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Review

Human Cardiac Organoids: Quantification and Qualification in Cardiovascular Studies

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Abstract: The human cardiac organoids (hCOs) represent a three-dimensional (3D) tissue model that mirrors in vivo cardiac conditions. Recent advancements underscore the immense potential of hCOs in several areas including studying early cardiogenesis, modeling heart diseases, screening potential drugs, and even exploring possibilities for cardiac regeneration. Recognizing the pivotal role hCOs play across various applications, this review examines the evolution of key metrics and tools for assessing cardiac organoids tailored for diverse research objectives. Moreover, it deliberates on the limitations of cardiac organoids and outlines the prospective avenues for future research applications of hCOs.

Keywords: 3D culture; cardiac organoids; self-assembling; drug-screening; disease modeling

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality globally, despite significant research efforts aimed at developing preventive interventions for heart diseases [1]. The reasons for this challenge include the following: (1) the lack of effective therapeutic strategies to help people survive heart attacks; (2) the shortage of reliable models for comprehensive investigations into the mechanisms of human cardiogenesis and maturation; (3) limited resources and techniques for extensive scale of drug screening. Two-dimensional (2D) in vitro models do not precisely mimic the complex in vivo environment that comprises cell-cell and cell-extracellular matrix (ECM) interactions, and regulatory factors [2]. Traditional three-dimensional (3D) cell cultures may fail to recapitulate some morphogenesis and pathological processes due to low genetic and phenotypical heterogeneity [3]. Animal models are currently the gold standards for many assays in modeling human physiological conditions, gene expression patterns and metabolic activities. However, the species distance between animals and humans restricts the direct extrapolation of animal research findings into human heart diseases [4]. 3D cardiac organoid model, which resembles the heart as it contains essential cardiac cell components, structures, and functions, is evolving as an attractive model for basic and pre-clinical CVD research [4].

Cardiac organoids are complex structures that autonomously organize into heart tissues or heart organ-like formations, encompassing a similar ratio of key cardiac cell types like cardiomyocytes (CMs), cardiac fibroblasts (CFs), and endothelial cells (ECs) to the cellular composition of the heart that are cultivated in vitro from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) [5,6]. Consequently, cardiac organoids can effectively mimic cell-matrix dynamics, cell-cell interactions in human hearts, as well as both physiological and pathological human cardiac development in vivo [7–9]. The term “cardiac organoid” was initially introduced around two decades before [10], gaining popularity quickly in 2017 [11,12]. However, the development and utilization of hCOs have significantly lagged behind other types of organoids such as those of the lung [13], small intestine [14], pancreas [15], liver [16], prostate [17], brain [18], and

tumor tissue [19]. The construction of hCOs faces several obstacles associated with the diverse cellular composition and intricate micro-environments [20].

In contrast to 2D cultures, cardiac organoids can replicate 3D morphogenesis and pathological processes in the hearts [8], shedding light on the potential of cardiac organoid as a promising platform for systematic investigations into cardiac self-organization, congenital heart defects, and translational researches. The recently established state-of-art hCO platform resembling the embryonic heart with subcompartments and multiple chamber-like cavities fulfills the specific requirements of drug screening in individual hCOs [21].

The analysis of complex multicellular organoids is becoming feasible due to the evolution of the cutting-edge technologies, including human-induced pluripotent stem cells (hiPSCs), single-cell analysis [22], and multi-photon microscopy. The emergence of optimized iPSCs and cardiac differentiation protocols has accelerated the construction of cardiac organoids in vitro [23]. In this review, we will present recent significant advances in the realm of cardiac organoids and their diverse applications. The various methods of assessing essential functions and features of cardiac organoid are also discussed. Finally, strategies for overcoming existing challenges and future directions are discussed.

2. Classification of Human Cardiac Organoids

The heart is the first functional organ developed in human embryos, composed of multiple cell types and regulated by highly hierarchized signaling pathways. CMs contribute 70–85% to the heart by volume but only represent 25–35% of the heart cell populations [24,25]. Non-CM cells, including vascular endothelial cells, vascular smooth muscle cells, CFs, neurons, and immune cells, constitute 65–75% of the total cell population in the heart [26]. Surrounding the cardiac cells and vessels, 3D extracellular matrix (ECM), containing laminin proteins, fibronectin, interstitial collagen, cytokines, growth factors, proteases, and glycoproteins, constitutes the micro-environment to support cells in anisotropic alignment, biochemical signaling, and mechanical strength [27]. There is no globally agreed definition for hCOs, but a typical hCO should be a 3D structure containing major heart cell types and recapitulating the critical heart structures and function [28]. An ideal hCO can model the complex in vivo cardiac microenvironment under both physiological and pathological conditions. The names of "organoid" and "spheroid" are frequently used interchangeably, in certain cases, the 3D heart constructs with scaffolds are also called hCOs. Therefore, we classify the hCOs into two categories: (1) the engineered heart tissue (EHT) and (2) self-assembling cardiac organoids (Figure 1).

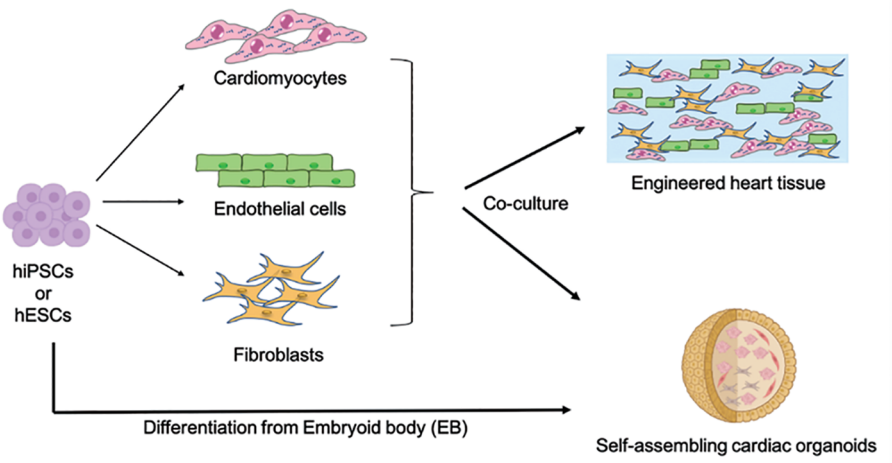

Figure 1. Classification of human cardiac organoids.

2.1. Engineered Heart Tissue

The objective of in vitro EHT is to create systems that simulate the physiological structure and function of an in vivo heart. The EHTs are primarily produced by integration of engineering methods and biomaterials

rather than natural cardiac developmental mechanisms [28]. Thus, one of the advantages of EHT is that various cardiac cell types derived from stem cells can be co-cultured and introduced onto scaffolds, biomaterials, extracellular matrix (ECM), or bioengineered devices to mold the desired three-dimensional (3D) structure for specific cardiac physiological tests, such as contraction and electrophysiology. The first reported EHTs were simply constructed by fostering intercellular adhesion of co-cultured one or two types of heart cells from rats and mice to form a loose spherical structure referred to as a cardiac spheroid [10,29]. The first creation of functional human EHT using iPSCs was reported later, which involved generating 3D cardiac tissue from these stem cells. This technique has enabled the study of human heart disease and the testing of potential therapies in a more physiologically relevant context [30]. EHTs serve as ideal models for analyzing cardiac contractility and electrophysiology as they can simulate certain aspects of adult cardiac tissue, however, they do not fully replicate the intricate morphology and early developmental patterns observed in natural hearts and lack cell type diversity. Additionally, the construction of EHTs is complex, requiring specialized equipment, making it expensive and challenging to be applied for high-throughput screening.

2.2. Self-Assembling Cardiac Organoids

Self-assembling cardiac organoids are cell aggregates that form heart-like structures with minimal external intervention. Congenital heart diseases are the most prevalent type of birth defects. Self-assembling cardiac organoids closely represent the developmental and spatiotemporal patterns of the embryonic heart, facilitating our understanding of early cardiogenesis [31]. Silva et al. constructed an organoid model using multiple lineages derived from human iPSCs to mimic the cellular and structural complexity during early embryogenesis, from which they discovered that the endoderm tissue (gut/intestine) in the organoids promoted the development of cardiac-like tissues, such as an internal CM core and epithelial-like structures [32]. However, this model is not fit for the investigation of the cardiac morphogenesis, as the accelerated development of primitive cystic structures inhibit the cardiac morphogenesis. While multi-lineage embryonic organoids offer advantages for studying the co-development of the foregut and heart, their limitations restrict their application in cardiac-specific physiological function investigations. These limitations include the absence of mature cardiac structures like ventricles and vessels, as well as the presence of additional non-cardiac cell types.

To address the specific needs of cardiac research, scientists have developed lineage-specific cardiac organoids. These organoids consist exclusively of heart-relevant cell types, mimicking the structure and function of the human heart more closely. Unlike embryonic organoids, lineage-specific cardiac organoids reserve their capability of continuous cell specification and structural transformations in vitro, resembling in vivo hearts better [33]. In 2021, a self-assembled lineage-specific hCO model named ‘Cardioids’ was reported. The model comprised signaling pathway factors that modulate the CM and endothelium separation, which endowed the cardioids chamber-like structures with cavities. These cardioids show distinct myocardial and endothelial layers interacting with epicardium, resembling early cardiac chamber development. Meanwhile, the critical WNT-BMP signaling pathway in mesoderm and its downstream target HAND1, a transcription factor associated with congenital heart septal defects, was identified to govern the self-organizing of cardioid [8]. In general, lineage-specific cardiac organoids offer a powerful platform for investigating the mechanisms of human cardiogenesis, endothelial and epicardial morphogenesis processes, and modelling of congenital heart diseases.

3. Evaluations of Cardiac Organoids: Qualification and Quantification

As a newly evolved model for hearts, a guideline on the exanimation and evaluation of the hCOs should be developed to widen their application in either basic or preclinical investigations. To establish a guideline for assessing the hCO quantifications and qualifications, we summarize the available characterization methods for hCOs in terms of morphology, electrophysiology, metabolism, and gene expressions (Figure 2).

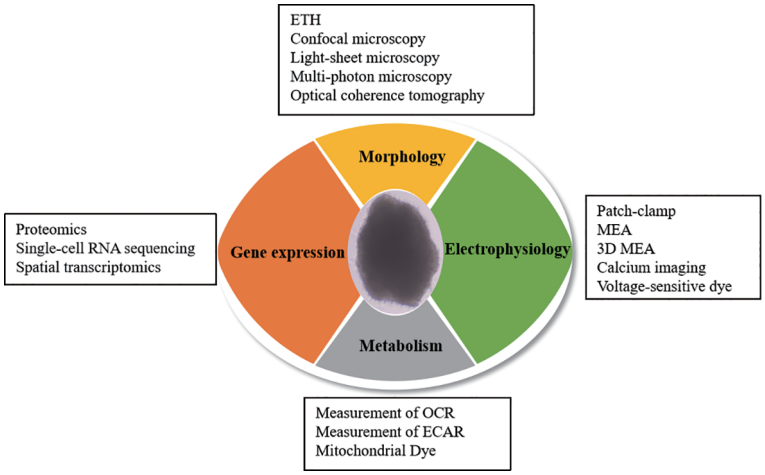

Figure 2. Guideline for human cardiac organoids evaluations [34–45].

3.1. Morphological Evaluation

The shape, size, surface and interior structures of an organoid, cell composition and viability, motions and contractions are all critical morphological parameters to evaluate in organoid studies [46].

Microscopy is the fundamental strategy examining the morphological indicators of an organoid. Surface observations can be achieved by using traditional white balanced or fluorescence microscopy on quantifying the organoids' sizes, shapes, as well as the process of cell aggregation. However, in contrast to 2D cell cultures, the compacted organoid tissue texture increases the challenges in observing the interior morphological features inside cardiac organoids. Therefore, advanced imaging techniques, like the confocal, light-sheet and multi-photon microscopy, need to be applied to observe alive or crosslinked hCOs. Due to photon scattering caused by dense structure of hCOs, confocal microscopy is limited in viewing the internal structures of thick organoid tissues [47]. One limitation to live imaging of hCOs is the extended scan time caused by pronounced light scattering. This scattering prevents researchers from capturing the inner core regions of cardiac organoids within a single heartbeat. Future advancements in imaging techniques are needed to overcome this challenge [48]. Light-sheet microscopy, characterized by a relatively shorter point scanning time is an alternative in imaging hCOs, but it is constrained by the penetration depth of 3 μm into cardiac organoids due to scattering. To solve this problem, Richards and colleagues combined light-sheet microscopy with a customized two-photon microscopy to achieve reduced scattering due to longer wavelength in scanning deeper regions of hCOs. This approach successfully captured calcium transient profiles in live cardiac organoids [49]. Recently, Ming and colleagues successfully observed the internal structures of cardiac organoids longitudinally using optical coherence tomography without sectioning or staining [49].

To evaluate the morphology of crosslinked hCOs, tissue fixation and sectioning is adopted as it allows extended scan times and higher spatial resolution of imaging. However, hCOs can be nearly invisible after embedding in paraffin or optimal cutting temperature compounds (OCT) due to small sizes and colorlessness [8]. Therefore, the use of tissue clearing reagents or strategies may improve the imaging quality of hCOs with high spatial resolution by enhancing their tissue transparency [46]. However, clearing may cause deformations to the hCOs, therefore, the choice of a clearing method should be carefully chosen based on the morphological parameters of interest within the organoid [50].

For cardiac organoids, contraction is a key morphological parameter to be examined. The measurement of contractile force of EHTs is in real-time and directly quantified, through two elastic posts posited at both sides of hCOs. In contrast, the easiest and cost-effective method of quantifying the contractions of cardiac organoids is the application of motion mapping and analysis of video captured by microscopes [51]. These analyses provide necessary information on contraction rate, duration, magnitude and regularity. Of note, interference from changes in position and orientation due to the robust beating of hCOs required extra consideration to avoid miscalculations.

3.2. Electrophysiological Evaluation

The electrophysiology of cardiomyocytes is an irreplaceable parameter for gaining insights into cardiac function, mechanisms of diseases, and the development of potential therapeutic interventions [52]. In animal studies and monolayer cultures, patch-clamp and multielectrode array (MEA) techniques are used for electrophysiological characterization of CMs. Patch-clamp is a powerful and wide applied method, it records quantitative action potentials and relevant ionic currents at a single-cell level; however, it is not efficient for high throughput projects [53,54]. In contrast, MEA detects action potential propagation and extracellular field potential and can be applied to multiple wells simultaneously, making it more suitable for high throughput investigations [54].

However, the current patch-clamp and MEA assays are mainly designed to assess 2D cultures, it is still a challenge to quantify the action potential of 3D hCOs. One optional method of assessing the action potential of hCOs is to dissociate the organoids into single cells and perform patch-clamp on the cells seeded on glass coverslips. Although this approach facilitates the detection of chamber-specific action potential, the readout may vary from the original electrophysiological features in hCOs after dissociation and culturing on 2D glass coverslips. Additionally, conventional MEAs only record the outer edges of hCOs due to the planar electrodes. Current improvements of MEAs include the design of bendable thin-film MEAs to fully cover the hCOs and 3D MEAs using mesh nanoelectronics or stretchable arrays [55]. Mesh electrodes embedded uniformly in hCOs enable synchronously record of electrophysiology across the whole structure, but we note that the insertion of these mesh electrodes into hCOs may interfere with the development of hCOs, which is especially obvious in long-term studies [56].

The detection of calcium current is important in studying hCOs' electrophysiology, and to fulfill this, calcium imaging based on fluorescent indicators is employed [7]. For example, Archon1, a recently developed genetically encoded voltage indicators, allows simultaneous detection of changes of calcium concentration across multiple cells induced by action potential within hCOs [7]. However, calcium imaging has limited capability in capture dynamic membrane voltage or specific physiological and pathological action potential waveforms individually [57]. Voltage-sensitive dye was specifically designed to solve this problem, and its intensity changes based on alterations of membrane potential. By this characteristic, the presence of ventricular- and atrial-like regions was identified by distinct action potential durations (APDs) in hCOs [58]. Nevertheless, like the mesh electrodes, the dye-mediated cytotoxicity limits its application in long-term study and need further improvements.

3.3. Evaluation of Metabolism

Since the heart's function relies on converting chemical energy into mechanical energy, a well-developed metabolism is critical for the successful application of hCOs. Research suggests that hiPSC differentiation into CMs and subsequent self-organization of hCOs are accompanied by a metabolic shift from anaerobic glycolysis to mitochondrial respiration [59]. Measuring concentrations or metabolic fluxes of metabolites involved in metabolic reactions is a well-established approach for evaluating hCOs' metabolism [60]. The readouts offer valuable insights into the activation or inhibition of specific metabolic pathways as well as changes in the energy demand of hCOs under physiological or pathological conditions. Specifically, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are two robust indicators of metabolic fluxes. The OCR is an indicator of mitochondrial respiration while ECAR indicates proton excretion through glycolysis. Fluctuations in OCR and ECAR are tightly associated with the ATP turnover, and can reflect the metabolism status of hCOs [61]. Of note, multiple types of cells contained in hCOs can interfere with the interpretation of OCR and ECAR caused by the distinct metabolic baseline of each cell-type. Moreover, mitochondrial staining is emerging an effective method of evaluating hCOs' metabolism which assesses the mitochondrial properties and functions [62].

3.4. Evaluation of Gene Expression

Gene expression is another crucial indicator for characterizing the phenotypes of hCOs. Quantitative RT-qPCR of gene markers was utilized to compare 2D cardiomyocytes and hCOs at different time points (Day 14 and Day 21). It was observed that hCOs provide a microenvironment that sustains the various cardiovascular

cell types [63]. However, it is difficult to quantify the expression of genes in hCOs. For example, multi-omic analysis of hCOs proteomics can reveal the activity and abundance of a particular protein, as well as its post-translational modifications [64]. Given the diverse cell types within hCOs, most researchers prefer single-cell sequencing (scRNA-seq) to analyzing the bulk population to obtain comprehensive information about the cell's composition, metabolism, and differentiation trajectories. Notably, scRNA-seq can detect low-abundance cell types but offers lower sequencing depth and coverage compared to bulk sequencing. Of note, single-cell dissociation for sequencing resulted in the loss of spatial information. To overcome this problem, spatial transcriptomics has been introduced owing to its ability to identify cellular differentiation during morphogenesis [65]. Despite the development of these sequencing techniques, their application is challenging compared with conventional gene expression assays, when considering the cost and spatial information preservation.

4. Applications of Human Cardiac Organoids

The development of strategies and guidelines for quantifying and qualifying hCOs models has accelerated our ability to understand their complex structures or maturity as well as investigate heart diseases and cardiac development. The available evaluation techniques facilitate the application of cardiac organoids in drug and toxicity screening, disease modeling, and organ transplantation (Figure 3).

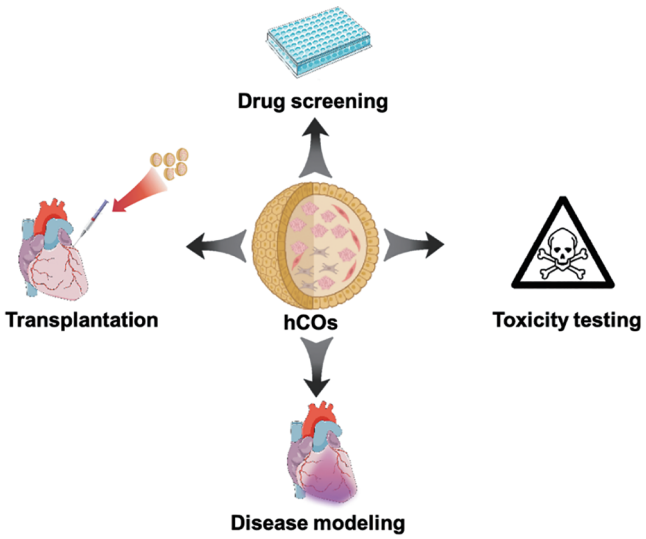

Figure 3. Application of human cardiac organoids (hCOs).

4.1. Cardiac Organoids in Drug and Toxicity Screening

Drug-induced cardiovascular toxicity damages the structure and function of heart, and this stands as a primary cause of drug failure in clinical trials, causing substantial waste of research expenditure [34].

hCOs are emerging as a state-of-art model for preclinical drug and toxicity screening. EHTs has been utilized in drug test to measure the contractile force [35]. EHTs are constructed by encapsulating hiPSC-derived cardiomyocytes with or without other cell types like fibroblasts into hydrogels (e. g., fibrin and collagen I). The nature of EHTs restricts their ability to maintain tissue integrity without inclusion of protease inhibitors in the culture media; otherwise, they will be easily broken by the pre-tensioning force from two silicone posts at both ends of the ETH [36]. In additional to the low representativeness of EHTs in modelling the whole heart as an organ, the preparation of EHTs with casting molds and huge number of cells restrict their application in high throughput drugs test [37]. A high-throughput platform utilizing lineage-specific cardiac organoids has been reported recently [38]. The hCOs in this report contains functional contractile tissues resembling the biological properties of a natural heart. Screening 105 small molecules using the hCOs platform yielded surprising results. Compared to traditional 2D assays, the hCOs platform revealed notable differences. Importantly, this approach identified two compounds that effectively promote cardiac cell

proliferation while minimizing their effects on heart rhythm and contractility [38]. The development of hiPSCs makes the personalized medicine possible by assessing drug efficacy and safety with specified hiPSCs from desired patients, recapitulating the genetic features matched to individuals. Despite significant advancements, fully replicating a real heart's complexity with hCOs remains a long-term challenge [39]. Continuous efforts are advocated to refine the hCOs thereby improve the prediction power of drug screening with in vitro methods in clinical purposes.

4.2. Cardiac Organoids as Disease Models

Disease modelling is an intricate process for unraveling the mechanisms of heart development heart diseases. The hCOs recapitulate the complex microenvironment in the heart more accurately in comparison with 2D models. Many human cardiac diseases arise from inherited genetic mutations. hCOs can improve our understanding of the pathophysiology and the underlying genetic causes of these conditions [40]. For instance, dilated cardiomyopathy (DCM) is the leading cause of death among patients with Duchenne Muscular Dystrophy (DMD), however, the conventional cellular and animal models are not sufficient and predicable enough to indicate the pathogenesis of this disease in human. Long-term culture of hCOs self-organized from iPSCs derived from a DMD patient revealed five key miRNAs associated with this genetic heart disease. These hCOs successfully mimicked the pathological features of DMD-related cardiomyopathy, including limited initial proliferation, fibrosis, and deterioration of cardiomyocytes with abnormal adipogenesis [41]. In overall, around 1% of all live births are both with congenital heart defects (CHDs); and there is still lack of representative in vitro models for them. The hCO serves as a feasible model for expanding our understanding of these defects that occur during early cardiogenesis. For example, diabetes in the initial trimester of pregnancy increases the incidence of congenital heart defects in their offspring [42]. To better examine its underlying mechanisms, hCOs were constructed to mimic heart development in the early trimester and model the impact of pregestational diabetes on the embryonic heart development [42].

Utilization of CRISPR/Cas9 in the construction of hCOs enables the establishment of disease-associated mutations carrying models and correction of mutated genes in hCOs. hCOs constructed with hiPSC-CM carrying mutated myotonic dystrophy protein showed evident contractile dysfunction, and only correct 30% to 50% of cardiomyocytes using the CRISPR/Cas9 system could reverse the pathological phenotype [43]. However, a current limitation of hCOs is that they are primarily made up of immature cardiomyocytes and lack immune cells. This hinders our ability to model age-related heart disease and conditions involving immune cells.

4.3. Cardiac Organoids for Transplantation

Terminally differentiated adult CMs feature limited regeneration, and the renewal rate of CMs is estimated 0.5% and 2% of CMs in the heart, contributing to the fatality of myocardial infarction and heart failure [44,45]. Currently, heart transplantation serves as the sole viable long-term treatment option available. Nevertheless, the scarcity of suitable donor hearts and the substantial risks associated with surgery underscore the pressing need to explore alternative solutions. The application of organoids containing similar cell types to the target organs is becoming a viable option for transplantation. For example, brain organoids derived from human embryonic stem cells (hESCs) were proved to be transplantable into the adult mouse brain, exhibiting progressive neuronal maturation and spread of axons in the host brain [66]. Similarly, more than 80% of hiPSC-derived intestinal organoids has been reported to be engrafted into the mouse mesentery 10 weeks after transplantation [67]. Even though cardiac organoid is anticipated to be transplantable based on the experiences from other kind of organoids, this approach is still in its early stage. Heterotopic implantation of hCOs into mice peritoneal cavity induced neovascularization and the CM from this 3D structure showed enhanced maturity compared with directly implanted CM [68]. Moreover, direct transplantation of hiPSC-derived CMs can serve as an alternative cell therapy for the renewal of myocardium. For instance, the transplantation of hiPSC-derived CMs into the infarcted hearts of mice yielded significant functional improvement [69, 70]. However, as mentioned above, the transplanted hiPSC-derived CMs or COs are immature, and thus cause arrhythmias in host hearts [41,71]. Therefore, it is important to develop effective approaches for accelerating the maturation of cardiac organoids. Moreover, good manufacturing practices

(GMPs)-compatible materials that replace animal-derived materials like Matrigel and basement membrane extract need to be designed to facilitate commercial production and application of hCOs in transplantation [72].

5. Current Limitations

The 3D hCOs are promising alternatives to traditional 2D cellular models and animal models which can be used to investigate cardiac diseases, heart regeneration and drug tests. However, majority of the hCOs are heart tissue-like compositions and lack complex cardiac-specific features, such as left-right asymmetry and conductance properties. Meanwhile, hCOs lack critical cellular elements, like immune cells. Furthermore, the culture medium for multicellular organoids is generally optimized for the predominant cell type, but it may not be ideal for all the cell types present within the organoids [46]. The existing techniques for building hCOs involve the usage of multiple growth factors, which are generally expensive and disrupt the natural morphogen gradients within the tissues [20]. Moreover, the available hCOs are not mature enough to represent the features of the adult human heart, and also lower in complexity in mimicking the embryonic heart developments. The other limitation is that there are still insufficiencies of well-certified or qualified technologies or protocols for analyzing and evaluating hCOs in all aspects of phenotypes and functions. Extensive effort and studies are required to address these challenges to accelerate investigations into cardiac disease and development of therapies.

6. Future Perspectives

Overall, integrating cutting-edge hCOs technology offers a robust and adaptable platform for biomedical research, drug discovery, human disease modeling, and preclinical studies. Despite the various limitations and challenges, hCOs technology is evolving rapidly and increasingly applied in various health fields to improve our understanding of cardiovascular diseases. Further research on 3D organoid models is advocated to refine the current hCO models and enhance their application in basic, preclinical, and clinical studies in the future.

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