photograph of the heart-on-chip bioreactor. (C) whole mount image of a cardioid(left) and fluorescent (right); red, TNNT2; cyan, PECAM1; green, Epicardium; white, DAPI. Scale bar, 200 mm. (D). Left: Representative immunofluorescence images of CM- (TNNI, green), EC- (CD31, grey) and fibroblast- (COL1A1, red) markers in microtissues, as indicated. Scale bar: 50 μm. Right: Representative immunofluorescence images (top) and digital images (bottom) showing TNN1+ (green), COL1A1+ (red), and CD31+ (grey) cells in spherical microtissues. (A), Reprinted from Hinson et al[72] with permission of the publisher. Copy right, AAAS. (B), Reprinted from Zhang et al[38] with permission of the publisher. Copy right, Elsevier. (C), Reprinted from Hofbauer et al[56] with permission of the publisher. Copy right, Elsevier. (D), Reprinted from Giacomelli et al[37] with permission of the publisher. Copy right, Elsevier.

of this field. These works include geometric confinement of hPSCs in micropatterns[52] and generation of precardiac organoids using mouse PSCs.[53] However, these 3D models failed to show the tissue interactions in embryonic heart development. Several recent studies generated cardiac organoids, such as heart-forming organoids (HFOs),[54] human heart organoids (hHOs),[55] cardioids[56] and multilineage organoid that recapitulates cooperative cardiac and gut development.[57] Their contributions have significantly developed the culture system. In contrast to EHTs, cardiac organoids are constructed by self-organization of hPSCs responding to defined factors that govern lineage commitment. These organoids could reflect the embryonic heart structure and spatio-temporal patterning of early cardio-genesis.[54-57] Therefore, the cardiac organoid platform is ideal to study early human cardio-genesis, developmental diseases such as congenital cardiac malformations[54] and regeneration after injury.[56]

Scaffold-free culture methods such as spherical microtissues, could be derived from hanging drops or in 96 well plates. Spherical microtissues were usually formed from hPSC-CM alone (mono-culture microtissues) or in combination with stromal cells (multi-cellular microtissues). Compared to CM mono-culture microtissues, multi-cellular microtissues showed more mature contractile phenotype and drug response.[58-60] The co-cultured cardiac microtissue was initially constructed using hiPSCs derived cardiomyocytes together with primary stromal cells such as Human Umbilical Vein Endothelial Cells (HUVEC) and Human cardiac fibroblasts,[61,62] however, primary stromal cells were unable to represent genetic signature of specific patient, limiting the accuracy of disease modeling. To solve this problem, recent studies combined hiPSCs derived cardiomyocytes (CMs), cardiac fibroblasts (CFs) and cardiac endothelial cells (ECs) to construct 3D cardiac microtissues.[37,63] Interestingly, microtissues derived from healthy CMs and patient CFs were able to recapitulate phenotypes of arrhythmogenic cardiomyopathy,[37] suggesting that non-CMs and the inter-cellular cross-talk play important roles in cardiomyopathy. Therefore,

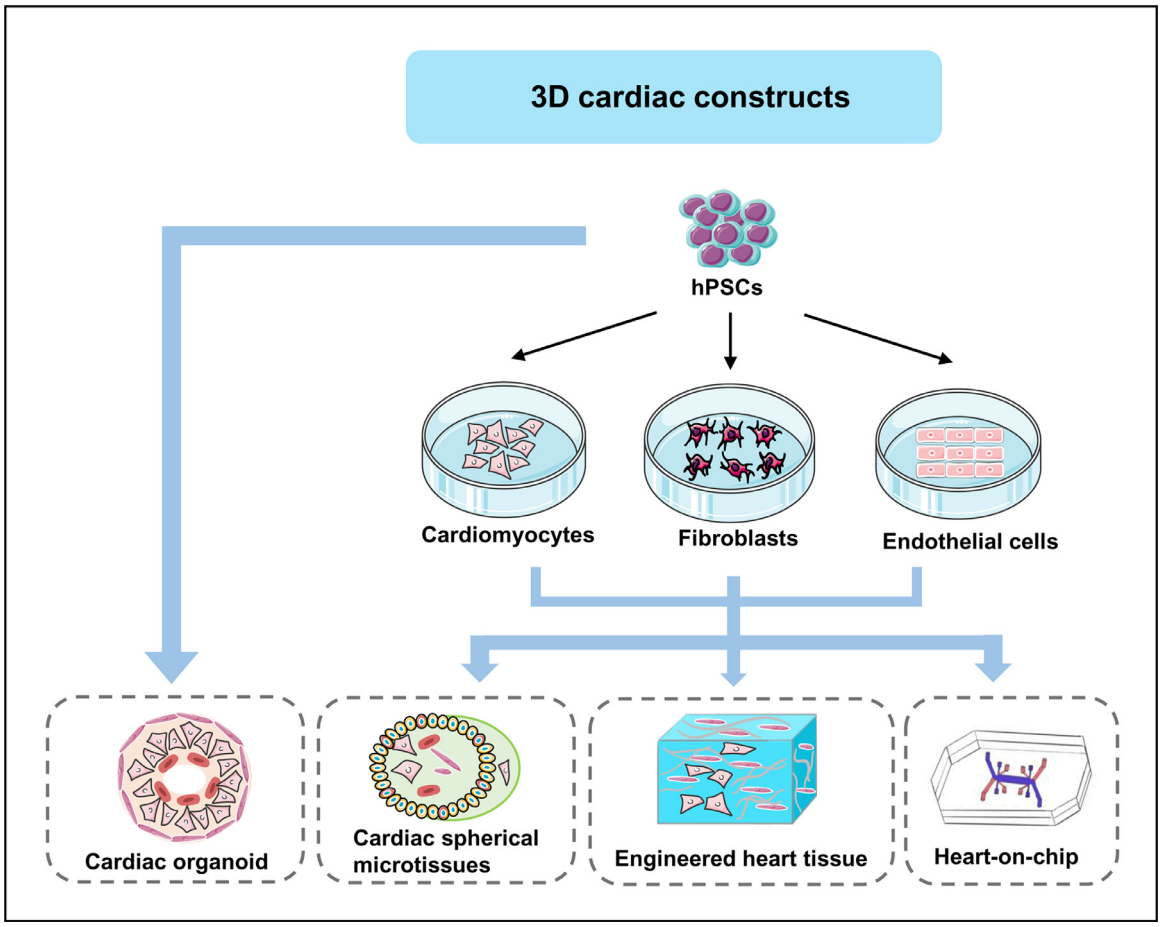

Figure 2. Tissue engineering strategies to build human hearts *in vitro*. Illustration of all the strategies and major cell types derived from hPSCs that are used to generate 3D cardiac constructs.

the construction of cardiac organoids based on iPSC derived cardiac cells permits a specific control over cell type ratio and functional study of disease-causing mutations in a particular cell-type.

The delicate microfluidic heart-on-chip enables perfusion which could mimic blood flow and cellular micro-environment *in vivo*. This circulating fluid flow facilitates the delivery of oxygen, nutrients, and drugs to cardiomyocytes.[64-66] On the other hand, the Muscular Thin Film heart-on-chip technology has been applicated in modeling of Barth syndrome disease[67] and ischemia reperfusion injury (IRI)[68], and showed advantages in real-time multiple channel analysis of myocardial drug response.[69] Heart-on-chip enables precise monitoring and high-throughput analysis of diverse physicochemical parameters found in cardiac tissue, such as chemical diffusive gradient and certain physical forces. Therefore, heart-on-chip could be used as a highly efficient drug screening system. In a combination of 3D bioprinting with heart-on-chip technology, pre-vascularized myocardium could be subjected to long time perfusion, cultured in a well-designed microbioreactor.[70] Moreover, by connecting different micro-organs-on-chip using circulating flow to construct a so-called body-on-chip,[71] it is possible to co-culture multiple organs and analyze drug metabolism in a more comprehensive manner.

Applications of cardiac microtissues in cardiac disease study

Disease modeling

So far, 3D cardiac constructs have been applied to disease modeling, drug testing, multi-omics study, gene editing, study of pathological mechanisms and cardiac regenerative therapies (Figure 3). As mentioned above, EHT constructed from hPSC-derived cardiac cells have been used to study heart disease caused by genetic mutation, such as hypertrophic cardiomyopathy (HCM)[45,48] and dilated cardiomyopathy (DCM)[72] (Table 1). Although non-genetic cardiac damages and diseases, such as IRI and maladaptive hypertrophy, are some of the major causes of death and have greater social impact worldwide, their detailed pathogenic

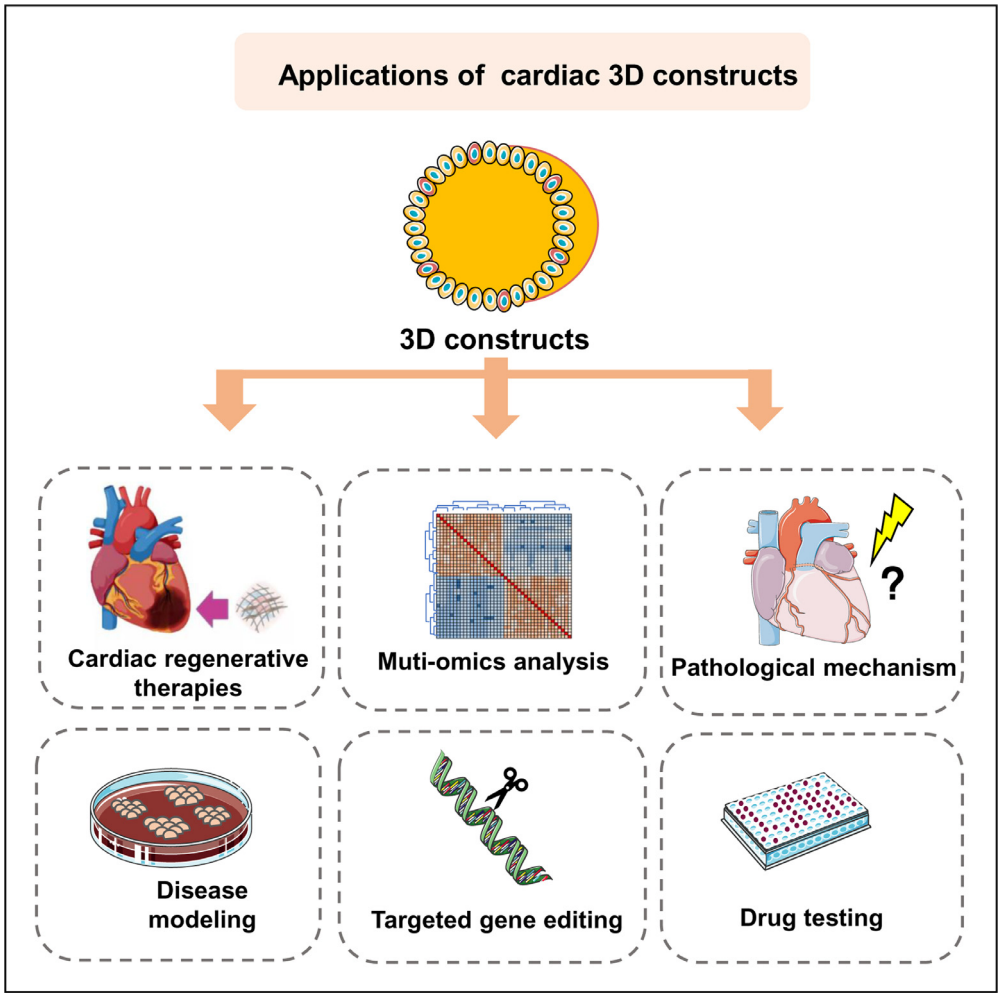

Figure 3. Applications of 3D cardiac constructs. The applications of 3D cardiac constructs in establishing patient specific library, multi-omics study, pathological mechanism study, disease modeling, gene editing and drug testing and cardiac regenerative therapies.

mechanism and possible protective strategies are less understood. Compared to genetic diseases, which have defined molecular causes and reproducible phenotypes, it is more challenging to construct non-genetic disease model using hPSCs-CMs and microtissues. Modeling IRI or hormone induced damage or disease phenotype with hPSCs-CMs is hindered by cellular immaturity, which leads to non-responsivity to hypoxia condition[73] or unstable response to adrenaline stimulation.[74] Compared to cultured monolayer cardiomyocytes, cardiac microtissues could provide a more complex *in vitro* disease model mimicking physiological and pathological responses of human heart with unprecedented precision and advanced cellular maturity. Therefore, tissue engineering and organoid culture system have been proved to be better platforms for predictive drug testing of IRI and maladaptive hypertrophy. There are several studies that use IRI and myocardial infarction (MI) model on EHT, organoid and heart-on-chip (Table 1). Dynamic control over oxygen concentration in culture condition was used to construct the IRI model.[68,75] In case of developing MI and heart failure models, strategies such as chronic norepinephrine (NE) treatment,[76,77] oxygen depletion[61,70] and cryo-injury[56] are used to simulate pathogenic condition *in vitro*. For maladaptive hypertrophy, methods including afterload enhancement[78] or hormone stimulation[44] are often applied for disease modeling on 3D constructs. Moreover, patient drug toxicity responses are also successfully recapitulated on cardiac microtissue.[38,71]

Diabetic cardiomyopathy is one of the major complications of diabetes, a disease that affects hundreds of millions of patients worldwide. To replicate human diabetic cardiomyopathy *in vitro*, hPSC-CMs were exposed to chronic hyperglycemic stress and showed pathological hypertrophy and reduced contractility.[30]

Sorted by	Modeling strategies	Microtissue types	Disease models	Applications	Phenotypes	Potential targets	References
Disease modeling	Tafazzin mutation	Heart-on-chip	Barth syndrome	Genetic disease modeling	Sparse and irregular sarcomeres, contracted weakly	Tafazzin	67
Disease modeling	BRAF mutation	EHT	Hypertrophic cardiomyopathy	Genetic disease modeling	Significantly increased tissue size, twitch force, and ANP gene expression	BRAF	45
Disease modeling	PRKAG2 mutation	EHT	Left ventricular hypertrophy	Genetic disease modeling	Increased microtissue twitch force and enhanced myocyte survival	AMPK signaling	48
Disease modeling	Stimulated by Angiotensin-II, Endothelin-1 and Isoproterenol	EHT	Pathological hypertrophy	Modeling cardiac development and disease	Physiological responses to Isoproterenol and recapitulating pathological hypertrophy	N/A	44
Disease modeling	Chronic NE treatment	EHT	Heart failure	Disease modeling and heart repair	Contractile dysfunction, cardiomyocyte hypertrophy, cardiomyocyte death, and N-terminal pro B-type natriuretic peptide release	N/A	77
Disease modeling	NKX2.5 loss	Organoid	Cardiac malformations	Genetic disease modeling	Loss of tissue compactness and a decreased cell adhesion. Significant enlargement of NKX2.5-KO-derived cells	NKX2.5	54
Disease modeling	Increased afterload exposure	EHT	Afterload enhancement	Functional study of lncRNA H19 in cardiac hypertrophy	Significant contractile force reduction	NFAT signaling	78
Omics study	Compound stimulation	EHT	Compound stimulated proliferation	RNA-seq and proteomics	Cell cycle activation	Mevalonate Pathway	83
Omics study	Cryo-injury	Organoid	MI	Single cell RNA-seq	Fibrosis	N/A	56
Omics study	PKP2 mutation	Organoid	Arrhythmogenic cardiomyopathy	Single cell RNA-seq	Arrhythmogenic cardiomyopathy	PKP2 and CX43	37
Gene editing	TTN mutation	EHT	DCM	CRISPR/Cas9 gene editing	Sarcomere insufficiency, impaired responses to mechanical and β-adrenergic stress, and attenuated growth factor and cell signaling activation.	TTN	72

Table 1 *(Continued)*

Sorted by	Modeling strategies	Microtissue types	Disease models	Applications	Phenotypes	Potential targets	References
Gene editing	X-linked dystrophin gene mutation	EHT	DMD	Correction of DMD mutations by exon skipping as myoediting	Lacking dystrophin expression, contractile dysfunction	Mutations in the X-linked dystrophin gene	[50]
Gene editing	Genetic mutation	EHT	Long QT syndrome type 3 and DCM	Insertion of mutation into an hiPSC line via CRISPR-Cas9 gene editing	Enhanced oxidative and glycolytic metabolism; increased contractile deficit caused by mutations under metabolic maturation culture conditions	N/A	[88]
Mechanism	Reduced oxygen level in 3D microtissues	Organoid	MI	Explore the critical pathways involved in cardiac pathophysiology	Response to chemical, physiological and pathological stimuli	VEGFA and IL6	[61]
Mechanism	6 hours ischemic condition (sodium lactate treatment and low O2), 3 hours reperfusion (complete culture medium, normal O2)	EHT	IRI	Studying key aspects of IRI	Increased cell death and opening of MPTP	Opening of MPTP	[75]
Mechanism	Hyperglycaemia and inflammatory cytokines	Organoid	Diabetic vasculopathy	Identifying the regulators of diabetic vasculopathy	Thickening of the vascular basement membrane	DLL4 and NOTCH3	[79]
Mechanism	Palmitate treatment	EHT	Cardiomyocytes proliferative block caused by maturation	Fatty acid metabolic mechanism of cardiac maturation	Screening for optimal metabolic substrate treatment	β-catenin and YAP1	[42]
Drug testing	Compounds treatment	EHT	Drug response	Multiple drug testing	Response to drugs such as pharmacological regulators of inotropy, mediators of pacemaking, modulators of ion-channel currents and proarrhythmic compounds	N/A	[96]
Drug testing	Dox treatment	EHT, heart-on-chip	Dox induced cardiotoxicity	Personalized drug testing	Dose-dependent responses towards DOX	N/A	[38]
Drug testing	Oxidative stress induced by H2O2 and controlled oxygen level	EHT	MI	Platform for drug testing after MI	Functional and pharmacological deterioration specific to aged myocardium	Epoxide hydrolase	[70]

Table 1 *(Continued)*

Sorted by	Modeling strategies	Microtissue types	Disease models	Applications	Phenotypes	Potential targets	References
Drug testing	Perfusion with micro-peristaltic pump	Heart and body on chip	Anti-cancer drug induced cardiac toxicity	Drug toxicity testing	Different toxicity outcomes in response to capecitabine and cyclophosphamide depending whether liver organoids are present	N/A	[71]
Drug testing	Reduced oxygen level in 3D microtissues along with stimulation by NE	Organoid	AMI	Model of hypoxia-enhanced drug cardiotoxicity test	Pre-existing hypoxic cardiac injury exacerbates the cardiotoxicity of DOX	N/A	[76]
Drug testing	3 hours ischemic treatment (sodium lactate treatment and 1% O2), 1.5 hours reperfusion (complete culture medium, 21% O2)	Heart-on-chip	IRI	Test the protective effect of Endothelial extracellular vesicles on IRI	50% cell death, cessation of tissue contraction	AMPK signaling, Antioxidant, Glycolysis, UPR, Redox, HSPs, Calcium homeostasis	[39]
Drug testing	Transport and subsequent cardiac effect of drug across a vascular endothelial barrier along with co-exposure of TNF-α	Heart-on-chip	Inflammation	Multiple parallel drug testing	TNF-α accelerated the passive transport and cardiac effect of Isradipine across the endothelial tissue	TNF-α	[69]

***Table 1*: Cardiac disease models based on microtissue culture system.**

AMI, Acute myocardial infarction; AMPK, Adenosine Monophosphate Activated Protein Kinase; ANP, atrial natriuretic peptide; CX43, connexin43; DCM, Dilated cardiomyopathy; DLL4, Delta like canonical Notch ligand 4; DMD, Diverse muscle dystrophy; DOX, doxorubicin; EHT, Engineered heart tissue; HSPs, Heat Shock Proteins; IL6, Inter Leukin 6; IRI, Ischemia reperfusion injury; MI, Myocardial infarction; MPTP, Mitochondrial Permeability Transition Pore; NFAT, Nuclear factor of activated T cells; NE, noradrenaline; NOTCH3, notch receptor 3; PKP2, Plakophilin 2;TNF-α, tumor necrosis factor-α; TTN, Titin; VEGFA, Vascular Endothelial Growth Factor A; UPR, unfolded protein response.

However, there is a lack of 3D co-culture model of human diabetic cardiomyopathy. A recent study successfully built human blood vessel organoids comprised of microvascular networks enveloped by basement membrane.[79] Interestingly, exposure of these blood vessel organoids to hyperglycemic environment induces phenotypes that resemble the microvascular changes found in patients with diabetes. These results indicate that human cardiac organoids modeling diabetic cardiomyopathy could display changes in their microvascular networks. Therefore, vascularized cardiac organoid served as a suitable model to study microvasculature mechanism and cellular crosstalk in diabetic cardiomyopathy.

3D models are unique in disease simulation. For example, it is particularly suitable for studying the interaction of different cells in the heart during the pathological process. Moreover, live imaging of cardiac organoids could provide dynamic observation of the pathological progression, which is not applicable to real human heart organs. In 3D microtissue, the system of CF, EC and CM co-culture can promote the maturation of CM, which was shown by the growth of sarcomere, the increase of mitochondria, the enhancement of contractility and mitochondrial respiration. The advantage of 3D disease model also lies in the fact that some disease phenotypes cannot be simulated at the 2D level. For example, Titin mutant iPSC-CM showed no phenotypic difference compared with WT in 2D model. While in 3D EHT, the mutant tissues strip showed obvious insufficient contractile force under the elastic resistance exerted by silicone posts.[72]

Despite the advantages on disease modeling, the maturity of 3D models is still not fully comparable with that of adult human cardiomyocytes. For example, the PDE subtype in 3D models is different from that of adult human cardiomyocytes: the main PDE subtype in adult human cardiomyocytes is PDE3, while the PDE subtype in EHT is PDE4.[10,80] This may cause differences in drug response, and indicates a gap in the maturity of CM in EHT compared with adult human cardiomyocytes. Take that into consideration, microtissues are more suitable for simulating disease in early development. Of course, this is not to say that microtissues cannot simulate adult heart disease, only that the construction of adult disease model requires more careful assessment of the pathogenic phenotype in microtissues. Determination of whether the adult disease phenotype can be reproduced in the 3D microtissues is of pivotal importance in this situation.

Multi-omics study

An important step in understanding the molecular complexity of cardiac pathogenesis is depicting the molecular landscape of human heart. With recent advances in multi-omics study, including bulk-transcriptomics, proteomics and single cell transcriptomics, possible causative mechanisms of several cardiac diseases are being addressed. Bulk-RNA sequencing can provide a comprehensive gene expression profile of microtissues, which is helpful to extract key characteristic gene expression changes and describe the features of microtissues.[81] The single cell transcriptomic analysis could be used to reveal molecular distinction in different cardiac cell types, both in microtissues[37] and cardioids.[56] This state-of-art technology is an ideal tool to demonstrate the spatiotemporal patterning of early cardio-genesis and cellular cross talk in cardiac microtissues. Moreover, single-cell RNA-Seq can also be used to explore the molecular mechanism in disease models. In an EHT model of pulmonary atresia with intact septum (PAIVS), single cell transcriptome suggested that the phenotypes of reduced contractile and prolonged contractile kinetics were associated with abnormal expression of cardiac contractile apparatus genes.[82] In addition to transcriptomics, proteomics based on mass spectrometry (MS) is capable of measuring variations in global protein expressions which may not be indicative of the transcriptome alone. Comprehensive proteomic analysis of pathological cardiac model revealed alterations in key metabolic and signaling pathways.[68,83]

The power of multi-omics resides in the ability to provide full spectrum analysis of molecular events in disease models. Multi-omics analysis could be applied on in-depth mining of candidate pathogenic factors. By integrating transcriptomic and proteomic data, multiple sequential events of disease occurrence are analyzed. Moreover, therapeutic targets and pathways were identified according to the changes of candidate factors at multiple dimensions. For example, compound 65, a TGFBR inhibitor that can promote the proliferation of cardiomyocytes, was screened out in EHT model. The detailed molecular changes upon compound 65 treatment were analyzed through the combined analysis of transcriptome and proteome. Combined analysis revealed the involvement of mevalonate pathway in proliferation of cardiomyocytes.[83]

Multi-omics analysis could also be used to construct gene regulation network. The interaction between mRNA, regulatory factors and proteins in the organism forms a network. In order to clarify the regulation between various molecules, it is necessary to build a gene regulation network to connect them with each other, in order to have a deeper understanding of the molecular mechanism of complex phenotypes in diseases. The construction of gene regulatory network also helps to screen out key regulatory genes.[84] Taken together, a thorough examination and a subsequent representation of the transcriptome and proteome of cardiac microtissue addresses questions regarding the molecular characteristics of the human heart and provides insights into unknown drug targets.

Despite the huge power of multi-omics technique, its application in cardiac 3D constructs still faces

challenges: Due to the limitation of cell number in the microtissues, the current research on cardiac 3D constructs mainly focuses on single-cell RNA sequencing, bulk-RNA sequencing, and proteomics. Combined analysis of modified proteomics (such as phosphorylated proteomics) with proteomics is rare. The development of micro proteomics and modified proteomics will fill this gap. It is expected that multi-omics combined analysis will be promoted to the single microtissue resolution in the future.

Gene editing

Comprised of the Cas9 nuclease and customizable single guide RNA (sgRNA), CRISPR/Cas9 system is designed to induce DNA double-strand break (DSB) at a desired genomic locus. This technology has been broadly used in gene editing, drug discovery and therapy.[85] When the homologous template DNA is absent, the DSB is repaired through an error-prone nonhomologous end-joining (NHEJ) pathway, resulting in small indel and dysfunction of the gene.[86] In the presence of a donor DNA containing sequence homology with the targeted locus, the DSB can be repaired using homology-directed repair (HDR), which leads to insertion or point editing of the desired genomic locus.

The CRISPR-Cas9 gene editing technologies have revolutionized functional investigation of cardiac disease associated genes.[87] CRISPR/Cas9 technology has been applied on cardiac 3D constructs to construct isogenic gene-modified iPSC and microtissues. The complexity of human genetic background had a great impact on the phenotype of iPSC-CM. To solve this problem, isogenic mutant iPSCs were constructed via CRISPR/Cas9 gene editing on wild type iPSC.[47,67,72,88] On the other hand, isogenic control iPSCs were established by CRISPR/Cas9 correcting the pathogenic mutation in patient-derived iPSCs.[49] Isogenic control and mutant microtissues were then constructed, and their phenotypes were compared to confirm that the disease phenotype was indeed caused by the mutation of the target genes.

Gene editing can also be used to modify the regulatory sites associated with pathogenic mutations. For example, modification of the CaMKII phosphorylation site serine 2814 on RYR2 (RYR2-S2814) by gene editing to replace S2814 with alanine (RYR2-S2814A) could reverse abnormal diastolic Ca^{2+} level in MTF.[89] In EHTs generated from Duchenne muscular dystrophy (DMD) patient iPSCs, gene editing on RNA splice sites allowed skipping of the mutant or out-of-frame DMD exons[50] and restored the cardiomyocyte function in gene-corrected EHTs. These observation shows the possibility of reversion of the cardiac abnormalities by targeting of the regulatory sites associated with pathogenic mutations.

The Cas9 nuclease activity could be deactivated by mutagenesis, leading to catalytically deficient Cas9 (dCas9).[90] dCas9 can be modified by fusing with transcription repressors such as KRAB repression domain[91] to inhibit the expression of gene of interest (CRISPRi) in iPSC derived cardiomyocytes. Moreover, dCas9 fused with transcription activators such as VP64 transcriptional activation domain[92] could be used to activate target gene expression (CRISPRa). CRISPR-screening based on CRISPR-Cas9 gene knockout or CRISPRi/a transcriptional repression/activation offer powerful tools to manipulate gene expression on a large-scale. Taken together, CRISPR-Cas9 enables precise knock in, knock out, inhibition and activation of the gene of interest. By combining hPSC and CRISPR-Cas9 gene editing, cardiac microtissue with the desired pathogenic mutation can be generated, and disease related genes could be removed from genome in patient specific microtissues. CRISPR-Cas9 technology can also be used on a genomic scale for functional screening of hiPSC derived cardiomyocytes.[93] In the future, application of CRISPR-screening technology on hPSC-CM pathological models will facilitate in establishing associations between gene expression and cardiac pathogenesis.

Studies on pathological mechanisms

Elucidation of the molecular mechanisms behind the phenotype of a disease or drug is a complex process, and it requires a variety of techniques to find possible targets and verify the hypothesis. Taking advantage of transcriptome analysis, potential therapeutic targets have been identified, such as phosphodiesterase (PDE2A, PDE3A)[74] and PDGF receptor beta (PDGFRB)[94] in familial dilated cardiomyopathy (DCM). Cardiac multi-cellular microtissues which contain multiple cell types serve as an ideal model for analyzing the cell-cell interaction during pathogenesis. With the help of single cell RNA sequencing, mechanism of enhanced hiPSC-CM maturation in microtissue was targeted on cAMP signaling pathway and CX43 gap junction gene.[37]

The next step of mechanism study is functional validation via activation/inhibition of target pathways by small molecule activators/inhibitors. Small molecule compounds have specific targets, and the concentration and duration of treatment can be accurately controlled. Therefore, they can achieve precise regulation of target pathways. Small molecule compounds are proved to be powerful tools for pathway function verification. For example, antioxidant NAC (N-acetyl-L-cysteine) treatment[27] and AMPK activation by AICAR, a pharmacological activator of AMPK[26] could successfully alleviate drug-induced cardiotoxicity. Persistent activation of cAMP pathway by exogenous addition of dibutyryl cAMP could recapitulate enhanced maturation of cardiomyocyte.[37] Cyclosporine A (CsA) can decrease IRI in the human microtissue model through inhibition of MPTP opening.[75]

Functional experimental verification can also be achieved through knockout or overexpression of target gene. For example, pathway correction by CRISPR/

Cas9-mediated gene editing[28] overexpressing PPP3CC could restore impaired cardiomyocyte function. Fatty acid metabolism-imposed proliferation barrier in cardiac microtissues can be rescued by overexpression of both β-catenin and YAP1.[42] Loss of function studies of the target gene CX43 in the iPSC-derived cardiac fibroblasts followed by 3D microtissue construction[37] validated the role of CX43 in promoting cardiomyocyte maturation. NOTCH3 mutation generated by CRISPR-Cas9 rescued the thickening of the vascular basement membrane in blood vessels organoids.[79]

Taken together, these findings suggest that iPSC-CM 3D models could be used as efficient tools for identification of therapeutic targets, and could act as testing platforms for potential therapeutic interventions such as chemical interference or gene editing.

Drug screening and testing

Cardiotoxicity is one of the most important reasons of drug withdrawal. Drug induced cardiovascular toxicity can lead to both functional deteriorations such as arrhythmia and alteration of the contractile force of the heart, and structural injury to heart tissue. However, since rodents and humans have significant differences with respect to electrical repolarization and myofilament dominant composition,[33] traditional preclinical rodent models to test cardiac toxicity or efficacy of compounds have been observed to be less predictive/effective. In contrast, EHT from hiPSC-CMs shows a lower repolarization reserve compared with human myocardium, and could serve as a sensitive *in vitro* platform for repolarization studies.[95] Moreover, primary CMs from preclinical animals such as non-human primates are not suitable to be subjected to high throughput drug screening due to ethical concerns in terms of the 3R principles (reduce, refine, and replace animal experimentation) and high costs. Therefore, platforms for modeling the human cardiac tissue are of particular interest for drug testing. Cardiac organoid platform, such as cardiac microchamber, is highly suitable for testing the side effects of drugs during early developmental stages of heart morphogenesis.[52] In contrast, metabolically mature EHT facilitates modeling of adult-onset disease[88] and analysis of drug response by measuring contractile force(inotropic) and heart beat rate (chronotropic).[96] Moreover, EHT could also be used as a platform for high-throughput screening to optimize metabolic substrate treatment[42] and chemical compounds promoting cardiomyocyte proliferation and regeneration.[83] Cardiac microtissues could provide a high throughput platform for detection of drug induced changes in cardiac structure.[62] In addition, microtissues constructed using genetic mutants of iPSC derived cardiac fibroblast cells faithfully recapitulate the arrhythmogenic cardiomyopathy phenotype,[37] and consequently could have an application in patient-specific drug testing. Also, microtissues mimicking myocardial infarction showed pre-existing hypoxic cardiac injury exacerbated by the cardiotoxicity of anti-cancer drugs,[76] suggesting the expansibility and modifiability of this platform. Heart-on-chip platform with flexible strain gauge embedded in 24-well plate provides precise and multiple parallel measurement of the contractile stress and beat rate of engineered heart tissue,[69] thus allowing high throughput drug testing and precise measurement. By analyzing cell death, drug toxicity could also be accessed with the heart-on-chip platform.[71] In conclusion, current research sufficiently demonstrates the power of cardiac 3D constructs in applications such as drug discovery on human cardiac diseases.

Cardiac regenerative therapies

hiPSC-derived cardiac cells could be used as regenerable resource for cell replacement of infarcted cardiac tissue. However, current attempts in transplanting these cells demonstrated the cellular immaturity of the injected cells, resulting in arrhythmias in the grafted animal.[97-99] Recently, EHT patches transplanted in animal hosts have successfully repaired heart injury in guinea pigs,[100] and demonstrated the technical feasibility in rats[101] and pigs.[102] Interestingly, EHT patches have never been shown to induce arrhythmias after transplantation, which may be because of their insufficient coupling with host tissue.[100-102] The limitation of graft-host tissue integration is therefore a barrier and a hot research topic for the development of functional tissue repair therapy. Also, prolonged culture and maturation time of immune compatible patient-specific iPSC-CM patch largely hinders the successful implementation of this technology. Therefore, it is of great importance to establish functionally mature and immune-evasive human cardiac tissue in regenerative medicine. Immune-evasive human islet organoids have been successfully built by overexpression of PD-L1.[103] These universally immune compatible islet organoids avoid immunological rejection in mice with normal immunity and can maintain glucose homeostasis. Another example is hypoimmunogenic iPSCs, established by inactivating major histocompatibility complex (MHC) class I and II genes and over-expressing CD47.[104] These hypoimmunogenic iPSCs derived cardiomyocytes could evade immune rejection in MHC-mismatched recipients and survive without immunosuppression. Therefore, by combining the mechanical training enhanced maturation, 3D printing, size modifications and immune evasive technology, functionally suitable cardiac grafts can be engineered for universal transplantation in the future.

Future perspectives

Chronic pathogenic condition is a whole-body event that involves cross-talk between different organs. With

development of multi-organ co-culture system, and the so-called body-on-chip technology, it is possible to connect multiple microtissues by circulating flow which mimics the interaction and exchange of metabolites between tissues in the body. Multi-organ systems are mostly used to study the absorption, distribution, metabolism, excretion, and toxicity of drugs. Interestingly, integrated body-on-chip platform connecting heart and liver organoids showed different cardiac toxicity response towards anti-cancer drugs such as capecitabine and cyclophosphamide with or without the presence of liver organoids.[105] These multi-organ systems integrated with force sensors or fluorescent reporters could be used for real-time recording of organ responses. In the future, these multi-organ systems will be expected to be maintained *in vitro* for extended culture periods for representing dynamic pathogenesis.

Closing remarks

Although huge progress has been made in this field, there are still several technical challenges remaining, such as reproducibility of the microtissue with respect to cellular composition and architecture.[56] It is also challenging to generate immune cell infiltrated or geriatric pathogenic 3D cardiac constructs due to the limitation of microtissue assembly and culture system. Although cardioids could to some extent recapitulate the chambered structure of the heart *in vitro*, it requires a deeper understanding of the developmental cues to pinpoint the drivers of the multi-chamber structure formation to generate a more complex structure (4 chambers) fully resembling the human heart. In addition, construction of chamber-specific EHT model is of particular interest in atrial-selective antiarrhythmic drug development.[106,107] Differences in drug response between atrial and ventricular EHTs have been shown by Goldfracht et al.[106] However, the effect of vernakalant on APD_{90} in atrial EHTs contrasts with results obtained in human atrial tissue and indicates immaturity of atrial hESC-CMs.[107] Therefore, further refinement of atrial differentiation protocol is required for fully functional atrium-specific EHT model. Moreover, the limited imaging depth and drug penetration caused by larger microtissue size must be considered. Thanks to growing imaging technologies with deeper imaging capacity and generation of multiple fluorescent reporter cell lines, the imaging challenges are being solved. And with the development of vascularized cardiac organoid,[38] the drug penetration will be greatly improved. With the progresses of specific culture protocols, exact cellular composition ratios and standard microtissue forming molds, it will be possible to increase the reproducibility of the main features of 3D constructs in the future. Increasing the understanding of bioengineering techniques that enable better control of tissue composition and extracellular environment will be pivotal in moving 3D cardiac constructs research forward. Overall, with the creation of robust and functional cardiac 3D constructs, we will be able to unveil the complicated human cardiac pathologies in a meaningful way. Advancements in human cardiac constructs will certainly speed up drug development and precision medicine.

Outstanding questions

Since the EHT patches cannot efficiently couple with host tissue, could it be possible to enhance the graft-host tissue integration of the EHT patches by genetic manipulation? For example, enhancing the expression of gap junction protein?

Could it be possible to generate immune cell infiltrated or geriatric pathogenic human 3D cardiac constructs in vitro? If so, could this platform be used to study the cellular cross-talk in the heart under physical or pathological condition?

Search strategy and selection criteria

Data from this review were identified by searches of PubMed and references from relevant articles using the following keywords, alone or in combination: pluripotent stem cells derived cardiomyocytes, cardiac 3D constructs, spheroids, cardiac organoid, microtissue, engineered heart tissue, heart-on-chip, drug screening and cardiovascular disease model. Only articles published in English were included. Abstracts and reports from meetings were excluded. Articles focused on human cardiac 3D constructs published from 2014 to 2021 are included.

Declaration of interests

The authors declare no competing interests.

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Author contributions

J.X. had the idea for the article. C.L. and X.F. performed the literature search and analysis. C.L., G.L., P.G. and J.

X. drafted and critically revised the article. All authors read and approved the final version of the manuscript, and ensure it is the case.

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