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Engineering cardiology with miniature hearts

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ABSTRACT

Cardiac organoids offer sophisticated 3D structures that emulate key aspects of human heart development and function. This review traces the evolution of cardiac organoid technology, from early stem cell differentiation protocols to advanced bioengineering approaches. We discuss the methodologies for creating cardiac organoids, including self-organization techniques, biomaterial-based scaffolds, 3D bioprinting, and organ-on-chip platforms, which have significantly enhanced the structural complexity and physiological relevance of *in vitro* cardiac models. We examine their applications in fundamental research and medical innovations, highlighting their potential to transform our understanding of cardiac biology and pathology. The integration of multiple cell types, vascularization strategies, and maturation protocols has led to more faithful representations of the adult human heart. However, challenges remain in achieving full functional maturity and scalability. We critically assess the current limitations and outline future directions for advancing cardiac organoid technology. By providing a comprehensive analysis of the field, this review aims to catalyze further innovation in cardiac tissue engineering and facilitate its translation to clinical applications.

1. Introduction

Cardiovascular disease is the primary global cause of mortality, claiming over 17 million lives annually. Ischemic heart disease, caused by obstructions or spasms in the coronary arteries, is the main contributor to this mortality, resulting in myocardial ischemia and the permanent loss of contractile tissue [1]. The heart's limited capacity for self-regeneration exacerbates these issues, leading to impaired cardiac function and heart failure. Heart transplantation remains the ultimate treatment for end-stage heart failure; however, the severe shortage of donor organs underscores the urgent need for alternative solutions. Approaches such as mechanical assist devices, regenerative therapies, and bioengineered tissues are being explored to address this gap. Among these, cardiac organoids present a promising avenue by providing a platform for generating functional cardiac tissue that could potentially replace damaged myocardium or support heart regeneration.

Cardiac organoid research began in 2000 with *in vitro* differentiation of cardiomyocytes from human embryonic stem cells (hESCs) [2]. This field advanced significantly in 2006 with human induced pluripotent stem cells (hiPSCs) [3], which reprogram adult somatic cells into a pluripotent state. iPSCs bypass ethical concerns associated with hESCs and provide an unlimited source of patient-specific cardiac cells. Combining iPSCs with bioengineering techniques, such as 3D scaffolds, bioprinting, and organ-on-chip platforms, has dramatically enhanced cardiac model functionality [4]. Scaffolding technologies provide the 3D architecture necessary for cell growth, mimicking the native extracellular matrix (ECM) to strengthen cellular interactions [5]. 3D bioprinting enables precise cells and scaffolds placement, recreating cardiac tissue's architecture and vascular networks [6]. Organ-on-a-chip technology integrates these components into microfluidic devices that replicate the heart's physiological microenvironment, critical for studying cardiac function and drug responses in a human-relevant context [7].

Cardiac organoids address the limitations of traditional animal models, which often fail to accurately reflect human cardiac physiology. Cardiac organoids are 3D, multicellular constructs derived from

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pluripotent stem cells that self-organize or are engineered to replicate key structural, cellular, and functional aspects of the human heart. These organoids emulate the heart's microarchitecture, including the arrangement of cardiomyocytes, fibroblasts, endothelial cells, and other cell types, and demonstrate physiological functions such as spontaneous contraction, electrical conductivity, and responsiveness to stimuli. These innovative *in vitro* models are valuable for exploring cardiogenesis, modeling cardiovascular diseases, and exploring novel therapeutics. This review gives a comprehensive analysis of the methodologies for creating cardiac organoids, covering cell sourcing, differentiation protocols, and biofabrication techniques. It discusses the current applications in cardiovascular biology, disease mechanisms, and potential clinical use. Furthermore, it assesses current model limitations and outlines key scientific and technical challenges that must be addressed to fully harness cardiac organoids in biomedical research and regenerative medicine. By synthesizing recent advancements and suggesting future directions, this review aims to spur further innovation in this rapidly evolving field.

2. Techniques in cardiac organoid biofabrication

In this section, four primary methodologies for the fabrication of cardiac organoids are introduced: self-organizing systems, 3D biomaterial-based approaches, 3D bioprinting technology, and heart-on-a-chip platforms. We critically examine the fundamental principles, recent advancements, and future potentials to transform cardiovascular research and therapeutic development. Various methodologies employed in the fabrication of cardiac organoids derived from hiPSCs are summarized in Table 1.

2.1. Self-organizing cardiac organoids

Self-organizing cardiac organoids utilize the innate ability of stem cells to form complex 3D structures that mimic native heart tissue, removing the need for external scaffolds or templates. This approach depends on the precise regulation of key developmental signaling pathways [28]. Critical pathways in heart development are shown in Fig. 1, highlighting the WNT signaling cascade as a central player. Other key components include activin/nodal signaling, bone morphogenetic protein (BMP) pathway, fibroblast growth factor (FGF) signaling (particularly FGF2), and glycogen synthase kinase 3-β (GSK3β) inhibition. Temporal and spatial regulation of these pathways guides pluripotent stem cells through mesoderm induction, cardiac specification, and the formation of diverse cardiac cell types, containing atrial and ventricular cardiomyocytes [28–31].

The first functional 3D heart model *in vitro* was established in 2020 using mouse ESCs [32]. By fine-tuning the FGF4-FGF1 axis, the differentiation of ESCs into cardiomyocytes and other cardiac cell types was well balanced, resulting in organoids that recapitulated both atrial and ventricular structures. Building on these findings, more functional cardiomyocytes are derived from human iPSCs by harnessing developmental processes, particularly through gradient activation of the WNT signaling pathway [33–35]. The conventional two-step WNT approach involves introducing CHIR to stimulate mesoderm induction at the oneset and adding IWP2 to suppress differentiation at the mesodermal stage. This method has been shown to effectively facilitate cardiac mesoderm differentiation. Building on this approach, Drakhlis et al. developed self-organizing human heart-forming organoids by co-inducing cardiac mesoderm and foregut endoderm from human PSCs [12]. Their resulting organoids demonstrated early chamber formation and contained both cardiac and foregut tissues, highlighting the interplay between cardiac and non-cardiac lineages during heart development. Similarly, Rossi et al. employed a gastruloid-based approach to generate cardiac from hiPSCs [36]. By mimicking early embryonic gastrulation through controlled activation of WNT and Nodal signaling pathways, they produced organoids that recapitulate key aspects of cardiogenesis, including the formation of primitive heart tubes and the specification of cardiac cell types. This method captures early embryonic development stages, offers valuable insights into the spatial and temporal dynamics of early heart development.

Hofbauer et al. subsequently reported the first self-organizing human heart-like organ capable of autonomous beating and featuring a structured heart chamber [11]. By removing redundant embryonic development factors and analyzing RNA-sequencing gene expression data, the WNT-BMP-HAND1 axis was identified as a critical regulator of cavity morphogenesis, with HAND1 being an essential transcription factor associated with developmental ventricular defects. While Hofbauer et al. employed an externally derived epicardial induction through an optimized WNT signaling protocol, a novel three-step WNT modulation protocol was utilized to achieve self-organizing human heart organoids with in situ epicardium formation. The three-step WNT protocol for generating more mature cardiac organoid was provided by Lewis-Israeli and Volmert et al. as follows [8,9]: Days 0–7: the two step WNT signaling axis is modulated to promoting early embryonic heart formation from hiPSCs/ESCs through two-step WNT signaling axis modulation. On day 7, a single dose of 2μM CHIR for 1h again transiently activates WNT, stimulating epicardial production; Day 7 onwards: Transferring cells to RPMI/B27 medium for continued cultivation; From day 20: Inducing metabolic shift by lowering glucose concentration to 4 mM and supplementing with fatty acids and L-carnitine to mimic fetal heart metabolism; Additional supplements: Adding triiodothyronine and IGF-1 to promote heart growth and maturation, along with ascorbic acid to mitigate oxidative stress. This comprehensive approach yielded cardiac organoids equivalent to 6–10 weeks of human gestation, marking a significant advancement allow for more accurate modeling of epicardial contributions to heart development and disease. Collectively, above methodologies expand our understanding of cardiac organogenesis and offer valuable platforms for studying congenital heart disease and therapeutic interventions.

2.2. 3D biomaterial-based cardiac organoids

The self-organization of stem cells into cardiac organoids faces reproducibility challenges due to complexity and environmental sensitivity. 3D scaffolds have been shown to induce 3D cell assemblies resembling *in vivo* tissues [5], offering a more controlled and reproducible approach to organoid generation. Cardiac bio-scaffolds can be categorized into decellularized ECM (dECM) and synthetic hydrogels (Fig. 2A), both supporting cell activities with bioactive molecules for growth and integration [37].

The ECM, a complex network of macromolecules, serves as the natural scaffold of tissues [38], providing structural support, organizing tissues, regulating cellular functions, and imparting rigidity and elasticity under mechanical forces [39]. Ott et al. demonstrated the potential of dECM by decellularizing mouse hearts, preserving ECM and architecture, and recellularizing them with neonatal cardiac cells, which contracted and responded to stimuli after 8 days [40]. This method's success has extended to large mammalian hearts [41]. Other work showed the assembly of the laminin/entactin complex with dECM to further reduce mechanical obstruction and enhance tissue organization [32]. dECM has been shown to reduce pathogenic activation by hiPSC-derived fibroblasts, enhancing myocardium development in hiPSC organoids [42]. Cardiac dECM exhibits therapeutic effects in fibrosis reduction and vascular regeneration. In a myocardial infarction mouse model, dECM in a hydrogel reduced wall thinning and enhanced vascularization [43]. Synthetic hydrogels, on the other hand, are highly hydrophilic 3D network structures that can rapidly absorb and retain large volumes of water, mimicking ECM's aqueous environment. They allow diffusion of small molecules, crucial for cell function and are customizable via polymers and crosslinkers for specific purposes [38,44]. For instance, VEGF-bound hydrogels implanted in rats induced significant neovascularization at the graft-host interface [45].

Table 1
Overview of cardiac organoid fabrication techniques, including details on cell sources, methodologies used, structural features of the organoids, and their functional outcomes, and highlighting the diversity in approaches and the resulting characteristics of the organoids.

Year	Cell source	Methodology	Structural features	Functional outcomes	Ref.
2021	hiPSC	Three-step WNT	1. 58.80 ± 1.53 % CMs, 12.49 ± 1.01 % FBs, 13.82 ± 1.54 % Endocardial cells, 1.63 ± 0.21 % Endothelial cells; 2. Endocardial lined chamber; 3. A network of ECs; 4. Well-defined sarcomeres, gap junctions, reminiscent of T-tubules.	1. Robust beating, well-defined action potential waves reminiscent of QRS complexes, T and P waves; 2. Strong regular calcium waves typical of cardiac muscle.	[8,9]
2021	hiPSC	1.Two-step WNT;2.adv. DMEM/F12+Ascrobic Acid	1. 91 % ACMs, 1 % VCs, 43.89 % FBs, 4 % sub-epicardial and capillary endothelium/pericytes/ adventitia cells; 2. Multilineage of cardiac and gut tissues 3. Sarcomere length reached 1.75 ± 0.13 mm.	1. A decrease in spontaneous calcium activity; 2. Respond to higher frequencies of electrical stimulation; 3. Multilineage organoids promote atrial/nodal CM specification and maturation.	[10]
2021	hiPSC	1.WNT-BMP-HAND1; 2. VEGF	1. Containing CMs, ECs and fibroblast-like cells, CMs/ ECs = 41 %(MYL7+) ~53 %(CDH5+); 2. Early left ventricular chamber-like; 3. Organized sarcomeres, interconnected via intercalated discs.	1. Beating at a similar rate and frequency at least 3 months; 2. Injury response.	[11]
2021	hiPSC	1.Matrigel-embedded hiPSC aggregates; 2. Two-step WNT.	1. 2 mm diameter; 2. NKX2.5–eGFP pattern>80 %; 3. 5.7 % atrial-like, 75.5 % ventricular-like; 4. Structured into an IC surrounded by a dense ML, which is covered by loosely arranged CMs and ST-like cells, and ultimately enclosed by MSC.	1. Contraction was typically observed on d7–d10. HFOs showed distinct contraction patterns revealed by calcium imaging.	[12, 13]
2022	hiPSC	Two-step WNT	1. Epicardium and myocardium; 2. Chamber formation (81.9 ± 4.2 %); 3. CMs (60.3 ± 2.0 %) + non CMs (39.7 ± 2.0 %); 4. Sarcomere (11.90 ± 0.14) μm.	1. Vascularization after *in vivo* transplantation; 2. Reproducible high beating efficiency (95.5 ± 2.3 %); 3. A regular and dynamic beating pattern of systolic/diastolic coupling.	[14]
2022	hiPSC, PE/ STM/PFH	1.Three-step WNT; 2. Co-culture.	1. A self-organized heart organoid comprising an epicardium-like layer that fully surrounds a myocardium-like tissue.	1. Isoproterenol, E-4031 and verapamil drug reactivity.	[15]
2023	hiPSC	Two-step WNT	1. Recapitulate the development of all major embryonic heart compartments, including right and left ventricles, atria, outflow tract, and atrioventricular canal.	1. Specific CMs were electrophysiologically homogeneous; 2. Atrial region becoming dominant in pacing.	[16]
2023	hiPSC	Two step WNT	1. Self-paced vascularized human cardiac organoids.	1. Electro-mitochondrial coupling was driven by mitochondrial Ca^{2+} oscillations driving respiration cycles; 2. Epinephrine reactivity.	[17]
2023	hiPSC, hiESC	Three step WNT	1. 17 % VCMs, 17 % ACMs, 3 % VCs, 17 % PEDCs, 1 % EPCs, 18 % SCs, 10 % CPCs, 5 % CCs, and 1 % ECs.	1. Mitochondrial maturation and oxidative metabolism↑; 2. Beating rates 60–80 bpm; 3. Ventricular-like AP.	[18]
2016	hiPSC-CMs	heart-on-a-chip	1. A instrumented cardiac microphysiological device via multi-material 3D printing; 2. Sarcomere length 1.8 μm; 3. Four cell layers	1. Verapamil and isoproterenol reactivity; 2. Anisotropic cardiac tissue.	[19]
2017	hiPSC-CM, hFBs, HUVECs, hADSCs	1.The agarose hydrogel mold; 2. Mixed cell suspensions	1. hiPSC-CMs: cFBs: HUVECs = 5:4:1 or 3:6:1; hADSCs: hiPSC-CMs: cFBs: HUVECs: hADSCs = 50:29:14:7; 2. Z-line formation; 3. Lumen-like structures; 4. MYL2/ MYL7↑; 5. ECM production.	1. Spontaneous beating; 2. The contraction amplitude 0.61 ± 0.25 %; 3. Beat rates: 36.9 ± 8.0 bpm; 4. Verapamil and isoproterenol reactivity.	[20]
2019	hiPSC-CM, hiPSC-FBs. HUVECs	SWIFT	1. Replicate the LAD coronary artery for perfusion; 2. 79 ± 6 % CMs (cardiac troponin T-positive, cTnT+) and 19 ± 6 % stromal cells (cTnT-, Vimentin+); 3. Cell density: 240 million cells/ml, CMs density: 180 million cells/ml.	1. Day 7: beats spontaneously and synchronously; 2. Day 8: the cardiac tissue developed a pervasive sarcomeric architecture; 3. Isoproterenol reactivity.	[21]
2019	hiPSC-CMs, hiPSC-FBs, C2C12	FRESH 2.0	1. Multiscale vasculature (8–50) mm diameter; 2. Tri-leaflet valves, 3. 98 % CMs, 2 % cardiac FBs; 4. Sarcomeric α-actinin + myofibrils.	1. Synchronized contractions, directional APD, and wall thickening up to 14 %; 2. Longitudinal calcium wave ~2 cm/s and anisotropy ratio of ~1.5; 3. Baseline spontaneous beat rate of ~0.5 Hz; 4. Cultured for up to 28 days.	[22]
2019	hiPSC-CMs, hiPSC-ECs	3D sacrificial printing	1. Height: 20 mm; diameter: 14 mm; 2. Including the major blood vessels (LAD, RAD); 3. Chamber: the right and left ventricles; 4. Elongated CMs with massive actinin striation.	1.Signal propagation>10 cm/s; 2. Long-term culturing; 3. ECM remolding.	[23]
2020	hiPSC	3D bioprinting, in situ differentiation	1. Two chambers and a vessel inlet and outlet.	1. Electromechanical coupling. 2. Chambered muscle pumps demonstrated macroscale beating and continuous APD with responsiveness to drugs and pacing;	[24]
2023	hiPSC-CMs NRVMs	3D bioprinting	1. Shear-induced alignment of fibres during ink extrusion provides microscale geometric cues; 2. Scaffold: 8 wt% fibre, 3.4 wt% gelatin and 2.4 wt% alginate.	1. Robust electrical coupling along the direction of printing; 2. Maintained for 14 days in culture with a beat rate of ~0.71 bpm; 3. Synchronized contractions; 4. Ventricular volume 5.94 ± 1.66 % (between contraction and relaxation).	[25]
2023	hiPSC/NRVCs	Sequential printing in a reversible ink template	1. hiPSC expansion and differentiation in situ. 2. Vascularized ventricle (diameter 2 cm, thickness 4 mm); 3. A hierarchical vascular network; 4. Sarcomeres and cellular interconnection.	1. Day 14: cardiac organoids beating; 2. Oxygen transfer efficiency↑; 3. Viability↑.	[26]
2023	hiPSC-CMs	Direct 3D bioprinting	1. Centimeter-sized functional ventricle-shaped (height: 14 mm, diameter: 8 mm); 2. typical striated patterns of α-actinin, ssTnI, and cTnI.	1. Day 3: beating synchronicity; Day 28: up to 20 bpm; 2. Pacing center; 3. Verapamil reactivity; 4. Cultivation for up to 100 days.	[27]

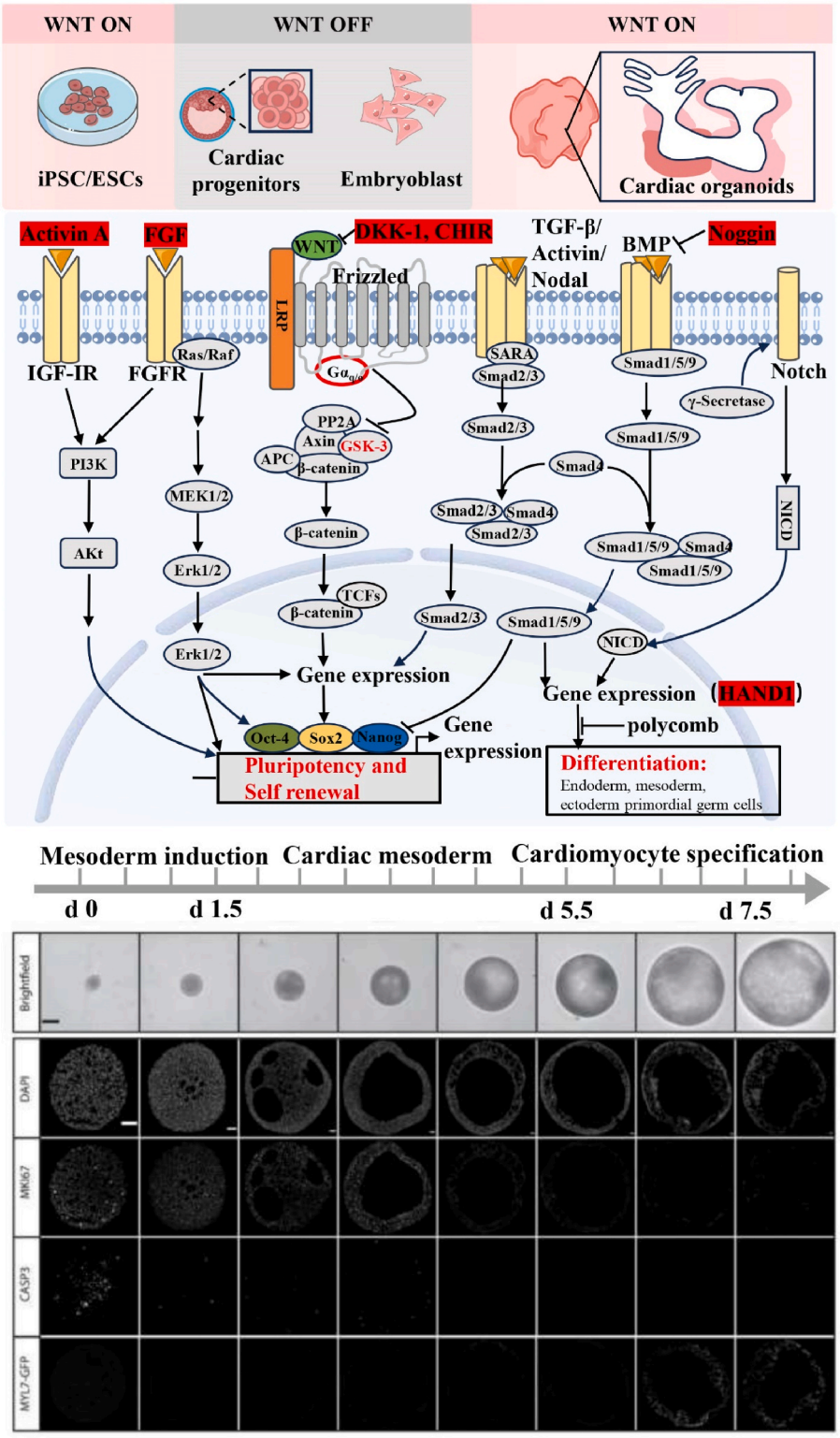

Fig. 1. The molecular and cellular dynamics involved in generating cardiomyocytes from pluripotent stem cells. WNT ON promotes pluripotency and self-renewal, then WNT OFF facilitates cardiomyocyte differentiation into cardiac progenitors and embryoblast, and WNT ON supports cardiac organoids maturation. WNT pathway is central to cardiogenesis, modulating β-catenin-mediated transcription, and Activin A/Nodal, FGF, BMP, TGF-β regulate the differentiation via Akt, Smad and NICD, enabling the sequential specification of hiPSCs to mesodermal, cardiac mesodermal, and beating cardiac progenitors. Oct-4, Sox2 and Nanog are pluripotency genes, HAND1 is a differentiation gene. WNT-BMP-HAND1 axis governs the self-organization of the cavity structures in cardiac organoids. Sequential images under time points (Day -1 to Day 7.5) visualized the cellular progression from pluripotent stem cells to differentiated cardiomyocytes, reprinted from Ref. [11] with permission. DAPI: nuclear stain; MKI67: proliferation marker; CASP3: apoptosis marker; MYL7-GFP: WNT pathway activity.

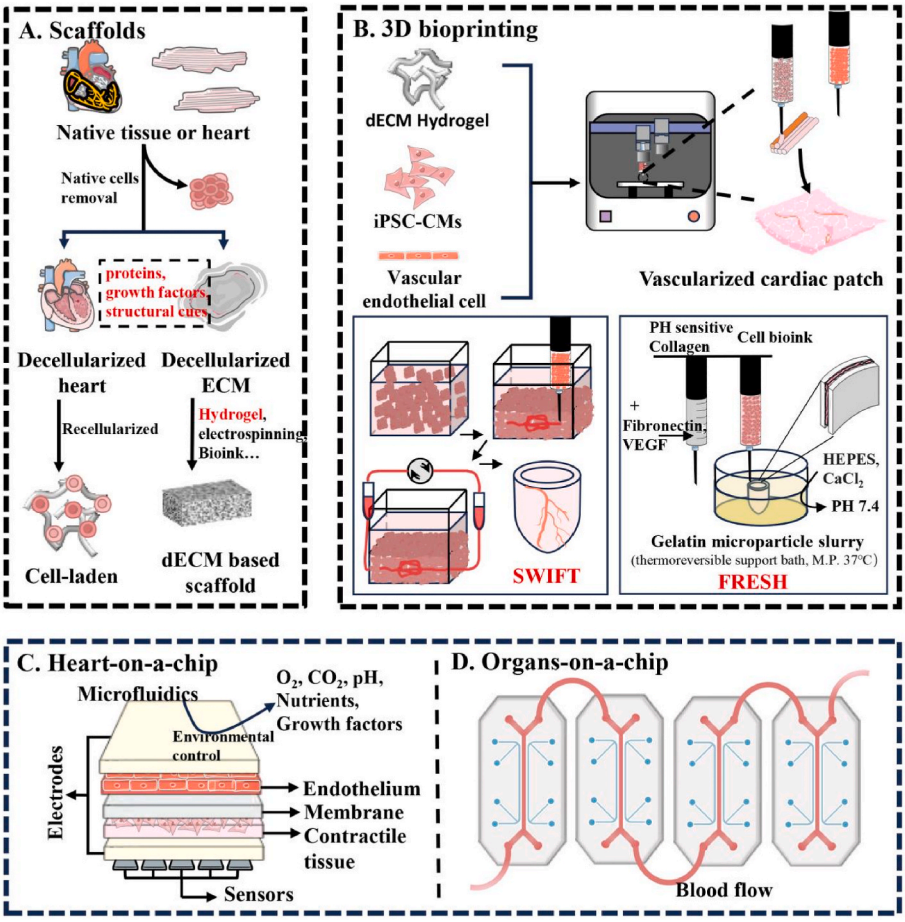

Fig. 2. Advanced techniques in cardiac tissue engineering. (A) Scaffolds: The decellularized ECM (dECM) scaffolds derived from native cardiac tissues retain essential proteins, growth factors, and structural components. (B) 3D Bioprinting: dECM hydrogels are combined with iPSC-CMs and vascular endothelial cells to fabricate vascularized cardiac patches. SWIFT embed cells within a matrix to form perfusable vascular networks, while FRESH employs a gelatin slurry as a supportive bath during printing to maintain structural integrity. (C) Heart-on-a-Chip: Microfluidic heart-on-a-chip devices simulate the cardiac tissue microenvironment by precisely controlling parameters such as O_2, CO_2, pH, nutrients, and growth factors. These platforms incorporate components like endothelial-lined channels, flexible membranes, and contractile cardiac tissues, integrated with sensors and electrodes to monitor physiological responses and tissue function in real time. (D) Organs-on-a-Chip: Extending the heart-on-a-chip concept, organs-on-a-chip systems mimic physiological conditions across multiple organ systems by simulating blood flow and inter-organ interactions. These platforms enable the study of systemic responses and the effects of cardiac function on other tissues within a controlled microfluidic environment.

2.3. 3D bioprinted cardiac organoids

3D bioprinting combines computer-aided design with biomaterials to fabricate structures that closely mimic native cardiac tissue architecture and function [46]. As shown in Fig. 2B, this technique involves the layer-by-layer deposition of bioinks, which are specialized materials containing living cells and supporting biomolecules. In 2019, the first miniature, perfusable heart was created by 3D bioprinting [23]. These bioinks typically include cardiac cells, such as cardiomyocytes and endothelial cells, along with ECM components like collagen and hydrogels. Utilizing high-resolution imaging data, these models are precisely arranged to replicate native heart tissue structure [47].

However, the bioprinting of myocardial tissues often results in smaller, thinner constructs due to challenges in reproducing the complex vascular network necessary for high cell density [48]. Traditional methods struggle to recreate the heart's coronary system, prompting the development of embedded bioprinting techniques with low-viscosity bioinks such as collagen, alginate, and gelatin [49]. Two notable approaches in this field are sacrificial writing into functional tissue (SWIFT) and freeform reversible embedding of suspended hydrogels (FRESH) (Fig. 2B). Gelatin was used as a sacrificial ink in the SWIFT, melting at 37°C to form blood perfusion chambers. Cardiomyocytes showed contractile activity, but tissue was prone to tearing. To overcome this, oxidized sucrose was added to enhance tissue robustness through a Schiff base "click" reaction with native ECM amine groups [50]. Skylar-Scott et al. utilized this by using iPSCs with SWIFT to print cardiac tissues at 200 million cells per milliliter, replicating coronary artery structure, boosting cell viability [21].

FRESH utilizes a pH-driven collagen gel for better control than temperature-sensitive gels, facilitating heart model creation at various scales by leveraging cell remodeling abilities to construct vessel walls [22]. FRESH bio-ink can be optimized by incorporating laminin-111 and fibronectin to optimize stem cell proliferation and differentiation, resulting in heart chamber-like structures viable for over six weeks [24]. A multiscale embedded printing method was recently developed by Zhang et al., which successfully produced full-size human heart models on a decimeter scale using a thermosensitive hydrogel and nanoclay bath for stable support, addressing vessel collapse issues [51]. However, hydrogels provide only temporary support for blood vessels due to their soft nature, leading to potential tissue remodeling and vessel collapse [48]. Ongoing research aims to enhance support systems and printed vascular network stability.

2.4. Cardiac organoids modeled by heart-on-a-chip

Heart-on-a-chip technology has emerged as a powerful tool for modeling human cardiac physiology and pathology *in vitro,* as it replicates key aspects of cardiac structure and function within a microfluidic environment, providing with real-time biological data through integrated feedback systems [52]. It has evolved from simple 2D cardiomyocyte cultures to complex 3D cardiac tissue models, using fabrication methods like 3D printing, electrospinning, and thermoplastic micro-milling to create biomimetic scaffolds [53].

As illustrated in Fig. 2C and D, a cardiac organ-on-a-chip system comprises three main components: on-chip cardiac tissue, microfluidic channels, and integrated sensors. This setup allows for the simulation of human vascular circulation and the replication of electrical, mechanical, and biochemical signals present in the cardiac microenvironment [54,55]. These systems overcome traditional methodological limitations, providing precise *in vitro* simulation of cardiomyocyte stimulation and capturing organ-level contractions with high fidelity [56]. Innovative visualization techniques, such as color-changing hydrogels inspired by chameleons, allow real-time observation of myocardial contractions [57]. Additionally, high-frequency ultrasound adds to these systems' capabilities, enabling non-invasive quantitative studies of systolic kinetics in cardiac chips [58]. To address the inherent softness of biomaterials like hydrogels in constructing vascular-rich organ chips that mimic muscle contraction, bio-scaffolds can be embedded into vessels at the microscale [59].

3. Variance in applications with diverse cardiac organoids

Self-assembled cardiac organoids closely mimic natural developmental processes and capture the cellular heterogeneity of the heart, making them ideal for studying cardiac ontogeny and complex multicellular interactions. In contrast, scaffold-based cardiac organoids offer greater reproducibility and allow for the incorporation of specific design elements such as vascularization and electrical stimulation. Both approaches are designed to replicate cardiac function and serve various applications, as illustrated in Fig. 3. The applications of cardiac organoids are outlined in Table 2.

3.1. Precision disease modeling

Cardiac organoids derived hiPSCs offer superior relevance over traditional animal models due to their accurate replication of human-specific cellular architectures. These *in vitro* models, developed through differentiation and self-assembly of hiPSCs, can intricately replicate the cardiogenesis process [11,36]. By employing single-cell RNA sequencing, the development of congenital heart diseases (CHD) and related mutations in non-coding regions can be meticulously tracked throughout cardiac development [16]. This level of detail was previously unattainable with conventional methodologies. For example, Schmidt et al. developed a human heart model platform that maps the development of the embryonic heart's major chambers, outflow tract, and atrioventricular canal, offering insights into interventricular interactions, signaling pathways, and contraction propagation, and illustrating the effects of mutations, teratogens, and pharmaceuticals on the developing heart, helping to identify how these factors lead to defects [17]. By visualizing cardiac developmental trajectories in cardiac organoids, Kostina et al. successfully reproduced *in vitro* the pathological features of pregestational diabetes induced CHDs, including endoplasmic reticulum stress with abnormalities of ultra-long-chain fatty acid lipid profiles [74]. Furthermore, they demonstrated for the first time a direct role of IRE1-RIDD in lipid metabolism, which could be ameliorated by targeting IRE1.

The integration of CRISPR-Cas9 technology into cardiac organoid allows for introduction or elimination of specific mutations, effectively modeling various CHDs, including construal defects, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), mitochondrial cardiomyopathy, and Duchenne muscular dystrophy (DMD)-associated

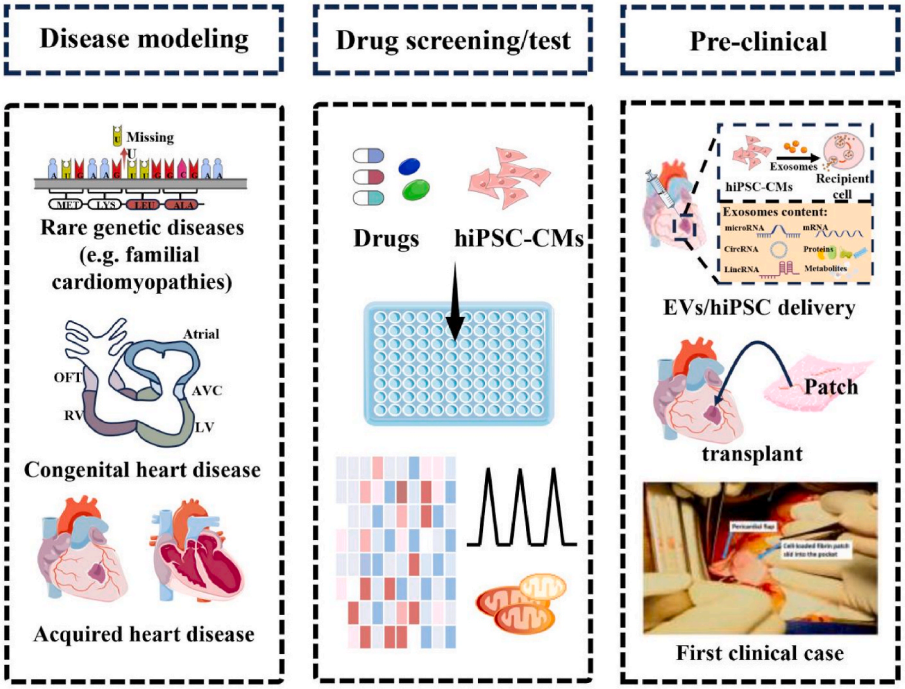

Fig. 3. Various applications of cardiac organoids. Disease modeling: focuses on rare familial cardiomyopathies, congenital heart diseases (including outflow tract, OFT; atrioventricular canal, AVC; right ventricle, RV; left ventricle, LV), and acquired heart diseases. Drug test: utilizes high-throughput screening methods to evaluate the efficacy of various drugs on hiPSC-CMs by characterizing cellular function, gene expression and mitochondrial activity. Pre-clinical applications: explores the use of hiPSC-CMs and their exosomes to repair damaged heart tissue. The first clinical case involving a cell-loaded fibrin patch demonstrates the transition from pre-clinical research to clinical application, reprinted from Ref. [60] with permission.

Table 2
Applications of cardiac organoids in disease modeling and therapeutics, including information on specific diseases or conditions modeled, and key findings from the studies, emphasizing the role of cardiac organoids in understanding disease mechanisms, evaluating drug efficacy, and exploring regenerative therapies, showcasing their significance in cardiovascular research and drug development.

Applications	Year	Experimental condition	Key findings	Ref.
Disease modeling	2014	The mitochondrial cardiomyopathy of barth syndrome	1. Modeling the mitochondrial cardiomyopathy of Barth syndrome by heart-on-a-chip; 2. TAZ mutation causes the phenotypes, impairing sarcomere assembly and contractile stress generation.	[61]
	2018	The Short QT Syndrome	1. Established the short QT syndrome model;	[62]
	2018	MYBPC3 deficiency	1. MYBPC3(-) and environmental stresses synergistically lead to contractile deficits.	[63]
	2020	Myocardial infarction	1. Established human cardiac organoids to model of the post-MI tissue; 2. Pathological metabolic shift towards glycolytic metabolism; 3. JQ1;	[64]
	2021	Acquired arrhythmias	1. A 3D human model of cardiac arrhythmia-on-a-chip;	[65]
	2022	Dilated cardiomyopathy	1. Gö 6976 combined with SB 203580, rescued contractile dysfunction in DCM (TNNT2, TTN, LMNA, PLN, TPM1, LAMA2); 2. Mediated by ATF4 and its downstream effector genes, PHGDH and TRIB.	[66]
	2023	Cardiac fibroblast heterogeneity	1. hiPSC-derived organoid fibroblast population displays a high degree of heterogeneity that approximates the heterogeneity of populations in both the normal and diseased human heart; 2. Fibroblasts possessing reparative features;	[67]
	2024	Dilated cardiomyopathies	1. C10orf71 as a causal gene for DCM by contributing to the contractile function of cardiomyocytes.	[68]
Drug screening platform	2017	Tyrosine kinase inhibitor cardiotoxicity screening	1. Cardiac safety index; 2. VEGFR2/PDGFR-inhibiting TKIs elicit a compensatory increase in cardioprotective insulin/IGF1 signaling (phosphorylation).	[69]
	2019	Drug screening in identifies Pro-proliferative	1. Functional screening of 2/5000 small molecules with pro-regenerative without impacting force. (PLK1, cyclin D1); 2. Pro-proliferative compounds synergistic activate the mevalonate pathway and a cell-cycle network.	[70]
	2020	fluidically coupled vascularized organ chips	1. Physiological PK modelling of first-pass drug absorption, metabolism and excretion; 2. Orally administered nicotine (using gut, liver and kidney chips) and for intravenously injected cisplatin (using coupled bone marrow, liver and kidney chips);	[71]
	2022	A heart-liver-bone-skin chip	1. Maintained their molecular, structural and functional phenotypes over 4 weeks of culture; 2. Recapitulated the pharmacokinetic and pharmacodynamic profiles of doxorubicin in humans; 3. Allowed for the identification of early miRNA biomarkers of cardiotoxicity;	[72]
	2024	Intestine-liver-heart-lung cancer microphysiological drug screening system	1. Using the models evaluated the independent drug efficacy of four first-line oral anti-lung cancer drugs, their side effects on multiple organs in a multi-organ system, and the actual drug efficacy after absorption by organs; 2. Model includes a total of 304 microwell channels, enabling the simultaneous on-chip culture of 304 spheroids of the same type for testing four different drugs.	[73]
	2024	Pregestational diabetes-like conditions	1. Targeting IRE1 or restoring lipid levels partially reversed the effects of pregestational diabetes; 2. ROS-mediated stress and CM hypertrophy.	[74]
In vivo transplantation	2012	1.hESC-CMs; 2. Guinea-pig MI model	1. Infarct size: (13.2 ± 0.9 % of the left ventricle in hESC-CM, 14.8 ± 1.4 % in non-CM, and 15.3 ± 1.9 % in PSC-only recipients); 2. Perfusion by the host coronary circulation; 3. Echocardiography: left-ventricle dimensions↑, fractional shortening↓; 4. Arrhythmias: Ventricular tachycardia.	[75]
	2014	1.hESC-CMs; 2. non-human primate MI model	1. Infarct size↓, a-actinin: 98 %; 2. Microcomputed tomography: hESC-CM grafts are successfully perfused by host vasculature and are viable long term; 3. Arrhythmias: premature ventricular contractions and runs of ventricular tachycardia with rate 180 bmp, accelerated idioventricular rhythm.	[76]
	2015	1. Isl-1+ SSEA-1+ hiPSC-CMs; 2. fibrin scaffold; 3.a 68-year-old patient suffering from severe heart failure	1. Over the 3-month follow-up, no complications have occurred; 2. NYHA Class 1 with a LVEF of 36 % and a reduction of both LV end-diastolic and end-systolic volumes (from 161 to 134 ml and from 117 to 84 ml, respectively) without any strengthening of her drug regimen; 3. The akinetic infarct zone which has been cell patch-treated but not revascularized has now become moderately hypokinetic; 4. Rejection reaction.	[60]
	2018	1.hiPSC-CMs, smooth muscle cells, ECs; 2. Fibrin scaffold; 3. Porcine MI model.	1. The engraftment rate was 10.9 ± 1.8 % at 4 weeks after the transplantation.; 2. LVEF↑, LVEDV↓, Infarct size↓; 3. Arterioles: interspersed with αSMA+ and CD31+; 4. Reverse some MI-associated changes in sarcomeric regulatory protein phosphorylation.	[77]
	2023	1.hiPSC-CMs, ECs; 2. Non-Human Primates MI model	1. Maturity: pan-cadherin↑, host-graft structure↑, sarcomeric length↑; 2. EF: 8.893 ± 1.326 % (P = 0.0005); 3. Arterioles: CD31↑; 4. Arrhythmias: Ventricular tachycardia.	[78]
	2024	1. A clinical-grade hiPSC-CM patch; 2. Porcine MI model	1. LVEF: 4 weeks (61.1 ± 5.7 % vs. 46.3 ± 2.3 %, P < 0.01), 8 weeks (60.1 ± 7.5 % vs. 48.6 ± 6.1 %, P < 0.05), and 12 weeks (63.0 ± 6.7 % vs.39.6 ± 9.8 %, P < 0.01); 2. MRI: cardiac function improvements occurred in the infarct-border zone rather than in the infarct zone. 3. ΔIMR: PL (-20.0 ± 28.2 vs. 38.4 ± 12.2, P < 0.05), obtuse marginal branch (-17.0 ± 11.1 vs. 34.3 ± 23.7, P < 0.05); 4. Arterioles: (2542.2 ± 465.3/mm^2 vs. 1732.4 ± 405.2/mm^2, P < 0.05).	[79]

cardiomyopathy [61,63,80,81]. The development of CRISPR-Cas9-mediated homology-independent organoid transgenesis enables efficient generation of human organoids with specific gene knockouts [82]. hiPSCs from both healthy individuals and patients with specific diseases or genetic mutations can be used to construct disease models [83]. Shinnawi et al. integrated patient-specific hiPSC-CMs, CRISPR-Cas9 technology, and tissue engineering to create a novel tool for studying short QT syndrome caused by a single gene mutation leading to arrhythmias [62].

The prevalence of acquired heart disease is significantly higher in models of heart disease and related conditions, particularly with advancements in the cardiac organoid platforms. By integrating atrial-specific cardiomyocytes with fibroblasts and pacemaker cells, O'Hern et al. utilized heart assembloids to model atrial fibrillation, one of the most common cardiac arrhythmias affecting millions of individuals [84]. Besides, Williams et al. explored cardiac conduction abnormalities by using methylβ-cyclodextrin to disrupt gap junctions between hiPSC-CMs, affecting intracellular signaling and Ca^{2+} handling, leading to decreased conduction speed and altered action potentials [65]. Similarly, advanced electrophysiological techniques with hiPSC-CMs models, such as multi-electrode arrays, optical mapping and patch clamping, have greatly enhanced myocardial electrical activity assessment and arrhythmia risk prediction [85–87]. Fernandes et al. demonstrated that epicardial cells could differentiate into fibroblasts through epithelial-mesenchymal transformation, responding to pathological stimuli and mimicking fibroblastic changes in a failing heart [67]. By comparing control organoids with those exposed to stimuli like hypoxia and TGFβ1, they identified several fibroblast subtypes, highlighting the complexity of cardiac fibrosis and identifying targets for anti-fibrotic therapy. Electrical coupling between fibroblasts and cardiomyocytes facilitates signal sharing, potentially impacting cardiac electrophysiology and contributing to arrhythmias [88,89]. By altering experimental conditions, such as inducing cryoinjury, myocardial infarction organoid models have been established [90]. Richards et al. advanced this by creating an oxygen gradient and chronic adrenergic stimulation in cardiac organoids, replicating the infarcted, border and distal heart zones, offering a unique opportunity to study immediate post-myocardial infarction changes [64].

3.2. Redefining cardiac drugs discovery

Cardiotoxicity, characterized by electrophysiology dysfunction or myocardial damage, remains a major cause of failure in drug development. Numerous drug classes, such as antineoplastics, anti-inflammatories, and cardiovascular agents, can trigger cardiotoxic events [91]. hiPSC-CMs offer a human-specific platform for early cardiotoxicity detection. The integration of wireless monitoring systems into these platforms allows for continuous, real-time observation of myocardial contraction and drug effects [92]. Hence, the Cardiac Safety Index (CSI) has been developed to assess drug safety, by evaluating parameters such as cardiomyocyte viability, contractility, electrophysiological properties, Ca^{2+}-handling capacity, and signaling pathways [69,93].

Cardiac organoids have transformed the evaluation of cardiotoxicity in cancer therapies known for cardiotoxic potential. hiPSC-derived cardiac organoids have shown promise in assessing the cardiac safety of chemotherapeutics, including doxorubicin and kinase inhibitors [94]. Chen et al. successfully reproduced doxorubicin-induced cardiac dysfunction in organoids, revealing insights into chemotherapy-induced cardiotoxicity and informing therapeutic strategies [95]. Subsequent studies have explored mitigating doxorubicin's cardiotoxicity through telomerase and targeted gene therapies involving p53 and SLC28A3 [96–98]. The scalability and reproducibility of cardiac organoids significantly benefit high-throughput drug screening, streamlining drug discovery. For example, Mills et al. identified 105 pro-proliferative compounds via the mevalonate pathway from a library of 5000 compounds using a high-throughput platform with human heart organoids [70]. This approach not only accelerates compound screening but also aids in identifying novel therapeutic targets. Perea-Gil et al. used iPSCs with a TNNT2 gene mutation to create DCM models, discovering tyrosine kinase inhibitors Gö 6976 and SB 203580 that ameliorated contractile dysfunction [66].

In addition to their applications in oncology and rare genetic diseases, organoids have been successfully used in drug screening tests for common diseases. O'Hern et al. developed a platform for atrial fibrillation drug discovery, creating a controlled environment that facilitates the screening of anti-arrhythmic agents and investigation of patient-specific responses [84]. This study highlights the effectiveness of cardiac organoids in modeling common cardiac arrhythmias and aiding the development of targeted therapies. Furthermore, cardiac organoids present a promising approach for evaluating drug-induced developmental cardiotoxicity, addressing a critical gap in prenatal pharmacology. As mentioned earlier, Volmert et al. employed self-organizing cardiac organoids to enhance understanding of cardiac development while establishing a pharmacology safety testing platform. They specifically assessed the effects of antiemetic drugs during pregnancy, exposing cardiac organoids to commonly used antiemetics to evaluate the potential cardiotoxic effects on fetal heart development [9].

Innovations in multi-organ platforms now simulate complex physiological interactions to enhance predictive accuracy. Ronaldson-Bouchard et al. developed an organs-on-a-chip system that interconnects heart, liver, bone and skin tissues through a vascular network, effectively replicating human drug pharmacokinetics and the multifaceted effects of doxorubicin [72]. Moreover, Herland et al. combined microfluidics with gut, liver and kidney organoids, achieving predictive outcomes that closely align with patient data [71]. Lee et al. further enhanced this technology by introducing a mesh-assisted, vascularized tissue microfluidic platform capable of accommodating organoids of various sizes, enabling precise analysis of drug responses and improving drug safety assessments within environments that mimic human physiological conditions [99].

3.3. In vivo transplantation

Both hiPSC-CMs and hiPSC-derived fibroblasts can form mechanical and electrical connections with the host myocardium while enhancing myocardial function through paracrine signaling mechanisms [100,101]. These cells secrete growth factors, cytokines, and extracellular vehicles (EVs) that enhance the microenvironment by promoting angiogenesis, reducing fibrosis and decreasing inflammation. A preclinical study has demonstrated hiPSC-CMs-derived EVs improve cardiac function in a myocardial infarction mouse model by delivering microRNAs and proteins to recipient cells, there by increasing vascularization and inhibiting fibrosis and inflammation [102].

Unlike direct injection of hiPSC-CMs or its EVs, cardiac patches allow for precise placement and secure attachment to the heart, improving cell retention and localized treatment efficacy. Integrating biomaterials such as fibrin, collagen and synthetic polymers into these patches enhances their mechanical strength and promotes vascularization and tissue integration. Preclinical trials show significant improvements in cardiac function when hiPSC-CMs are combined with biomaterials for heart repair in myocardial infarction models, including enhanced left ventricular function, decreased infarct size and improved angiogenesis [103,104]. Besides, biomaterials can provide a scaffold for delivering a high density of organized cardiac cells to the damaged myocardium, enhancing cell retention and survival. Miyagawa et al. developed a biodegradable cardiac patch seeded with hiPSC-CMs and applied it to porcine models of myocardial infarction [79]. The patch improved left ventricular function, reduced infarct size, and promoted angiogenesis. A pioneering clinical case involved a 68-year-old patient with severe heart failure who received a fibrin scaffold graft embedded with cardiac progenitor cells, resulting in significant improvement without adverse

effects [60]. In vivo transplantation of hiPSC-CMs offers the potential to remuscularize infarcted myocardium and improve cardiac function.

Advancements have been made in developing 3D cardiac patches that incorporate multiple cell types to enhance therapeutic outcomes. Including hiPSC-derived endothelial cells and mesenchymal stem cells (MSCs) in the patches promotes vasculogenesis and angiogenesis post-infarction [105]. Gao et al. engineered a tri-cellular cardiac patch comprising hiPSC-CMs, endothelial cells, and smooth muscle cells on a fibrin scaffold [77]. Transplantation in rat models led to significant improvements in cardiac function and vascularization. Similarly, Cheng et al. transplanted cardiac organoids composed of hiPSC-CMs and endothelial cells into non-human primates of myocardial infarction [78]. The organoids facilitated neovascularization, reduced fibrosis, and improved cardiac function. Additionally, to ameliorate patch under-vascularization, Sun et al., utilizes adipose-derived revascularized constructs, which significantly increased the survival rate of hiPSC-CMs and improved cardiac function post-infarction in rat models [106].

Functional integration, alongside cell survival, is crucial for the clinical translation of cardiac patches, yet it presents a significant challenge. Electromechanical mismatches between the patch and native tissue can lead to severe complications, including life-threatening arrhythmias. Chong et al. transplanted hESC-CMs into a non-human primate model of myocardial infarction [76]. The transplanted cells survived, engrafted and electrically coupled with the host myocardium, leading to partial restoration of contractile function. However, episodes of ventricular arrhythmias were observed, highlighting a critical safety limitation of this approach. Similarly, Shiba et al. demonstrated that transplanted hESC-CMs could mature and integrate structurally and functionally with the host heart tissue in macaque monkeys, but still suffered from the arrhythmias [75]. In an innovative attempt to address this issue, Tan Y et al. combined cardiac organoids engineered with electrically conductive silicon nanowires, significantly enhancing the formation of an electrically conductive network [107]. This design enhanced electrical signal propagation and cell-cell communication, potentially providing an effective solution to poor eletromechanical coupling. Nonetheless, the long-term biocompatibility of nanomaterials still requires further investigation.

4. Significant challenges in achieving a fully functional engineered heart

As illustrated in Fig. 4, mature cardiomyocytes are structurally and functionally specialized, exhibiting greater efficiency compared to hiPSC-CMs. They possess well-developed myofibrils, organized sarcomeres, and an extensive transverse-tubule (T-tubule) system that facilitates efficient electrical conduction and synchronized contraction. The mature sarcoplasmic reticulum supports calcium-induced calcium release (CICR) effectively, while abundant gap junctions enable rapid intercellular signal transmission. In contrast, hiPSC-CMs have sparse myofibrils, an underdeveloped sarcoplasmic reticulum, and rudimentary or absent T-tubules. Moreover, mature cardiomyocytes are multinucleated, whereas hiPSC-CMs are predominantly mononucleated. The proliferative potential of hiPSC-CMs induces the risk of teratoma or tumor formation upon transplantation [108]. Consequently, their clinical application carries a significant risk of teratoma development. Additionally, prolonged culture increases the likelihood of genomic instability and potential senescence. To mitigate risks associated with immature hiPSC-CMs, stringent differentiation protocols and purification methods are essential. Advanced sorting techniques, such as fluorescence-activated cell sorting and magnetic-activated cell sorting, can enhance cell population purity [109–111]. Furthermore, the introduction of 'suicide genes' by CRISPR-Cas9, which effectively triggers apoptosis, represents a promising approach for eliminating immature cells in cardiac organoids [112].

Reflecting on these cellular-level challenges at the tissue level, hiPSC-CM-based organoids face multiple challenges, as shown in Fig. 5. Morphologically, the undirected cellular arrangement within organoids fails to achieve the synchronized contractions. Electrophysiologically, the electrical signaling and calcium dynamics in hiPSC-CM organoids do not accurately replicate the intricate excitation-contraction coupling of the human heart. Metabolically, hiPSC-CMs organoids predominantly rely on glycolysis rather than the oxidative phosphorylation pathway, resulting in less efficient energy production [113]. Furthermore, recent single-cell transcriptomic analyses have revealed that the heart comprises multiple cell types, including immune cells, adipocytes, mesothelial cells, and neuronal cells [114]. Cardiac organogenesis and function necessitate not only mechanical interactions between diverse cell typers but also biochemical signaling that determines cell fate. Thus, the current spatial cellular organization within cardiac organoids may not accurately reflect the cellular diversity of native cardiac tissue [115]. For instance, most cardiac organoids lack resident cardiac macrophages, which are critical to myocardial injury and repair processes [116]. Hamidzada et al. successfully integrated macrophages into engineered contractile human cardiac microtissues [117]. These macrophages can ingest apoptotic cells during cardiac stress, facilitating matrix formation and degradation. Additionally, they contribute to the maturation of cardiomyocyte sarcomeric proteins, enhance contractility and improve relaxation kinetics. O'Hern et al. combined cardiac organoids with macrophages to recreate physiological immuno-cardiac interactions, highlighted the role of NLRP3 inflammasome in macrophages in the development of atrial fibrillation [84].

Incorporating macrophages into organoid models could provide deeper insights into cardiac regeneration and inflammatory responses. Recent studies have also demonstrated the potential of macrophages to address the dearth of vascularization observed in cardiac organoids. Landau et al. incorporated hiPSC-derived macrophages into heart-on-a-chip, observing the formation of stable and perfusable microvasculature within cardiac tissue [118]. RNA-seq analysis revealed an upregulation of pro-angiogenic and cardiac maturation markers. Vascularization within organoids is critical for supporting cell survival and function over extended periods. However, maintaining the long-term viability of large and complex cardiac organoids poses challenges due to limitations in nutrient and oxygen diffusion, leading to necrotic core formation. Strategies to enhance vascularization include co-culturing with endothelial cells to promote the formation of microvascular networks and incorporating angiogenic growth factors. Bioprinting techniques that allow for the creation of pre-designed vascular channels within organoids are also being developed.

To address these challenges, recent efforts have focused on enhancing the maturation and functionality of cardiac organoids, as illustrated in Fig. 6. Incorporating features such as microgrooves or nanopatterned surfaces promotes cell alignment, organization and contractile function [119]. Earlier attempts at the biological scaffold mentioned above, dECM-based biomimetic scaffold retains native tissue architecture and biochemical cues, several limitations hinder its application. One challenge is that the decellularization process can lead to immunogenic components residue, which may elicit immune responses upon implantation [120]. Additionally, scalability and reproducibility are difficult due to variability in source tissues and differences in decellularization efficiency [121]. Integration of dECM-based constructs with host tissue is often limited by differences in mechanical properties and degradation rates. Given the high oxygen consumption demands of the heart, there is a pressing need for more effective decellularization techniques to create a comprehensive cardiac vascular network that meet the metabolic demands of cardiomyocytes. Moreover, regulatory hurdles and high production costs further complicate the clinical translation of dECM-based therapies.

Despite these challenges, recent advancements in emerging technologies offer promising avenues for improvement. For example, a leaf vein-inspired design has been used to guide cell and fiber organization for action potential transmission [122]. Compared to the control, tubular hiPSC-CMs structures in hydrogels exhibit longer sarcomeres,

higher levels of connexin 43, maturity-related cardiac markers, electrical coupling-related genes and metabolic genes. After 17 days, these tissues demonstrated synchronized contractions, mimicking organized muscle structure and vascular functionality. 3D bioprinting, such as electrospinning and laser assisted 3D bioprinting, provides higher precision 3D spatial cell tissue structure and formation, potentially overcoming the limitations of myocardial anisotropy [123,124]. Furthermore, electrospinning into aligned nanofibres mimics the muscle ECM, directing muscle tissue alignment, and its inherent hyperelastic properties also allow for muscle compliance. Adding silicon nanowires into the cardiac spheroids not only gives topological cues, but also forms an electrically conductive network and strength synchronized contraction, resulting in structural and functional maturation.

Electrical-pacing-induced contraction training has been demonstrated to strengthen the electromechanical properties of hiPSC-CMs [113]. The development of the "Biowire" platform employs electrical stimulation to significantly improve the maturation of hiPSC-CMs [125,126]. Increasing stimulation frequency from 1 Hz to 6 Hz resulted in more refined sarcomere structures, mature electrophysiological properties, and enhanced Ca^{2+} handling capabilities in regulating cardiac muscle contraction. Then, Radisic et al. fabricated a scalable "Biowire II" platform by an automated workflow [127]. Substrates comprising carbon electrodes were subjected to heat pressing in order to create patterned micropores for tissue cultivation. Thermoplastic elastomer/quantum dot (TPE/QD) microfilaments, fabricated through 3D printing, were employed to provide anchoring for the generation of anisotropic myocardial tissue. The Biowire II device combines electrical stimulation and topographical cues to promote the maturation of hiPSC-CMs. Mechanical stretching could also facilitate the maturation of hiPSC-CMs [128]. Polydimethylsiloxane (PDMS), a polymer commonly utilized in the fabrication of organ-on-a-chip devices, offers flexibility and plasticity beneficial for cell culture and in situ measurements of cardiomyocyte contractility. In a microfluidic system combined with a pneumatic pressure channel, the PDMS membrane functions as a strain transducer, flexing in response to mechanical stress [129]. This configuration provides a well-defined mechanical microenvironment for hiPSC-CMs maturation. Replicating the full complexity of native cardiac tissue remains challenging, Ronaldson-Bouchard et al. developed an intensity training platform spanning over four weeks, combining electrical pacing with mechanical stretch to produce more mature cardiomyocytes [113]. The timeline comprises several key stages: Initial Differentiation (Days 0–12): hiPSCs are guided to differentiate into cardiomyocytes through the modulation of the WNT signaling pathway. During this phase, the nascent hiPSC-CMs begin to exhibit spontaneous contractions. Electrical and mechanical stimulation (begin at Days 12): The cells undergo dynamic mechanical stretch and electrical pacing, with the stimulation frequency increasing 0.33 Hz per day, from 2 Hz to 6 Hz. Indicators of maturity include elongated cell morphology, aligned sarcomeres with increased length (up to ~2.2 μm, comparable to adult cardiomyocytes), higher expression of adult isoforms of cardiac proteins (an increase in MYH7 relative to MYH6), and a shift towards oxidative phosphorylation for energy production. As a result, the contractility of the cardiomyocytes improves significantly, along with notable enhancements in their electrophysiological properties. However, several limitations and challenges remain. The extended culture period raises concerns about genomic instability and the potential for cellular senescence. Additionally, the protocol's complexity may impact scalability and reproducibility in different laboratory settings.

Various biomolecules have also been shown to promote metabolic and functional maturation. Using fatty acid-rich maturation media in microfluidic systems or introducing exogenous inhibitors to suppress lactate-stimulated glycolysis can induce a metabolic switch towards oxidative phosphorylation, thereby enabling cardiomyocytes to adapt to energy-intensive work. These strategies have improved mitochondrial structure and Ca^{2+} handling capacity of hiPSC-CMs, leading to more stable beating rates and action potential durations [130,131]. Moreover, glucose deregulation inhibits excessive proliferation of hiPSC-CMs [132]. Hormones play a crucial role in regulating cell maturation. β-adrenergic receptor stimulation by isoproterenol or norepinephrine promotes T-tubule formation and enhances contractility [133]. The

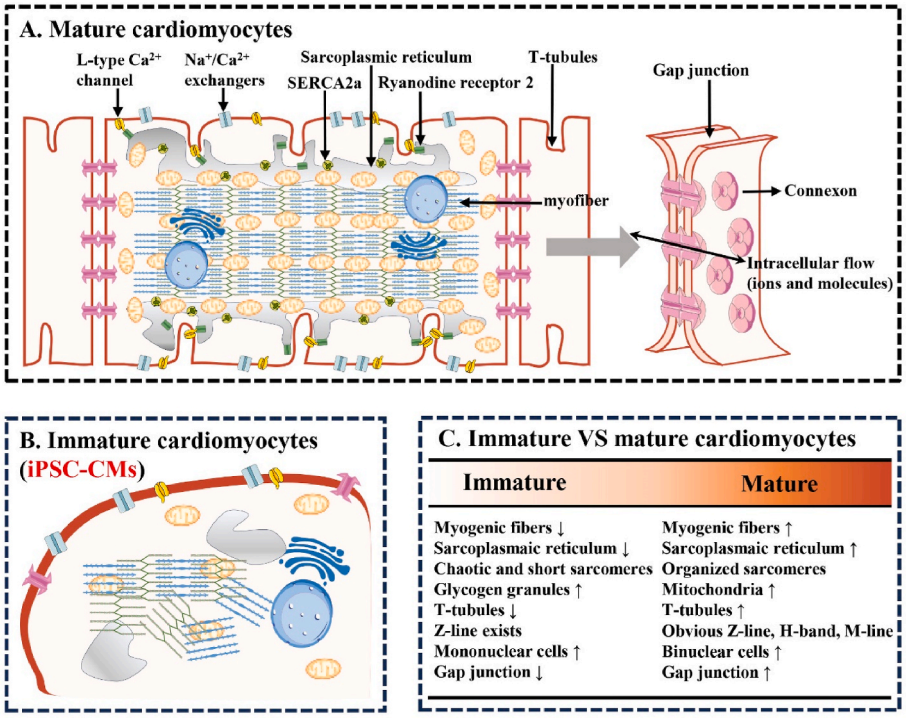

Fig. 4. Comparison between hiPSC-CMs and mature cardiomyocytes. (A) mature cardiomyocytes possess well-organized sarcomeres, extensive T-tubule networks, and efficient calcium-handling systems, enabling synchronized contraction and efficient mechanical performance. (B) hiPSC-CMs exhibit disorganized sarcomere structures, underdeveloped sarcoplasmic reticulum, and lack of T-tubules, leading to compromised electrical conduction and contractile function.

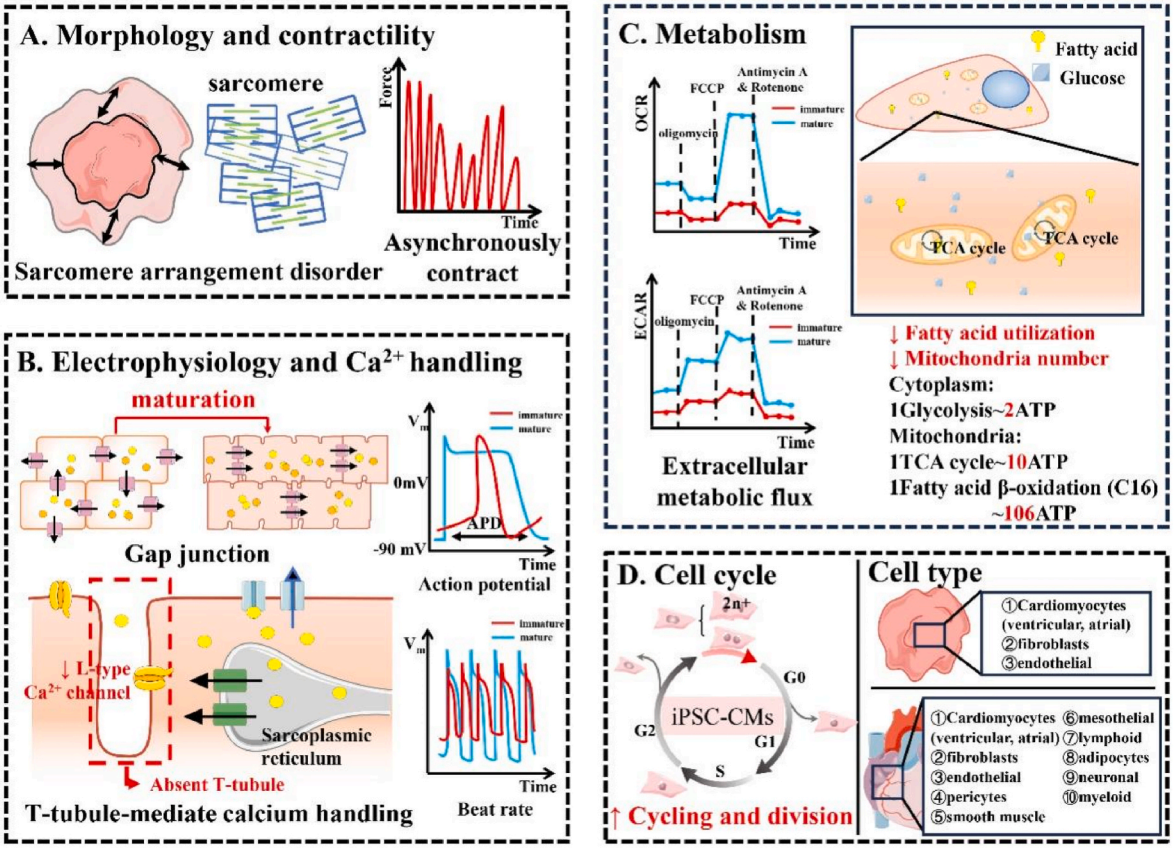

Fig. 5. Multifaceted challenges in maturing hiPSC-CMs organoids into fully functional cardiac tissues. (A) Morphology and Contractility: sarcomere arrangement disorder leads asynchronous contraction and reduced force generation. (B) Electrophysiology and calcium handling: immature action potentials and beat rates are depicted, with deficiencies in gap junction and T-tubule-mediated calcium handling, impacting excitation-contraction coupling. (C) Metabolism: hiPSC-CMs organoids reduce fatty acid utilization and mitochondrial numbers, emphasizing the reliance on glycolysis over oxidative phosphorylation. (D) Cell cycle and cell types: immature cardiomyocytes exhibit increased cycling and division, whereas mature cardiomyocytes exit the cell cycle, achieving a stable, non-proliferative state. The human heart is comprised of ten major cell types, yet most existing heart organoids have only three.

combination of triiodothyronine (T3) and glucocorticoids supports T-tubule development and enhances CICR and excitation-contraction coupling [134]. T3 treatment further increases mitochondrial maximal respiratory capacity and reserve capability of hiPSC-CMs [135]. Paracrine factors secreted from neighboring cells also regulate hiPSC-CM maturation. In co-culture with soluble factors from MSCs, hiPSC-CMs showed more organized myofibers and improved survival during transplantation [136]. Integrating fibroblasts into hiPSC-CM organoids improved electromechanical properties and synchronized mechanical contractions [137]. Future research may still focus on integrating multiple cardiac cell types to better mimic the heart's cellular diversity and on developing biofabrication techniques with precise spatial organization and functional vascular networks. Synergistically combining mechanical, electrical, metabolic conditions and biochemical stimuli may further promote cardiomyocyte maturation.

Deficiencies at the technical level constitute a significant obstacle to the advancement of cardiac organoids. The complexity of cardiac organoid culture protocols affects scalability and reproducibility, and batch-to-batch variability can arise from differences in cell sourcing, differentiation efficiency, and culture conditions [138]. Therefore, standardization of protocols is essential to achieve consistent results. Automation of fabrication processes using bioreactors and robotic systems can improve scalability and reduce human-induced variability. For example, Prondzynski et al. developed a stirred suspension system integrated with a computer-controlled freezer, capable of producing cardiomyocytes with a purity and survival rate exceeding 90% [139].

The intricate 3D structure of organoids at a miniaturized scale complicates detailed characterization using standard microscopy and electrophysiological techniques. Current morphological analysis methodologies primarily rely on thin tissue sections and bioinformatics, lacking intuitive and convenient means of characterization. Recent advances aim to overcome these hurdles. Ming et al. developed a customized spectral-domain optical coherence tomography (OCT) system, enabling nondestructive longitudinal imaging and the propagation of Ca^{2+} signals within the wild-type hiPSC-derived organoid with Ca^{2+} indicator GCaMP6f [140]. Building on this, Hao et al. further refined the approach by integrating dynamic contrast OCT with fluorescence imaging into a dual-modality imaging system [141]. This innovation facilitated the study of the structural contraction and relaxation of beating organoids with high spatial resolution *in vitro*. To conduct functional analyses of organoids, Lachetta et al. introduced a high-throughput platform comprising multielectrode arrays (MEA), capable of recoding the long-term changes in transmembrane ion currents, thus providing suitable for organoid toxicity testing [142]. Expanding on the MEA high-throughput platform, Yin er al. integrated MEA with a resistive skin sensor that directly 'bited' at surface of the cardiac organoid, enabling real-time electromechanical measurements [143]. Additionally, Fang G et al. introduced biocompatible and compressible hollow microlasers to realize all-optical assessments of cellular stress within organoids [144]. Strohm EM et al. developed a noninvasive platform that combined ultrasound with traction force microscopy, allowing for high-throughput and time-resolution analysis of myocardial contractile function in pore plates [58]. The maturation of organoids can be predicted based on the findings from both

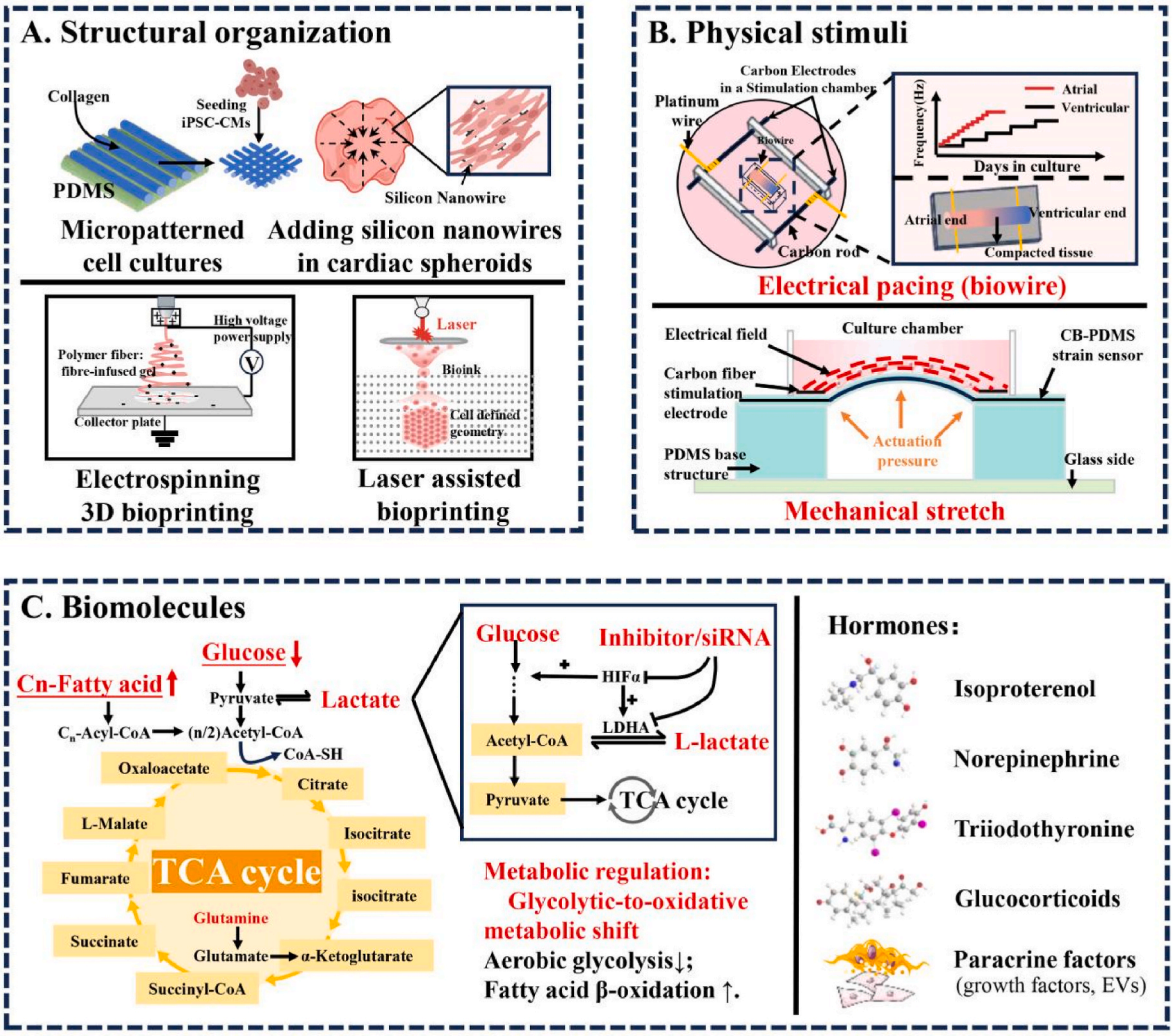

Fig. 6. Strategies for enhancing the maturation and functionality of hiPSC-CM organoids. (A) Structural organization using microgrooves, nanopatterned surfaces and precise bioprinting trajectories to promote cell alignment. (B) Physical stimulation platforms like the "Biowire" or mechanical stretch to refine sarcomere structure and improve electrophysiological properties. (C) Applying biomolecules such as fatty acid-rich media, metabolic inhibitors, and novel hormones (isoproterenol, norepinephrine, triiodothyronine, glucocorticoids and paracrine factors) further to induce metabolic and functional maturation.

morphological and functional analyses.

5. Conclusion

We have outlined the significant advancements in organoid fabrication techniques: self-organization, biomaterial scaffolds, 3D bioprinting, organ-on-chip technologies, and their multiple applications. Despite significant strides, critical challenges remain, including achieving full functional maturity of cardiomyocytes, integrating diverse cell types to reflect the heart's cellular heterogeneity, establishing effective vascularization for sustained nutrient and oxygen delivery, and replicating the complex architectures of native cardiac tissue. The future envisions creating fully mature, functionally accurate, and clinically relevant heart models capable of self-repair and adaptability, closely mimicking the dynamic nature of cardiac tissue. Potential breakthroughs involve enhanced maturation protocols, multicellular integration, effective vascularization, and replication of complex heart structures. Further research should focus on improving electrophysiological integration, metabolic maturation, immune system interactions, and personalized disease modeling and drug screening. Addressing these challenges requires interdisciplinary collaboration, standardization of protocols, advancement of biofabrication technologies, ethical and regulatory frameworks. By overcoming these obstacles through dedicated research and collaborative efforts, cardiac organoids hold the promise of transforming our understanding and treatment of heart diseases, paving the way for breakthroughs in regenerative medicine, ultimately offering renewed hope for patients worldwide.

CRediT authorship contribution statement

Xiaojun Xia: Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Conceptualization. **Miner Hu:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Conceptualization. **Wenyan Zhou:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Conceptualization. **Yunpeng Jin:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Conceptualization. **Xudong Yao:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

The data and material that support this review are openly available.

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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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Data availability

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

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