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Cardiac organoid: multiple construction approaches and potential applications

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The human cardiac organoid (hCO) is three-dimensional tissue model that is similar to an *in vivo* organ and has great potential on heart development biology, disease modeling, drug screening and regenerative medicine. However, the construction of hCO presents a unique challenge compared with other organoids such as the lung, small intestine, pancreas, liver. Since heart disease is the dominant cause of death and the treatment of such disease is one of the most unmet medical needs worldwide, developing technologies for the construction and application of hCO is a critical task for the scientific community. In this review, we discuss the current classification and construction methods of hCO. In addition, we describe its applications in drug screening, disease modeling, and regenerative medicine. Finally, we propose the limitations of the cardiac organoid and future research directions. A detailed understanding of hCO will provide ways to improve its construction and expand its applications.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in humans. According to the World Health Organization (WHO), CVD accounted for 17.9 million deaths worldwide in 2019.[1] Although the heart and CVD have been widely studied, developing therapies that effectively tackle and reverse heart disease remain far from satisfaction. The reasons are manifold and include the lack of valid models that can adequately outline the mechanisms underlying human cardiogenesis and diseases and can be used for disease modeling and therapeutic testing. Despite the common application of non-representative conventional two-dimensional (2D) cultures or nontranslational animal models to explore CVD etiology, there is a pressing need for human-derived models to fully translate these findings into clinical settings.[2] The 2D cell model lacks cell–cell and cell–extracellular matrix (ECM) interactions and experiences foreign surface topography and soluble bioregulatory factors; thus, it is difficult to reflect the complexity of *in vivo* tissue. In addition, cells are genetically and phenotypically highly altered, and heterogeneity is disrupted. Moreover, they cannot reflect three-dimensional (3D) morphogenesis and pathological processes.[3] Although animal models can simulate the *in vivo* physiological environment, they have limited applicability in addressing the onset and development of the human heart and CVD because of significant differences between animal and human hearts in terms of gene expression patterns, metabolic activity, and inflammatory cells. Therefore, the *in vitro* construction of 3D cardiac models that mimic the key features of the human cardiac cell composition, structure, and functions is vital for advancing basic and pre-clinical CVD research.[4]

Organoids, which are *in vitro* 3D tissues derived from organ-restricted embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (ASCs), have attracted intensive attention in the field of biological and medical research. Compared to 2D cultures, organoids contain self-renewal stem cells that self-organize into complex tissues and organ-like structures, and are thus able to simulate human tissue-like cell–cell and cell–matrix interactions, as well as organ physiology and pathology *in vivo*.[5] Organoids have been recently applied to model various organs and disease conditions. However, the construction and application of human cardiac organoids (hCOs) have been lagging significantly in comparison to those of other organoid types, such as the lung,[6] small intestine,[7] pancreas,[8] liver,[9] prostate,[10] brain,[11] and tumor tissue.[12] The construction of hCOs presents a unique challenge because of the diverse cellular population and complicated microenvironments.[13] To date, several successful constructions of hCOs have been reported in the literature in recent years, owing to the rapid development of organoid technology.

This article describes the status of research on hCOs, including their classification and construction methods for

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hCOs, and outlines their application areas, including disease modeling, drug development, and regenerative medicine. Finally, the limitations and future directions of hCOs are discussed.

2. Types of human cardiac organoids

The human heart is one of the most structurally and functionally complex organs in the body, and consists of several cell types with different developmental origins. Cardiomyocytes (CMs) make up 70–85% of the natural heart volume, but account for only 25–35% of the total number of heart cells.[12] Non-CMs, including vascular endothelial cells, vascular smooth muscle cells, fibroblasts (FBs), neurons, and immune cells,[14] account for 65–75% of the total cells. Cardiac cells interact with the vascular system and are surrounded by a 3D ECM network. The ECM is a complex microenvironment containing laminin proteins, fibronectin, interstitial collagen, cytokines, growth factors, proteases, and glycoproteins,[15] which provide anisotropic alignment, biochemical signaling, and mechanical support for cells. Ideally, hCOs should reproduce the morphology, structure, metabolism, and function of the natural cardiac environment, as well as complex microenvironments, *in vivo*, such as CMs, non-CMs, vasculature, and ECM networks, to study cell–cell and cell–matrix interactions under normal and pathological conditions.

There is no standardized definition for hCOs.[16] In general, hCOs are three-dimensional constructs that reproduce part of the shape or function of heart tissue, and contain major heart cell types, such as CMs, cardiac fibroblasts (CFs), and endothelial cells.[17] The terms ''organoid'' and ''spheroid'' are frequently used interchangeably, and in the context of hCOs, organoids are widely used to refer to 3D cardiac microstructure.[17] In this article, we refer to tissues cultured using tissue engineering techniques and self-organization methods as organoids because both methods attempt to reconstruct 3D *in vitro* culture models with heart-like structures and functions. Human embryonic stem cells (hESCs), adult stem cells, and human-induced pluripotent stem cells (hiPSCs) can be used to create hCO models. In this review, hCOs are classified into two categories: engineered heart tissue (EHT) and self-assembling cardiac organoids.

2.1. EHTs

The goal of *in vitro* EHTs is to create systems that mimic the physiological structure and function of the natural heart *in vivo*. The production of EHTs does not rely primarily on the developmental mechanisms of the natural heart, but instead combines engineering methods and biomaterials.[18] Thus, multiple cardiac cell types derived from stem cells can be co-cultured and inoculated onto scaffolds, biomaterials, ECM, or bioengineered devices to design the desired 3D structure for specific cardiac physiological tests (*e.g.*, contraction and electrophysiology). The earliest EHTs were created using one or two types of heart cells from rats and mice *via* co-culturing and intercellular adhesion to form a loose spherical structure known as a cardiac spheroid.[19,20] Cardiac spheres or spherical microtissues are generally formed by mixing pre-differentiated CMs, endothelial cells, and CFs in proportions similar to the cellular composition of the heart.[17] Non-spherical structures can also be formed using a 3D micropattern matri.[21] Although early EHTs can be used for disease modeling, they do not recapitulate the complex morphology and developmental processes of the heart, and lack many cardiac cell types. Recently, EHTs have undergone significant innovations. EHTs have improved their complexity and modeling capabilities by generating a structure more similar to *in vivo* ones and a maturation state more similar to that of *in vivo* CMs.[22–24]

EHTs are ideal models for analyzing cardiac contractility and electrophysiology because they can mimic certain aspects of adult cardiac tissue. More importantly, EHTs enable disease phenotype simulations, drug testing, cardiac pathogenesis studies, and therapeutic applications. For instance, EHTs have been used to study heart diseases induced by genetic mutations, such as hypertrophic cardiomyopathy (HCM) caused by rapid accelerated fibrosarcoma B-type (BRAF) mutations[25] and recombinant actinin alpha 2(ACTN2) mutations.[26] Miniaturized EHTs can be used for *in vitro* drug development,[27] and EHTs can perform real-time analysis of cardiac contraction parameters.[28] However, they cannot reproduce early developmental patterns of the natural hearts, thus reducing their extent of applicability. Furthermore, the construction of an EHT is complex because of the requirement for specialized equipment, making it difficult to scale up to a standard 96-well plate, and thus limiting its high-throughput applicability.

2.2. Self-assembling cardiac organoids

Self-organizing cardiac organoids are generally created by inducing cell aggregates to differentiate and organize into heart-like structures with minimal external intervention. Self-assembling cardiac organoids can be divided into self-assembling embryonic organoids and self-assembling cardiac lineage-specific cardiac organoids.

Self-assembling embryonic organoids mirror the embryonic heart structure and spatiotemporal patterns of early cardiogenesis, allowing the research on cardiac development in the context of co-development with the foregut endoderm and ectoderm.[18] Silva *et al.*[29] generated multi-lineage organoids from early definitive mesodermal progenitors after 5 days of differentiation, which maintained cellular complexity. The multi-lineage organoids revealed key structural features of the developing human heart and gut at 5 weeks of gestation, including an internal CM core, external epicardial-like cells, and epithelial-like structures. However, estimating the degree of cardiac morphogenesis in multilineage organoids is challenging because the accelerated development of primitive cystic structures in intestinal organoids disrupts the cardiac morphogenesis initially presented in the culture system. Rossi *et al.*[30] induced mouse ESCs to form embryonic organoids and directed them through the basic steps of early cardiac organogenesis. Embryonic organoids mimic embryonic development and form progenitor cells. These progenitor cells self-organize into an anterior region resembling a cardiac crescent, and then

co-develop with a primitive intestinal lumen separated by an endocardial layer. Although this platform can be used to investigate heart development, it is currently unable to reproduce the formation of heart tubes and ventricles *in vivo*, which resemble foregut contractions. To create sophisticated and highly structured 3D heart-forming organoids (HFOs), Drakhlis *et al.*[31] embedded aggregates of hiPSCs in Matrigel and modulated the biphasic WNT pathway with small molecules. HFOs are primarily composed of the foregut endoderm, fibroblasts, CMs, and endothelial cells (ECs). In particular, HFOs are mainly composed of a myocardial layer arranged by endocardial-like cells, and also contain vascular networks and foregut endodermal tissues, as well as the septum-transversum-like anlagen surrounding the HFOs. The structure of HFOs closely resembles aspects of the natural heart before the formation of the heart tube, mimicking the early development of the human heart, but still has no major cardiac structures, such as ventricles. In general, non-cardiac cells found in embryonic organoids can be used to explore the mechanisms of the foregut and heart co-development. However, the absence of ventricles and tubes and the presence of non-cardiac cells make it challenging to analyze cardiac-specific physiological functions.

The lineage-specific cardiac organoid represents a type of organoid that contains only heart tissue cells, but no non-cardiac tissue cells. Because of the absence of spatial constraints and interactions with other embryonic tissues, lineage-specific cardiac organoids can autonomously form and transform the structure *in vivo* like those of natural hearts. Lineage-specific cardiac organoids use pluripotent stem cells (PSCs) or ASCs induced by signals to form tissue-like structures and organ-specific cell types that can be used to study patterning and morphogenesis in specific organs.[32] In addition to the human heart, self-organizing lineage-specific organoids have been reported for all major organs. In 2021, Hofbauer *et al.*[33] created cardioids, a self-assembled lineage-specific hCO model made by adding signaling pathway factors indicative of the separation of CMs and endothelium without non-cardiac tissues (such as endoderm and ectoderm derivatives) or exogenous ECM. The cardioids had chamber-like structures with cavities, and separated myocardial and endothelial layers that interacted with the migrating and differentiating epicardium, similar to the early cardiac chamber development. The cardioid model identifies the signaling mechanisms that control and coordinate the self-organization and lumen development of the cardiac mesoderm, as well as the pathways *via* which these processes are disrupted by mutant cardiac transcription factors. Therefore, human cardioids can be utilized to study the mechanisms underlying human cardiogenesis, development, and congenital heart disease, as they depict early myocardial, endothelial, and epicardial morphogenesis processes. Lee *et al.*[34] successfully obtained chambered hCOs by modulating the Wnt signaling pathway *via* sprinkling a mixture of hiPSCs and matrigel into an ultra-low adherence culture dish. This study verified the structural similarity of hCOs to the natural heart, such as both having similar areas of atrium/ventricle, chamber, and epicardium/myocardium. The method used in this study allowed for the easy construction of hCOs that reproduces the morphology and function of the heart and produces vascularization after transplantation into the body.

3. Methods for constructing human cardiac organoids

The methods of hCOs construction can be generally classified into three categories: tissue engineering method, self-organization method, and 3D printing method (Fig. 1).

3.1. Tissue engineering methods

Tissue engineering methods include the application of tissue engineering technology and biomaterials to create 3D models of tissues and organs. These methods consist of three main components: (1) seed cells such as implanted cells, iPSCs, ESCs or progenitor cells; (2) scaffolds to support the cells; and (3) active factors to regulate the cells.[18]

3D scaffolds that support cells are important features of tissue engineering. The principle of the 3D scaffold is to mimic the natural cardiac ECM using an exogenous matrix. The cardiac ECM is a critical component that not only provides structural integrity, but also plays a key role in a variety of genetic and metabolic diseases.[35] Moreover, the ECM creates a natural 3D environment favorable for cell and tissue growth, maturation and repair, with corresponding mechanical and biochemical properties that regulate cellular functions, such as cell migration and lineage qualification.[36] Cardiac ECM scaffolds mainly contain collagen I, III and IV, glycosaminoglycan, fibronectin, laminin and a small number of growth factors.[37] The scaffold biomaterials should possess the following properties to mimic the natural ECM: the materials should be biocompatible, elastic and mechanically strong; reproduce the environment of the natural cardiac ECM as closely as possible; facilitate the establishment of mechanical and electrical coupling; and not interfere with cell proliferation and differentiation.[38] The commonly used scaffolds for constructing hCOs are summarized in Table 1. Hydrogels, decellularized ECM (dECM), and synthetic polymers are the primary biomaterials used for scaffolds in tissue engineering methods.[39]

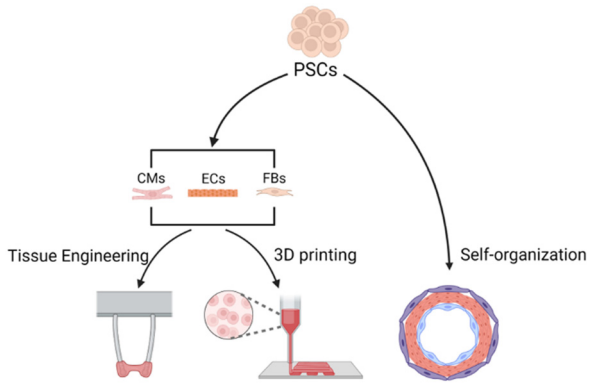

Fig. 1 Methods for the construction of human cardiac organoids.

Table 1 Cardiac organoids by tissue engineering methods: scaffolds, cells, species and applications

Scaffolds	Cardiac cells	Supporting cells	Species	Applications	Ref.
Collagen type I/Matrigel	hESC-CMs hiPSC-CMs	Fibroblasts	Human	Disease modeling Regenerative medicine	23
	hPSC-CMs	Fibroblasts	Human	Disease modeling	24
	hiPSC-CMs	Stromal cells	Human	Disease modeling	26
	hPSC-CMs	Stromal cells	Human	Drug discovery	28
	hPSC-CMs	Stromal cells	Human	Drug discovery	44
Fibrinogen	hiPSC-CMs	None	Human	Drug discovery	25
Gelatin-chitosan/polycaprolactone	Neonatal CMs	None	Rat	Regenerative medicine	46
Native cardiac extracellular matrix	hiPSC-CMs	None	Human	Regenerative medicine	48
DVS fibers ECM	iPSC-CMs	None		Regenerative medicine	54
Fibrin hydrogels	hPSC-CMs	Dermal fibroblasts	Human	Disease modeling	67
Low-collagen hydrogels	hiPSC-CMs	None	Human	Disease modeling Regenerative medicine	68
dECM-fibrin hydrogels	H9c2	Fibroblasts	Rat	Drug discovery	71
ECM	hiPSC-CMs	hiPS-ECs	Human	Drug discovery	82
Collagen type I/geltrex	hESC-CMs	hES-epicardium	Human	Regenerative medicine	111
Fibrinogen/Matrigel	hiPSC-CMs	Smooth muscle cells/fibroblasts	Human	Regenerative medicine	113

Approximately 75–90% of the cardiac ECM is fibrillar collagen, which is highly conserved across species and can be easily isolated from a variety of animal tissues.[40] As a result, most techniques for cardiac tissue engineering are based on hydrogel scaffolds containing collagen and Matrigel or ECM scaffolds from the *in vivo* environment. Hydrogels, which are hydrophilic polymers with a 3D network that can absorb large amounts of liquid, exhibit good biocompatibility and diffusion properties, allowing for uniform seeding of cells.[41] Cells encapsulated in hydrogels can be organized into specific geometries to obtain tissues with complex geometries and 3D cell–cell interactions.[42] The molecular weight and density of the hydrogel can also be adjusted by introducing cross-linked polymers to mimic the mechanical properties of the natural heart.[38] These advantages render hydrogels with outstanding features for suitability as scaffolds for cardiac tissue engineering. Collagen hydrogels are pioneering biomaterials for constructing engineered heart tissue.[43] Richard *et al.*[44] encapsulated 70% of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and 30% of stromal cells into collagen I and Matrigel on ice. Subsequently, they pipetted the mixture into the Heart-Dyno device, which is a 96-well screening platform where each well had a culture insert containing an elliptical seeding hole with two elastic columns that provide mechanical resistance to the contraction of the hCOs. To ensure that hCOs were formed at the center of the column, the apparatus was then centrifuged. With minimal cells and reagents, this device can automatically construct dense muscle bundles around built-in rods. It can also automatically analyze contraction forces without the need for tissue processing. Therefore, collagen-containing hydrogel scaffolds provide abundant natural heart-like ligands for embedded cells, and promote cell-mediated tissue structural remodeling and compaction.[45] Although natural hydrogels are highly bioactive and biodegradable, they have limited mechanical strength compared to natural tissues. Seokwon *et al.*[46] developed a polycaprolactone (PCL) stent containing a gelatin–chitosan hydrogel by combining various scaffold materials, which partially solved the disadvantage of weak mechanical strength. This scaffold allowed cardiac cells to seed and migrate in a bionic environment, while maintaining a sufficiently strong tensile strength.

The dECM, which is extracted from the decellularized tissues of human and animal heart samples, has become one of the most popular candidates as 3D scaffolds for cardiac biotissue engineering owing to its capability to inherit the intrinsic cues from a native heart ECM. Theoretically, dECM is most similar to the authentic tissue microenvironment. Thus, it is an ideal biomaterial to support the seeding and transplantation of hiPSC-CMs, and can be used to construct a variety of complex myocardial tissues.[47] Guyette *et al.*[48] partially recellularised the dECM of a human native heart using hiPSC-CMs. The seeded constructs formed force-producing human myocardial tissue and exhibited electrical conductivity, left ventricular pressure development and metabolic functions. Although both natural hydrogels and dECM have advantages, such as good biocompatibility and adequately mimicking the composition of natural ECM, their widespread applications were limited by their low mechanical strength and batch-to-batch variability.[49] Synthetic polymers are also commonly used to construct engineered heart tissues.[50] Given their excellent biodegradability, biocompatibility, and reproducible mechanical, chemical, and physical properties, they can be well matched for specific requirements and commercialized due to the precise control of their structural and physical properties, including electrical conductivity, hardness, porosity and shrinkage.[51] Synthetic polymers such as polyethylene glycol (PEG), PCL, polylactic acid-glycolic acid copolymer (PLGA), and polyurethane (PU) have been widely used as scaffolds for cardiac tissue engineering.[42,52,53] DePalma *et al.*[54] fabricated a fibrous extracellular matrix consisting of electrospun dextran vinyl sulfone (DVS) fibers. These highly aligned fibers of the scaffold have tunable mechanical properties and low stiffness, optimally facilitating the assembly and contraction of engineered cardiac tissue. However, synthetic polymers usually typically exhibit poor transduction and cell adhesion.[55] Molecular modifications, combination of proteins and growth factors, the addition of short peptides may resolve these issues.[56,57]

Currently, most human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) *in vitro* are developmentally and functionally immature, thus having obvious deference in structure, metabolism and gene expression. For instance, hPSC-CMs have spherical morphology and small size, which are similar to immature fetal CMs. It lacks the clear T-tube, and has chaotic and short sarcomes with the length of only 1.6 μm. Its metabolism is highly dependent on glycolysis.[58,59] In contrast, mature CMs are rod-shaped with clearly discernible elongated sarcomeres (around 2.2 μm). Fatty acids are their main energy substrates.[58,60,61] For the gene expression, hPSC-CMs highly expressed myosin heavy chain 6 (MYH6), cardiac titin N2A isoform and alpha-myosin heavy chain (α-MHC). However, mature CMs expressed high levels of myosin heavy chain 7 (MYH7), N2B isoform of cardiac titin and beta-myosin heavy chain (β-MHC).[58] Furthermore, mature CMs have lower resting membrane potential, faster depolarization rate, longer duration plateau period present after depolarization and stronger calcium handling than hPSC-CMs in terms of action potential.[62] Enhancing the maturation of hPSC-CMs to a level like that of adult CMs is a challenge in cardiac tissue engineering. Previous studies have demonstrated that exogenous stimuli, such as mechanical and electrical stimuli, can further induce maturation of cells and tissues. For example, Vivas *et al.*[63] developed a heart-on-chip device that can apply the electrical stimulation to promote the maturation of CMs in the EHT. The mechanical load is one of the most important factors regulating the development, maturation, and aging of the heart.[64] In recent years, most cardiac tissue engineering techniques have used an auxiliary mechanical strain to mimic the force of CMs contraction in the myocardium.[65,66] Ronaldson-Bouchard *et al.*[67] co-cultured 75% hiPSC-CMs and 25% dermal fibroblasts, and embedded them in fibrin hydrogels to generate heart-like tissues in a polycarbonate-based tissue bioreactor. The platform had 12 tissue culture wells, each containing a flexible elastic column. The human heart tissue showed enhanced the cardiac ultrastructure, oxidative metabolism, T-tube formation and calcium handling dynamics, as well as a mature "adult-like" CM phenotype after being subjected to progressively increasing electromechanical stimulation for 21 days, which demonstrates the potential of this system to mimic adult heart disease. Lu *et al.*[68] assembled hiPSC-CMs into low-collagen hydrogels at a high cell density to generate EHTs, and induced the maturation and growth of EHTs through three weeks of continuous electrical stimulation, medium agitation and gradual stretching (Fig. 2). The hiPSC-CMs after stretching stimulation exhibited structural improvements in cell volume, linearity, and myonodal length (2.19 ± 0.1 μm), and the change in expression of α-MHC to β-MHC was observed. Furthermore, the expression of specific genes of adult CMs was upregulated overall. Zhao *et al.*[24] used the Biowire II platform to create myocardial tissue by mixing hiPSC-CMs (ventricular, atrial, or both) and CFs in a 10 : 1 ratio with hydrogel inside the microwells of the Biowire II platform. The Biowire II platform consists of a series of micropores (5 mm × 1 mm × 0.3 mm) patterned on a polystyrene plate capable of growing thin cylindrical tissues suspended between two parallel lines that can quantify both force and Ca^{2+} transients.

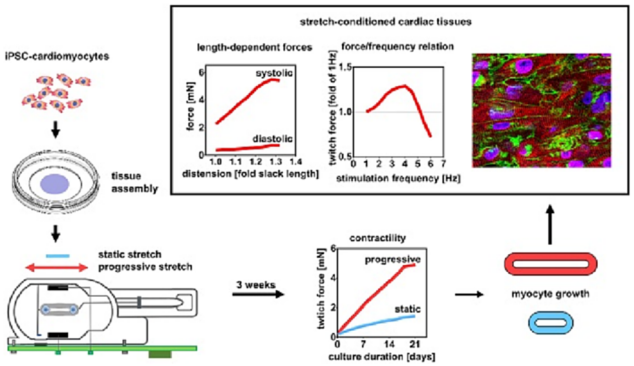

Fig. 2 Representative tissue engineering methods. hiPSC-CMs were assembled into low collagen hydrogels at high cell density to generate EHTs. Reprinted with permission from ref. 68. Copyright 2021, Theranostics.

Li *et al.*[69] found that the spontaneous re-entry waves generated by cardiac rings resulted in denser CMs myofilaments, longer myofilaments, maximized cardiac gene expression, and the upregulated expression of mature CMs-related genes, such as MYH7 and genes involved in sarcomere structures (ACTN2 and DES), ventricular structures (MYL2 and MYL3), ER-Ca^{2+} function (PLN), myoglobin (MB), and β1-adrenoceptor (ADRB1), in addition to Ca^{2+} handling properties and mitochondrial respiration, which suggested that re-entry waves promote maturation of circularly EHTs. It is well known that the arrangement of CMs and fibers has an important effect on the physiology, metabolism and electromechanical function of heart tissue.[70] In addition, the main stress and strain directions form an obvious helical structure at the macroscopic level. Navaee *et al.*[71] performed periodic uniaxial mechanical stretching of the pre-aligned EHT to simulate the helical arrangement of tissues *in vivo*, which demonstrated that mechanical stimuli and surface morphology increased the neonatal CMs maturation markers α-actinin and connexin-43, and promoted their maturation and functions. Recently, Takada *et al.*[72] constructed EHT with more uniform arrangement of CMs by implanting hiPSC-CMs into microprocessed fibronectin gels. They showed that 4.3×10^5 unaligned CMs produced a contractile force of 1 mN, whereas the same contractile force only needed 2.5×10^5 uniformly arranged CMs. Therefore, uniformly aligned CMs significantly increased the contractility, synchronization, and electrical integration of EHT. In addition, Huebsch *et al.*[73] inoculated 80% hiPSC-CMs and 20% stromal cells on microfluidic chips to form EHT, and found that microfluidic chips enhanced the arrangement of CMs and extracellular matrix production. It was also found that a maturation medium rich in fatty acids promoted the maturation of CMs. Therefore, EHTs have immense potential for measuring the strength, contractility, and electrophysiology of cardiac tissue. However, the EHTs fail to fully recapitulate the anatomy of adult heart tissue. Additionally, EHTs cannot serve as an ideal disease model because it contains only a few cell types and the genesis and developmental processes of EHT. Furthermore, their physiological responses to injury are not identical to those of the natural heart.[2] More importantly, the construction process is complex and requires the use of biological materials, specialized machinery and infrastructure, thereby

reducing reproducibility, model predictability, broad applicability, and high throughput scalability.

3.2. Self-organization methods

Self-organization methods, which generally use cells with a high differentiation potential and specific developmental signaling molecules to create hCOs, have become available. By directing stem cells into the cardiac mesoderm pathway, self-organization produces a more diverse cell population than the co-culture protocols, and promotes more physiologically relevant cell interactions to obtain the shape and function of the heart, thereby revealing the genesis and development of the natural heart.[2]

Lee *et al.*[74] used laminin–endothelial inhibitor complexes and FGF4 to stimulate mouse ESC-derived embryoid bodies to form mouse cardiac organoids (mCOs). The mCOs displayed continuous morphological changes, including cardiac crescentic structures, heart-tube formation, heart-tube annulation, and chamber formation. The resulting mCOs had atrial and ventricular components containing myocardium, conduction tissue, smooth muscle, and endothelial cells, and the myocardium of organoids can autonomously contract and generate action potentials, which closely mimic the ultrastructural, histochemical, and gene expression characteristics of the developing heart *in vivo*. The pro-epicardium (PE) organ containing epicardial membrane derived from epicardial progenitor cells is an important structure of cardiac embryo development. The developmental route of PE is still unclear. More recently, Branco *et al.*[75] generated a self-organizing multilineage embryonic organoids with an early embryonic structure including the PE, septum transversum mesenchyme (STM) and liver buds by regulating the Wnt pathway of iPSC. Subsequently, the constructed organoids were co-cultured with CM aggregates to produce epicardium-myocardial hCOs containing WT1+ epicardial-like layer that completely surrounds cTnT+ myocardium-like tissue, which demonstrated the function of PE/STM cells in hCOs. Lewis *et al.*[76] generated self-assembled developmentally relevant hCOs using a three-step Wnt signaling modulation strategy (activation/inhibition/activation) with chemical inhibitors and growth factors (Fig. 3A and B). The hCOs show physiologically representative developmental stages, with multiple cardiac cell types, spontaneously formed vessels, and complex internal chambers that recapitulate heart zone formation and atrial specification. The hCOs are very similar to human fetal heart tissues at the transcriptomic, structural, and cellular levels. Although a chamber-like hCO with a central cavity has recently been reported, the reason for the chamber formation has not been clearly revealed. Liang *et al.*[77] successfully co-differentiated iPSCs into hCOs which contained CMs and VE-cadherin (VE-cad)+ cardiac endothelial-like cells by reactivating Wnt signaling at the cardiac progenitor stage. It was found that VE-cad+ endothelial-like cells formed the cardiac endothelial-bounded chamber, thus forming the chamber-like hCO with a central cavity. The membrane potential of chamber-like hCO was more mature than that of traditional hCOs only composed of CMs. Xuan *et al.*[78] used human cardiovascular

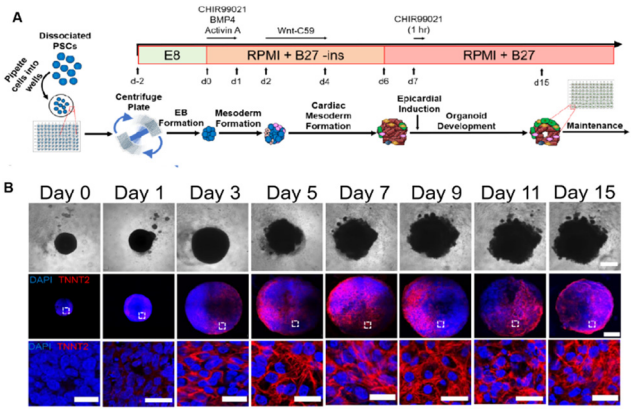

Fig. 3 Representative Self-organization Methods. (A) and (B) Lewis *et al.*[76] generated self-assembled developmentally relevant hCOs using a three-step Wnt signaling modulation strategy (activation/inhibition/activation) with chemical inhibitors and growth factors. Reprinted with permission from ref. 76. Copyright 2021, Nature Communications.

progenitors (CVPs) to induce the differentiation of hESCs into chambered hCOs by regulating the Wnt/ß-catenin pathway of CVPs amplification, which demonstrated that CVPs were essential for the formation of the heart chamber.

Although self-organization methods have greatly improved our ability to study cardiac development and disease *in vitro*,[79] they still have some limitations. Firstly, self-organization methods are not uniform and do not allow for the precise control of cell proportions, overall composition and the resulting tissue structure. Secondly, self-assembly is more fragile than tissue engineering technique because of the lack of supporting materials and the 3D constructs. Finally, the tissues and cells presented in self-assembled hCOs are immature. Therefore, further studies should be conducted to promote the maturation of tissues and cells in self-assembled hCOs. Tables 2 and 3 detail the markers and ultrastructures used to evaluate the maturation of hCOs.

3.3. 3D printing methods

3D bioprinting is a layer-by-layer manufacturing technique that allows for the precise control of tissue structure and cell distribution to replicate the complex geometry of the heart. 3D bioprinting can be used to create perfusing vascular networks[80,81] and various cardiac tissue structures, such as heart valves, blood vessels, and myocardium. Noor *et al.*[82] fabricated a bioink through integrating hiPSC-CMs, ECs and ECM to construct cardiac parenchymal tissues and blood vessels. Additionally, they created functional cardiac patches, which were thick, perfusion-vascularizable and perfectly matched the patient's immunological, cellular, biochemical, and anatomical properties using 3D bioprinting. Lee *et al.*[83] utilized collagen as an ink to print the shell, while using high-concentration cells (human embryonic stem cells differentiated cardiomyocytes (hESC-CMs) and 2% CFs) to print a human ventricular model between the ventricular walls (Fig. 4A). The ventricular model showed synchronous contractions and directional action potential propagation, and the ventricular wall thickened up to 14% during systole after 7

Table 2 Ultrastructures of Mature hCOs[58–62]

	Mature CMs	Immature CMs
CMs ultrastructures	High myogenic fibers	Low myogenic fibers
	Sarcoplasmic reticulum is distributed around the myogenic fibers	Sarcoplasmic reticulum is underdeveloped and mainly distributed in the periphery of the cytoplasm
	Highly organized sarcomes (2.2 μm)	Chaotic and short sarcomes (1.6 μm)
	Rich mitochondria	Rich glycogen granules
	Rich T-tubules	Little T-tubules
	Obvious Z-line, H-band, M-line	Z-line exists
	More binuclear cells	More mononuclear cells
	Cells form tight connections with intercalated discs	Cells are loosely connected in a punctate pattern

Table 3 Markers of Mature hCOs[58,67,69,88]

Function	Cardiac specific genes	Increase or decrease
Contractile apparatus genes	β-MHC	Increase
	cTNT	
	MYH7	
	titin N2B	
	MLC2v	
Adult-like conduction	ITPR3	Increase
	KCNH2	
	HCN4	Decrease
Maturation	NPPB	Increase
	MAPK1	
	PRKACA	
Ultrastructure	MYH7	Increase
	GJA1	
	TNNI3	
	AKAP6	
	GJA5	
	JPH2	
Energetics	AKAP1	Increase
	TFAM	
	PPARGC1A	
Ca^{2+} handling genes	RYR2	Increase
	CAV3	
	BIN1	
	ATP2A2	
	ITPR3	
	SERCA2a	
	CACNA1C	
	CASQ2	
Ion channel genes	KCNA4	Increase
	KCNJ2	
	KCNH2	

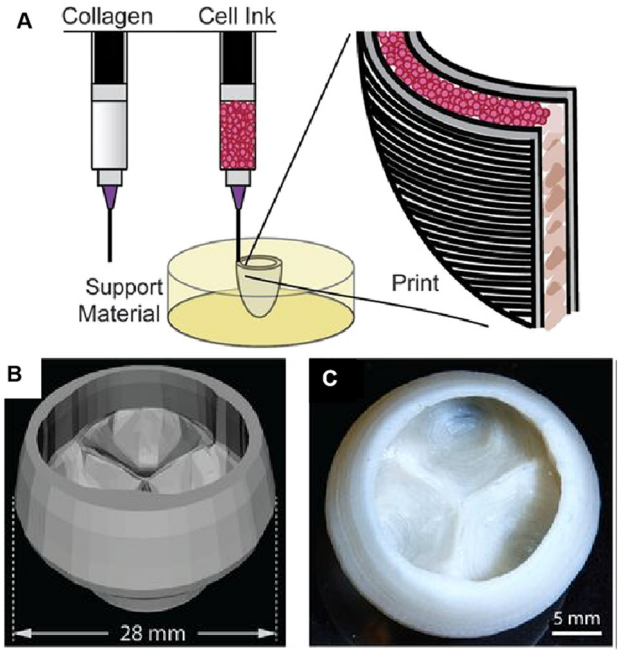

Fig. 4 Representative 3D Printing Methods. (A) Lee *et al.*[83] utilized collagen as ink to print the shell, while using high-concentration cells (hESC-CMs and 2% CFs) to print a human ventricular model between the ventricular walls. Reprinted with permission from ref. 83. Copyright 2019, Science. (B) and (C) Lee *et al.*[83] also printed a trilobular collagen heart valve (diameter: 28 mm) and improved its mechanical properties using a decellularized pig heart valve fixation protocol. Reprinted with permission from ref. 83. Copyright 2019, Science.

days. Lee *et al.*[83] also printed a trilobular collagen heart valve (diameter: 28 mm) and improved its mechanical properties using a decellularized pig heart valve fixation protocol (Fig. 4B and C). The collagen valves possessed well-separated leaflets, were sufficiently strong to be handled in air and displayed the mechanical integrity and function of collagenous constructs on an adult scale. Kupfer *et al.*[84] printed constructs containing two chambers, one vascular inlet and one outflow using an optimized ECM protein bioink. The ECM allowed iPSCs to proliferate and fill the gaps of the structures. When the iPSCs are sufficiently plentiful, differentiation of the cells within the structure occurs, resulting in the formation of discrete vessels and closed chambers.

The human ventricular muscle pump shows massive beating and continuous action potential propagation, and responds to drugs and pacing. This type of human chamber organoid may be useful for cardiac medical device testing and regenerative medicine. Infarcted heart muscle tissue can also be printed using 3D bioprinting technology. Basara *et al.*[85] prepared a bioink by mixing hiPSC-CMs with human cardiac fibroblasts (hCFs) and printed the infarct boundary area to simulate an infarct area surrounded by healthy tissue. Gelatin methacryloyl-methacrylated hyaluronic acid-decellularized human heart ECM (GelMA-MeHA-dhECM) hydrogels were used to achieve a variety of mechanical properties to better model the infarcted region. This resulted in the model achieving an order of magnitude difference in stiffness between healthy and scar tissue, but it was unable to achieve the physiologically accurate stiffness of myocardial tissue.

3D bioprinting technology has become a versatile and powerful method for rapidly constructing patient-specific perfusing tissues of arbitrary volume and shape, creating EHT with the structural, mechanical, and biological properties of the human heart. However, this method has several limitations. Firstly, 3D bioprinting is expensive and has not yet been achieved on the manufacturing scale. Secondly, 3D bioprinting requires billions of cells to print large tissues because cell loss often occurs when cells are mixed into a bioink.[15,86] Thirdly, existing 3D printing methods cannot print both the external geometry and the internal microstructures of organoids, such as vascularization. Recently, Fang *et al.* solved this problem by printing ventricular models with perfusable vascular networks using microgel-based biphasic (MB) bioinks by shear thinning, self-healing behavior and sequential printing in reversible ink templates (SPIRIT).[87]

4. Applications of human cardiac organoids

With the rapid advance of the hCO technique, the hCO models have become increasingly mature and complex, which not only help us to better explore the mysteries of cardiac and cardiac disease development, but also promote their applications for drug screening, disease modeling and organic transplantation (Fig. 5).

4.1. Drug Screening

Cardiotoxicity is one of the main reasons for drug withdrawal from clinical trials and the market. Drug-induced cardiovascular toxicity can result in structural damage to the cardiac tissue and deterioration of cardiac function, including arrhythmias and changes in cardiac contractility.[89] Adverse drug reactions typically occur during the clinical phase and incur high costs. Traditional preclinical drug cardiotoxicity and efficacy tests are performed in 2D cell cultures and animal models. However, 2D cell culture does not accurately recapitulate the 3D cellular microenvironment of *in vivo* organs, whereas animal models provide a limited success when translating results to humans because of the obvious distinction in terms of metabolic activity and gene expression patterns between humans and animals. The hCOs can mimic the microenvironment of the natural heart, and thus may be a superior model for preclinical drug screening.

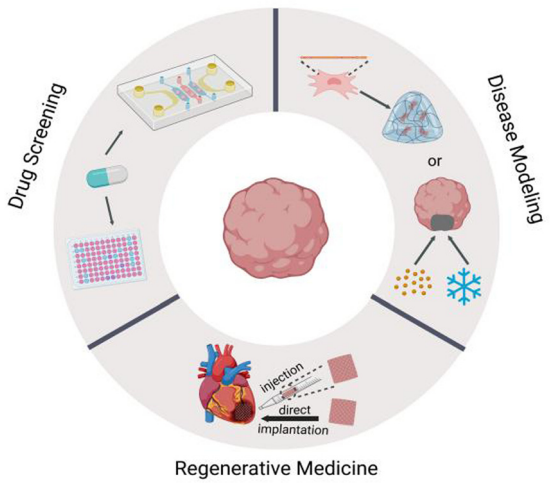

Fig. 5 Application of human cardiac organoids: drug screening, disease modeling and regenerative medicine.

Mills *et al.*[44] developed a high-throughput platform using bioengineered hCOs containing functional contractile tissues resembling the biological properties of the natural heart. They screened 105 small molecules with the potential for regeneration using the hCO platform. They demonstrated striking discrepancies between the hCO system and traditional 2D assay, and identified two compounds that promote cardiac proliferation, but have less of an effect on rhythm and contractility. The hCO platform can also be used to screen for drugs that promote organoid growth, maturation, and functionality. Tyler *et al.*[90] used the hCOs to evaluate the direct cardiotoxicity of trametinib, which is commonly used to treat BRAF-mutated metastatic melanoma. Trametinib was added to the medium for 6 days, and it was found that the diameter and contractile force of the hCOs treated with Trametinib were decreased in comparison to those in the untreated control group, which was consistent with *in vivo* data. There is a pressing task to move from the current stratified CVD management into a better personalized cardiovascular medicine that benefits each individual to improve clinical outcomes.[91] An advantage of iPSCs is that they are derived from the somatic cells of patients, making them a genetical match with the individual patient. The iPSCs-derived hCOs can inherit all genomics involved in drug responses, thereby offering an unprecedented opportunity to precisely evaluate and compare the drug efficacy and safety in each patient.

The development of complicated organoids to mimic the organ–organ crosstalk when screening drug candidates has become an urgent task. Skardal *et al.*[92] developed a multi-organ on-chip platform containing the liver, heart, lung, blood vessels, testis, colon, and brain using 3D organoid technology derived from human primary cells and stem cells, which not only survived for at least 28 days *in vitro*, but also maintained viability and functionality in the long term. The multi-organ on-chip platform was utilized to screen a group of drugs recalled from the market by the Food and Drug Administration (FDA), and to model the hepatotoxicity of troglitazone and mibefradil and the cardiotoxicity of astemizole, pegaolet, and terodiline.

Although hCOs are powerful tools for testing drugs, they are still far from a natural heart.[93] The reliability of drug screening can be improved by increasing the structural and functional similarity of organoids to the natural heart, and by exploring the influence of the cardiac microenvironment and other organs on cardiac drug toxicity.

4.2. Disease modeling

Elucidating disease mechanisms is a complex process because various techniques are required to identify possible targets and

test hypotheses. Multicellular hCOs can be used to analyze cell–cell interactions in pathogenesis, thus paving the way for disease investigation.

Human heart diseases are divided into inherited and non-inherited disorders. Inherited diseases are caused by CMs carrying genomic mutations that trigger abnormal heart function over time and the development of chronic disease.[94] HCM, dilated cardiomyopathy (DCM), and arrhythmogenic right ventricular cardiomyopathy are examples of inherited cardiomyopathies. Using hCOs as models for inherited diseases can facilitate a better understanding of the pathophysiology and genetic origins of these diseases. DCM is the primary cause of death in patients with Duchenne Muscular Dystrophy (DMD). The pathophysiology of the disease is unclear, and current cellular and animal models can not accurately reflect the human phenotypes. Marini *et al.*[95] used DMD patient-derived iPSCs for self-organization into hCOs (DMD-hCOs) and performed long-term culture. DMD-hCOs lacked initial proliferative capacity. Furthermore, DMD-related cardiomyopathy and disease progression phenotypes, such as cardiomyocyte deterioration, fibrosis and abnormal adipogenesis, were observed in long-term culture. Five miRNAs essential for this genetic heart disease have been identified. Yang *et al.*[96] examined the function of a new myosin heavy chain 7 mutation (MYH7 E848G) observed in inherited cardiomyopathies, using hCOs constructed from patient-specific hiPSC-CMs. They discovered that patient-specific hiPSC-CMs with MYH7 E848G-induced systolic dysfunction and controls made of homologous cells with genome-editing both recapitulated the systolic dysfunction of cardiomyopathy. Mutations in the titin gene are the most frequent genetic cause of DCM, heart failure, and premature death. John *et al.*[97] assessed the pathogenicity of titin gene variants using hCOs. Titin gene variants in hCOs impair contractile performance. However, contractile dysfunction is not detected in 2D cultures of hiPSC-CMs carrying titin variants. Therefore, it is possible that hCOs can accentuate disease phenotypes that are not obvious in 2D cultures.

The clustered regularly interspaced short palindromic repeats (CRISPR) system was first applied in mice and human gut organoids in 2013.[98] Since then, several applications of the CRISPR systems in heart organoids have been reported. Long *et al.*[99] found that hCOs constructed with myotonic dystrophy protein-mutant hiPSC-CMs exhibited significant contractile dysfunction. However, the mutation could be corrected using CRISPR associated protein9(CRISPR/Cas9) to achieve tissue-specific therapeutic effects. Yang *et al.*[96] created an EHT by co-culturing bone marrow stromal cells and familial cardiomyopathy patient-specific hiPSC-CMs (containing the MYH7 E848G mutation) in polydimethylsiloxane molds. The pathogenicity of the E848G mutation was confirmed by the significantly diminished contractile function seen in EHT with the MYH7 mutation, as well as the contractile dysfunction seen in homozygous cells edited using CRISPR/Cas9.

The pathology of nongenetic diseases develops over time. Diet, interactions with other organs, and organ aging influences the development of non-genetic diseases. Common nongenetic heart diseases include myocardial infarction, heart failure, and cardiac hypertrophy. Myocardial infarction is caused by blockage of the coronary artery, which prevents the flow of oxygenated blood to the downstream myocardium, causing myocardial cell death owing to local blood flow constriction. This results in ischemia of the region, which reduces the ability of the heart to pump blood throughout the body. Consequently, the nervous system compensates for cardiac output.[100,101] Positive feedback can result in cardiac dysfunction and ultimately heart failure, as the damaged heart cannot be fully compensated for or regenerated. The first organoid model of CVD was established as an organoid model of myocardial infarction or injury.[94] Voges *et al.*[102] employed a dry ice probe to induce localized cryogenic damage hCOs, which simulated the process of myocardial infarction. The regenerative capacity of hCOs was fully restored within two weeks after injury, as the surrounding cells remained viable. Lee *et al.*[103] used the same cryogenic injury approach to build a myocardial infarction model and found that the overall function of the heart organoid was inhibited. Richards *et al.*[104] replicated the structure of the human heart after myocardial infarction by simulating infarct, border, and distal zones. They also replicated the hallmarks of myocardial infarction including pathological metabolic shifts, fibrosis, and calcium imbalance at the transcriptional, structural, and functional levels using the neurotransmitter norepinephrine to stimulate hCOs to incorporate an oxygen diffusion gradient. Tiburcy *et al.*[23] used chronic catecholamines to stimulate EHT, to reproduce the heart failure phenotype, including diminished β-adrenergic responses and CM hypertrophy after neurohumoral hyperstimulation, demonstrating the applicability of EHM in an *in vitro* model of simulated heart failure. However, current models of myocardial infarction and heart failure lack immune cells and use immature CMs, which do not accurately reproduce the functions of immune cells and mature CMs in myocardial infarction and heart failure.

4.3. Regenerative medicine

Myocardial infarction and heart failure are leading causes of death worldwide. The ability of adult mammals to regenerate CMs from their hearts is extremely limited. The CM renewal rate was found to be between 0.5% and 2%,[105–107] and decreased with age, damaged heart muscles and the formation of fibrotic scars. Remuscularization of the injured myocardium has long been a problem for cardiovascular researchers. To date, heart transplantation is the only available long-term treatment option. However, there is an urgent need to seek alternative solutions due to the scarcity of viable donor hearts and the significant risks of surgery.[108–110] Regenerative strategies such as allogeneic transplantation, direct intramyocardial injection of stem cells, and CMs[110] have shown therapeutic potential in animal models, but suffer from insufficient healthy tissue donors, immune rejection, rapid clearance of cells, and low integration efficiency with the host. hCOs have a more complex spatial structure and cellular composition than stem cells or cardiomyocytes. Organoid technology may be a candidate for addressing these issues by enhancing survival rates,

inducing endogenous tissue remodeling, and promoting functional integration, as shown in studies on the transplantation of hCOs into rats, naked mice, pigs, and other animals.[88,111–114]

Cardiac patches are thin sheets of cardiac muscle and other cells that can be implanted in a stent or interconnected to form a layer.[115] The cardiac patches were anisotropic. Mechanical and/or electrical stimulation can be applied and extended. The size of the cardiac patch (7 mm × 7 mm) is more suitable for clinical studies, and has been used in the regenerative treatment of animal models of myocardial injury, such as rat[111] and pig models.[116] Zimmermann's team[112] implanted cardiac patches into myocardial infarcts in immunosuppressed rats and observed the formation of a thick layer of myocardium. The cardiac patches did not cause arrhythmias after 28 days, and exhibited the same electrical coupling as that of the natural heart. Shadrin *et al.*[113] encapsulated hiPSC-CMs in a hybrid hydrogel to create a cardiac patch and subsequently transplanted them onto the rat epicardium without increasing the incidence of arrhythmias. The cardiac patch displayed a long-term survival and high vascularization, but lacked electrical integration with host rat hearts. The existence of the fibrous layer between the graft and the host may explain the absence of electromechanical coupling. Epicardial cells are a promising adjuvant therapy for cardiac repair. Bargehr *et al.*[111] constructed heart patches by co-culturing human ESC-derived epicardial cells and CMs, which were then transplanted into the hearts of infarcted thoracic muscle-free rats. The transplanted epicardial cells have been found to create long-lasting fibroblast grafts in the infarcted heart, increase the size of the transplanted heart by 2.6-fold and the number of transplanted CMs by 2-fold, and enhance both graft and host vascularization, thereby improving the contractile function of the heart. Despite the development of engineered heart patches with clinically relevant dimensions, there remain challenges associated with the small size and slow flow of the prefabricated vascular network, insufficient graft thickness, lack of electromechanical coupling, and conduction velocity and force measurements, which from a quantitative standpoint, create demerits in comparison with those of the human myocardium. Varzideh *et al.*[88] mixed and co-cultured human ESC-derived cardiac progenitor cells, mesenchymal stem cells and human umbilical vein endothelial cells on hydrogels and converted them into 3D uniformly beating circular hCOs within three days. When the maximum beating was achieved, hCOs was encapsulated in collagen I and transferred to a PLA basket that was 3D printed before being sutured directly into the peritoneal cavity of a nude mouse. The CMs in hCOs were more mature at the transcriptional and ultrastructural levels one month after transplantation, and the expressions of cardio-specific genes such as cTNT, β-MHC, MLC2v, RYR2, SERCA2a, CACNA1C, and CASQ2, were higher than those of CMs only. Moreover, the hCOs possessed a primitive vascular system for energy, O_2 exchange and waste material removal.[76] Furthermore, pre-vascularized hCOs provided a microenvironment with multiple angiogenic growth factors that activate host ECs to migrate into hCOs.[88] Most hCOs currently in preclinical use require open-heart surgery for placement, cannot be implanted in a minimally invasive manner, and require long-term immunosuppression. Montgomery *et al.*[114] developed a cardiac patch (1 cm × 1 cm) that was delivered *via* a 1 mm small hole, can restore the initial morphology after injection, and has no impact on the viability or function of CMs. However, their patches currently can be used in only rat and pig animal models.

4.4. Others

In addition to drug screening, disease modeling and regenerative medicine, heart organoids can be used to study the developmental biology of embryos,[29,30,33,74] gene editing[96,99] and more. For example, the hCOs constructed by Hofbauer *et al.*[33] summarized the pathogenesis of early myocardial, endothelial and epicardial morphology, which can be used to study the pathogenesis and development of the human heart. Branco *et al.*[75] constructed hCOs with PE cells, epithelioid structure, and STM cells, and demonstrated the developmental route and function of PE/STM cells. Yang *et al.* used CRISPR/Cas9 technology to construct EHT of hiPSC-CMs containing the MYH7 E848G mutation, and demonstrated myocardial contraction disorders caused by mutations in this gene. It is also possible to provide patient-specific transplant donors for organ transplants in the future.

5. Conclusions and outlook

Organoids are a promising technology for constructing 3D organ models to bridge the gap between 2D cellular and animal models, and have greatly promoted the advancements in the biomedical fields, including tissue regeneration, drug screening and disease research. Although promising, the development of hCOs have been lagging significantly compared to other organoids due to the structural and functional complexity of hearts. The current hCOs are relatively simplified organ-like tissues, most of which do not have the complete cell composition, or the structure and function of the heart. Most of the current construction methods of hCOs lack complete and mature vascularization, which leads to the uneven distribution of oxygen and nutrients, resulting in a core necrosis of hCOs and limiting their size and long-term survival time. Additionally, the hCOs constructed by current construction methods remain immature, and most of them do not contain immune cells. Although the maturity can be improved by electrical and mechanical stimulus, the method should be optimized to accurately simulate the myocardial maturation *in vivo*. Notably, there are numerous existing construction methods for hCOs, but standardized and reproducible construction methods have been yet developed to fabricate highly efficient and stable hCOs. In addition, the current construction methods of hCOs require the addition of various growth factors in culture medium, which is costly, and the growth factors may impair the natural morphological gradient of tissues.[13]

The construction of hCOs can be inspired by other organoids: most organoids are constructed by incrementally adding growth factors, and share some core growth factors such as Wnt activators/inhibitors, BMP inhibitors, etc.[12] On this basis, signals or hormones needed during the development of the heart can be added to the culture medium to construct hCOs. Recent research has further improved the complexity of hCOs by focusing on integrating the frontier advantages of cell biology, genetics and materials science to construct more precise and faithful hCOs of those composition, with the spatial organization and vascularization closely resembling that of natural heart tissues. Overall, as organoid technology continues to develop, more accurate hCO models can be expected to benefit the cardiac field.

Conflicts of interest

There are no conflicts to declare.

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