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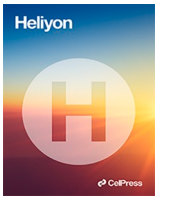

Review article

A review of protocols for engineering human cardiac organoids

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ABSTRACT

The use of human cardiac organoids (hCOs) as 3D in vitro models for cardiovascular research has shown great promise. Human pluripotent stem cells (hPSCs) have proven to be a potent source for engineering hCOs. However, various protocols for generating hCOs from hPSCs result in significant differences in heart development, maturity, complexity, vascularization, and spatial structure, all of which can influence their functional and physiological properties. This protocol review aims to highlight different strategies for generating hCOs using hPSCs while also critically discussing their challenges and limitations.

1. Introduction

Cardiovascular disease(CVD) is the foremost cause of global mortality [1]. An estimated 17.9 million people died from CVD in 2019, representing 32% of all global deaths [2]. To comprehend its pathogenesis and develop effective therapies, it is necessary to create more accurate models. However, current research models, for example, traditional 2D monolayer models and 3D tissue models, inadequately replicate the complexity and physiology of the human heart due to their simple cellular composition [3,4]. Human cardiac organoids (hCOs) derived from human pluripotent stem cells (hPSCs) are recent developments that offer a powerful tool for studying cardiac development, disease, and regeneration. hCOs are multicellular mixtures cultured in vitro in 3D. It has a cellular composition similar to the heart and a complex spatial structure. They have the potential to improve clinical outcomes for patients with cardiac disease and enhance our understanding of the human heart [5–7].

Since human cardiomyocytes (CMs) are not easily obtained from patients, hPSCs are the most viable cell source for generating hCOs. hPSCs can differentiate into all cell types found in the heart, making them an invaluable resource for developing hCOs [8,9]. Nonetheless, the techniques employed for generating hCOs from hPSCs vary significantly, resulting in differences in heart development, maturity, complexity, vascularization, and spatial structure [10,11].

This protocol review extensively examines the current state-of-the-art methods for engineering hCOs from hPSCs and critically evaluates their advantages and limitations. It is crucial to note that this review specifically focuses on hCOs, which have enormous potential in advancing our comprehension of heart development and disease, although other reviews of cardiac tissue are available [12,13].

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2. hCOs differentiation from hPSCs

A comprehensive understanding of the cellular and molecular events that occur during in vivo cardiogenesis [14] is necessary to efficiently produce hCOs from hPSCs in vitro. During embryonic development, pluripotent epiblast cells undergo a complex trajectory of heart development regulated by an evolutionarily conserved network of transcription factors and signaling pathways [15]. *TBX5*, *NKX2-5*, *GATA4*, and *TBX20* are crucial regulators of CMs, ECs, and FBs differentiation [16–18]. The sequential differentiation steps, including mesoderm, cardiogenic mesoderm, and cardiac progenitor cells, lead to the generation of functional CMs and other cardiac cells [19]. Signals induced by nodal, bone morphogenetic proteins (BMPs), Wnts, and fibroblast growth factors (FGFs) generate cardiac mesoderm-like cells expressing markers such as T (Brachyury) [20]. The heart field is established by the initial inhibition of the Wnt/β-catenin pathway, identified by the expression of mesoderm posterior 1 (MESP1) [21]. Retinoic acid (RA) signaling also controls cardiac development by establishing a posterior-to-anterior gradient of RA signaling, leading to normal patterning of both posterior and anterior heart segments [22]. Regulating the RA signaling pathway during in vitro cardiac differentiation can achieve ventricular/atrial CMs-specific differentiation [23]. Therefore, in vitro differentiation of hPSCs into hCOs should accurately replicate regulatory pathways by mimicking the sequential steps of in vivo embryonic heart development. To achieve efficient and controllable protocols for generating hCOs, small molecules (SMs) and growth factor screening tools have been developed. Two main strategies for hCOs generation exist - Monolayer-Based differentiation and 3D De novo differentiation-both of which are described below in detail (Fig. 1 and Fig. 2).

2.1. Monolayer-Based Differentiation of hCOs

Monolayer culture is a widely used method for differentiating hPSCs into CMs. The GiWi and CDM3 protocols are representative approaches that use small molecules to modulate the canonical Wnt/β-catenin signaling pathway, resulting in efficient hPSC differentiation into CMs [24]. The GiWi method uses CHIR99021 (1 day) and IWP2/4 (2 days) with a 2-day gap between the two small molecule treatments, while RPMI1640/B27 without insulin serves as the basal culture medium [25,26]. The CDM3 protocol is an

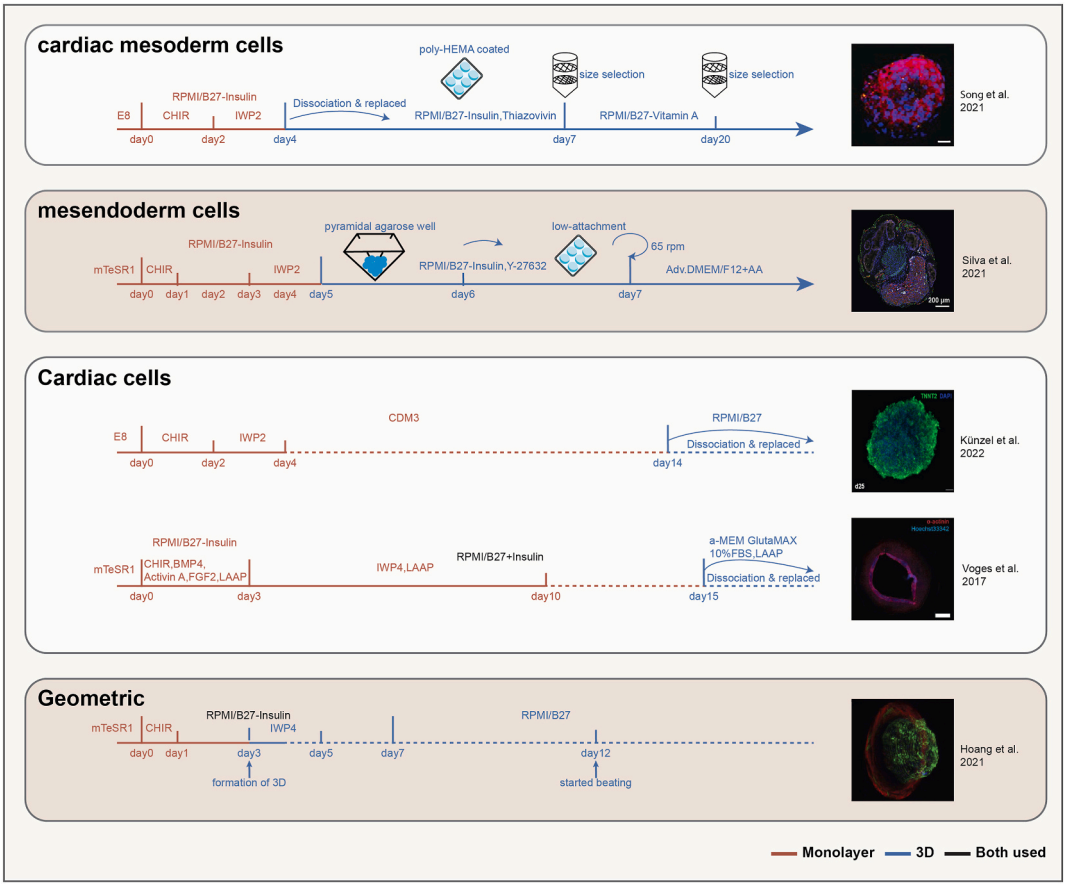

Fig. 1. Monolayer-Based Differentiation of hCOs. 3D starts with cardiac mesoderm cells, reproduced with permission [37]; 3D starts with mesendoderm cells, reproduced with permission [35]; 3D starts with cardiac cells, reproduced with permission [29,31]; Geometric to start 3D, reproduced with permission [39].

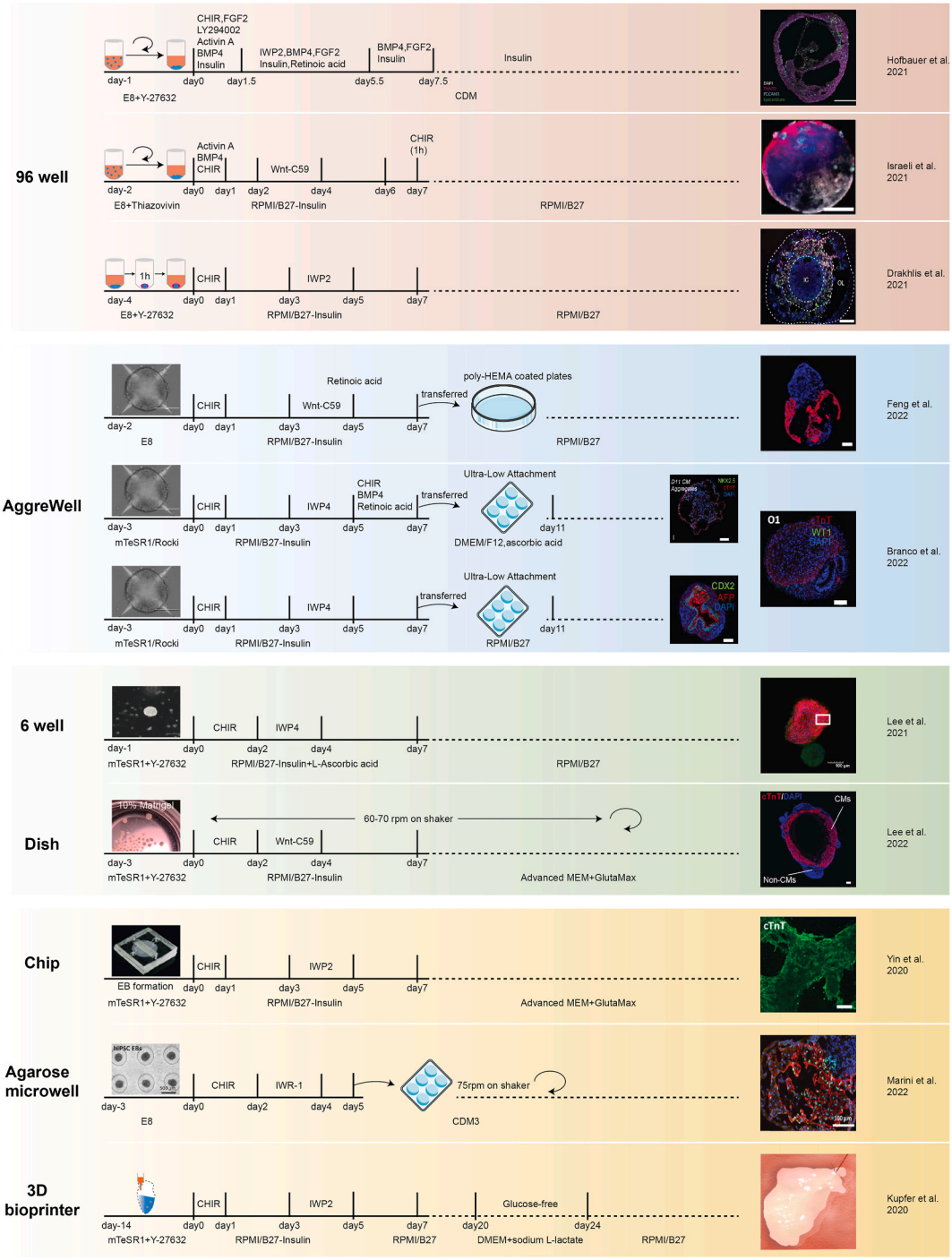

Fig. 2. Presents the 3D differentiation of in vitro human cardiac organoids using various methods, including low adherent 96-well and 6-well plates, agarose microwell chips, AggreWell plates, and dish-based methods, as well as a 3D bioprinter. All images have been authorized for redistribution, except for the image of the 3D bioprinter, which was generated by the authors.

optimized version of the GiWi method, wherein B27 components are progressively removed, identifying the essential differentiation factors and resulting in a CDM3 medium consisting of RPMI 1640 basal medium, L-ascorbic acid 2-phosphate (AA 2-P), and BSA. Differentiation in the CDM3 method uses CHIR99021 (2 days) followed by Wnt-C59 (2 days) [21]. The two methods differ slightly in regulating the Wnt signaling pathway and using inhibitors, but both are highly effective in inducing CM differentiation. During monolayer differentiation, hPSCs undergo sequential differentiation steps (i.e., mesoderm, cardiogenic mesoderm, and cardiac

progenitors) to generate functional beating CMs and other cardiac cells, as demonstrated by these classic methods [27].

2.1.1. 3D starts with cardiac cells

Cardiomyocytes, fibroblasts, and endothelial cells are the fundamental units of heart tissue [28]. To differentiate hPSCs into CMs, a common approach is monolayer culture [21,25]. hCOs are formed by assembling cardiac cells from a monolayer culture into a 3D structure. A protocol developed by Karolina Künzel uses CDM3, CHIR, and IWP2 in monolayer culture for 14 days, followed by single-cell digestion and transfer to 96-well U-shaped low-adhesion plates to form hCOs [29]. Although the hCOs contained 72.0% CMs, 11.1% FBs, and less than 1% ECs, the CDM3 medium is optimized for CMs and not for non-CMs' survival. As a result, RPMI1640 + B27 replaces the CDM3 medium during hCO culture. This method provides a robust 2D differentiation protocol that allows high-throughput experiments without a supporting scaffold. However, differentiation efficiency in the first 14 days varies among patient-derived iPSC lines, and further protocol optimization is necessary. The protocol also needs to increase ECs differentiation efficiency and introduce other cardiac lineage-specific cell types like endocardial and epicardial cells, SMCs, and immune system cells. While this approach involves fewer protocol steps than current methods, it has less sophisticated cardiac architecture, chamber-like structure development, and germ-layer interaction. The size of hCOs is limited because an inadequate or excessive number of resuspension cells will result in poor hCOs formation. A 96-well device called the HeartDyno is used by Richard J. Mills and Holly K. Voges for automatic formation of dense muscle bundles, with each hCOs generated by mixing 5 × 10^4 cardiac cells with collagen I. The resulting hCOs display immature human myocardium characteristics, with lower expression of adult sarcomeric isoforms like β-MHC, MLC2v, and TNNT2, and higher expression of fetal sarcomeric isoform (MLC2a) compared to native adult human heart tissue [30,31]. Monolayer differentiation of cardiac cells using the CDM3 and GiWi methods results in a high proportion of CMs, exceeding 90%. Even after introducing additional growth factors, the proportion of CMs remains as high as 70%. However, it is vital to note that the initial goal of this method was to obtain highly purified CMs, and further modifications are necessary to engineer hCOs that more accurately reflect the cell ratios of the human heart.

2.1.2. 3D starts with mesendoderm

The mesendoderm (ME) is a crucial structure in mammalian embryo development, giving rise to both mesoderm and endoderm [32]. In vitro, chemically defined formulations have been developed to induce human pluripotent stem cells (hPSCs) into ME, with Wnt and TGF-β signaling playing essential roles [33,34]. Ana C. Silva et al. recently developed a human multilineage iPSC-derived organoid that mimics the cooperative development and maturation of the heart and intestine [35]. Early mesodermal progenitor cells were generated through CHIR and IWP2 modulation of the Wnt signaling pathway in a monolayer differentiation. The derived ME was then cultured in 800 mm pyramidal agarose wells at a density of 2.4×10^4 cells/well in 10 mM Rocki medium before being transferred to low-adherence tissue culture plates and subjected to rotational suspension culture. On day 7, the authors observed that separating the multilineage and conventional media produced distinct organoids. The multilineage medium resulted in both cardiac and gut tissues, while the conventional medium only developed cardiac tissue. The multilineage organoids underwent progressive development of cardiac muscle cells over 30 days, followed by the emergence of endodermal cells, remaining in an early stage of development after 100 days of culture without spontaneous contractility. Electrical stimulation induced contractile behavior, but no luminal structures resembling chambers were observed, possibly due to the early stage of development. The authors did not explain the relationship between gut cell formation and the culture medium and further exploration is necessary to clarify this issue.

2.1.3. 3D starts with cardiac mesoderm cells

Vertebrate development involves differentiating cardiac myocytes (CMCs) from a mesodermal lineage, giving rise to the first and second heart fields (FHF and SHF) [36]. Myeong-Hwa Song developed a method for deriving cardiac organoids, called CMC-COs, from hPSC-derived CMCs [37]. This process induced CMC differentiation by using small molecules CHIR and IWP2 in hPSCs, followed by size selection and replating onto poly-HEMA-coated plates. Cultivating the CMC-COs in RPMI/B27-Vitamin A medium resulted in mature ventricular-like functions, including well-organized sarcomeres, mitochondria, T-tubule structures, and intercalated discs. However, the proportion of cell types in CMC-COs differed significantly from that of the natural heart [28,38]. Also, the organoids were smaller and lacked cavity structures. Notably, 3D culture can determine the fate of CM's subtypes. CMC-hCOs primarily composed of ventricular CMCs and obtained by replating at the CMC stage, had greater structural and functional maturity than CMs-hCOs, obtained by replating from CMC monolayers that continued differentiation. The author seeded 2×10^6 CMCs per poly-HEMA-coated 6-well to spontaneously form hCOs, which varied in size and required two additional filtrations using a cell strainer. These steps increased the complexity of the process, posing a risk of potential cell damage.

2.2. 3D de novo hCOs differentiation

Embryonic development in vivo is a complex 3D process [40]. To replicate this process in vitro, hPSCs are differentiated into hCOs within a 3D environment. This involves dissociating 2D-cultured hPSCs into single cells and using a stem cell medium, with or without a Rock inhibitor, to promote the formation of hPSC spheres or embryoid bodies (EBs). The critical difference between these methods is how the 3D structure is generated.

2.2.1. Aggregation

2.2.1.1. Self-aggregation. Fangchao Yin used a custom-designed chip to seed and cultivate hiPSCs, promoting spontaneous EB sphere formation using mTesR1 and Y27632 rock inhibitor in the lower channel. RB-medium, containing CHIR and IWP2, was used to generate hCOs [41]. Similarly, Su-Jin Lee used low-adhesion 6-well plates and replaced IWP2 with IWP4. LAAP and ZM447439 were added to RB-medium, with ZM447439 increasing the expression of non-CMs and CMs during hCO generation [42]. Seul-Gi Lee modified the protocol by introducing ECM components to support the organoid structure geometrically, to enable organoid scale-up in a 60 cm dish [43]. The authors induced EB formation using mTeSR™1 medium, 10 μM Y-27632, and 10% matrigel. C-59 was also used to inhibit the Wnt signaling pathway during hCO differentiation. The differentiation process was conducted on a shaker to prevent EB aggregation and sedimentation [43]. This approach, relatively easy to operate, leads to hCOs of varying sizes with a complex structure sacrificed. The authors classified the hCOs into five groups - degradation, large sphere, large oval, large cyst, and chamber formation. Interestingly, some of the hCOs showed chamber structures, which was interpreted as differences in the number of cells in the organoid formation process [43]. However, the use of the EB method leads to an inherent limitation of hCOs of varying sizes.

2.2.1.2. Forced-aggregation. Wei Feng utilized the AggreWell™800 plate to create uniform embryoid bodies (EBs), an array of standardized microwells with each well measuring 800 μm, and then introduced CHIR and C-59 to RB-medium to trigger hCOs formation from the EBs. The cell aggregates were transferred to tissue culture plates coated with 5% Poly (2-hydroxyethyl methacrylate) to form hCOs [44]. Mariana A. Branco also employed AggreWell to generate uniform EBs and used CHIR and IWP4 to produce hCOs, which were mainly composed of CMs. BMP4, CHIR, and RA were added from day 5 of the progenitor cells to day 7 of differentiation to form a multilineage pro-epicardium/foregut organoid, mimicking the co-emergence of pro-epicardium, septum transversum mesenchyme, and liver bud. After digesting the derived organoids into single cells, a self-organized hCOs containing an epicardium-like layer surrounding a myocardium-like tissue was formed. These hCOs showed structural similarities with early and transient embryonic structures containing PE, STM, and posterior foregut. The resulting hCOs were categorized into three types - intact, holes, and cavities - despite having no differences in cell number. The ratio of ventricular and atrial CMs was regulated by selectively supplementing with RA [45]. Multilineage heart organoids provide an opportunity to investigate cell-cell interactions between different germ layers during early heart development [46].

Pablo Hofbauer used 96-well U-shaped low-adhesion plates to regulate the size of EBs and control cardiac differentiation in a study published in Cell [47]. Hofbauer's differentiation approach involved temporal control of key cardiogenic signaling pathways, including Activin A, BMP, FGF, RA, and WNT, resulting in cardioids, which are cavity-containing cardiac structures. However, this method requires several costly molecules and lacks epicardial cells. Co-culturing with 2D-differentiated epicardial cells is necessary to simulate injured tissue. Additionally, the author found that extracellular matrix (ECM) was not required. Yonatan R. Lewis-Israeli simplified the differentiation process by reducing the number of factors used. He only utilized CHIR, Activin A, BMP4, and C-59 and did not co-culture the hCOs with epicardial cells. Epicardial cells were induced by treating the cells with CHIR for an hour on the seventh day of differentiation [48]. The resulting hCOs had smaller cavities compared to those produced by Hofbauer's method [47].

Lika Drakhlis reduced the number of molecules used to only CHIR and IWP2, with the Matrigel being necessary for EB formation [49,50]. Only 5000 hESC cells as the starting amount could successfully generate hCOs. The resulting hCOs contained a compact myocardial layer enclosing an eGFP-negative inner core and covered by an outer layer of NKX2.5-eGFP-positive and -negative cells forming a half-shell-like structure. The hCOs are composed of a myocardial layer lined by endocardial-like cells and surrounded by septum-transversum-like anlagen. The hCOs expressed 75% ventricular marker and 5.7% atrial marker, as well as 88% NKX2-5 positive cells. On the other hand, the hCOs contained 7.5% of the pluripotency genes. The percentage of fibroblasts was low. The hCOs significantly increased in size over time, owing to an increase in outer layer cells. However, after 146 days of culture, only CMs remained, implying that the current hCOs culture medium cannot support non-CMs for an extended period. Vittoria Marini used only CHIR and C-59 to generate cavity-containing hCOs, using agarose microwells to regulate the size of EBs [51]. To better resemble the actual morphology of the heart, Molly E. Kupfer developed an optimized bio-ink formulation, which can be 3D printed into complex geometries. The bio-ink contains hiPSCs at 15 million cells/ml with 10% GelMA, 0.25% ColMA, 93.75 μg/ml LN and FN, 0.5% LAP, and 5 μM ROCK inhibitor. After a 14-day culture period, the constructs were differentiated with CHIR and IWP2, resulting in hCOs with contiguous and thick muscularization, electrical connectivity, and pump function, closely resembling the actual structure of the heart with complex geometries [52].

The current protocols discussed allow for the efficient generation of structurally complex, functionally mature, and cell composition-rich hCOs using simple and inexpensive manipulations.

2.3. hCOs assembly from various cells

Mahesh Devarasetty utilized a combination of commercial hiPSC-CMs and human primary cardiac fibroblast (hCF) cells to generate hCOs. The cells were mixed in a 9:1 ratio and then seeded at 1000 cells/organoid into non-adherent, round-bottom, 96-well plates using CMs maintenance medium [53]. The resulting organoids reached stable diameters of approximately 250 μm and began spontaneously beating. These organoids were viable in culture for over four weeks and continued to beat over that time frame. Michele Filippo Buono combined healthy and HCM-associated hiPSC-CMs with human cardiac microvascular endothelial cells (HCMECs) and HCFs in a ratio of 3:2:5 and then pipetted 100,000 cells in 20 μL cell suspension into each well to form hanging drops, generating highly contractile triculture organoids. The resulting hCOs were transferred to Poly-L-Lysine-coated 12-well plates for long-term culture and

maintained in a CMs maintenance medium for 21 days. The authors observed that the longer the triculture period, the more contact between CMs and non-CMs was established, resulting in more physiological-like hCOs [54]. Dylan J. Richards and his colleagues used hiPSC-CMs, human ventricular cardiac fibroblasts (cFBs), and human umbilical vein endothelial cells (HUVECs) to fabricate hCOs. The cells were injected into agarose hydrogel molds according to preset cell ratios, with the medium mixed in the same ratio. Human adipose-derived stem cells (hADSCs) were added to promote endothelial function. hCOs with hADSCs were fabricated with a 50% hiPSC-CMs, 29% cFBs, 14% HUVECs, and 7% hADSCs ratio [55–57]. Although these methods are relatively simple to operate, the acquisition of commercial and primary human cells is limited, and the inconsistent genetic backgrounds of the different cells obtained in the resulting hCOs are a critical limitation.

Fengzhi Zhang developed a method for generating hCOs using hiPSC-derived CMs, ECs, and SMCs in a 3D ECM-rich gel with the addition of 20% Matrigel to maintain structural integrity and provide signals from the extracellular matrix [58]. Asher Kahn-Krell developed a 3D culture-based process using hiPSC-CMs spheres to create hCOs containing all four cardiac cell types [59]. However, scaffold-free construction resulted in hCOs that were structurally different from the human heart. Camila Hochman-Mendez developed a method for regenerating hCOs using decellularized whole rabbit heart scaffolds recellularized with hiPSC-ECs and hiPSC-derived cardiac cells, resulting in hCOs with a heart-like shape. However, there was significant cell death during long-term culture, and the decellularized scaffold thrombosed when transplanted into the femoral artery bed of an adult pig [60].

In summary, the methods discussed are relatively simple, although they often sacrifice the complexity of the structure of hCOs (Fig. 3). Using primary cells provides patient-specific cell phenotypes but creates challenges for obtaining cells with consistent genetic backgrounds and may face ethical barriers. Using hiPSCs for differentiation solves the problem of inconsistent genetic backgrounds but adds to experimental difficulty and time cost. Although there are limitations, researchers have begun to transplant hCOs into animals, representing a significant step towards organoid transplantation therapy for cardiovascular disease [58,60].

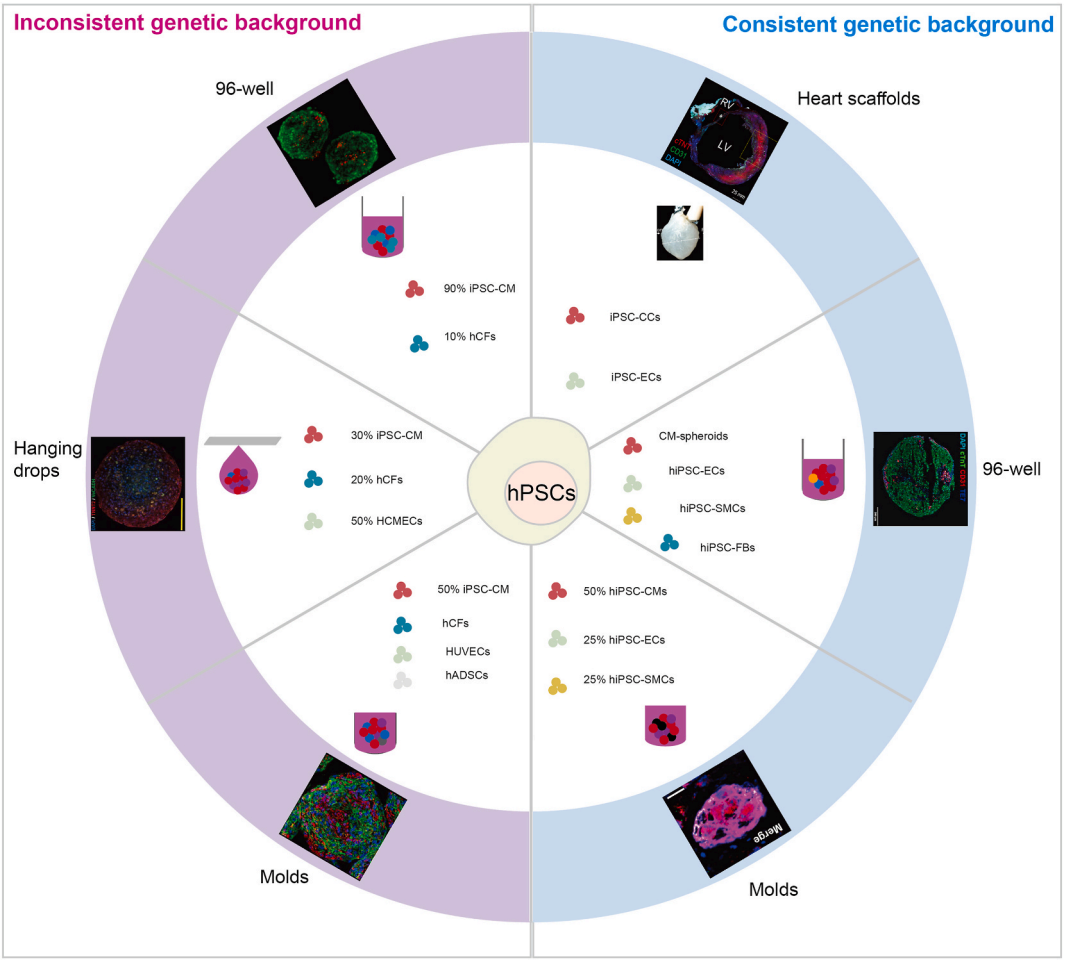

Fig. 3. Displays the assembly of in vitro human cardiac organoids from various cells with different genetic backgrounds on the left, and with genetically identical cells on the right. The left panel shows the techniques employed in creating such organoids, including the use of low adherent 96-well plates, hanging drops, and molds. In contrast, the right panel shows methods utilizing heart scaffolds, low adherent 96-well plates and molds, with cells having the same genetic background. All images have been authorized for redistribution.

Table 1
Overview of current protocols for in vitro hCOs generation.

Methodology	Cell resource	Small molecular	Beating initiation	Composition	Structure	Size (~)	Function assay	Ref
Monolayer-Force aggregation 96 well	hiPSC	CHIR、IWP2	Day 15	CMs/FBs /ECs/Others	Round shape intact	1000 μm	Doxorubicin toxicity tests	[29]
Monolayer-Force aggregation Heart-Dyno	hESC	LAAP/BMP4/ Activin A/FGF2/ CHIR/IWP4	Day 20	CMs/ECs/Stromal cells	Circular	NA	Pro-regenerative drug screen Cryoinjury	[30, 31]
Monolayer-Force aggregation pyramidal agarose well	hiPSC	CHIR/IWP2	Day 15	CMs/SMC/FBs/Gut cells/Proliferating cells/ enteroendocrine cells	Round shape intact	2000 μm	Embryonic heart and gut development	[35]
Monolayer-Self aggregation 6 well	hESC &hiPSC	CHIR/IWP2	Day 7	CMs/ECs/SMA FBs	Round shape intact	70–100 μm	Mature and ventricular hCOs	[37]
3D de novo EB 6well	hiPSC	CHIR/IWP4/ LAAP/ZM447439	Day 8	CMs/FBs/ECs	Round shape intact	400 μm	Nifedipine, tetrodotoxin (TTX), and E−4031 response	[42]
3D de novo EB chip	hiPSC	CHIR/IWP2	Day 10	CMs	Round shape intact	NA	Safety assessment of antidepressant drugs	[41]
3D de novo EB Dish	hiPSC	CHIR/C-59	Day 10	CMs/FBs/SMA/ Epicardial cells/ EPDCs	Degradation, large sphere, large oval, large cyst, and chamber formation	Up to 1700 μm	In vivo vascularization	[43]
3D de novo EB aggreWell	hiPSC	CHIR/C-59 R A	Day 15	CMs/FBs/epicardial cells/ECs/non-heart-cells	Intact, holes, and cavities	1000 μm	Chamber defects	[44]
3D de novo EB aggreWell	hESC &hiPSC	CHIR/IWP4/RA/ BMP4	Day 11	CMs/pro-epicardium cell/liver cell/ECs	Cavity	800 μm	Epicardium-myocardium interaction	[45]
3D de novo EB 96 well	hESC &hiPSC	CHIR/IWP2/ BMP4/Activin A/ RA/FGF2/ LY294002	Day 5	CMs/FBs/valvar cell/ECs	Cavity	2000 μm	Injury model	[47]
3D de novo EB 96 well	hESC &hiPSC	CHIR/C-59/ Activin A/BMP4	Day 6	CMs/epicardial cells/ECs/ endocardial cells	Cavity	1000–1500 μm	Pregestational diabetes induced CHD	[48]
3D de novo EB 96 well	hESC	CHIR/IWP2	Day 10	CMs/ECs/AFE progenitors/ mesenchymal cells/ Liver anlagen/PFE/ Pluripotent cells	Three layers: IC, ML and OL, Cavity	2000 μm	NKX2.5-KO phenotype	[49]
3D de novo EB Agarose microwell	hiPSC	CHIR/C-59	Day 8	CMs	Cavity	500 μm	Duchenne Muscular Dystrophy	[51]
3D de novo EB Bioprinted hChaMP	hiPSC	CHIR/IWP2	NA	CMs/SMA/ECs	Heat shape	1.3cm–2.3 cm	Macroscale chambered model	[52]
hCOs assembly from various cells	hiPSC-CM hCF	NA	Day 4	90% hiPSC-CM 10%hCF	Round shape intact	250 μm	Optical Tracking and Digital Quantification of Beating Behavior	[53]
	hiPSC hiPSC-CM hCF HCMECs	NA	Day 2	30%hiPSC-CM 20%hCF 50%HCMECs	Round shape intact	>400 μm	HCM model	[54]
	hiPSC-CM hCF HUVECs hADSCs	NA	Day 10	50% hiPSC-CMs and 50% non-myocyte (at a 4:2:1 ratio of hCF:HUVECs: hADSCs)	Round shape intact	200 μm	Myocardial infarction and drug cardiotoxicity	[55]

(*continued on next page*)

Table 1 (*continued*)

Methodology	Cell resource	Small molecular	Beating initiation	Composition	Structure	Size (~)	Function assay	Ref
	hiPSC	CM: CHIR/IWP2 ECs：CHIR/VEGF FGF2 SMCs：CHIR/PDGF TGF-β1	Day 3	50% hiPSC-CMs 25% hiPSC-ECs 25% hiPSC-SMCs	Cavity	50–200 μm	Transplantation	[58]
	hiPSC	CM: CHIR/IWR-1-endo ECs：STEMdiff Endothelial Differentiation Kit SMCs：PDGF-β、TGFβ hCF：CHIR/bFGF	Day 1	hiPSC-CMs:hiPSC-ECs:hiPSC-SMCs:hiPSC-CFs = 4:2:1:1	Round shape intact	Up to 1100 μm	Maturity	[59]
	hiPSC	hiPSC-CCs hiPSC-ECs	NA	hiPSC-CCs(~800 million) hiPSC-ECs(~100 million)	Heat shape	3.2cm–4.1 cm	Transplantation	[60]

CHIR: CHIR99021; LAAP, L-ascorbic acid 2-phosphate; BMP4, bone morphogenetic protein 4; FGF2, basic fibroblast growth factor; IWP2/4, inhibitor of Wnt production 2/4; C-59, Wnt-C59; RA, retinoic acid; CM, cardiomyocyte; ECs, Endothelial cells; VEGF, vascular endothelial growth factor; SMCs, Smooth muscle cells; TGF- β1, Transforming Growth Factor-Beta1; PDGF, Platelet-derived growth factor; hCF, human cardiac fibroblast; CCs, cardiac cells.

3. Conclusions and discussion

Currently, there are increasingly more methods for generating hCOs [61–63]. We should carefully choose the research model regarding to the research purpose. The assembly of hCOs from various cells is more suitable for drug screening and cell interactions studies in CVD, as the type and number of cells in their assembly are controllable. hCOs differentiation from hPSCs is more suitable for heart developmental studies to investigate the role of genes or signal pathway in cardiac development, and is also suitable for hereditary cardiomyopathies. However, the current methods used to engineer hCOs still have limitations and do not closely resemble the actual morphology of the heart or cover a large region of it(Table 1). While significant progress has been made, challenges remain to fully realize the potential of hCOs as models for studying heart disease and developing new therapies. Key challenges include improving cardiac cell differentiation efficiency from hPSCs, developing reliable methods for characterizing the physiological properties of hCOs, enhancing vascularization, scaling up production for high-throughput screening, and developing standards for hCOs to ensure their reliability and reproducibility across labs. Addressing these challenges will enable the use of hPSC-derived hCOs in cardiovascular research, revolutionizing our understanding of heart development, disease, and treatment.

Furthermore, while hCOs are a promising tool for studying human heart development and disease, there remain ethical and regulatory challenges for using these models in drug discovery and transplantation therapy. Also, there is a need for more significant investment in the development of alternative testing and discovery models for improved safety and ethical standards.

Finally, while hCOs have potential, they are likely limited in their ability to fully recapitulate the complexity and dynamics of in vivo heart development and disease. Human bodies are far more intricate, and cell interactions far more multifaceted than what current in vitro models can replicate. Therefore, the continued use and refinement of both hCO models and in vivo models will be necessary to expand our understanding of heart development and pathogenesis, and to develop novel therapies for patients with cardiovascular disease.

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Data availability statement

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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