Biomaterials Translational

Model construction and clinical therapeutic potential of engineered cardiac organoids for cardiovascular diseases

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Key Words:
cardiac organoids; cardiovascular diseases; clinical applications; scaffolds; self-assembly

From the Contents

ABSTRACT

Cardiovascular diseases cause significant morbidity and mortality worldwide. Engineered cardiac organoids are being developed and used to replicate cardiac tissues supporting cardiac morphogenesis and development. These organoids have applications in drug screening, cardiac disease models and regenerative medicine. Therefore, a thorough understanding of cardiac organoids and a comprehensive overview of their development are essential for cardiac tissue engineering. This review summarises different types of cardiac organoids used to explore cardiac function, including those based on co-culture, aggregation, scaffolds, and geometries. The self-assembly of monolayers, multilayers and aggravated cardiomyocytes forms biofunctional cell aggregates in cardiac organoids, elucidating the formation mechanism of scaffold-free cardiac organoids. In contrast, scaffolds such as decellularised extracellular matrices, three-dimensional hydrogels and bioprinting techniques provide a supportive framework for cardiac organoids, playing a crucial role in cardiac development. Different geometries are engineered to create cardiac organoids, facilitating the investigation of intrinsic communication between cardiac organoids and biomechanical pathways. Additionally, this review emphasises the relationship between cardiac organoids and the cardiac system, and evaluates their clinical applications. This review aims to provide valuable insights into the study of three-dimensional cardiac organoids and their clinical potential.

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Introduction

Cardiovascular diseases (CVDs) represent a major global health threat worldwide due to their high morbidity and mortality rates.[1-4] CVDs include hypertension, myocardial infarction (MI), and heart failure, etc.[5, 6] There is an increasing focus on understanding the pathogenesis of CVDs, improving diagnosis and treatment of CVDs and developing new drugs.[7] Traditionally, two-dimensional (2D) cell and animal models have been developed to explore the pathogenesis of CVDs.[4] However, these 2D models are not entirely suitable for replicating *in vivo* cardiac diseases.[8] 2D cell models fail to replicate the immune system, matrix components and organ-specific functions, resulting in the loss of genetic heterogeneity of the original cells after several generations and impacting the structural physiology in animal models of cardiac development and pathogenesis.[9]

Cardiac organoids are novel *in vitro* cell models with complex three-dimensional (3D) structures that mimic human heart tissue.[10-12] Research has shown that 3D cell clusters can be used to develop various organoids, including those for the intestine, colon, stomach, liver and bowel organoids, which exhibit similar structures and functions of these internal organs.[13-15] In a 3D culture system, embryonic stem cells and human induced pluripotent stem cells (hiPSCs) can differentiate randomly or directionally into different cell types, resulting in the formation of

cardiomyocytes.[16, 17] Cardiac organoids offer great potential for uncovering the fundamental mechanisms of cardiac development in both basic research and clinical applications.[18] They are used to mimic cardiac development and model CVD.[19] While cardiac organoids show promise in various diseases and drug screenings, their progress has been hindered by the complexity of early heart development.[20, 21] Recently, numerous publications have detailed the construction and application of cardiac organoids, underscoring their rapid evolution.[22, 23] The multi-chamber heart organoid model is also used to reveal the coordination mechanism of heart chamber interaction, which is helpful to study heart development and heart-related disease research. Therefore, a comprehensive and detail overview of the development of cardiac organoids is crucial for advancing engineered heart tissue.

This review summarises various types of cardiac organoids used to investigate cardiac functions, including co-culture, aggregation, scaffolds, and geometries (**Figure 1**). Multiple cell lines, such as cardiomyocytes, fibroblasts and endothelial cells (ECs), are co-cultured to create cardiac organoids that replicate the complex cardiac microenvironment *in vitro*. The self-assembly of monolayer, multilayer and aggravated cardiomyocytes forms biofunctional cell aggregates in cardiac organoids. Scaffolds made of decellularised extracellular matrix (dECMs), 3D hydrogels and bioprinting provide essential support frameworks for cardiac organoids and play a crucial role in cardiac development. Various geometries, including spheroids, patches, chambers and rings are established to create cardiac organoids for investigating intrinsic communication between cardiac organoids and biomechanical pathways. The relationship between cardiac organoids and the heart system is also emphasised to evaluate the underlying mechanism and interactions involved in cardiac development, cardiac diseases, drug screening, cardiac regeneration and repair. Furthermore, it evaluates the potential of cardiac organoids as *in vitro* cardiac models for clinical applications, investigating key functions and biological states such as contractility, morphogenesis, electrophysiology, metabolism and gene expression. This review aims to provide valuable insights into the 3D construction technologies of cardiac organoids and their clinical applications in CVDs.

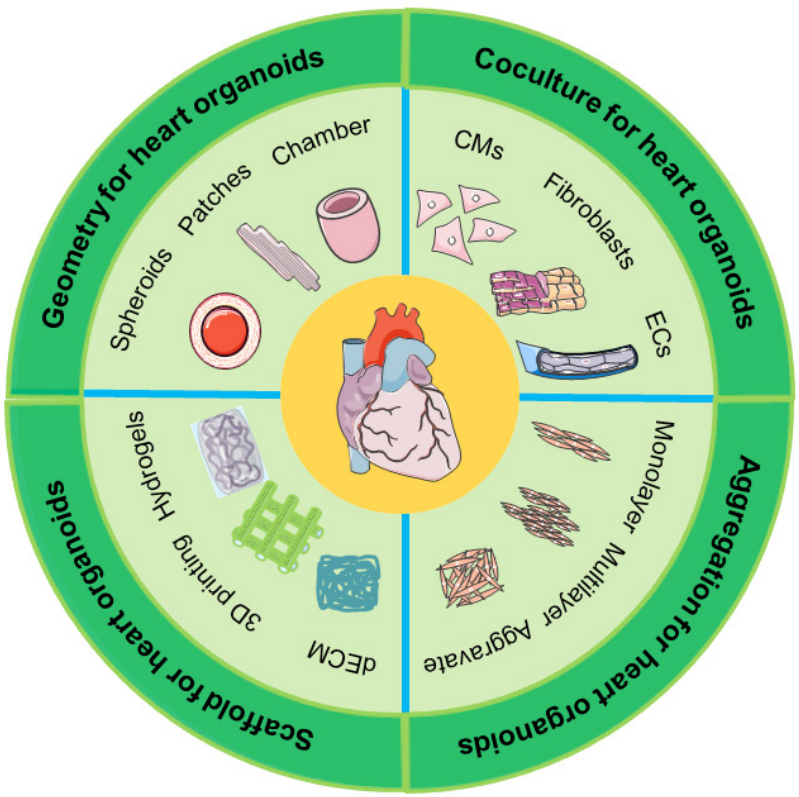

Figure 1. Overview of cardiac organoid construction technologies, including co-culture, aggregation, scaffolds and geometries. 3D: three-dimensional; CMs: cardiomyocyte; dECM: decellularised extracellular matrix; ECs: endothelial cell. Created with Microsoft PowerPoint 2010.

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Retrieval Strategy and Selection Criteria

Data for this review were identified by searching of PubMed and references of relevant articles using the search terms: "cardiac organoids", "cardiovascular diseases", "clinical therapeutic potential", "cardiac organoid construction", "co-culture", "aggregation", "scaffolds", "geometries", "decellularised extracellular matrices", "3D hydrogels", "bioprinting techniques", "cardiac development", "drug screening", "heart regeneration and repair", "morphogenesis", "metabolomics", and "gene expression". Only articles published in English from 1996 to 2024 were included.

Cardiac Organoids to Mimic Natural Human Hearts

Cardiotoxicity is the primary factor contributing to the failure of new drug candidates for CVDs.[24] Functional drugs targeting myocarditis, arrhythmias, hypertrophy and heart failure (HF) are specifically designed to mitigate cardiac diseases, and cardiotoxic effect of drugs can be evaluated in cardiac organoids.[25-29] During drug evaluation, human adult cardiomyocytes obtained from patients' cardiac biopsies are the preferred cell type for assessing cardiotoxicity during drug evaluation. However, the limited availability of human cardiac tissues restricts their widespread use as human cardiac models.[30, 31] To address these problems, human stem cells, including embryonic stem cells (SCs), and induced pluripotent stem cells (iPSCs) offer ideal alternatives for studying the physiological and pathological aspects of the cardiovascular system in toxicity assessment, drug screening and regenerative medicine.[32] Stem cells have the potential to differentiate into cardiomyocytes, offering a continuous source of cardiac cells for the treatment of CVDs. However, human pluripotent stem cells (hPSCs) differentiated into cardiomyocytes exhibit immature cardiomyocyte phenotypes in 2D monolayer systems to limit their applications. This immaturity affects their development in terms of spreading size, excitation-contraction coupling (T-tubules), active force stimuli (mature Ca^{2+} channels) and energy conversion efficiency (oxidative metabolism) when cultured in 2D system.[33]

A 3D cardiomyocyte culture system can closely mimic *in vivo* physiological and dynamic conditions for cardiovascular evaluation.[34] In this microenvironment, cardiomyocytes and other cardiac cells are cultured within solid biomaterials (such as scaffolds or hydrogels) to promote cardiac tissue formation and stimulate the physiological microenvironment of the heart.[35, 36] The geometric morphology of cardiomyocytes, myofibril expression and junction protein formation in a 3D system are significantly different from those in 2D cell culture. Additionally, 3D culture provides protection to cardiomyocytes against drug-induced mechanical stress and apoptosis.[37-39] Recently, organoids, as artificial cardiac tissues, have been developed to replicate cardiac structures and functions by modifying their size, shape and configuration. Human cardiac organoids are generated through aggregation-induced self-assembly of differentiating cardiomyocytes from hPSCs.[40] Therefore, cardiac organoids may be ideal models for mimicking native cardiac elements, including inflow-outflow territories, cardiac chamber architecture and heart-related regulation.[41]

Cardiac Organoids and Tissue Models

Cell aggregation for heart organoids

Aggregation

Human cardiac organoids are primarily constructed by inducing spontaneous self-assembly (aggregation) of specific cells under appropriate conditions. A prominent method for archiving this is the embryoid body 3D differentiation technique, widely used to induce organoid differentiation. Previous studies have focused more on cardiomyocyte production and differentiation rather than embryoid body development.[42] Controllable transcription markers for the first and second heart fields (FHF/SHF), regulated by bone morphogenetic protein and activin A, are used to generate distinct progenitor populations and precardiac spheroids. These spheroids exhibit similar *FHF*/*SHF* gene expression levels and potential for heart differentiation.[43] After 15-day culture, monolayers of cardiomyocytes with typical cardiac gene and protein expression profiles are formed to organise numerous sarcomeric structures. These early studies demonstrate that cardiac development is regulated by spontaneous cell self-assembly or aggregation. There is a desire for multifunctional cardiomyocyte aggregation under deep signalling stimuli and novel technologies, to evaluate the structures and outcomes of cardiomyocyte aggregation organoids through natural and artificial interventions (**Figure 2A–C**).[44-48]

Due to the intrinsic tendency of cardiomyocytes to aggregate into spherical or layered structure, cardiomyocyte aggregation facilitates the establishment of matrix-free heart tissue organoids for cardiovascular therapy.[46] Hanging drop methods and self-organisation are the simplest strategies for constructing cardiomyocyte aggregation organoids in anti-adhesion plates or scaffolds.[49, 50] In practical applications, heart organoids are successfully developed to protect cardiomyocytes against necrosis or apoptosis and promote directed differentiation towards the heart by expression of cardiac markers.[51, 52] These methods promote cell-cell communication and cell-extracellular matrix (ECM) interaction to induce cardiomyocyte aggregation for cardiac differentiation. However, the variability of organoid sizes and shapes is limited due to the voluntary self-aggregation.[53] To address these challenges, bioprinting technology has been developed to create cardiac organoids with complex and stereoscopic 3D structures.[54] Various geometries of heart organoids are formed using a 3D bioprinter and further refined with adjustable cell ratios, including hiPSC-cardiomyocytes, fibroblasts and ECs.[55] These 3D microtissue structures are used to construct cardiac structures and functions in an MI model (**Figure 2D**, and **E**).[47] A mixture of iPSCs and fibroblasts, at a cell ratio of 1:4, is created to serve as fibrotic cardiac tissues, which reduces the contractile force and alters beating rhythm in impaired organoids. Additionally, self-assembling human cardiac organoids derived from hPSCs have been reported to model heart development and congenital heart diseases. A three-step Wnt signalling modulation strategy, using chemical inhibitors and growth factors, is employed to develop complex internal chambers, recapitulate heart field and atrioventricular specification, build sophisticated vasculature, and present robust functional activity (**Figure 2F**).[48]

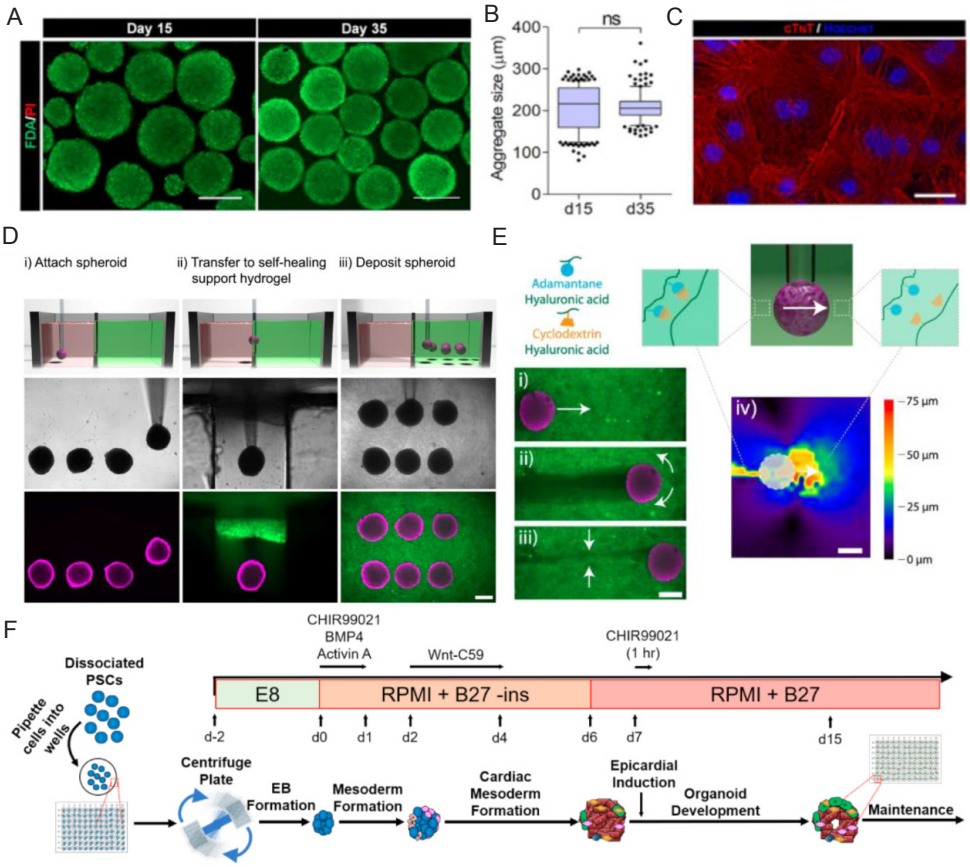

Figure 2. Spontaneous self-assembly (aggregation) of cardiac cells in appropriate states is used to form cardiac organoids. (A) Cell viability of hiPSC-cardiomyocytes at days 15 and 35. Scale bars: 200 μm. (B) Aggregate size of hiPSC-cardiomyocytes at days 15 and 35. (C) Immunofluorescence analysis of hiPSC-cardiomyocytes (from plated 3D aggregates) for cTnT (red). Scale bar: 30 μm. A–C are reprinted from Correia et al.[44] Copyright 2017, Wiley Periodicals, Inc. (D) Schematic (top), bright-field (middle), and fluorescence (bottom) images demonstrating, (i) MSC spheroid aspiration in a media reservoir, (ii) spheroid transfer into a self-healing support hydrogel (FITC-labelled), and (iii) spheroid deposition within the support hydrogel through removal of vacuum from the micropipette tip. (E) Reversible interactions between guest (adamantane, blue) and host (β-cyclodextrin, orange) modified hyaluronic acid of the support hydrogel (containing FITC-microparticles). Scale bars: 250 μm. D, and E are reprinted from Daly et al.[47] (F) A schematic diagram of the protocol used to differentiate TNNT2$^+$ cardiomyocytes in embryoid bodies. Reprinted from Lewis-Israeli et al.[48] 3D: three-dimensional; BMP4: bone morphogenetic protein 4; CHIR99021: a canonical Wnt pathway activator; cTnT: Troponin T; EB: embryoid body; FDA: fluorescein diacetate; FITC: fluorescein isothiocyanate; HOECHST: Hoechst 33342; ins: insulin; ns: not significant; PI: propidium iodide; PSC: pluripotent stem cell; RPMI: RPMI 1640 medium; TNNT2: troponin T2.

Co-culture

The complex cardiac microenvironment compromises not only cardiomyocytes but also the ECM and various cell types, such as ECs, vascular smooth muscle cells and pericytes, fibroblasts, neurons and immune cells.[56, 57] In the native cardiac microenvironment, cardiomyocytes are interconnected and play a crucial role in regulating the contractile performance of cardiac tissues. However, cardiomyocytes constitute only 25–35% of the myocardium by total cell amount. Other cardiac cells include atrioventricular nodal cells, ventricular cells, sinoatrial nodal cells, atrial cells, and Purkinje cells, each contributing to cardiac functions through interactions with cardiomyocytes.[58] To mimic the complex cardiac tissue in native microenvironment, cardiomyocytes are often combined with other cardiac cells in heart organoids to investigate their interactions within their local niches.[59, 60] Transcriptomic data from six heart regions have been done to elucidate the cellular composition of the mature heart.[61] Atrial and ventricular tissues exhibit different distributions of cell types, emphasising the intricate cell composition within the myocardium, as the evidence by the heterogeneity of gene expression among various cell populations.

Cell suspensions are mixed in appropriate ratios to investigate potential heart functions. ECs and fibroblasts are co-cultured under the same condition to promote the formation of cardiac spheroids.[62-64] ECs contribute to vasculogenesis within the heart organoids, while fibroblasts enhance interactions with adjacent cardiomyocytes and support the structural and functional development of hiPSC-cardiomyocytes in cardiac tissue. An engineered platform for heteropolar heart organoids has also been developed to respond to chamber-targeting drugs.[65] Human stem cells have been shown to differentiate

into norepinephrine-secreting sympathetic neurons when co-culture with cardiomyocytes, regulating heart rate in ventricular cardiomyocytes.[66] Although cardiomyocytes assist in neuronal maturation, the impact of neurocardiac co-culture on cardiac organoids and sympathetic CVDs remains to be further explored.[57] Additionally, cardiomyocytes are encapsulated within a sophisticated 3D microenvironment known as the ECM, which contains various functional proteins such as collagen, gelatin, laminin, fibronectin, fibrillin and elastin.[67-70] These proteins provide mechanical and viscoelastic properties, allowing cardiac cells to remodel their cytoskeletons for force-sensing adaptability in native heart tissue.[71] Consequently the myocardium perceives extracellular forces and responds to mechanical stimuli by increasing calcium concentration, aiding in the formation of heart organoids.[72, 73]

Scaffolds for heart organoids

Extracellular matrix scaffolds

The ECM serves as a cellular habitat, crucial for providing cells with mechanical support for maturation, and influences biochemical and electrical properties through parameter optimisation and mechanics tuning.[74-76] To utilise these ECM properties in cardiac organoids, decellularised ECM scaffolds are designed to maintain the complex structures and functions in the microenvironment of cardiac tissues by the determination of cell distribution, construction and function.[77, 78] Direct decellularised ECMs retain the native composition and architecture for tissue engineering applications in heart tissues. Human cadaveric hearts are used to construct decellularised native ECMs, and hiPSC-cardiomyocytes are then seeded in these ECMs to establish human myocardial-like cardiac organoids.[79] In this process, cellular components are removed from cardiac tissues, while preserving ECM integrity.[80] However, bare ECMs exhibit low mechanical stability in cardiac organoids. To address this, supporting materials have been developed by using bioprinting methods. For instance, gelatin methacryloy and gelatin methacryloyl/methacrylated hyaluronic acid hydrogels are synthesised with the incorporation of decellularised ECMs to enhance mechanical properties for cardiac tissue organoids (**Figure 3**).[81] Despite improvement in ECM mechanics, significant challenges persist such as ECM integrity, native construction and complete cell removal, due to the addition of supplementary materials. Creating cardiac organoids using decellularised ECMs is still time-consuming and labour-intensive with current technologies.

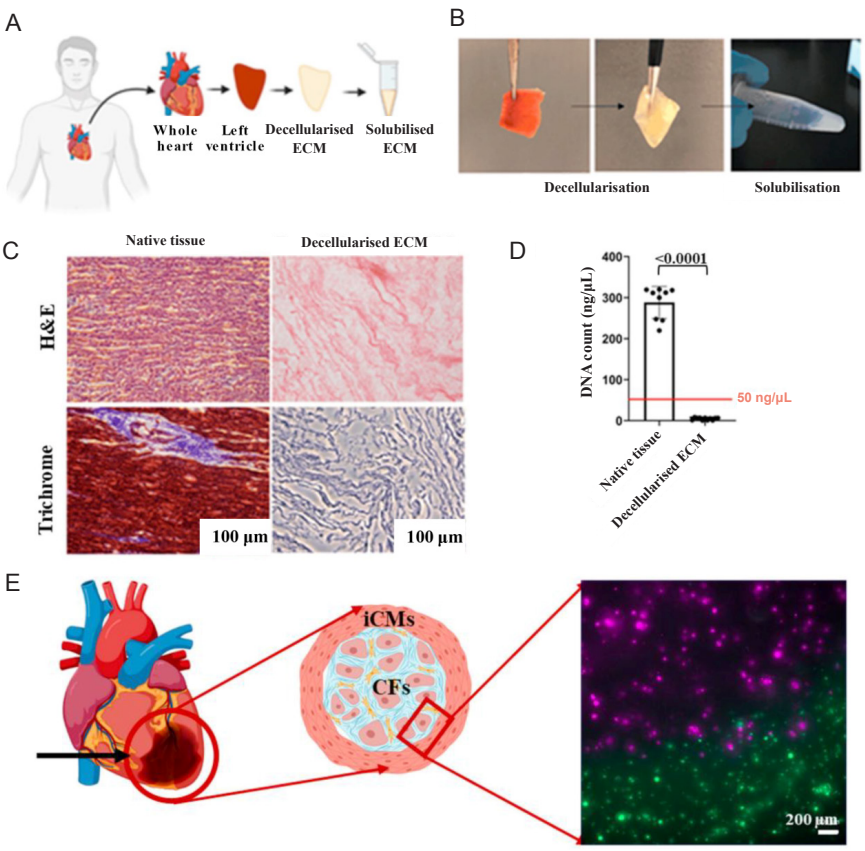

Figure 3. Decellularised ECM scaffolds for cardiac organoids. (A) Schematic illustration of heart decellularisation process. (B) Representative images demonstrating the workflow of decellularised ECM scaffolds. (C) H&E and Masson's trichrome staining of native tissue and decellularised ECM scaffolds. Scale bars: 100 μm. (D) Quantitative DNA measurements. (E) Infarct region with cardiomyocytes (green) and CFs (magenta). Scale bar: 200 μm. Reprinted from Basara et al.[81] CF: cardiac fibroblast; ECM: extracellular matrix; H&E: haematoxylin & eosin; iCM: induced pluripotent stem cell derived cardiomyocyte.

Hydrogel scaffolds

Hydrogels have been used to establish heart tissue-like 3D networks for cardiac organoids due to their hydrophilic nature, which facilitates the absorption of significant amounts of fluids, particularly water and interstitial fluid.[82, 83] Their excellent biocompatibility, high bioactivity, typical biodegradability, and good diffusion and permeability for oxygen, nutrients, growth factors and water-soluble products make hydrogels ideal adjustable biological scaffolds for cardiac tissue engineering. The mechanical properties of hydrogels can be modified by biochemical and biophysical cross-linking to match the mechanical characteristics of heart tissues.[84]

Cardiomyocytes are incorporated into hydrogel solutions, and the uniform cardiomyocyte-hydrogel mixture is dropped into a designed mould to adjust the geometric morphology and mimic cardiac tissues. Following hydrogel polymerisation, bulk hydrogel organoids containing cardiomyocytes and other cardiac cells are successfully constructed to study cell-cell and cell-ECM interactions in a complex 3D microenvironment.[85] Injectable hydrogels can deliver desired drugs or genes into heart in a minimally invasive manner for cardiac repair. For example, hydrogels loaded with hiPSC-cardiomyocytes are directly injected into hearts for ischaemic myocardial regeneration and repair.[86] Delatin fibre-infused gel inks have been developed for free-standing 3D-printed tissue scaffolds with cellular alignment cues.[87] Various biomaterials, including natural and synthetic polymers are used to design hydrogel scaffolds for cardiac organoids. Initially, hydrogel organoids were created using natural biomaterials such as collagen, gelatin, chitosan, fibrin, hyaluronic acid and matrigel.[88] Collagen-based hydrogels are engineered to control their elastic modulus, matching the mechanical stiffness of cardiac tissue (6–10 kPa).[89] These hydrogels can induce embryonic SCs to undergo cardiomyogenic differentiation, whereas soft hydrogels with a stiffness of 0.2 kPa fail to support cardiomyogenic lineage differentiation due to inefficient mechanical signal transmission.

Although natural biomaterials exhibit excellent biocompatibility and biodegradability to serve as ECM matrices, their stability and structural fragility limit their application in cardiac organoids. Alternatively, synthetic hydrogels or polymers, including poly(vinyl alcohol), poly(ethylene glycol), polycaprolactone, polylactic acid, polyglycolic acid, polylactic-coglycolide, gelatin methacryloyl and poly(2-hydroxyethyl methacrylate), are used to create engineered cardiac organoids.[90-92] Small molecules and peptides are modified into these synthetic polymers to stimulate the native cardiac microenvironment, enhancing cell-cell and cell-ECM interactions.[93] Electrospun dextran vinyl sulfone fibres are prepared to form fibrous cardiac-like matrices and construct contractile-layer organoids with the addition of iPSC-cardiomyocytes.[94] The contractility of cardiac organoids is enhanced by mechanical stimulation of synthesised hydrogels, promoting the maturation of hiPSC-cardiomyocytes through the regulation of cell junctions and T-tubule structures.[95] Electrical stimulation is reported to increase force formation, enhance cell alignment and improve junction production.[96-98] Mechanical properties combined with electrical stimulation are used to investigate their combined effects on cardiac organoids.[99, 100] Bioactive materials in synthesised hydrogels induce cell adhesion and differentiation, leading to efficient vascularisation in cardiac organoids.[101] Hydrogel-free immobilisation is facilitated for fast and blur-free imaging on heart organoid-chips and serves as a potential microfluidic platform for high-throughput and high-resolution imaging of cardiac organoids (**Figure 4**).[102]

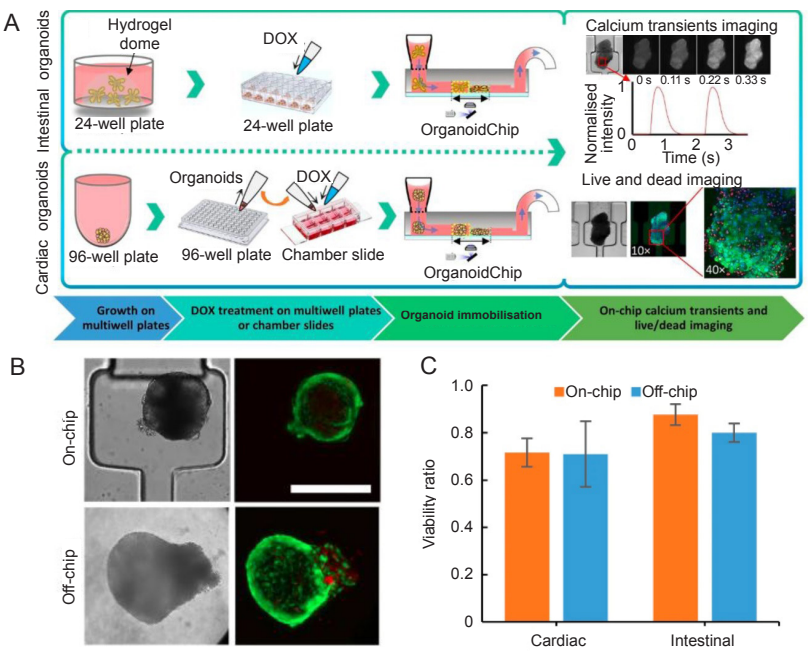

Figure 4. Hydrogel-based heart chips for cardiac organoids. (A) Flowchart for describing the main steps of the organoid-chip hydrogels. (B) Calcein AM and EthD-1 staining of cardiac organoids (20× original magnification). Scale bar: 400 μm. (C) Viability ratio of the organoids. Reprinted from Moshksayan et al.[102] AM: Calcein AM; DOX: doxorubicin; EthD-1: ethidium homodimer-1.

Geometry for heart organoids

Cardiac spheroids

Cardiac spheroids are multicellular, heart-functioning aggregates of cardiomyocytes within a cardiac microenvironment.[103] Three methods have been reported to form cardiac spheroids, including self-assembly of floating cells, hanging drop and microfluidic techniques.[104, 105] These methods are used for cardiac organoid development due to their small diameters and high permeability, which allow nutrient solutions and growth factors to pass through easily, facilitating the co-culture of cardiomyocytes and other cells.[106] The 3D co-culture involves incorporating cardiac fibroblasts and ECs into iPSC-cardiomyocytes to construct complex organoids within a cardiac microenvironment. Spheroids without scaffolds are established by mixing cardiomyocytes and fibroblasts at a 4:1 ratio, mimicking a native healthy heart.[107] The ultrastructural and electrophysiological features of co-culture spheroids are like those of fetal cardiomyocytes. Due to their heart-like properties, cardiac spheroids can serve as effective disease models for cardiovascular therapy. For example, cardiac organoids are designed by mixing ECs and primary human cardiac fibroblasts with hiPSC-cardiomyocytes in a 5:2:3 ratio.[108] These iPSC-cardiomyocytes are derived from various donors, including healthy individuals and hypertrophic cardiomyopathy patients.[109] The results show that hypertrophic cardiomyopathy spheroids exhibit arrhythmic pattering structures, while healthy spheroids present regular beating patterns. This cardiac organoid model demonstrates physiological relevance, depending on culture time duration for sustained viability. Cardiac spheroids are also used as building blocks to construct larger heart tissues.[110-113] A 3D bioprinting method for high cell-density heterogeneous tissue models is developed using spheroid fusion in self-healing hydrogels. Furthermore, aspiration-assisted freeform bioprinting is employed to create pre-fabricated tissue spheroids within a yield-stress gel (**Figure 5**).[114]

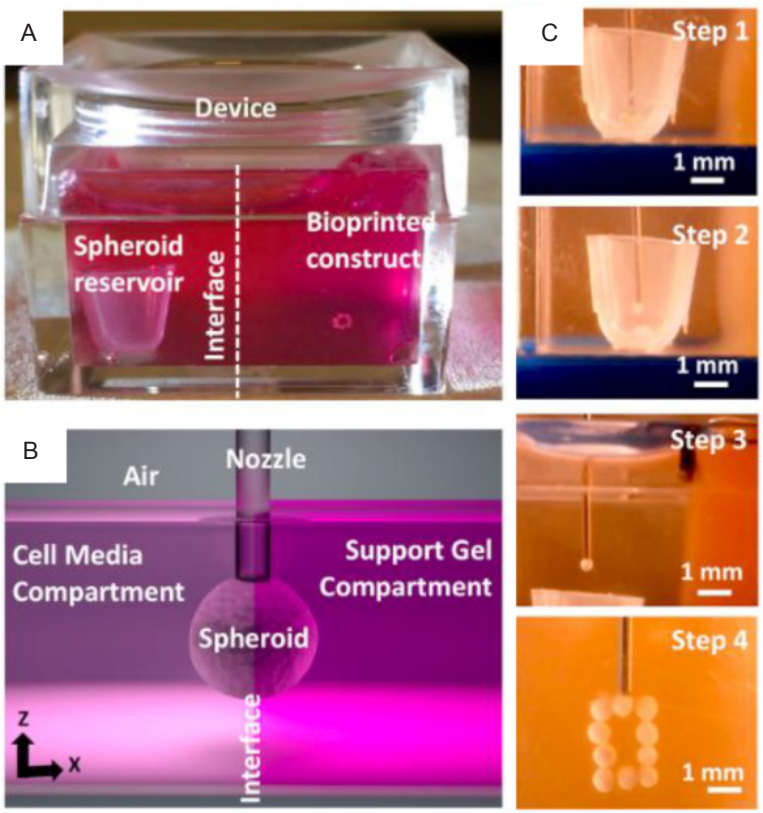

Figure 5. Geometries of cardiac organoids. (A) The bioprinting setup. (B) A schematic showing the process of spheroid traversal across the yield-stress gel and media compartment. (C) A step-by-step illustration of the process. Scale bars: 1 mm. Reprinted from Ayan et al.[114]

Cardiac chamber

Cardiac chambers are designed to function as pumping units using decellularised heart tissues and scaffold models.[115] These complex structures are suitable for setting clinical trial parameters, including ejection fraction, pressure overload, cardiac output, and stroke volume.[116] This platform of cardiac chambers is useful for constructing an adjustable heart niche to mimic the cardiac microenvironment *in vitro*. Tissue engineered hearts and stem cells have been used to design simplified models of human cardiac muscles or aligned monolayers, helping to bridge the gap between experimental animals and clinical trials.[117, 118] However, direct measures of cardiac performance, as a pump, have not been developed for human heart system *in vitro*. A next-generation *in vitro*

biomimetic model of pumping human heart chamber has been created to demonstrate its capability for pharmaceutical testing.[119] From hPSC-cardiomyocyte embedded in collagen-based ECM hydrogel, the 3D electro-mechanically coupled, fluid-ejecting miniature human ventricle-like cardiac organoid chamber can be successfully formed. Transcriptomic and RNA sequencing analysis reveals upregulation of key Ca^{2+}-handling, ion channel, and cardiac-specific proteins in human ventricle-like cardiac organoid chamber compared to lower-order 2D and 3D cultures of the same constituent cells. For clinical studies, the physiologically complex contractile ability is measured, and human ventricle-like cardiac organoid chamber displays key molecular and physiological characteristics of the native ventricle, showing expected mechanical and electrophysiological responses, including positive and negative inotropes.[120] This "human-heart-in-a-jar" technology could enhance drug discovery by providing human-specific preclinical data during early stage of drug development.

Human cardiac scaffolds are partially decellularised and reseeded with pluripotent stem cell (PSC)-cardiomyocytes as cardiac chamber in a custom cardiac bioreactor to provide coronary perfusion and left ventricular wall mechanical stimuli.[121] This native human cardiac matrix is then repopulated to form 3D complex cardiac tissues. Functional myocardial tissue at a human scale is established on this platform after 120 days of culture. Additionally, the seeded cardiac-like structures develop force-related human myocardial tissue and exhibit electrical conductivity, left ventricular pressure formation, and metabolic functions similar to native hearts. In another study, a tissue-engineered nanoscale fibrous scaffold model of the heart ventricle is created to induce native-like anisotropic myocardial tissue genesis and chamber-level contractile function for cardiac chamber organoids.[122] The diastolic chamber volume of tissue-engineered ventricles is approximately 500 μL, which is 250 times smaller than a human ventricle, serving as a proof-of-concept disease model for structural arrhythmia.

Other cardiac geometries

In addition to cardiac spheroids and chambers, several other cardiac geometries are utilised to generate heart organoids, including cardiac patches, cardiac strips and cardiac rings. Cardiac patches consist of thin hydrogel and scaffold sheets with cardiomyocytes and other cardiac cells to form shape-controlled layered patches.[123] Clinical sizes for cardiac patches, such as 4 cm × 4 cm and 3.5 cm × 3.4 cm, are designed for cardiac organoids.[124] Cardiac strips are formed in rectangular molds with a wire or two flexible posts for 3D engineered cardiac tissue organoids.[125, 126] These moulds encapsulate the cells in a hydrogel or scaffold for anisotropic structure formation.[127, 128] Cardiac rings are constructed in circular molds for 3D engineered cardiac tissue culture.[129-131] The circular shapes promote homogeneous tension distribution in engineered cardiac tissues, which is beneficial for heart organoids.[132-134]

Relationship Between Cardiac Organoids and Heart System

Cardiac development

Cardiac organoids have been widely used in cardiac developmental research to investigate the communication and interaction between organoids and their complex cardiac microenvironment.[135] These organoids serve as efficient bioreactors to replicate cardiac-like developmental; process, including organ formation and cellular heterogeneity *in vitro*, without forming complete hearts in all aspects. The emergence of heart tissue organoids, with cellular and structural complexity, has shown promise in enhancing cardiomyocyte maturation in human iPSC-derived spheroids. The presence of endoderm tissue in cardiac organoids supports the development of cardiac-like tissues, including cardiomyocyte expansion, compartmentalisation, enrichment of atrial/nodal cells, myocardial compaction, and fetal-like functional maturation. Combined tissues from various germ lineages are generated and matured in the same organoid model to examine multi-tissue relationships during cardiac development and physiological ontogenesis.[136] Cardiac organoids are useful models for exploring heart development, physiology and heart-related diseases. Fundamental processes of early organogenesis are studied using axially patterned embryonic organoids to investigate embryonic development and the evolution of cardiovascular progenitors.[137] These progenitors self-assemble into an anterior domain resembling a cardiac crescent before forming beating cardiac tissue near a primitive gut-like tube with an endocardial-like layer. Cardiogenesis is examined in mimicking embryonic organoids.

Organoid models have been designed to examine early tissue development. Structurally sophisticated 3D heart-forming organoids are created by incorporating hPSC aggregation into cardiac patch platform and inducing cardiac maturation through robust electromechanical coupling, consistent H-zones and I-bands for T-tubules and M-bands (**Figure 6**).[138] A myocardial layer is formed by endocardial-like cells and septum-transversum-like anlagen in heart-forming organoids. The heart-forming organoids are used to investigate genetic defects *in vitro* with NKX2.5-knockout organoids, presenting cardiac malformation phenotype in transgenic mice. Cardiac organoids facilitate in-depth studies of molecular mechanisms to reveal human heart development.[139] Stem cell biological methods, omics, gene editing, microfluidics and biosensors are also applied to induce cardiac development and serve as *in vitro* devices and platforms for studying cardiac development in heart organoids.

Cardiac diseases

Various cardiac disease models have been constructed to explore the biological mechanism and interaction between cardiac organoids and early human cardiac diseases.[140] Cardiac 3D organoids can avoid the cross-contamination risks associated with traditional 2D cultures and better mimic the complex cardiac microenvironment under physiological and pathological conditions. Therefore, the cardiac organoids can be used to produce large quantities of cardiac-like tissues *in vitro*, providing valuable biomaterials, sequencing, and advancing the understanding of biological etiologies and therapeutic technologies.[141, 142]

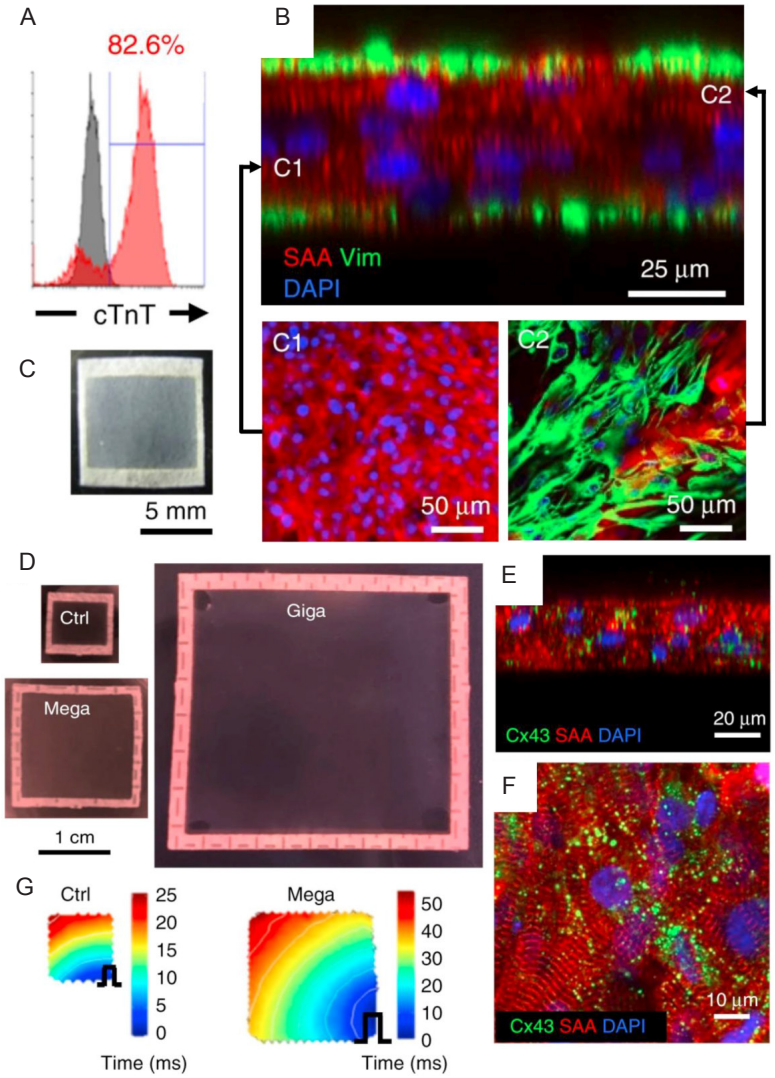

Figure 6. Regulation of cardiac organoids in cardiac maturation and development. (A) Flow cytometry histogram from hiPSC-cardiomyocytes after differentiation. (B) Photo of a 7 mm × 7 mm hiPSC-cardiomyocyte-derived tissue patch (human "cardiopatch"). (C) Cross-sectional confocal image of 3-week-old cardiopatch demonstrating several layers of densely packed SAA^+ hiPSC-cardiomyocytes (C1) surrounded by a layer of Vim^+ fibroblasts (C2). Scale bars: 25 µm (B), 5 mm (C), 50 µm (C1, C2). (D) Representative images of ctrl (7 mm × 7 mm), Mega (15 mm × 15 mm) and Giga (36 mm × 36 mm) cardiopatches. Scale bar: 1 cm. (E, F) Representative confocal images of Giga cardiopatches stained for Cx43 and SAA, as seen in confocal cross-sections (E) or in the XY plane in the middle of the patch (F). Scale bars: 20 µm (E), 10 µm (F). (G) Representative activation maps of ctrl, Mega, and Giga cardiopatches following point stimulation from bottom right corner (pulse sign). Reprinted from Shadrin et al.[138] ctrl: control; Cx43: connexin-43; DAPI: 4′,6-diamidino-2-phenylindole; hiPSC: human induced pluripotent stem cell; SAA: sarcomeric α-actinin; Vim: vimentin.

MI occurs due to coronary artery damage by ischaemic blockage, limiting blood delivery to the downstream myocardium, resulting in cardiomyocyte death and dysfunctional hearts.[143] Human cardiac model developed using cardiac organoids are utilised to study MI and injury in CVDs. These organoids exhibit traits similar to fetal hearts and can reverse heart functions, promote efficient proliferation and reduce cardiac fibrosis or hypertrophy, indicating increased regenerative performance and a mature cardiac tissue phenotype. hiPSC-derived multicellular cardiac organoids are modelled to address acute MI and heart fibrosis.[144] Self-organising heart organoids, including cardiomyocytes, fibroblasts and ECs, mimic the cellular composition of human cardiac tissue. Techniques such as immunohistochemistry, quantitative polymerase chain reaction (q-PCR), flow cytometry and single-cell RNA sequencing are used to analyse the multicellular composition of cardiac organoids. These organoids can alleviate acute MI by reducing cardiomyocyte death, functional deficits and Ca^{2+} alterations. To further explore the function of cardiac organoids, hypoxia-induced ischaemia and ischaemia reperfusion (IR) are used to culture cardiac organoids, stimulating a damaged heart in an *in vitro* model.[145] IR-induced cardiac organoids exhibit

cardiac fibrosis, accelerate collagen accumulation, disrupt Ca^{2+} alterations, and cause electrophysiological anomalies to emulate heart disease. The disease models, resembling *in vivo* 3D hearts, show promising potential to replace animal experiments in cardiac diseases and provide a platform for drug screening of therapeutic target identification.[146]

Cardiac organoids are also applied to study cardiac hypertrophy and heart failure, helping to regulate underlying mechanisms of *in vitro* heart repair. Engineered human myocardial organoids are developed to study the structural morphology and mature function of cardiomyocytes.[147] These engineered organoids, prepared with bovine collagen without serum and insulin, show advanced maturation of cardiac cells, similar to fetal hearts at 13 weeks of gestation. Matrigel can mix with progenitor cells and cardiomyocytes, and is designed to form cardiac organoids for cardiac chamber formation. Single-cell transcriptomic analysis reveals different cardiac lineages, such as cardiomyocytes, ECs and other cardiac cells. Additionally, cardiac organoids with 100 ng/mL endothelin-1 (a cardiac hypertrophy inducer) for 28 days result in increased chamber wall, reduced ejection fraction and tachycardia, consistent with clinical observations in patients with cardiac hypertrophy.[148]

Drug screening and evaluation

Due to their availability, large-scale feasibility and genetic manipulability, cardiac organoids have been extensively applied in high-throughput drug screening for drug discovery and analysis (**Figure 7**).[149] The heart is sensitive to the side effects of drugs, and toxicity should be systematically examined to broaden their development and application. Current strategies for drug screening have been developed for therapeutic compounds and approved for extensive use in practice. However, results from preclinical models *in vivo* or *in vitro* often provide erroneous data and cannot precisely epitomise human physiology.[150] To overcome these difficulties, a human primary cell- and stem cell-derived 3D organoids are used as drug screening panels, which include U.S. Food and Drug Administration-approved drugs from market production.[151] Multiple tissue organoid types are prepared on this platform to remain viable *in vitro* for 28-day culture. Organoids are exposed to non-toxic compounds and remain viable at clinically relevant doses. Additionally, integrated multi-organoid systems including liver, cardiac, lung, vascular, testis, colon and brain organoids, are used in other assays. These integrated organoids also show viability and express functional long-term cardiac markers. Examples of multi-organoid 'body-on-a-chip' systems serve as model frontier of dependent metabolism and the downstream effects of drugs in an organoid platform. 3D *in vitro* organoids present physiologically relevant model for drug screening, reducing the cost and failure rates for new drug discovery.

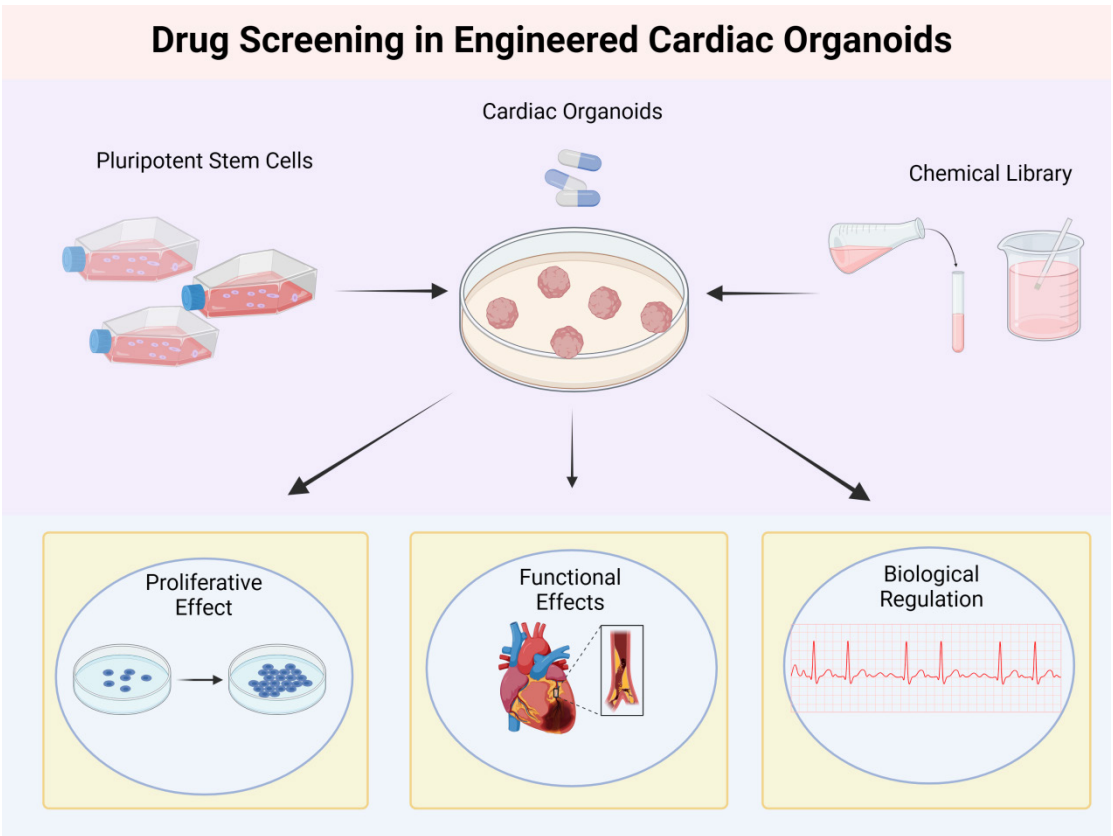

Figure 7. Cardiac organoids are used to regulate high-throughput drug screening for drug discovery and analysis. Created with BioRender.com.

Functional cardiac organoids are further developed to explore the molecular mechanisms of drug screening. Drug screening in human PSC-cardiac organoids can identify pro-proliferative compounds.[152] A high-throughput bioengineered human cardiac organoid platform has been fabricated to provide contractile cardiac tissue with biological properties similar to native heart tissues. Additionally, functional drug screening of 105 small molecules with pro-regenerative potential was performed. High-throughput proteomics in cardiac organoids can reveal synergistic stimuli of the mevalonate pathway and a cell-cycle network by pro-proliferative compounds. The assessment of doxorubicin toxicity is evaluated using human cardiac organoids for drug cardiotoxicity.[153] Both traditional 2D monolayer cell models and 3D animal models have shown limitations and cannot fully mimic human heart physiology or pathology. A human cardiac organoid model promoting directed differentiation of human embryonic SCs is successfully created through 3D self-organised structures, capturing the biological characteristics and functions of heart tissue. This cardiac organoid model can recapitulate early myocardial development stages and accurately characterise the cardiotoxic damage caused by anticancer drug doxorubicin, including clinical cardiac injury and cardiac function indicators, cell apoptosis, inflammation and fibrosis. Cardiac organoid models are extensively used to evaluate drug cardiotoxicity, opening new avenues for drug screening and discovery.[154]

Heart regeneration and repair

Cardiac organoids are emerged in tissue engineering as a potential therapeutic tactic for damaged hearts, aiming to achieve cardiac regeneration for treating cardiac diseases.[155] Currently, available donors and allografts are the main methods for regenerative medicine, but the shortage of human hearts and rejection response limit the application of regenerative medicine in cardiac repair.[156] Cardiomyocytes can improve cardiac function and increase the expression of maturation markers in human cardiac organoids.[157] Human cardiac patches have been developed and implanted into pig hearts for MI repair. The cardiac organoids can increase cell survival, promote cardiac function, and reduce infarct areas post-transplantation.[158] Based on these results, cardiac organoids may pave the way for viable cardiac regeneration and repair and provide crucial parameters for cardiac pathological studies, drug screening and regenerative medicine.[159]

The Hippo signalling pathway plays a crucial role in tissue engineering and regenerative medicine for cardiac repair.[160] Certain cell signals stimuli induce the phosphorylation of transcriptional cofactors YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif), causing YAP nuclear translocation for mitosis. The Rockefeller University lats inhibitor (TRULI), a small molecule to block large tumor suppressor homolog 1 (Lasts1) and large tumor suppressor homolog 2 (Lasts2) kinases in Hippo pathway, promotes the proliferation of some cell types for cardiac regeneration.[161] Additionally, a chemical compound TDI-011536 has been identified as an efficient Lats kinase blocker, enhancing cardiomyocyte proliferation in hPSC organoids.[161] TDI-011536 inhibits YAP phosphorylation and stimulates the transcriptional activation YAP-targeting genes in the heart. TRULI also benefits cardiomyocytes proliferation and promotes cardiac regeneration in organoids. Cardiac fibroblast heterogeneity from hPSC-derived epicardial cells is modeled to create cardiac organoids for cardiac regeneration.[162] Single cell transcriptomics reveal that the hPSC-derived organoid fibroblast population exhibits a high degree of heterogeneity, approximating the population heterogeneity in both normal and diseased cardiac tissues. Cardiac organoids play an important role in cardiac development, maturation and regeneration.

Clinical Potentials of Heart Organoids

The assessment of cardiac organoids in clinical applications is crucial for investigating their decisive functions and biological conditions. Currently, many studies have reported the construction methods of cardiac organoids and their applications in drug screening experiments, disease models, regenerative medicine and treatment.[163] Cardiac organoids retain the phenotype of organs *in vivo* while being operable *in vitro*, allowing for accurate study of human diseases pathogenesis and avoiding the limitations of traditional cell culture methods and animal models.[164] Organoids can simulate organ development and related diseases and they hold significant potential in drug screening and disease treatment. Although cardiac organoids have been developed for scientific research, progress in the clinical field has lagged, partly due to the complexity of early heart development. A series of documents detailing the construction and application of cardiac organoids have been published, highlighting their rapid development.[165] Previous studies have evaluated 3D cardiac organoids in various practices to reveal their clinical value and factors related to organoids, including contractile ability, morphogenesis, electrophysiology, metabolomics and gene expression levels.

For morphogenesis and contractility, engineered cardiac organoids can be controlled using non-contact and real-time analysis methods to mimic the contractility and morphological features of heart tissues.[166] Cardiac contractility is identified by changes of cardiac morphogenesis, particularly through the morphological observation of microscopy videos using a motion vector algorithm program to regulate cardiac contractility. Additionally, cardiac organoids are evaluated using echocardiograms from ultrasound waves to enhance the temporal and spatial resolution.[167] Ca^{2+} transient content in cardiac organoids is assessed using a customised two-photon scanned light sheet microscope to study the contractility of live heart organoids.[168] Clinically, the evaluation of morphogenesis and contractile ability requires high spatial resolution and deep tissue transparency for the accurate observation of cardiac organoids.[169]

The electrophysiology of cardiomyocytes and cardiac tissues is another crucial factor in analysing the clinical applications of cardiac organoids.[170] This aspect is related to heart diseases and therapy in cardiac organoids. Manual patch-clamp and multielectrode array techniques are used to monitor cardiac action potentials by increasing the spatial resolution in 2D cultures.[171, 172] The electrophysiology of cardiac organoids is

also studied using Ca^{2+} imaging and voltage-sensitive dyes in 3D culture to distinguish high-resolution 3D morphogenesis of cardiac organoids.[173, 174] Applying these methods to heart organoids will enable the monitoring of electrophysiological characteristics during heart development, which is beneficial for clinical practice.

Cardiac metabolism regulates adenosine triphosphate production to support heart functions through energy-related substances such as glucose, fatty acids, and proteins. Mitochondrial oxidative phosphorylation is the primary pathway for energy production in cardiac metabolism.[175] Metabolites are examined to evaluate the metabolic level of cardiac organoids via the tricarboxylic acid cycle and the mitochondrial electron transport chain.[152, 176] adenosine triphosphate turnover, from production to consumption, is related to the oxygen consumption rate and extracellular acidification rate in the metabolism of cardiac organoids.[177] As cardiomyocytes mature, oxygen consumption rate and extracellular acidification rate are enhanced in cardiac organoids. Because the external environment of cardiac organoids constantly changes, the metabolic reaction must be accurately regulated to maintain the stability of each component in cardiomyocytes, ensuring the homeostasis of the body.[178] Metabolic regulation also enables organoids to respond to external signals and interact with their microenvironment.

The phenotypes of cardiac organoids are assessed by gene expression of cardiomyocytes to display the physiological and pathological status in these organoids.[179] Cardiac organoids often involve multiple cell types, and gene expression is measured using single-cell sequencing analysis.[180] This analysis reveals maturation, differentiation, apoptosis, and metabolism, exploring biomarkers of various cell types through single-cell RNA sequencing.[181] Ligand-receptor pairs identified in single-cell transcriptional data elucidate intercellular communication and interaction, regulating morphological features in cardiac organoids.[182] Additionally, single-cell multi-omics provide transcriptomic, epigenetic and proteomic information for cardiac organoids.[183] Spatial transcriptomics can also be used to avoid uncertain locations in clinical applications.[184, 185] In addition, this review merely focuses on the model construction and clinical therapeutic potential of engineered cardiac organoids. There are some limitations for cardiac organoids in this review, including biomaterial choice tactics, interaction of cardiomyocytes and organoids, and cardiac development mechanism *in vitro*.

Conclusions and Future Perspectives

Engineered cardiac tissues are modelled as cardiac organoids for heart-related studies. Cardiac organoids are considered as ideal heart models to mimic natural cardiac tissues. Various methods of developing cardiac organoids are employed to explore cardiac functions, including co-culture, aggregation, scaffolds and geometry. Multiple cell lines, such as cardiomyocytes, fibroblasts and ECs, are co-cultured in cardiac organoids to stimulate the complex microenvironment *in vitro*. Monolayer, multilayer and aggravated cardiomyocytes self-assemble to form biofunctional cell aggregates in cardiac organoids. Scaffolds, including dECMs, 3D hydrogels and bioprinting, provide the supporting framework for cardiac organoids. Various geometries, such as spheroids, patches, chambers and rings are constructed to develop cardiac organoids. The relationship between cardiac organoids and heart system is evaluated to reveal the underlying mechanisms and interactions involved in cardiac development, cardiac diseases, drug screening, heart regeneration and repair. Additionally, the assessment of cardiac organoids in clinical applications as *in vitro* heart models is crucial for investigating the decisive functions and biological status, including contractility, morphogenesis, electrophysiology, metabolomics, and gene expression. Heart organoids are assessed to have prospectively clinical potentials in drug screening, disease model construction, regenerative medicine and therapy.

Cardiac organoids have the potential to develop into more complex and native-like engineered cardiac tissues that mimic human hearts in the future. The combination of PSC-cardiomyocyte methods with microfabrication techniques is essential for creating the next generation of engineered cardiac organoids with various structures and geometries to restore cardiac functions *in vitro*. Additionally, different technologies are being explored for engineered cardiac organoids, with a focus on their utility in drug screening evaluation and cardiac regeneration. These technologies aim to reveal the molecular mechanisms of maturation, differentiation and apoptosis of cardiac cells in both natural and artificial heart organoids or matrices. Specifically, the manipulation of biomechanical and biochemical inputs is considered as a crucial factor for force-related signalling transduction and the downstream morphogenesis of cells or tissues. In cardiac organoids, the production of camber-like structures is inspected by morphogen alternation, lineage regulation, fate evaluation and spatial transcription in 3D engineered heart tissues. Therefore, heart organoids are flourishing and evolving in cardiac development and disease evaluation, promoting the repaid formation of more native cardiac organoids *in vitro*.

Author contributions

YW and JX conceptualised and designed the review, drafted the manuscript, and revised the manuscript. YW, YH, and TH created all the figures and tables. YW, MG, GL, DC and JX critically revised and edited the review. All authors reviewed and approved the final version of the manuscript.

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Conflicts of interest statement

The authors declare no conflict of interest.

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