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REVIEW

Pluripotent stem cell-derived cardiomyocyte transplantation: marching from bench to bedside

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Cardiovascular diseases such as myocardial infarction, heart failure, and cardiomyopathy, persist as a leading global cause of death. Current treatment options have inherent limitations, particularly in terms of cardiac regeneration due to the limited regenerative capacity of adult human hearts. The transplantation of pluripotent stem cell-derived cardiomyocytes (PSC-CMs) has emerged as a promising and potential solution to address this challenge. This review aims to summarize the latest advancements and prospects of PSC-CM transplantation (PCT), along with the existing constraints, such as immune rejection and engraftment arrhythmias, and corresponding solutions. Encompassing a comprehensive range from fundamental research findings and preclinical experiments to ongoing clinical trials, we hope to offer insights into PCT from bench to bedside.

transplantation | cardiomyocytes | stem cell | engraftment arrhythmia | immunogenicity | clinical trial | engineered heart tissue

Introduction

Cardiovascular diseases (CVDs), such as myocardial infarction (MI), heart failure (HF), cardiomyopathy, and congenital heart diseases (CHD), significantly contribute to global morbidity and mortality (Roth et al., 2020). The adult human heart's limited regenerative capacity poses the risk of irreversible cardiac damage following the loss of cardiomyocytes (CMs) (Ali et al., 2014). Despite advancements in cardiovascular medicine, fully replacing lost CMs remains unattained. Allogeneic heart transplantation, a last-resort option for end-stage HF, faces challenges such as donor scarcity and side effects of immunosuppressive therapy (IST) (Weber et al., 2017).

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offer promise in regenerative medicine. PSCs have the ability to differentiate into various cell lineages, including but not limited to CMs (Lian et al., 2012), intestinal cells (Spence et al., 2011), and endothelial cells (Wang et al., 2020a). PSC-derived cells can also be constructed into 3D biological structures with various organ-specific cell types, such as organoids (Vandana et al., 2023) and blastoids (Rivron et al., 2018; Wu et al., 2024). These PSC-derived products provide excellent platforms for drug screening, disease modeling, and embryology research (Shi et al., 2017). More importantly, PSC-derived product transplantation has been considered and tested as a regenerative therapeutics for various diseases. In particular, iPSCs, reprogrammable from a patient's somatic cells, can generate autologous grafts, potentially eliminating the need for IST. Among various fields of regenerative medicine (Shi et al., 2017), PSC-derived cardiomyocytes (PSC-CMs) and their transplantation have shown potential for heart regeneration.

This review comprehensively summarizes PSC-CM transplantation (PCT) advancements, starting with the generation and maturation of PSC-CMs, and the methodology of PCT. Insights into challenges like immune rejection, engraftment arrhythmias (EAs), graft-host integration, and tumorigenicity are also provided. Finally, we systematically summarize recent PCT clinical trials. This review offers not only full-range knowledge of PCT but also practical guidance for those who endeavor to participate in this promising field (Figure 1).

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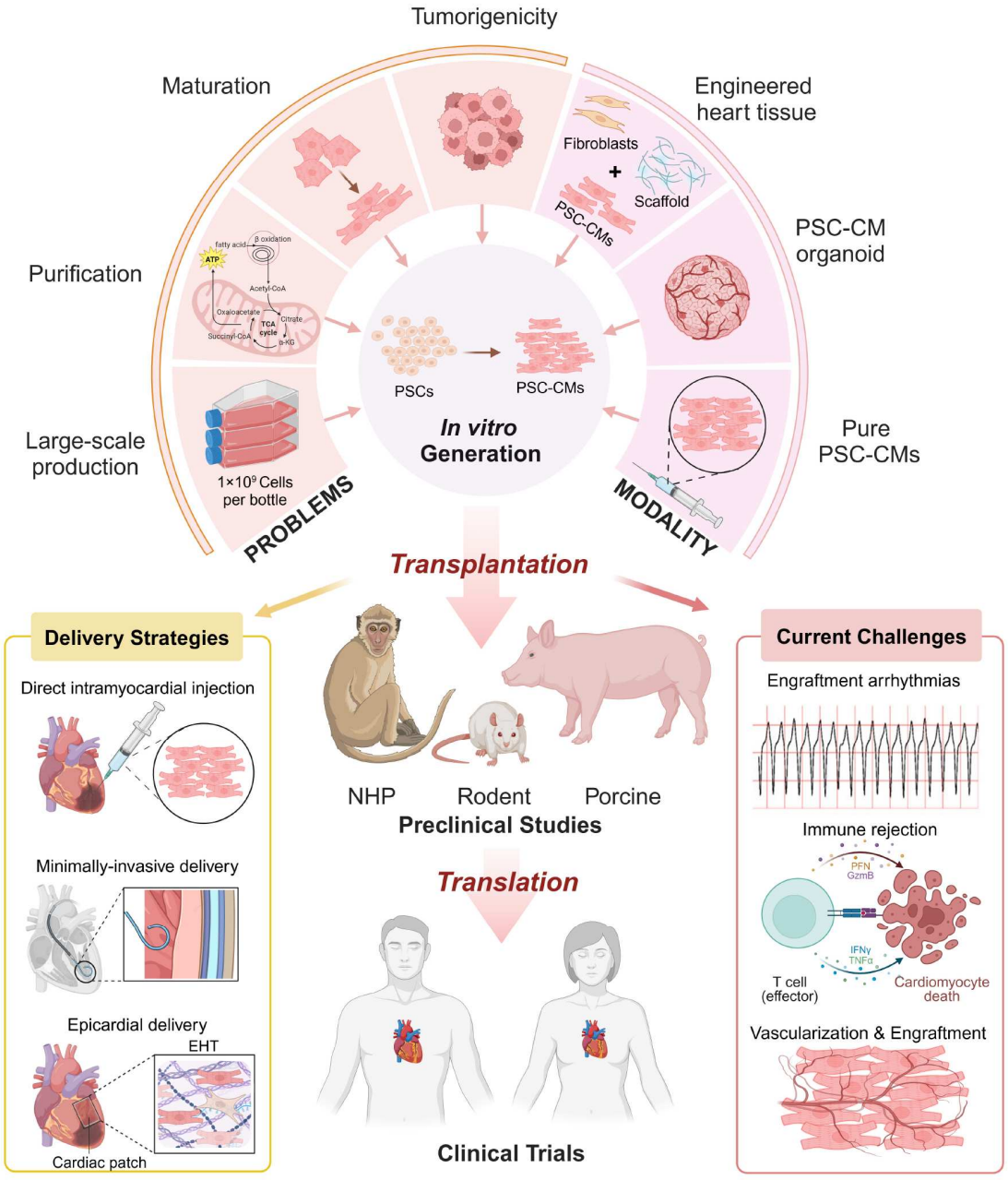

Figure 1. Overview of PCT from bench to bedside. Three steps are required for the bench-to-bedside translation of PSC-CMs. The *in vitro* generation of PSC-CMs constitutes the very first step, which can result in grafts of multiple modalities, including pure PSC-CMs, organoids, and EHT. Meanwhile, PSC-CMs must surpass the challenge of tumorigenicity, maturation, purification, and large-scale production to achieve transplantation. The preclinical animal study is the second step, in which NHP, rodent, and porcine are commonly used. PSC-CMs can be delivered through intramyocardial injection, transcatheter delivery, or cardiac patch placement, with severe challenges such as engraftment arrhythmias, immune rejection, vascularization, and engraftments hindering the final translation. The final stage is human clinical trials of different stages, to determine the final safety and efficacy of PCT. EHT, engineered heart tissue. NHP, non-human primate. PCT, PSC-CM transplantation. PSC-CM, pluripotent stem cell-derived CM.

Generation and maturation of PSC-CMs

Generation of PSC-CMs

Successful PCT relies on the *in-vitro* generation of functional PSC-CMs. In humans, four key signaling pathways: Wnt, BMP, TGF-β, and FGF, play a significant role in early CM differentiation (Parikh et al., 2015). Thus, the *in-vitro* differentiation of PSC-CMs involves introducing crucial growth factors (GFs) to modulate these signal pathways.

The initial protocols of PSC-CM differentiation utilized embryoid bodies (EBs) formed from ESCs, with subsequent CM purification (Kehat et al., 2001; Mummery et al., 2002). However, early protocols were relatively insufficient and unstable (Jiang et al., 2012). Improved methods, such as combining GSK3 inhibitors and Wnt signaling pathway inhibitors (Jiang et al., 2012), achieved up to 98% cTnT$^+$ CMs across various hPSC lines (Lian et al., 2012; Lian et al., 2013).

Further refinements, like removing heterogeneous components such as bovine serum albumin (BSA) (Lian et al., 2015), and utilizing activin A and BMP4 (bone morphogenetic protein 4) (Kattman et al., 2011), have enhanced cardiac differentiation. Purification of PSC-CMs, often with lactate-rich substrate, minimizes the risk of teratoma formation post-transplantation, further ensuring safety (Hattori et al., 2010; Tohyama et al., 2013) (Figure 2).

Notably, hPSC-CMs encompass various cardiac cell lines, including ventricular CMs, atrial CMs, and pacemaker cells (Devalla and Passier, 2018). Studies have explored subtle gene expression differences to generate specific cardiac cell lines, such as sinoatrial node-like cells, offering possibilities for biological pacemaker creation through modulating retinoic acid and FGF (fibroblast growth factor) signaling pathways combined with metabolic screening (Darche et al., 2022; Hou et al., 2022).

Mass production of PSC-CMs

PCT necessitates a substantial cell quantity because each acute myocardial infarction (AMI) incident can claim approximately 1 billion CMs in humans (Laflamme and Murry, 2005). For mice, pigs, and NHP, PCT requires around 1×10^6, 1×10^8 (Marchiano et al., 2023), and 1×10^9 PSC-CMs per heart, respectively (Chong et al., 2014), highlighting the need for mass production.

Several strategies have been proposed for mass PSC-CM production. One approach involves generating clinically relevant-sized cardiac patches (CPs). Gao et al., Shadrin et al., and Fukuda et al. have all reported successful generation of large CPs, some exceeding 4 cm×4 cm, with over 4 million PSC-CMs of high purity (Gao et al., 2018; Shadrin et al., 2017), while others produced CPs consisting of 7.2×10^8 hiPSC-CMs per patch (Tohyama et al., 2017). Quite recently, another team led by

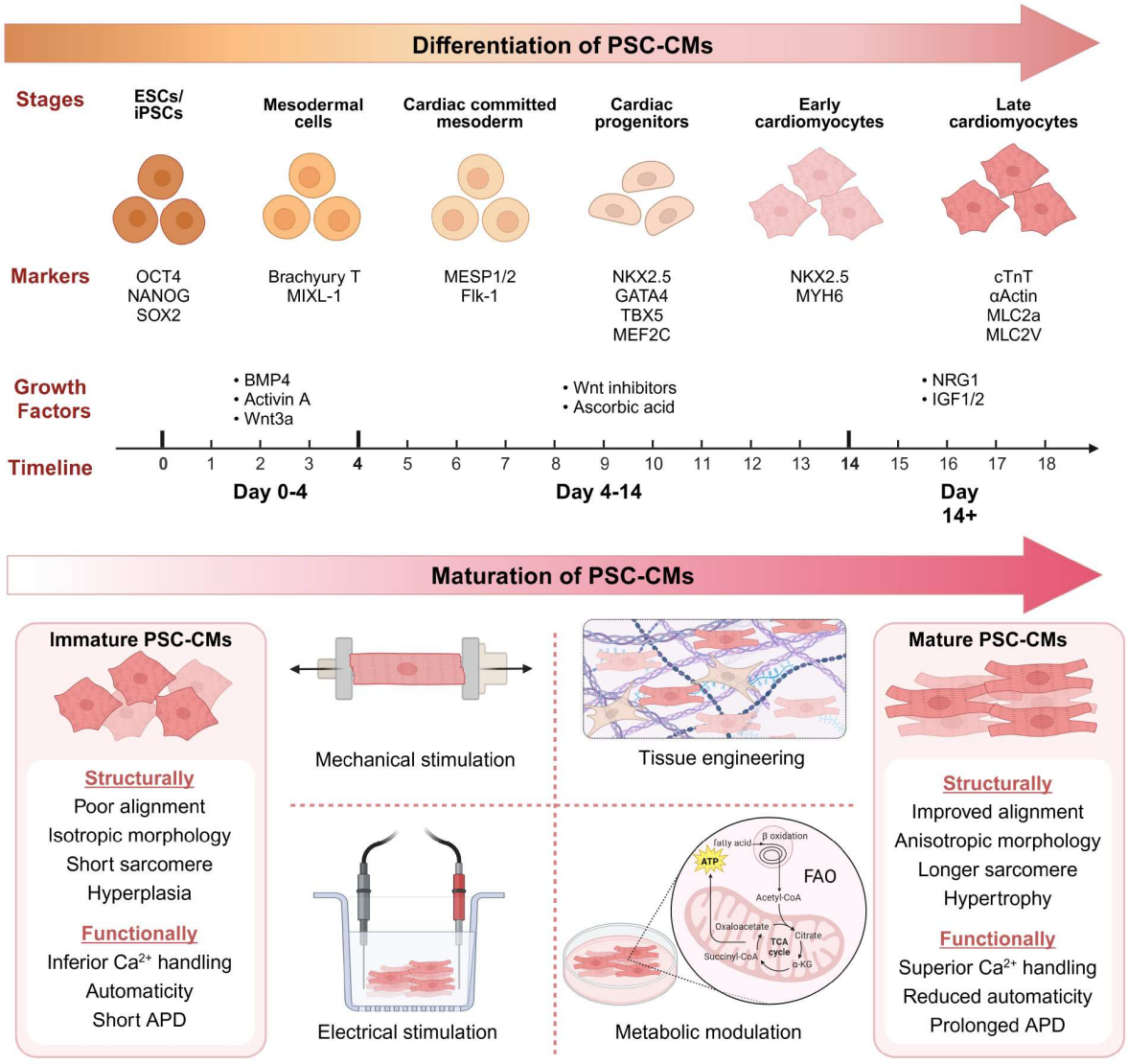

Figure 2. Differentiation and maturation of PSC-CMs. The differentiation of PSC-CMs includes multiple steps, starting from iPSCs/ESCs to mesoderm cells, cardiac progenitors, and finally, CMs. During differentiation, different growth factors are needed to modulate the cell lineage. The product cells of each stage have specific markers that allow researchers to identify, such as cTnT for late CMs. The newly-differentiated PSC-CMs present immature structurality and functionality and thus must undergo *in vitro* maturation before transplantation. Commonly adopted methods and evaluations for maturation are also listed in the figure. CM, cardiomyocyte. cTnT, cardiac troponin T. ESC, embryonic stem cell. PSC-CM, pluripotent stem cell-derived cardiomyocyte.

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Coulombe generated a mega-CP of 6.5 cm×7.5 cm with 1 billion hiPSC-CMs seeded, demonstrating feasibility in manufacture, surgical transplantation, and preclinical treatment (Dwyer et al., 2023). An alternative solution involves differentiating PSC-CMs in specialized platforms, such as stirred-tank bioreactors (Pandey et al., 2019), microfabric vessels (Miwa et al., 2021), and PDMS (polydimethylsiloxane)-lined roller bottles (Dhahri et al., 2022), all capable of producing large quantities of PSC-CMs.

However, not all protocols are clinically valuable or feasible. Evaluation criteria include economic efficiency, biocompatibility, and the incorporation of PSC-CM purification and maturation. Future comparative studies are crucial to establish optimal protocols for large-scale PSC-CM production, meeting clinical demands effectively.

Maturation of PSC-CMs

One of the most unique properties of PSC-CMs is that they present with phenotypes that closely resemble those of early fetal CMs rather than their adult counterparts. Structurally, PSC-CMs are more round-shaped and underaligned, with relatively immature sarcomere. Functionally, PSC-CMs present with automaticity and inferior Ca^{2+} handling (Scuderi and Butcher, 2017). These properties potentially impede the efficacy of PCT, calling for efficient maturation of PSC-CMs (Gomez-Garcia et al., 2021; Hong et al., 2023; Scuderi and Butcher, 2017).

Strategies to promote the maturation of PSC-CMs

The process of native CM maturation is intricately influenced by the microenvironment of CM development (Yang et al., 2014). Replicating this microenvironment *in vitro* has been shown to improve CM maturation, such as applying electrical stimulation, mechanical stretch, and metabolic manipulation (Hong et al., 2023; Sun and Nunes, 2016). For instance, Lu et al. applied progression stretch to PSC-CM-based heart tissue and observed an increase in the maximum contractile force and sarcomere length, almost comparable to that of adult human CMs (Lu et al., 2021). Crestani et al. (2020) on the other hand, discovered that applying electrical stimulation in the beginning stages of PSC-CM differentiation resulted in enhanced PSC-CM maturation in aspects of gene expression, formation of gap junction, faster depolarization, and improved Ca^{2+} transients. Biochemical manipulation of proteins and signal pathways has also been widely investigated. As an example, Chirico et al. and Liu et al. reported improved maturation with the treatment of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) activators asiatic acid and GW501516 (Chirico et al., 2022), and ZLN005 (Liu et al., 2020), respectively. Of note, engineered heart tissue (EHT) has emerged as an important platform for PSC-CM maturation by incorporating various strategies (Hong et al., 2023; Sun and Nunes, 2016), including engineering the substrates, co-culturing with other cardiac-specific cells, introducing electrical stimulation, and mechanical stretch (Kumar et al., 2023; Patel et al., 2023; Ruan et al., 2016; Song et al., 2022b). Collectively, these cues replicate the essential components responsible for the maturity of PSC-CMs. However, current maturation strategies still cannot generate fully-matured PSC-CMs *in vitro*, whereas the immature electrophysiology is considered a prime reason for side effects such as EAs (see "Engraftment arrhythmias"). Future efforts are needed to address this limitation, possibly by combining multiple strategies to facilitate the maturation process (Figure 2).

Parameters to evaluate the maturation of PSC-CMs

The maturation of PSC-CMs can be evaluated with various parameters, including biochemical, structural, and biophysical ones (Hong et al., 2023). At the genetic and molecular levels, maturation involves shifts in gene expression and protein isoforms, such as the downregulation of pluripotency transcription factors and upregulation of mesodermal and cardiac markers. Key transitions include the modulation of sarcomeric proteins like cTnT and myosin heavy chain (MHC) (Hunkeler et al., 1991; Opitz et al., 2004; Xu et al., 2009) and the titin isoform shift from N2BA (3,200–3,700 kD) to N2B (3,000 kD) (Opitz et al., 2004). These changes are identified through RNA sequencing (RNA-seq), ATAC sequencing (ATAC-seq), and single-cell RNA sequencing (scRNA-seq), providing a comprehensive toolkit for assessing PSC-CM maturity (Murphy et al., 2021; van den Berg et al., 2015).

Morphologically, early-stage PSC-CMs are smaller and rounder, with an average sarcomere length of 1.65 μm, while late-stage cells become more elliptical and develop a highly organized sarcomere structure of about 2.2 μm in length (Awasthi et al., 2012; Kehat et al., 2001; Yang et al., 2014). Despite these changes, late-stage PSC-CMs lack transverse tubules and multinucleation (Pioner et al., 2019; Robertson et al., 2013). Metabolically, PSC-CMs transition from glycolysis to oxidative metabolism, with mitochondria volume increasing significantly during maturation (Giordano, 2005; Hattori et al., 2010; Hu et al., 2018; Lopaschuk and Jaswal, 2010; Mills et al., 2017). Adult CMs rely primarily on fatty acid oxidation, while PSC-CMs depend more on glycolysis (Lopaschuk and Jaswal, 2010).

Electrophysiologically, PSC-CM maturation is marked by improved action potential profiles and increased force generation. Immature cells have slower upstroke velocities (~50 V s^{-1}) and less negative resting membrane potentials (−50 – −60 mV), compared with mature cells (~250 V s^{-1}, −80 – −90 mV) (Goversen et al., 2018; Kim et al., 2015; Yang et al., 2014). The conduction velocity increases from ~0.1 to 0.3 – 1.0 m s^{-1} in mature myocardium (Yang et al., 2014). PSC-CMs start spontaneous beating early in differentiation and maintain rhythmic activity for over a year, with rates from 21 to 52 beats per minute (Brito-Martins et al., 2008; Burridge et al., 2011; Karakikes et al., 2015; Mummery et al., 2012; Otsuji et al., 2010). These cells exhibit major ion currents typical of adult CMs, though often at abnormal levels, and their contractile force increases from the nanonewton to the micronewton range (Yang et al., 2014).

Another significant parameter of CM maturation is the improved Ca^{2+} handling, which is substantial in bridging electrophysiology and mechanical contraction (also known as excitation-contraction coupling) (Ernst et al., 2023; Karbassi et al., 2020). For adult mature CMs, the cell depolarization triggers the Ca^{2+} influx into the cells through L-type calcium channels (LTCC), which then results in the release of Ca^{2+} from the ryanodine receptors (RyR) on the sarcoplasmic reticulum. Finally, Ca^{2+} that has been released binds with troponin C, initiating muscle contraction (Setterberg et al., 2021). For immature PSC-CMs, however, the calcium dynamics are slower, and the expression of calcium handling-related proteins, such as LTCC and RyR, is significantly lower than that of adult ventricular CMs (Ernst et al., 2023; Karbassi et al., 2020).

Therefore, the improved calcium transient, signified by an increase in Ca^{2+} amplitude, upstroke velocity, and recovery time, also suggests the maturation of PSC-CMs (Ernst et al., 2023; Hwang et al., 2015). By using precise parameters to quantify PSC-CM maturation, we can enhance the consistency and effectiveness of cell therapies, contributing to better treatment outcomes for cardiovascular diseases.

Methodology of PSC-CM transplantation

Graft modalities

Dispersed PSC-CMs

Dispersed PSC-CMs are one of the most widely adopted modalities of PSC-CM graft transplantation, for they can be easily obtained, preserved, and purified (Chong et al., 2014). Generally, dispersed PSC-CMs can be harvested once the cells are well-differentiated *in vitro* (with spontaneous contractions), and subject to direct intramyocardial injection. The application and limitation of dispersed PSC-CMs will be further elaborated in the section "Direct intramyocardial injection".

Engineered heart tissue

EHT refers to 2D or 3D structures composed of various cells and biomaterials, such as fibrin, hydrogel, and decellularized extracellular matrix (ECM), mimicking the function and structure of natural cardiac tissue (Jiang et al., 2021; Riegler et al., 2015; Wendel et al., 2015). Apart from PSC-CMs, EHT can include cardiac fibroblasts (CFs), endothelial cells (ECs), smooth muscle cells (SMCs), and mesenchymal stem cells (MSCs) (Lou et al., 2023; Pinto et al., 2016; Zhang et al., 2018). With diverse combinations of cells, biomaterials, and construction strategies, EHT can manifest in various modalities, with 2D PSC-CM cardiac patches (CPs or "cell sheets") and 3D PSC-CM spheroids being the most commonly used.

PSC-CM CPs, configured as cord-like or mesh-like constructs, are usually placed on the epicardium, and offer controlled generation and transplantation, making them widely used in clinically relevant studies. Large-scale PSC-CM CPs have shown success in injured pig hearts (Gao et al., 2018; Querdel et al., 2021; Weinberger et al., 2016) and clinical cases of end-stage HF patients (NYHA class III to class I, LVEF from 26% to 56%) (Menasché et al., 2015), resulting in improved cardiac function. PSC-CM spheroids, another frequently used modality, have demonstrated heart recovery in MI (Mattapally et al., 2018b) and HF (Kawaguchi et al., 2021) models. Compared with CPs, PSC-CM spheroids, being 3D-structured, provide additional dimensions for tissue engineering, enhancing the maturity and function of PSC-CMs (Beauchamp et al., 2020; Veldhuizen et al., 2020). Their injectable size also allows minimally invasive delivery, reducing transplantation-associated damage (Kim et al., 2023).

Given the complexity of EHTs with multiple cell types, oxygen supply is crucial for their survival. However, EHT vascularization remains insufficient during manufacture and post-transplantation (Liu et al., 2018), necessitating strategies such as cytokine application, co-transplantation of vascular organoids, or 3D printing vascularized and perfusable cardiac tissues (Noor et al., 2019; Silberman et al., 2023) to address this challenge. These strategies require extensive, clinically relevant experiments to validate their effectiveness before further clinical translation can be considered.

Cardiac organoid

Cardiac organoids (COs) can be considered a special type of 3D EHT. Different from traditional EHT, which requires artificial construction in special cultures or platforms with engineering, COs can be formed simply by self-organization, without the need for mold during tissue formation (Silberman et al., 2023). More distinctively, EHT can encompass any cell lines, whereas COs are mainly constituted of heart-specific cell types (such as CMs, CFs, and ECs) (Cho et al., 2022; Lancaster and Knoblich, 2014).

Ideally, mature organoids can serve as the basic structural and functional units of hearts. While this has not been fully accomplished, researchers have successfully generated 3D COs composed of 70% hiPSC-CMs, 15% hiPSC-ECs, and 15% hiPSC-CFs, replicating the natural percentage of different cell lines in hearts (Campostrini et al., 2021). Several other research teams have also generated self-organizing COs with different compositions and in different organ development stages (Drakhlis et al., 2021; Hofbauer et al., 2021; Lewis-Israeli et al., 2021). However, COs generated in previous studies were mainly used for developmental studies as well as disease modeling and drug screening (Richards et al., 2020). To further expand the application of COs, Ying Mei's team developed nanowired COs consisting of hPSC-CMs, CFs, ECs, stromal cells, and e-SiNWs (electrically conductive silicon nanowires). As these nanowired COs were transplanted *in vivo*, they presented significant vasculogenesis and cell retention (cell retention rate on D7: COs *vs.* dispersed hPSC-CMs, ~30% *vs.* <1%–10%) (Tan et al., 2023). However, this experiment, as pioneering as it was, used only rats. It will be necessary to further explore the therapeutic and regenerative potential of COs in larger animals, with a special focus on the retention rate and incidence of EAs.

Delivery strategies

Direct intramyocardial injection

Direct intramyocardial injection (DII) stands as one of the most widely utilized strategies for PSC-CM delivery, involving the loading of dispersed PSC-CMs, cardiac spheroids, or COs into syringes and injecting them directly into the infarct or peri-infarct zone of the host myocardium (Chong et al., 2014; Liu et al., 2018). DII can be accomplished through direct vision under thoracotomy, catheter-based methods, or ultrasound-guided percutaneous delivery, offering flexibility for diverse scenarios. However, DII comes with limitations, notably a low early cell retention rate ranging from 2.89% to 11.19% (Chong et al., 2014; Liu et al., 2018). The hostile environment and poor vascularization during early ischemic injury contribute to this suboptimal efficacy.

Various efforts have been made to enhance early cell retention, such as overexpressing Cyclin D2 (Zhao et al., 2021), co-transplanting hiPSC-ECs with hiPSC-CMs (Cheng et al., 2023a), co-transplantation of microvessels (Sun et al., 2020), administration of Tb4 (thymosin β4) (Tan et al., 2021), and overexpression of CDH2 (N-cadherin) (Lou et al., 2020) and Ang-1 (angiopoietin-1) (Tao et al., 2021). However, challenges persist in controlling the injection site, depth, and graft area, impacting treatment outcomes and hindering clinical translation. For instance, research has indicated that the injection site, i.e., intra-infarct injection or peri-infarct injection, plays an important role in the formation of EAs, as well as electromechanical integration (Gibbs et al., 2023). These limitations call for further

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development of more exquisite and accurate injection devices, such as reported by Tabei et al. (2019).

Despite the challenges, DII facilitates direct cell-cell contact between the host myocardium and graft, fostering electromechanical integration. While this improves cardiac function and directly restores lost CMs, the combination of electromechanical integration and the immature electrophysiology of PSC-CMs can lead to severe side effects, particularly EAs, raising significant safety concerns (see "Engraftment arrhythmias").

Epicardial delivery

Epicardial transplantation mainly applies to PSC-CM CPs, by directly placing the patch on the epicardium via thoracotomy. Different from DII, epicardial transplantation does not result in direct cell-cell contact between the graft and host CMs since the patches do not directly extend into the myocardium (Shadrin et al., 2017; Weinberger et al., 2016). Instead of remuscularization, CPs from epicardial delivery (ED) can improve the function of injured hearts via the paracrine effects of the patch cells. Interestingly, compared with DII, epicardial delivery of CPs demonstrates superior cell retention, with around 10% to 25% of the transplanted cells preserved within 4 weeks (Gao et al., 2018; Gao et al., 2017; Riegler et al., 2015). This is likely because CPs provide a stable substrate for cell retention, minimizing cell leakage. However, commonly used epicardial delivery strategies involve thoracotomy to expose the site on which patches are transplanted, which is relatively invasive and traumatic. In clinical practice, this invasive delivery strategy is likely to be used only during coronary artery bypass graft (CABG) surgery (Gao et al., 2018; Gao et al., 2017). More minimally invasive delivery strategies would further expand the implication of CPs.

Minimally-invasive delivery

Currently, the majority of MI without left main occlusion is treated with minimally invasive procedures, such as percutaneous catheter intervention (PCI) (Byrne et al., 2023). Other possible indications of PSC-CMs, such as HF and cardiomyopathy, are also generally treated non-surgically. Therefore, it is important to develop minimally invasive delivery (MID) strategies for PSC-CM grafts, to coordinate with clinical treatment options.

Previous attempts of MID mainly involve transcatheter delivery into the endocardium or coronary artery. Professor C. Murry's lab (Marchiano et al., 2023; Nakamura et al., 2021) has done extensive studies on catheter-guided percutaneous endocardial delivery to transplant dispersed PSC-CMs into pigs. On the other hand, Wang et al. (2021c) constructed an injectable shape-memory CP with a micromesh structure, which can be delivered to the endocardium via catheters. These strategies avoid the need for thoracotomy, resulting in better recovery and broader clinical applications. Notably, Kobayashi et al. (2023) attempted to deliver PSC-CM spheroids of different sizes to cynomolgus monkeys through intracoronary injection. Unfortunately, they discovered it was insufficient, as spheroids of small sizes are washed out quickly into the circulation system, and those of medium and large sizes result in coronary occlusion following ischemic injury and scar formation. Interestingly, they also observed partial cell engraftment in 2 out of 6 animals, suggesting transplanted PSC-CMs can migrate through the vessel wall (Kobayashi et al., 2023). Further experiments are needed to optimize the delivery strategy of transplanted grafts, to ensure both the safety and cell retention.

Comparison between the aforementioned strategies can be seen in Table 1.

Evaluating graft fixation

In various forms of PSC-CM transplantation, establishing effective graft fixation techniques is crucial for ensuring the stability and integration of the graft. The most commonly used methods are histology analysis and immunostaining of cardiac markers such as cTnT (Chong et al., 2014; Liu et al., 2018; Romagnuolo et al., 2019; Shiba et al., 2012). While such an approach can directly visualize the graft size, graft retention, and host-graft integration, it requires sacrificing the host and extracting the host heart, limiting their use in clinical settings. To monitor the position and status of grafts post-transplantation, a range of imaging techniques are utilized. For small animal models like mice, bioluminescence imaging effectively tracks the engraftment of iPSC-CMs (Funakoshi et al., 2016). In models with greater tissue depth or in clinical settings, MRI (magnetic resonance imaging) serves as a valuable non-invasive tool. Contrast-enhanced MRI (CE-MRI) allows researchers to localize and assess myocardial cells within scar tissue non-invasively (Cook et al., 2024; Liu et al., 2018). Quantitative analysis using fractional anisotropy (FA) and mean diffusivity (MD) reflects the microstructural properties of the graft. Studies show that the FA and MD values of mature PSC-CM grafts are comparable to healthy myocardium, indicating similar structural anisotropy (Cook et al., 2024). Additionally, diffusion tensor imaging (DTI) parameters can distinguish graft tissue from surrounding scar tissue, as scar tissue typically exhibits low anisotropy (Cook et al., 2024; Sosnovik et al., 2014). Comparing these DTI parameters to those of healthy myocardium can also determine the extent of graft absorption by the host, with similar parameters suggesting successful integration and absorption of the graft (Cook et al., 2024; Sosnovik et al., 2014).

Table 1. Comparison of different delivery strategies

Strategy	Modality	Delivery	Cell retention	Mechanism	Limitations	References
DII	Dispersed PSC-CMs, cardiac spheroids, COs	Injection to the infarct site under thoracotomy or ultrasound-guided delivery	Limited (2.89%–11.19%)	Direct electromechanical integration	Prone to EAs; limited cell retention rate	(Chong et al., 2014; Gibbs et al., 2023; Liu et al., 2018)
ED	PSC-CM cell patches or cell sheets	Implantation onto the epicardium via thoracotomy	Medium (10%–25%)	Paracrine effects from the cardiac patches or sheets	Need for invasive thoracotomy	(Gao et al., 2018; Gao et al., 2017; Shadrin et al., 2017; Weinberger et al., 2016)
MID	Dispersed PSC-CMs, cardiac spheroids, injectable cell patches	Catheter-guided percutaneous delivery	Varies based on specific delivery strategies	Direct electromechanical integration	Inability to directly visualize the position of delivery	(Marchiano et al., 2023; Nakamura et al., 2021; Wang et al., 2021c)

Animal models

In-vivo studies using animal models are crucial for evaluating the efficacy and safety of PCT, providing essential insights for subsequent clinical translation. Selecting appropriate animal and disease models is a significant consideration. Factors such as feasibility, cost-effectiveness, and researcher expertise play significant roles in this choice. Commonly employed animals for PCT range from rodents (mice, rats, and guinea pigs) to larger animals (porcine and NHP).

Initial PSC-CM studies typically employ immunocompromised rodents (athymic rats, SCID mice) due to their cost-effectiveness and feasibility, requiring only a small cell quantity (~1×10^6 PSC-CMs per animal). However, their cardiac structure and electrophysiology differences from humans pose challenges in predicting transplant outcomes (Rai et al., 2017). Porcine is preferred in preclinical studies, given their cardiac similarities to humans. NHPs, being phylogenetically closer to humans, are also utilized in preclinical studies, despite the associated higher costs and the need for a substantial cell quantity (~1×10^8 to 10^9 PSC-CMs per animal), which can be impractical in many experiments.

A unique consideration in animal selection is the occurrence of EAs, which are more prevalent in large animal models like porcine and NHPs and rarely observed in small animals like mice and rats, as discussed in the "Engraftment arrhythmias" section. Preventing EAs in large animal models can be as important as preventing immune rejection. Recent PCT studies and their adopted animal models and methods are summarized in Table 2.

Current obstacles and solutions

Immune rejection

Brief overview of allograft rejection

Currently, allograft rejection remains a prominent concern in both transplant medicine and regenerative medicine. Allograft rejection is mediated by a range of adaptive and innate immune responses (Figure 3). In PCT, the main contributors of immune rejections are $CD8^+$ cytotoxic T cell-mediated adaptive immune response, and natural killer cell (NK)-mediated innate immune response. The $CD8^+$ T-cell immune response originates from the recognition of allogeneic peptides presented in highly polymorphic MHC (major histocompatibility complex, referred to as human leukocyte antigen (HLA) in humans) class I (expressed in nearly all nucleated cells). On the other hand, the activation of NKs results from an imbalance between activating and inhibitory signals. NK-mediated rejection is particularly significant in PSC-derived graft transplantation because PSCs and their derivatives have been shown to express low levels of inhibitory MHC class I (Araki et al., 2013; Kruse et al., 2015) and upregulate activating ligands like CD155 (Chimienti et al., 2022; Kruse et al., 2015). Additionally, NK infiltration and NK-mediated rejection have been reported not only in allogeneic but also in syngeneic transplantation (Bogomiakova et al., 2023; Kruse et al., 2015; Nakamura et al., 2019).

Current strategies to reduce immune rejection

Since both allogeneic and syngeneic PSC and PSC-derived grafts have been found to suffer from graft rejection, it is necessary to develop safe strategies to reduce immune response without causing related immunological complications. So far, substantial efforts have been made to promote the immune tolerance of PCT, as summarized in Table 3.

(1) Co-transplantation of mesenchymal stem cells. MSCs, derived from bone marrow, cord blood, and PSCs, exhibit multipotent capabilities, participating in wound healing and the inflammatory response. Notably, MSCs possess immune privilege and unique immunomodulatory abilities (Götherström et al., 2004), inhibiting T cell proliferation and promoting regulatory T cell (T_{reg}) expansion (Kimbrel et al., 2014). Co-transplantation of MSCs has been explored to reduce T cell-mediated graft rejection.

Previous studies demonstrated that simultaneous injection of MSCs during allogeneic heart transplantation significantly prolonged graft survival and inhibited immune rejection (Chen et al., 2021; Gao et al., 2020; Wang et al., 2021b; Wang et al., 2014), which is also observed in allogeneic islet (Xu et al., 2012) and trachea (Khan et al., 2019) transplantation. Subcutaneous co-transplantation of syngeneic MSCs with iPSC-CMs reduced immune rejection in allogeneic models, leading to nearly twofold improvement in graft survival. This effect involved the recruitment of T_{reg} cells to the graft site, upregulation of IL-10 and TGF-β, and $CD8^+$ T cell depletion (Yoshida et al., 2020). Systemic venous administration of syngeneic iPSC-MSCs before PCT in an MI model also yielded positive results (Sun et al., 2021).

Table 2. Methods used in PCT in animal models of myocardial infarction[a)]

Animal	Cell	Cell number ($\times10^6$)	Days after MI	Delivery method	References
Pigtail macaques*	hESC-CMs	1,000	14	TDII	(Chong et al., 2014)
Cynomolgus macaques*	MHC-matched allogeneic iPSC-CMs	400	14	TDII	(Shiba et al., 2016)
Pigtail macaques*	hESC-CMs	90.3	12–15	TDII	(Liu et al., 2018)
Minipigs	iPSC-CM sheets	100	30	TDED	(Ishida et al., 2019)
Pigs	hESC-CMs+pro-survival cocktail	1,000	21	TDED	(Romagnuolo et al., 2019)
Mice	CPs	1 per mL	Chronic MI model	TDED	(Cui et al., 2020)
Rats	hiPSC-CMs	10	10	TDII	(Guan et al., 2020)
Rats	Engineered heart tissue	7–10	4	TDED	(Munarin et al., 2020)
Minipigs	CPs	7 per cm^3 (D=25 mm; T=1.2 mm)	7	Transcatheter delivery	(Wang et al., 2021c)
Cynomolgus macaques*	Cardiac spheroids	5–30	>5	Trans-coronary delivery	(Kobayashi et al., 2023)
Pigs	MEDUSA hESC-CMs	150	4	TDII	(Marchiano et al., 2023)

a) *D*, diameter. hESC, human embryonic stem cell. hESC-CM, hESC-derived cardiomyocyte. *T*, thickness. TDED, thoracotomy and epicardial delivery. TDII, thoracotomy and direct intramyocardial injection. *, non-human primates, NHP.

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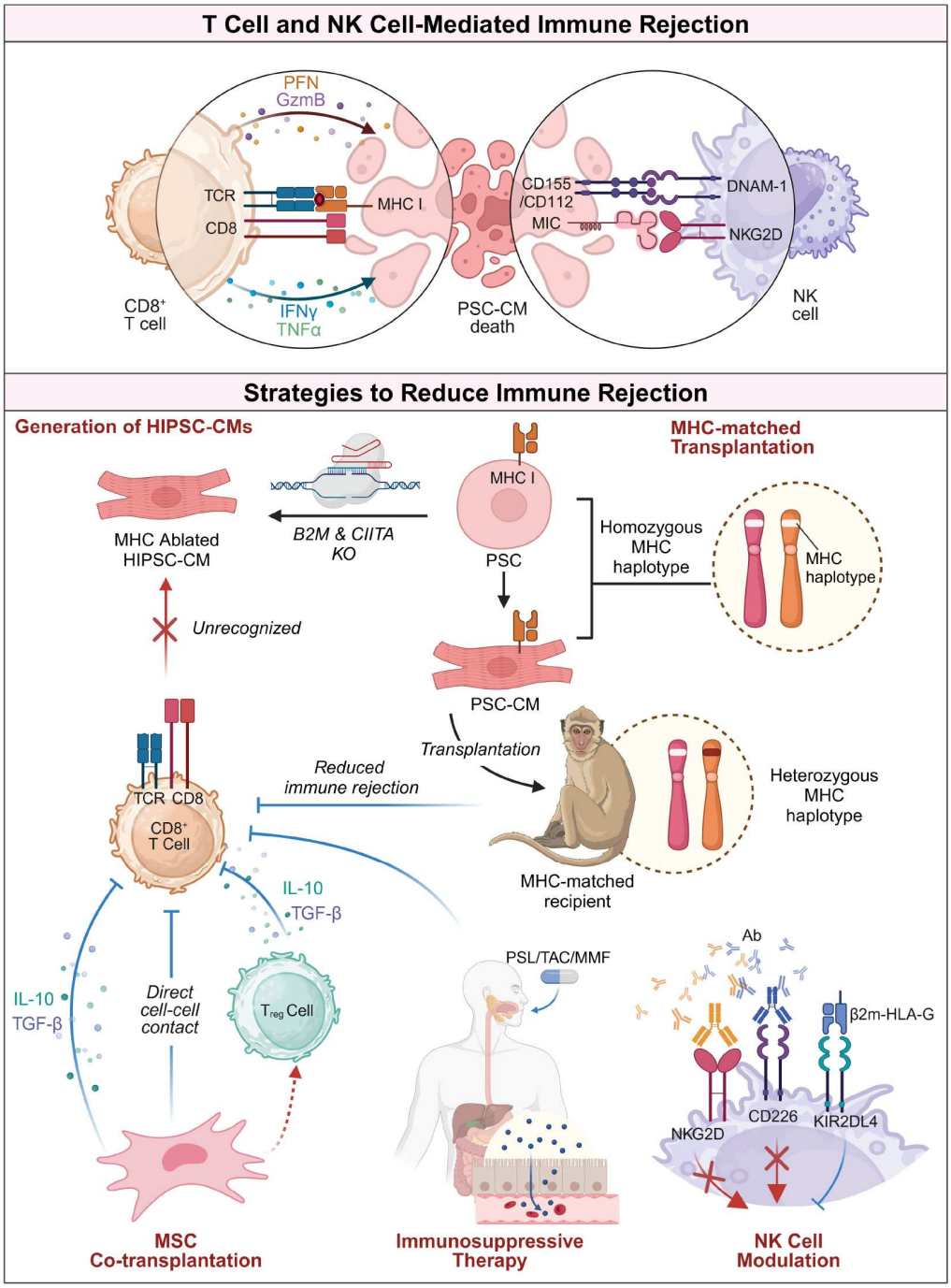

Figure 3. Mechanisms and solutions for immune rejection in PCT. Immune rejection constitutes one of the most serious challenges hindering PCT. The immune rejection for PSC-CMs is mostly mediated by $CD8^+$ cytotoxic T cells (due to the recognition of allogeneic MHC-I molecules) and NK cells (due to the loss of balance between activating and inhibitory signals). Various strategies have been applied to reduce immune rejection, including transplanting MHC-matched grafts, generating HIPSC-CMs without MHC expression, co-transplanting MSCs to modulate immune reaction, administering immunosuppressive agents, as well as modulating NK signals. HIPSC-CM, hypoimmune PSC-CM. MHC, major histocompatibility complex. MSC, mesenchymal stem cell. NK, natural killer cell. PCT, PSC-CM transplantation. PSC-CM, pluripotent stem cell-derived cardiomyocyte.

While the precise mechanisms underlying MSC-induced immune tolerance are not fully understood, current research emphasizes the crucial role of recruited T_{reg} cells (Sun et al., 2021; Wang et al., 2014; Yoshida et al., 2020). Additionally, MSC co-transplantation and the use of MSC-conditioned medium have been shown to improve integration between iPSC-CMs and the host myocardium, enhancing cardiac function, possibly through paracrine effects (Kc et al., 2020; Neef et al., 2022; Rubach et al., 2014). Taken together, co-transplantation of MSCs or the utilization of MSC-derived exosomes or cytokines holds significant potential in mitigating immune rejection and enhancing graft integration.

Table 3. Overview of immunomodulating strategies to reduce immune rejection in recent PCT studies[a)]

Immunomodulating strategy	Graft	Animal model	Immunity	Transp. strategy	Outcome	References
			Co-transplantation of PSC-CMs with MSCs			
Localized co-transp. of syngeneic BALB/c MSCs	C57BL/6 iPSC-CM sheets	Untreated BALB/c mice	Allo.	Subcutaneous	Graft survival on D7: iPSC-CM/MSC *vs.* iPSC-CM: 61%±7% *vs.* 34%±5% (P=0.008)	(Yoshida et al., 2020)
IV administration of hiPSC-MSCs	hiPSC-CMs	MI ICR mice	Xeno.	Intramyocardial	Graft survival on D7: hiPSC-CM/hiPSC-MSC *vs.* hiPSC-CM: 29.15% *vs.* 15.51% (P<0.01)	(Sun et al., 2021)
			Transplantation of MHC-matched PSC-CMs			
MHC-matched allo. transp. + PSL/MMF/TAC	MHC-HT1 homo. iPSC-CM sheets	MHC-HT1 hetero., untreated cynomolgus macaques	Allo.	Subcutaneous and intramyocardial	MHC-matched: higher graft fluorescence intensity in 1 and 2 M (P<0.05); lower T cell infiltration (P<0.05)	(Kawamura et al., 2016)
MHC-matched allo. transp. + methyl-PSL/TAC	MHC-HT4 homo. iPSC-CMs	MHC-HT4 hetero., MI cynomolgus macaques	Allo.	Intramyocardial	MHC-mismatched: severe rejection with T cell infiltration by 4 W; MHC-matched: No obvious immune rejection by W12, with graft remuscularization	(Shiba et al., 2016)
			Transplantation of HIPSC-CMs			
B2M and CIITA KO	MHC-I/II KO hiPSC-CMs	NA	NA	NA	T cell activation: MHC-I/II KO hiPSC-CMs *vs.* WT-hiPSC-CMs: 21% vs. 75%; Inhibition of HLA-E and HLA-F, but not HLA-G	(Mattapally et al., 2018a)
B2M and CIITA KO, CD47 OE	*B2m*$^{-/-}$ *Ciita*$^{-/-}$ Cd47 miPSC-CMs/ECs	Immunocompetent, ischemic heart failure BALB/c mice	Allo.	Intramyocardial	Survival: WT: total rejection by D14; *B2m*$^{-/-}$ *Ciita*$^{-/-}$ Cd47: stable survival by D28; SV: *B2m*$^{-/-}$ *Ciita*$^{-/-}$ Cd47 *vs.* WT: ~30 μL *vs.* ~18 μL (P<0.0001)	(Deuse et al., 2021)
B2M and CIITA KO, CD47 OE	*B2m*$^{-/-}$ *Ciita*$^{-/-}$ Cd47 miPSC-CMs; *B2M*$^{-/-}$ *CIITA*$^{-/-}$ CD47 hiPSC-CMs	BALB/c mice Humanized NSG-SGM3 mice	Allo.	Intraperitoneal	In BALB/c mice: WT: total rejection by D35; *B2m*$^{-/-}$ *Ciita*$^{-/-}$ Cd47: 100% survival by D50; In NSG-SGM3 mice: WT: slow rejection; *B2M*$^{-/-}$ *CIITA*$^{-/-}$ CD47: 100% survival by D50	(Deuse et al., 2019)
			Modulation of NK cell ligand-receptor interaction			
Anti-NK1.1 antibody treatment; IFN-γ-induced MHC-I upregulation + antibody-induced CD226 and NKG2D blockage	C57BL/6 miPSC-CM sheets	Untreated C57BL/6 mice	Syn.	Subcutaneous	Apoptosis index: Control *vs.* NKC-depleted: 22.2% ±2.9% *vs.* 8.1%±3.8%; Control *vs.* IFN-γ(+)/Anti-CD226 Ab/Anti-NKG2D Ab: 31.6%±2.4% *vs.* 6.2%±1.6%	(Nakamura et al., 2019)
Biallelic KI of HLA-G1 into *B2M* loci (*B2M*^{mHLAG}) to create β2m-HLA-G1 fusion protein; OE of soluble form of fusion protein (*B2M*$^{m/sHLAG}$)	*B2M*^{mHLAG} and *B2M*$^{m/sHLAG}$ hESC-CMs	NA	NA	NA	APA: WT, *B2M*^{mHLAG}, *B2M*$^{m/sHLAG}$: 101.0, 95.52, 100.9 mV; APD_{90}: WT, *B2M*^{mHLAG}, *B2M*$^{m/sHLAG}$: 221.6, 278.9, 191.7 ms	(Chen et al., 2023)
Generation of *B2M*^{mHLAG} and *B2M*$^{m/sHLAG}$ hESCs (see above)	*B2M*^{mHLAG} and *B2M*$^{m/sHLAG}$ hESC-CMs	NA	NA	NA	Cytotoxicity of NK cells (co-cultured with hESC-CMs): WT, *B2M*^{mHLAG}, *B2M*$^{m/sHLAG}$: ~15%, ~10%, ~7% (P<0.01)	(Shi et al., 2020)

a) D0 is defined as the day of transplantation, Dx is defined as x day(s) after D0. Ab, antibody. Allo., allogeneic. APA, action potential amplitude. APD, action potential duration. B2M, beta-2-microglobulin. CTIIA, class II MHC transactivator. hESC, human embryonic stem cell. hESC-CM, hESC-derived cardiomyocyte. Hetero., heterozygous. hiPSC, human induced pluripotent stem cell. hiPSC-CM, hiPSC-derived cardiomyocyte. hiPSC-MSC, hiPSC-derived mesenchymal stem cell. Homo., homozygous. HT, haplotype. IFN, interferon. IV, intravenous. KI, knock-in. KO, knock-out. M(s), month(s). miPSC, mouse induced pluripotent stem cell. MMF, mycophenolate mofetil. NA, not applicable. NKC, natural killer cell. OE, overexpression. PSL, prednisolone. SV, stroke volume. Syn., syngeneic. TAC, tacrolimus. Transp., transplantation. W(s), week(s). WT, wild type. Xeno., xenogeneic.

(2) Generation of MHC-matched PSC-CMs. Adaptive immunity-mediated graft rejection is primarily based on the recognition of non-self MHC molecules (Duneton et al., 2022). Initial hypotheses and subsequent studies demonstrated that when the donor and recipient share partially matching MHC, the immunogenicity of iPSCs (Mizukami et al., 2014) or iPSC-CM (Kawamura et al., 2016) allografts can be significantly reduced without inducing tumorigenicity (Ishigaki et al., 2021). For instance, iPSC-CMs with homozygous MHC haplotype HT4 (or HT1) survived without obvious immune rejection in heterozygous HT4 (or HT1) recipients when coupled with ISTs (Kawamura et al., 2016; Shiba et al., 2016). While MHC-matching grafts induced lower immune responses, MHC-mismatched grafts still improved cardiac function similarly (Kashiyama et al., 2019). The concept of using PSCs with homozygous MHC haplotypes as an "off-the-shelf" cell source with acceptable immunogenicity is supported by research findings (Taylor et al., 2012). However, practical implementation would require establishing cell banks with different homozygous MHC haplotypes, addressing challenges posed by regional and

population-specific MHC polymorphisms.

CRISPR-based gene editing has been employed to manipulate HLA class I genes, generating iPSCs with homozygous-like MHC haplotypes. Researchers have successfully developed immune-compatible HLA-I-matching iPSC-ECs (expressing HLA-A*11:01) (Song et al., 2022a), and hypoimmune, gene-edited ESCs with HLA compatibility in around 40% of the Asian-Pacific population (Kim et al., 2021). Chinese HLA typing-based cell lines have also been created, with the disruption of HLA-B, -C, and *CIITA*, while preserving HLA-A*11:01. The authors estimate that this cell line covers approximately 21% of the Chinese population (Ji et al., 2023).

Although the long-term safety of gene editing for generating homozygous-like MHC haplotypes requires further investigation, these approaches hold promise for future clinical translation and may serve as a potential "off-the-shelf" stem cell bank. However, current studies indicate that IST is still needed even with MHC-matching allogeneic graft transplants.

(3) Engineering of MHC expression. As previously discussed, the rejection of PSCs in allografts is primarily based on the recognition of allogeneic MHC (HLA) molecules. Cells with low HLA expression, such as MSCs, enjoy immune privilege, particularly with adaptive immunity. Therefore, there has been a significant effort to create hypoimmune pluripotent stem cells (HIPSCs) by disrupting the expression patterns of HLA class I and/or II molecules.

HIPSCs are commonly generated by manipulating the HLA class I complex, either through complete ablation of *B2M* (Karabekian et al., 2015; Kitano et al., 2022; Suzuki et al., 2020; Wang et al., 2015), a necessary component of the HLA class I complex, or allele-specific genome editing (Jang et al., 2019; Ji et al., 2023; Kim et al., 2021; Xu et al., 2019). The former results in complete HLA deficiency, while the latter allows more precise manipulation of specific HLA alleles. Inactivating HLA class II can also be achieved by inactivating *CIITA* (MHC II transactivator) (Deuse et al., 2019; Deuse et al., 2021; Hu et al., 2023; Hu et al., 2024; Mattapally et al., 2018a; Thongsin et al., 2023; Wang et al., 2021a; Wang et al., 2020b). HIPSCs edited for HLA class I and/or II molecules exhibit resistance to $CD8^+$ cytotoxic T cells and other anti-HLA immune responses.

Combining multiple manipulations of HLA-associated genes can optimize immune properties. For instance, HIPSCs generated by ablating HLA-A/-B/-C and HLA class II, and overexpressing immunomodulatory factors PD-LA, HLA-G, and CD47, are insensitive to T cells, NK cells, and macrophages (Han et al., 2019). HIPSCs, with the biallelic knockout of B2M or forced expression of membrane-bound or secretory β2m-HLA-G fusion protein, remain pluripotent and generate fully functioning cells with minimal immunogenicity (Chen et al., 2023). Recently, HIPSC-derived CMs were developed, evading NKs and T cells with downregulated expression of STAT-1 and NF-κB via the SHP-1 pathway, surviving in post-MI rat hearts with minimal IST (Fang et al., 2023).

Compared to iPSCs with homozygous MHC haplotypes, HIPSCs typically do not require MHC matching and exhibit more robust immune privilege with proper surface editing of the cells. However, careful evaluation of potential risks associated with gene editing and tumorigenicity is crucial before their application in future preclinical and clinical studies.

(4) Modulation of NK ligand-receptor interaction. While MHC-matching and HIPSC-derived graft transplantation have largely avoided adaptive immune responses, challenges may still arise from NK-mediated innate immune responses, even in subjects receiving syngeneic grafts (Bogomiakova et al., 2023; Kruse et al., 2015). NK activation results from an imbalance of signals between activating and inhibitory NK ligand-receptor interactions (Bogomiakova et al., 2023). To achieve immune evasion from NK cells, one approach involves upregulating inhibitory NK ligand-receptor interactions while downregulating activating interactions.

MHC class I molecules, classic inhibitory ligands for NK cells, can be upregulated with IFN-γ stimulation. iPSCs (Kruse et al., 2015) and their derivatives (Bogomiakova et al., 2023; Nakamura et al., 2019) typically exhibit low expression of MHC class I molecules. Upregulating inhibitory MHC class I molecules through IFN-γ treatment has been shown to significantly reduce NK infiltration and NK-mediated lysis (Bogomiakova et al., 2023; Nakamura et al., 2019), leading to increased iPSC-CM engraftment in syngeneic mice. Forced expression of inhibitory NK ligands, such as HLA-E and HLA-G, in iPSCs and MSCs has also resulted in NK cell evasion by inhibiting NK cell activity (Gornalusse et al., 2017; Wang et al., 2021a; Zheng et al., 2022). Additionally, depleting activating NK ligands, such as CD155, in iPSC-derived T and B cells has protected the graft from NK-mediated lysis both *in vivo* and *in vitro* (Chimienti et al., 2022).

Genetically engineered HLA-disrupted HIPSCs exhibit low or absent expression of MHC class I, making them potentially susceptible to NK-mediated cytolysis (Guo et al., 2021). To induce immune tolerance in both lymphocytes and NK cells, gene editing has been applied to insert the sequence of inhibitory ligands into HLA alleles, generating fusion proteins like β2m-HLA-G and β2m-HLA-E (An et al., 2022; Chen et al., 2023; Gornalusse et al., 2017; Guo et al., 2021; Shi et al., 2020). These proteins serve as inhibitory ligands for NK cells while invading T cell recognition, thereby evading both T cell-mediated and NK-mediated cytolysis.

Despite these advances, iPSC-derived grafts may express activating ligands, such as CD112 (ligand for DNAM-1 receptor), CADM1 (ligand for CRTAM receptor), CD70 (ligand for CD27 receptor) (Bogomiakova et al., 2023), and MICA/B (ligands for NKG2D) (Kruse et al., 2015), suggesting that future research may focus on genetically modulating the expression of activating ligands or generating monoclonal antibodies to directly block activating ligands or receptors. However, it is crucial to carefully assess the safety of NK immune tolerance, considering that NK cells may also play a role in preventing undifferentiated iPSCs from forming teratomas or tumors (Gröschel et al., 2017).

(5) Immunosuppressive therapies. ISTs represent the primary approach for preventing immune rejection following allograft or organ transplantation (Strzelec et al., 2023). Mature IST protocols have been established for various allogeneic organ transplantations through extensive clinical trials and practice. For instance, the common regimen for heart transplant patients involves a triple immunosuppressant combination of cyclosporine (Cs) or tacrolimus (TAC), mycophenolate mofetil (MMF), and prednisolone (PSL) (Guethoff et al., 2013; Guethoff et al., 2015).

Studies have employed clinically relevant doses and types of immunosuppressants, such as methyl-PSL/TAC (Shiba et al., 2016), TAC/MMF/PSL (Kashiyama et al., 2019), and CsA/methyl-PSL/Abatacept (Chong et al., 2014), to induce immune tolerance in allografts (Table 4). IST is necessary even when

Table 4. Common immunosuppressive protocols for PCT[a)]

Animal model	Graft	Immunity	Immunosuppressive protocol	References
Pigtail macaques*	hESC-CMs	Xenogeneic	Methyl-PSL: Init. 500 mg on D0; Maint. 1.0–1.5 mg kg^{-1} d^{-1} CsA: Maint. serum conc. 200–250 μg L^{-1} (reached on −D5) Abatacept: Init. 12.5 mg kg^{-1} d^{-1} on D0; Maint. QOW	(Chong et al., 2014)
Cynomolgus macaques*	iPSC-CMs	Allogeneic, MHC-matched	Methyl-PSL: Init. 10 mg kg^{-1} d^{-1} from −D1 to D2; Maint. 1 mg kg^{-1} d^{-1} TAC: Maint. 0.1 mg kg^{-1} d^{-1} from −D2	(Shiba et al., 2016)
Cynomolgus macaques*	iPSC-CMs	Allogeneic, MHC-matched and -mismatched	PSL: Maint. 1 mg kg^{-1} d^{-1} from −D2 MMF: Maint. 40 mg kg^{-1} d^{-1} from −D2 TAC: Maint. 0.5 mg kg^{-1} d^{-1} from −D2, target serum conc. >10 ng mL^{-1}	(Kashiyama et al., 2019; Kawamura et al., 2016)
Pigtail macaques*	hESC-CMs	Xenogeneic	Methyl-PSL: Init. 30 mg kg^{-1} d^{-1} on −D1; Maint. 6 mg kg^{-1} d^{-1} for 2 d, then 3 mg kg^{-1} d^{-1} CsA: Maint. serum conc. 200–250 μg L^{-1} (reached on −D5) Abatacept: Init. 12.5 mg kg^{-1} d^{-1} on D0; Maint. QOW	(Liu et al., 2018)
Yorkshire pigs	hiPSC-CMs	Xenogeneic	CsA: Maint. 15 mg kg^{-1} d^{-1} from −D3	(Ye et al., 2014)
Yorkshire pigs	hESC-CMs	Xenogeneic	Methyl-PSL: Init. 250 mg on D0; Maint. 125 mg d^{-1} CsA: Maint. serum conc. 250 μg L^{-1} (reached by 10–16 mg kg^{-1} BID from −D5) Abatacept: Init. 12.5 mg kg^{-1} d^{-1} on D0; Maint. QOW	(Romagnuolo et al., 2019)
Minipigs	hiPSC-CMs	Xenogeneic	TAC: Maint. 0.6 mg kg^{-1} d^{-1} IV from −D5	(Kawamura et al., 2012)
Minipigs	hESC-CMs	Xenogeneic	Methyl-PSL: Init. 3 mg kg^{-1} d^{-1} from −D2 for 2 weeks; Maint. 1.0–1.5 mg kg^{-1} d^{-1} CsA: Maint. serum conc. >300–400 ng mL^{-1} (reached by 250–1,000 mg BID) Abatacept: Init. 12.5 mg kg^{-1} d^{-1} on D0; Maint. QOW	(Marchiano et al., 2023; Nakamura et al., 2021)
Yorkshire pigs	hiPSC-CMs	Xenogeneic	Methyl-PSL: Maint. 1.5 mg kg^{-1} d^{-1} CsA: Maint. 15 mg kg^{-1} d^{-1}	(Gao et al., 2018)
SD rats	hESC-CMs	Xenogeneic	Methyl-PSL: Maint. 2 mg kg^{-1} d^{-1} CsA: Maint. 15 mg kg^{-1} d^{-1}	(Caspi et al., 2007; Guan et al., 2020)
SD rats	hESC-CMs-constructed EHT	Xenogeneic	TAC: Maint. 8 mg kg^{-1} d^{-1} PO, BID, for 30 d	(Riegler et al., 2015)
SD rats	hiPSC-CMs	Xenogeneic	PSL (80 μg d^{-1}) for 1 month, then TAC (12 μg d^{-1}) and MMF (8 μg d^{-1}) for 1 month, tapering for 1 month	(Ito et al., 2023)

Recommendation: (methyl-)PSL: Maint. 1.0–1.5 mg kg^{-1} d^{-1} from −D2; MMF: Maint. 40 mg kg^{-1} d^{-1} from −D2; TAC: Maint. 0.1–0.5 mg kg^{-1} d^{-1} IV −D2, target serum conc. >10 ng mL^{-1}; Abatacept: Init. 12.5 mg kg^{-1} d^{-1} on D0; Maint. every 2 weeks; CsA: Maint. 15 mg kg^{-1} d^{-1} PO from −D3, target serum conc. >250–400 μg L^{-1}

a) D0 is defined as the day of transplantation, −D*x* is defined as *x* day(s) prior to D0, and D*x* is defined as *x* day(s) after D0. BID, bis in die/twice a day. CsA, cyclosporine A. hESC-CM, human embryonic stem cell-derived cardiomyocyte. hiPSC-CM, human induced pluripotent stem cell-derived cardiomyocyte. Init., initial dosage. Maint., maintenance dosage. (Methyl-)PSL, (methyl-)prednisolone. QOW, quaque omni week/every other week. TAC, tacrolimus. *, non-human primates, NHP.

donors and recipients share matching MHC, as it has been shown to inhibit graft rejection, prolong graft survival, and improve cardiac function (Kashiyama et al., 2019; Kawamura et al., 2016). However, the optimal IST protocol for PSC-derived grafts remains uncertain due to the significant differences in immunogenicity between iPSC and ESC-derived grafts, as well as between PSC grafts and solid organs (Ito et al., 2023; Nakamura et al., 2019).

Recent studies, such as the work by Ito et al. (2023) on an IST protocol for iPSC-CM patch transplantation in allogeneic rat MI models, highlight the importance of developing context-specific IST protocols. Despite the widespread use of ISTs in autologous, allogeneic, and xenogeneic transplantation of PSC-CMs and other stem cell derivatives, it is crucial to consider the potential side effects of long-term IST, including infections, nephrotoxicity, and malignancies (Söderlund and Rådegran, 2015). Future efforts should focus on identifying optimal IST protocols for PCT, particularly in different immunogenicity backgrounds, with a priority on minimizing immunosuppressant dosages to reduce the risk of graft rejection for the benefit of recipients.

(6) Other emerging strategies. Apart from the previously mentioned strategies, several recent studies have reported potentially promising strategies for future application. For example, Fang et al. (2020) harvested iPSCs reprogrammed from immune-privileged human amniotic fluid-derived stem cells (hAFSCs) and then differentiated them into functional hAFSC-iPSC-CMs, with minimal immunogenicity, with low expression of MHC class I and no expression of MHC class II. Another interesting discovery is related to the complement system. As previously mentioned, the complement system is a significant part of innate immunity, yet little is known about how it interacts with PSCs and PSC-derived cells. Recently, Gaykema et al. (2023) reported that overexpression of CD55 in hiPSCs and hiPSC-derived kidney organoids obstructed the activation of the complement system by inhibiting the key enzyme responsible for the complement cascade, C3 convertase. Future research may further uncover the important role that the complement system plays in stem cell-based cellular therapy, as well as proper strategies to modulate this system.

Immunogenicity in current clinical reports with iPSC-based therapy

Clinical case reports involving iPSC-based cell therapy have showcased promising results, addressing various medical conditions such as macular degeneration (Mandai et al., 2017; Sugita

et al., 2020; Takagi et al., 2019), Parkinson's disease (Schweitzer et al., 2020), and ICM. These reports include autologous iPSC-RPE transplantation for macular degeneration, autologous iPSC-derived dopamine progenitor transplantation for Parkinson's disease, and allogeneic iPSC-CM patch transplantation for ICM (Miyagawa et al., 2022). The last case, which involved epicardial transplantation of an allogeneic, clinical-grade hiPSC-CM patch, reported improvements in clinical symptoms, wall motion, and cardiac function without detectable complications such as arrhythmias, tumors, or immunosuppression-related issues (Miyagawa et al., 2022). Notably, the participants in these studies have not reported severe immune reactions so far.

While these initial findings are encouraging, long-term evaluations of graft function, immunogenicity, and graft-host integration are necessary to further validate the safety and efficacy of iPSC-based therapy. The use of immunosuppressants in iPSC-CM patch transplantation indicates the need for ongoing efforts to optimize IST. Autologous iPSC-derived therapies, while promising, currently face challenges related to the time-consuming and costly nature of their production. Achieving broader adoption of iPSC-based therapy may hinge on the establishment of a universal "off-the-shelf" HIPSC bank.

It is crucial to consider the varying immunogenicity of iPSC-derived grafts, with different cell lineages displaying distinct immunogenic profiles. For instance, iPSC-RPE exhibits relatively low immunogenicity (de Almeida et al., 2014; Guha et al., 2013), while iPSC-CMs may elicit intense immune responses (Araki et al., 2013; Kawamura et al., 2016). This variability underscores the importance of carefully evaluating the necessity for different levels of immunomodulation tailored to specific iPSC-derived grafts (Miyagawa et al., 2022). Overall, the evolving landscape of iPSC-based therapies holds promise, but ongoing research and clinical studies will be pivotal in refining protocols and expanding their applicability.

Engraftment arrhythmias

Overview of engraftment arrhythmias

EA is defined as transient but severe ventricular arrhythmias, typically sustained ventricular tachycardia (VT) and accelerated idioventricular rhythm (AIVR), occurring and peaking 1–2 weeks after PCT, and decreasing within 1–2 months (Nakamura et al., 2021).

EA has been widely reported in many studies and is considered one of the greatest safety concerns obstructing the clinical application of PCT. In comparison to small animal models (such as mice, rats, and guinea pigs), where apparent EAs have not been observed (likely masked by their rapid baseline heart rate) (George et al., 1990), almost all large animals, such as pigs (Marchiano et al., 2023; Nakamura et al., 2021; Romagnuolo et al., 2019) and NHP (Chong et al., 2014; Liu et al., 2018; Shiba et al., 2016), exhibit a heavy burden of EAs after PCT, reaching as high as approximately 90% of the time per day suffering from EAs. Interestingly, while NHPs, such as *Macaca nemestrina* (Chong et al., 2014; Liu et al., 2018) and *Macaca fascicularis* (Shiba et al., 2016), have shown relative tolerance to EAs without fatal distress, EAs can be much more severe, even fatal in pigs, causing sustained VTs with heart rates of 220–350 bpm (beats per minute), leading to ventricular fibrillation or acute HF (Marchiano et al., 2023; Nakamura et al., 2021; Romagnuolo et al., 2019). The occurrence of fatal EAs in pigs can be especially concerning due to the structural and functional similarities between pig and human hearts. Particularly, the sinus heart rate (HR) of pigs (approximately 90 bpm) is closest to that of humans (approximately 60–100 bpm), compared with NHPs and small animals (Romagnuolo et al., 2019) (Figure 4).

Mechanisms of engraftment arrhythmias

As mentioned previously, one of the main differences between hPSC-CMs and adult ventricular cardiomyocytes (vCMs) is that hPSC-CMs exhibit relatively immature electrophysiology. Particularly, hPSC-CMs present automaticity (the ability to spontaneously depolarize, generate action potentials, and contract), while adult vCMs are quiescent, only excited by the pacemaker cells. Therefore, the automaticity of immature hPSC-CM grafts has long been suspected to mediate EAs. Much evidence has emerged to support this suspicion. For instance, recipients of hPSC-CMs typically do not exhibit EAs right after transplantation. However, as the graft electromechanically integrates with the host myocardium, the burden and severity of EA started peaking, eventually waning down as the grafted hPSC-CMs matured *in vivo* 1–2 months post-transplantation (Chong et al., 2014; Liu et al., 2018; Romagnuolo et al., 2019; Shiba et al., 2016). Once the EA commenced, neither overdrive pacing nor electrical conversion successfully terminated EAs (Liu et al., 2018). More recently, electrical mapping revealed that EAs originate from a focal point source at the site of the hPSC-CM graft (Liu et al., 2018; Romagnuolo et al., 2019). Collectively, these findings suggest that EAs most likely result from the ectopic excitation originating from the automaticity of immature hPSC-CM grafts, rather than reentry mechanisms (Marchiano et al., 2023). Results from computational modeling for hPCT after MI are also in line with this conclusion (Gibbs et al., 2023; Yu et al., 2019).

Fundamentally, the automaticity of immature hPSC-CMs is driven by the high expression of depolarizing-related ion channels and the low expression of repolarizing-related ion channels (Marchiano et al., 2023). However, the spontaneous excitation of PSC-CMs does not seem to cover all the mechanisms for EAs, as the *in vitro* quiescent MEDUSA hESC-CMs (*HCN4*/*CACNA1H*/*SLC8A1* 3KO/*KCNJ2* KI) developed by Marchiano et al. (2023) still resulted in sporadic premature ventricular contractions and rare nonsustained ventricular tachycardia, suggesting more complex mechanisms may contributed to the appearance of EAs. Future investigations are necessary for a more complete understanding of how EAs are driven, and corresponding strategies to eliminate them.

Strategies to reduce engraftment arrhythmias

As the mechanisms underlying EA are gradually being revealed, EAs can potentially be mitigated, if not avoided completely, by targeting the mechanisms, particularly the automaticity of immature PSC-CM grafts. Here, we further summarize some of the most current attempts as well as possible future directions.

(1) Anti-arrhythmic drug therapies.Given that the automaticity of the graft is mainly responsible for EA, it is thought that drugs that suppress automaticity may mitigate EA, ensuring recipients' safety until the graft matures *in vivo* and automaticity diminishes. Nakamura et al. (2021) have reported that the combination of amiodarone (a class III anti-arrhythmic drug) and ivabradine (a selective inhibitor of I_f, one of the main

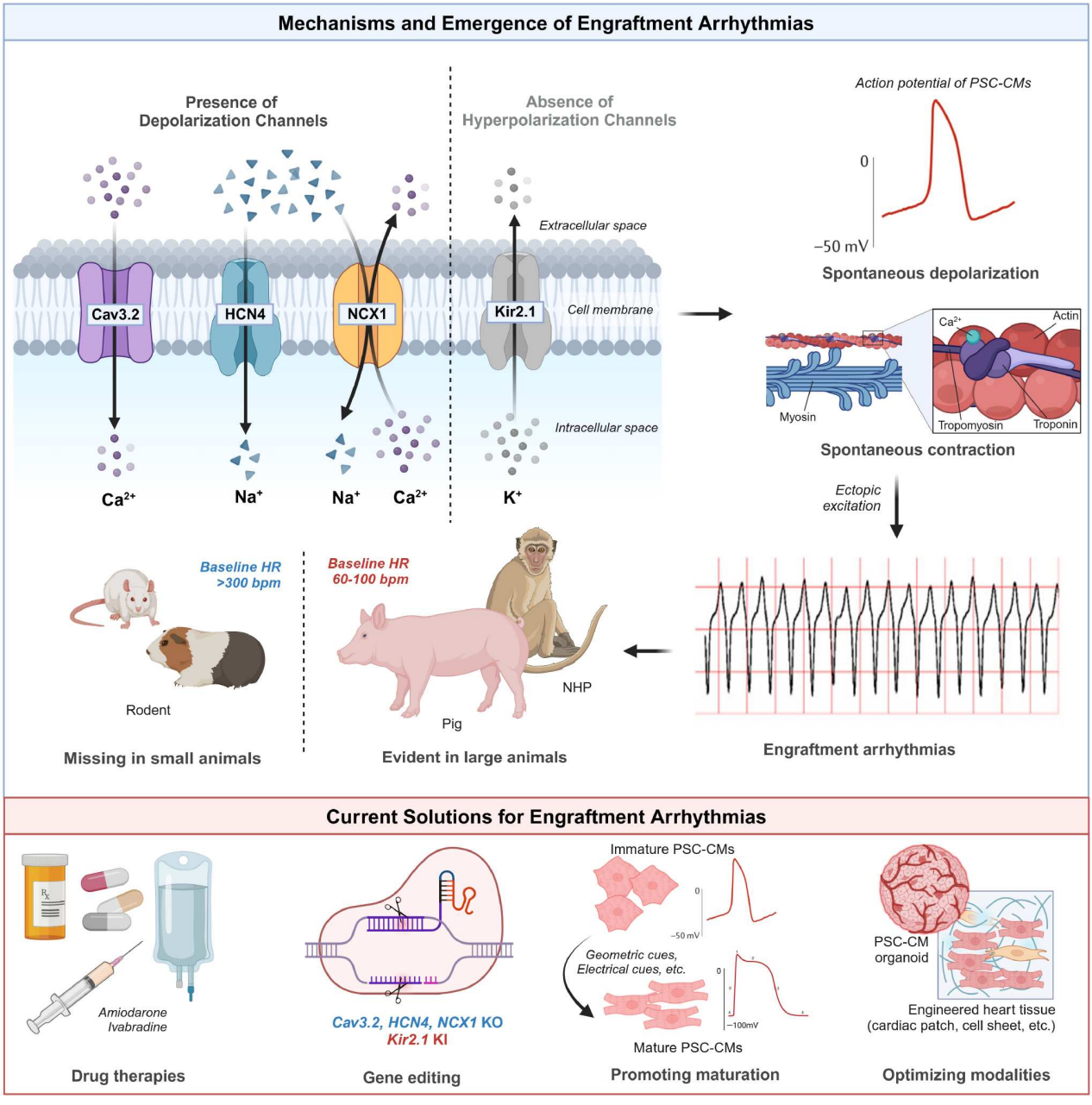

Figure 4. Mechanisms, emergence and current solutions of engraftment arrhythmias. EAs refer to the severe and constant ventricular arrhythmias that occur during early stages post-transplantation and are almost exclusively observed in large animals with relatively slower baseline HR. The fundamental mechanism contributing to EAs lies within the immature electrophysiology of PSC-CMs, as a consequence of immature ion channel expression. Due to the overexpression of depolarization channels, and low expression of hyperpolarization channels, PSC-CMs can spontaneously depolarize and contract, resulting in ectopic excitation, and present as EAs. Solutions for EAs include anti-arrhythmic drug therapy, gene-editing of ion channels, promoting PSC-CM maturation, and optimizing graft modalities. EA, engraftment arrhythmia. HR, heart rate. PSC-CM, pluripotent stem cell-derived cardiomyocyte.

currents responsible for the phase 4 spontaneous depolarization in cardiac pacemaker cells) effectively decreased the HR and EA burden, and increased survival after PCT in a pig MI model. However, the therapy failed to eliminate EAs entirely, with an EA burden of nearly 40% seven days post-PCT. Future attempts may focus on exploring more accurate dosages or effective combinations of drug therapy to further decrease or even eliminate the EA burden in the early stages post-PCT.

(2) Gene editing of ion channels. The expression pattern of ion channel genes mechanistically underlies the automaticity of hPSC-CMs, as well as that of pacemaker cells. In general, hPSC-CMs are found to have higher levels of expression for depolarization-associated genes and less hyperpolarization-associated ones, compared with vCMs (Nakamura et al., 2021). For instance, the hPSC-CMs exhibit extremely low expression levels of *KCNJ2*, which encodes Kir2.1, the ion channel mediating inward rectifying K^+ current (Nakamura et al., 2021). Therefore, modulating gene expression patterns towards quiescent vCMs may fundamentally limit the occurrence of EAs.

To this attempt, Marchiano et al. (2023) applied gene editing

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to knock out three depolarizing associated ion channel genes, *HCN4* (responsible for I_f), *CACNA1H* (responsible for I_{CaT}), and *SLC8A1* (encoding NCX1, which mediates Na^+/Ca^{2+} currents), and overexpressed hyperpolarizing associated ion channel gene *KCNJ2*, creating a quadruple gene-edited cell line, the MEDUSA (modification of electrophysiological DNA to understand and suppress arrhythmias) hPSC-CMs. The team confirmed that MEDUSA hPSC-CMs are quiescent yet excitable and contractable *in vitro*, and can decrease post-transplant EA burden by ~95% (P <0.001) in porcine (Marchiano et al., 2023). Nevertheless, even with quadruple gene-editing, porcine recipients of MEDUSA hPSC-CMs still presented sporadic, transient, and self-limited VTs at high dosages of cell transplantation (Marchiano et al., 2023), calling for deeper scoping of the mechanisms that may result in residual EAs. On top of that, the multiple gene editing strategy may raise concerns in regard to the safety and functionality of the cells. The concern of whether or not gene editing is necessary is especially important, as EAs can decrease over time while the editing of genes, as well as the matured cardiomyocytes, remain permanent. Functional modulation of genes through RNA delivery may also serve as a potential means for only temporal modulation.

(3) Promoting maturation of PSC-CMs. Since automaticity is characteristic only for immature PSC-CMs, and EAs gradually decrease as the graft matures correspondingly, it has been assumed that transplanting PSC-CMs that have undergone maturation *in vitro* may reduce EAs. In support of the hypothesis, Dhahri et al. (2022) reported that transplanting matured PSC-CMs resulted in less proarrhythmic behavior and improved integration and functionality. However, there has been no evidence of similar observations in large animal models, which calls for further investigation. Additionally, it would be beneficial to examine the effect of different maturation protocols. For instance, electrical stimulation, one of the most commonly used methods to enhance hPSC-CM maturation (Hong et al., 2023), has been observed to alter the expression of ion channels and electrophysiological properties of hPSC-CMs (Eng et al., 2016). Whether and how different maturation protocols may influence EA is a subject under further exploration.

(4) Optimizing graft modalities. The different graft modalities may also influence arrhythmic activity. For instance, Gao et al. (2018) reported that swine MI models receiving a human CM patch showed no spontaneous arrhythmias compared with controls. The authors suspected that the absence of arrhythmic activity was due to the improved electrophysiological maturation of hPSC-CMs, yet the low cell engraftment rate might also have contributed to the result (Gao et al., 2018). Another computational modeling research suggested that, although ectopic activation is rare due to improved cellular alignment, hPSC-CM cell sheets may serve as a substrate for reentrant VTs (Yu et al., 2019), though this has yet to be observed in animal studies.

In addition to the modalities for hPCT, the location (e.g., intramyocardial or epicardial), the spatial and electrical relationship between the graft and infarct scar tissue of the host myocardium (e.g., peri-infarct or intra-infarct), and the delivery strategy (e.g., DII, epicardial patch placement, or transcatheter delivery) may all influence in the arrhythmic episodes post-transplantation (Gibbs et al., 2023; Yu et al., 2019). An optimized protocol with the least proarrhythmic properties remains to be established to ensure the safety of clinical translation.

Graft-host integration

The degree of structural and electromechanical integration between PSC-CM grafts and host tissues significantly affects the safety and efficacy of PCT. For instance, immature graft-host integration has been linked to severe host-related complications, such as EAs, as previously described, while mature integration exhibits suboptimal graft survival (Cheng et al., 2023b; Shiba et al., 2016). Nonetheless, the electromechanical integration between host and graft is a prerequisite for restoring cardiac, especially contractile function in injured hearts (Shadrin et al., 2017). So far, studies in MI NHPs have generated the most promising outcomes, with effective electromechanical integration within a few weeks post-transplantation (Chong et al., 2014; Liu et al., 2018).

Traditional evaluation of graft-host integration involves immunohistology analysis, which limits real-time observation of integration in live subjects. Over the past years, cardiac live imaging technology has been developed to provide more dynamic observation of graft-host integration. One approach involves introducing the genetically encoded fluorescent calcium sensor GCaMP into the graft, enabling the visualization of cyclical cytosolic calcium transients (Zhu et al., 2014). This approach can effectively distinguish the PSC-CMs from the host myocardium, thus allowing for the quantitative evaluation of the presence and extent of host-graft electromechanical integration and the status of graft retention (Zhu et al., 2014). Another team innovatively introduced a dual-objective light-sheet fluorescence microscope (LSFM) system to achieve 3D imaging with high spatio-temporal resolution and limited photo-toxicity (Dvinskikh et al., 2023). Future attempts are needed to explore the optimal protocol that leads to superior graft-host integration, as well as means to monitor the integration status.

Tumorigenicity

Tumorigenicity remains a significant concern in PSC-based therapy, for their potential for infinite proliferation (Yamanaka, 2020). When compared with ESCs, iPSCs have shown a greater susceptibility to teratoma formation (Gutierrez-Aranda et al., 2010). However, iPSC-CMs have not been demonstrated to result in teratoma formation after *in vivo* transplantation in preclinical and clinical studies (Guan et al., 2020; Li et al., 2023; Liu et al., 2018; Miyagawa et al., 2022). These findings partly establish the safety rationale for broader clinical translation of PCT.

Nevertheless, as the long-term tumorigenicity of transplanted grafts remains unknown, and patients receiving allogeneic or even syngeneic PSC-CMs may require IST, there is still a need to establish rigorous protocols to minimize or eliminate tumorigenicity, generating clinical-grade iPSC-CMs. This protocol may encompass the detection and targeted eradication of undifferentiated or aberrantly proliferating iPSCs (Ito et al., 2019) and the introduction of apoptotic genes aimed at removing undifferentiated iPSCs (Itakura et al., 2017).

Clinical trials of PSC-CM transplantation

At present, PCT treatment is still in the preclinical and clinical trial stages. In the following sections, we will discuss clinical trials of dispersed PSC-CM and EHT transplantation, respectively, whether derived from ESCs or iPSCs. Table 5 summarizes the

Table 5. Overview of current clinical trials of PCT[a)]

Trial ID	Sponsor	Title	Condition	Intervention	Est. enroll.	Start date	Country
NCT05068674	Stanford University	Human Embryonic Stem Cell-Derived Cardiomyocyte Therapy for Chronic Ischemic Left Ventricular Dysfunction	Chronic ischemic left ventricular dysfunction	Injection of hESC-CMs	18	Mar. 2022	The U.S.
NCT02413450	Johns Hopkins University	Derivation of Human Induced Pluripotent Stem (iPS) Cells to Heritable Cardiac Arrhythmias	Inherited cardiac arrhythmias, LQTS, BrS, CPVT, ERS, AC, ARVD/C, HCM, DCM, muscular dystrophies (Duchenne, Becker, myotonic dystrophy), normal control subjects	Not provided	100	Aug. 2013	The U.S.
NCT03763136	Help Therapeutics	Treating Heart Failure With hPSC-CMs (HEAL-CHF)	Heart failure	Injection of allogeneic hPSC-CMs during CABG surgery. 200 million hPSC-CMs in 2.5–5 mL medium suspension will be injected into the myocardium	20	May 2019	China
NCT05223894	Help Therapeutics	Treating Heart Failure With hiPSC-CMs	Heart failure	Injection of allogeneic hPSC-CMs during CABG surgery. 100 million hPSC-CMs in 2.5–5 mL medium suspension will be injected into the myocardium	20	Apr. 2022	China
NCT04982081	Help Therapeutics	Treating Congestive HF With hiPSC-CMs Through Endocardial Injection	Cardiovascular diseases Congestive heart failure DCM	hiPSC-CM will be injected into the myocardium through a transcatheter endocardial injection system	20	Sept. 2021	China
NCT05566600	Help Therapeutics	Allogeneic iPSC-derived Cardiomyocyte Therapy in Patients With Worsening Ischemic Heart Failure	Ischemic heart failure Chronic heart failure	10–20 epicardial injections (0.25 mL each) of iPSC-CMs will be delivered in the border zone of the infarcted myocardium	32	Oct. 2022	China
NCT05647213	HeartWorks	Autologous Induced Pluripotent Stem Cells of Cardiac Lineage for Congenital Heart Disease	Univentricular heart Congenital heart disease Heart failure NYHA class III Heart failure NYHA class IV	Subjects in the treated arm will receive one dose of investigational product	50	Feb. 2023	The U.S.
NCT04696328	Osaka University	Clinical Trial of Human (Allogeneic) iPS Cell-derived Cardiomyocytes Sheet for ICM	Myocardial ischemia	Transplantation of allogeneic hiPSC-CM sheet	10	Dec. 2019	Japan
NCT04945018	Heartseed	A Study of iPS Cell-derived Cardiomyocyte Spheroids (HS-001) in Patients With Heart Failure (LAPiS Study) (LAPiS)	Heart failure Ischemic heart disease	Injection of allogeneic hiPSC-CM spheroids suspension	10	Apr. 2022	Japan
NCT04396899	University Medical Center Goettingen	Safety and Efficacy of Induced Pluripotent Stem Cell-derived Engineered Human Myocardium as Biological Ventricular Assist Tissue in Terminal Heart Failure (BioVAT-HF)	Heart failure	Implantation of EHM on dysfunctional left or right ventricular myocardium in patients with HFrEF (EF<35%)	53	Feb. 2020	German

a) AC, ARVD/C, arrhythmogenic cardiomyopathy. BrS, Brugada Syndrome. CPVT, catecholaminergic polymorphic ventricular tachycardia. DCM, dilated cardiomyopathy. EHM, engineered human myocardium. ERS, early repolarization syndrome. HCM, Hypertrophic cardiomyopathy. hESC, human embryonic stem cell. HFrEF, heart failure with reduced ejection fraction. hPSC, human pluripotent stem cell. LQTS, long QT syndrome.

most recent clinical trials of PCT.

Clinical trials of dispersed PSC-CM transplantation

While many preclinical experiments chose ESCs as the source of CMs, there are relatively few clinical trials involving the use of ESC-CMs. In a phase 1 dose-escalation pilot study, Joseph and his colleagues injected 50 million, 100 million, and 150 million ESC-CMs into the hearts of 18 patients to explore the optimal concentration for the treatment of chronic ischemic left ventricular dysfunction (CYG ID: NCT05068674).

In the case of iPSC-CMs, related research began as early as 2013. Andreas et al. collected somatic cells from patients with MI, reprogrammed and induced them into iPSC-CMs. These iPSC-CMs were originally used as the earliest disease models and research materials (CTGID: NCT02413450).

In May 2019, *Nature* reported two patients from China received iPSC-CMs injections during CABG surgery. Dongjin Wang, the surgeon, stated that the operation received a very positive effect, with neither of the two patients experiencing tumorigenesis, immune rejection, arrhythmias, or other complications. This marked the first known clinical application of dispersed iPSC-CMs for treating injured hearts (Mallapaty, 2020). The research group was subsequently granted approval to

expand their study to include 20 patients to further investigate the safety and efficacy of this protocol (CYGID: NCT03763136) (Zhang et al., 2022).

Help Therapeutics, the company that supplied the iPSC-CMs for Wang's study, subsequently collaborated with several Chinese hospitals. They conducted a series of trials involving the injection of different doses of iPSC-CMs into the endocardium and epicardium to determine the optimal treatment for HF (CYGID: NCT05223894, CYGID: NCT04982081, CYGID: NCT05566600). However, none of these trials have published their results so far.

In a study led by Timothy et al., researchers conducted a clinical trial using iPSC-CMs injection to treat CHDs (CYGID: NCT05647213). Nevertheless, no results from this trial have been published thus far.

Clinical trials of engineered heart tissue transplantation

In 2013, the earliest clinical trial involving EHT was conducted. In Philippe et al.'s study, 6 patients received a median dose of 8.2 million hESC-derived cardiovascular progenitors embedded in a fibrin patch. This patch was delivered epicardially during CABG. Although the hESCs were not directly induced into CMs, the progenitor cells were expected to continue differentiating into CMs, ECs, and SMCs *in vivo*. While two patients unexpectedly died (unrelated to the treatment), the remaining four patients assessed in a 1-year follow-up reported improved heart function. No teratomas, arrhythmias, or severe immune rejection were observed in these patients (Menasché et al., 2015; Menasché et al., 2018; Miller, 2018).

Clinical applications of iPSC-derived EHTs have been spearheaded by Japanese scientists. In January 2020, Sawa's team transplanted 3 allogeneic iPSC-CM patches (with 3.3×10^7 cells/patch) onto the epicardium of the anterior and lateral walls of the left ventricle of a patient with ICM. Researchers did not detect lethal arrhythmias or tumorigenesis after transplantation in this clinical case. Additionally, positron emission tomography (PET) revealed improved left ventricular wall motion following patch transplantation, accompanied by enhanced coronary flow reserve. These tests collectively demonstrated that the transplantation of iPSC-CM patches facilitated the functional recovery of the patient's injured heart (Miyagawa et al., 2022). Building on this success, a follow-up trial involving 10 patients is currently ongoing (CYGID: NCT04696328). In contrast to other clinical trials mentioned earlier, in this case, the transplant procedure is *not* performed simultaneously with other treatment methods such as CABG. This allows researchers to establish a clear link between the improvement in heart function to the transplantation of iPSC-CM patches.

Hreatseed, a Japanese company, conducted a phase 1/2 trial involving 10 patients. They aimed to evaluate the safety and efficacy of a type of iPSC-CM spheroids transplanted into patients with severe HF caused by underlying ischemic heart disease (CYGID: NCT04945018). The trial is still in the recruiting stage.

Tim et al. conducted a phase 1/2 trial to test an EHT constructed from defined mixtures of iPSC-CMs and stromal cells in bovine collagen type I hydrogel. The trial targets patients suffering from advanced HF with reduced ejection fraction and no realistic option for heart transplantation (CYGID: NCT04396899).

The primary indicators for evaluating the safety of PSC-CM transplantation (whether as dispersed PSC-CM or EHT transplantation) are the risk of tumorigenesis, immune rejection, and the potential to induce arrhythmias. Overall, there is currently no standardized protocol for safety assessment. The aforementioned Chinese study conducted the most comprehensive testing, with researchers planning a 12-month follow-up for patients. This follow-up will assess cardiac function and the incidence of arrhythmias through echocardiography, MRI, PET, electrocardiogram, exercise tolerance tests, and related questionnaires. Tumorigenesis will be evaluated through chest, abdominal, and pelvic CT scans and PET-CT scans. Immune rejection levels will be assessed via donor-specific antibody (DSA) testing and serological testing for anti-human leukocyte antigen (anti-HLA) antibodies/panel reactive antibodies (PRA) (NCT03763136).

Discussion

In recent years, the field of cardiovascular medicine has witnessed remarkable advancements driven by stem cell-based therapeutics in preclinical and clinical studies (Clavellina et al., 2023). For instance, the MSC-derived therapy has been shown to promote MI recovery (Ala, 2023; Barrère-Lemaire et al., 2024; Wang et al., 2021d), and alleviate arterial or pulmonary hypertension (Hansmann et al., 2022; Zhang et al., 2024). One of the most exciting and promising advancement is the application of PSC-CM for cardiac regeneration. The core of this approach is the transplantation of PSC-CMs into injured hearts, aiming to integrate and remuscularize with the host myocardium, replenishing lost adult CMs which are considered non-regenerable under physiological conditions, and ultimately enhancing cardiac function. Extensive preclinical studies have unveiled several challenges of PCT, including immune rejection, engraftment arrhythmias, tumorigenicity, and challenges in host-graft integration. So far, immune rejection has been properly managed by various strategies such as IST and MHC engineering. However, EAs still remain a huge challenge, as none of the current reports have completely eradicated EAs, even with quadruple gene editing (Marchiano et al., 2023). It is worth pointing out that individual clinical case reports revealed no proarrhythmic behaviors in patients receiving PSC-CM patch transplantation (Miyagawa et al., 2022).

Meanwhile, several current reports of PCTs entail transplanting hPSC-CMs into immuno-deficient animals (Cheng et al., 2023a; Lou et al., 2023; Mattapally et al., 2018b) or animals treated with immunosuppressants (Cheng et al., 2023a; Dhahri et al., 2022), which is essentially xenogeneic transplantation. Therefore, questions have been raised about whether the immune response and treatment effects observed in xenogeneic transplantation are comparable to allogeneic transplantation, which is of greater clinical relevance. So far, there have been no studies comparing immune responses and therapeutic effects of allogeneic versus xenogeneic transplantation of CMs, but relevant studies regarding other tissue transplantation can enlighten us on this matter. Erdag and Morgan reported that the human neonatal skin allografts were rejected between 5 and 9 d, whereas xenografts rejection occurred at 5–8 d. Allograft rejection was mediated by intense $CD3^+$, $CD4^+$, and $CD8^+$ T cell infiltration, and similar pathological changes were also observed in the xenograft models, suggesting comparable immune responses between xenograft and allograft rejection (Erdag and Morgan, 2004). Allogeneic and xenogeneic human embryonic

stem cell grafts (Grinnemo et al., 2006) and cartilage grafts (Uto et al., 2023) also induced similar immune responses. Both allogeneic and xenogeneic MSCs showed equal efficacy in acute cerebral infarction (Gutiérrez-Fernández et al., 2015), lung protection against ischemia-reperfusion injury (Lin et al., 2020), and soft tissue repair (Dong et al., 2020). However, Hwang et al. (2020) discovered that xenogeneic MSCs induced greater leukocyte and neutrophil infiltration than allogeneic MSCs, yet allogeneic MSCs attracted more microglia and macrophages. Shen et al. (1996) reported that neonatal intrathymic inoculation in recipients only abrogated allogeneic, but not xenogeneic rejection for heart transplantation. Interestingly, Choi et al. (2016) observed that human xenogeneic adipose tissue-derived MSCs prolonged the life of systemic lupus erythematosus mice even more than allogeneic MSCs, although inducing a greater humoral immune response. Taken together, xenogeneic and allogeneic tissue transplantation is comparable to a certain extent in regards to immune response and therapeutic efficacies, for which xenograft experiments can certainly guide the results and response of allograft transplantation. However, as the results of the comparison may vary from one tissue to another, it is still important to conduct comparative studies to further illuminate the similarities and differences between allogeneic and xenogeneic hPSC-CM transplantation.

As of now, no FDA-approved PSC-CM-based therapeutics are available globally. Nonetheless, many clinical trials have been undertaken, with some current reports suggesting that PCT demonstrates acceptable safety and efficacy. However, further investigations into the interplay between cell sources, transplantation methods, and potential risks and benefits are of great importance before achieving widespread clinical application.

Over the past two years, several cases of xenotransplantation of the heart (Schmauch et al., 2024), kidney (Montgomery et al., 2022), and liver (Mallapaty, 2024) have been reported (Fang et al., 2024). In these cases, functional organs of genetically engineered pigs (mainly α-1,3-galactosyl knockout) were transplanted into human recipients (Fang et al., 2024). However, many challenges remain so far. The first porcine kidney recipient died two months after the transplantation, with RNA-seq showing signs of antibody-mediated rejection (Pan et al., 2024). The first porcine heart recipient survived for 60 days, whereas the second survived for less than 6 weeks, also demonstrating molecular-level rejection and ischemic reperfusion injury (Schmauch et al., 2024). More importantly, whole-organ transplantation is a major surgery with various risks, and recipients are subject to life-long anti-rejection medication. These

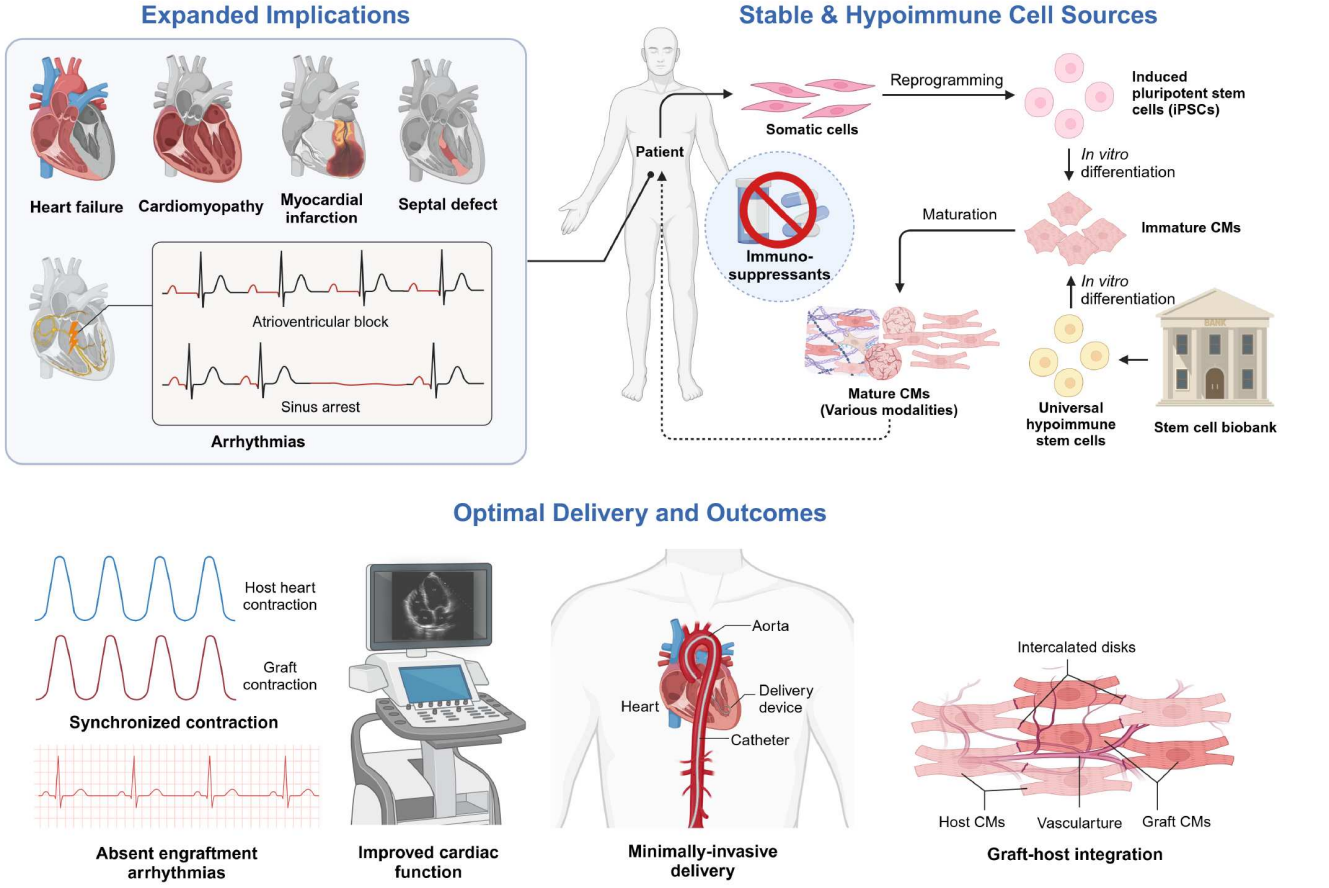

Figure 5. The future of PCT. The future of PCT mainly includes three areas. PCT is expected to expand its implications from MI to all CVDs which is caused by diseased CMs or can result in CM loss. Another area of interest is the robust cell source of PSC-CMs, either autologous PSC derived from patients' own cells, or HIPSCs from stem cell biobanks. With minimal immunogenicity, the need for immunosuppressants is avoided. At last, it will also be necessary to optimize the delivery modality and strategy, to achieve optimal outcomes, as exemplified by mature graft-host electromechanical integration, improved vascularization, synchronized contraction, and eventually result in improved cardiac function without engraftment arrhythmias. CM, cardiomyocyte. CVD, cardiovascular disease. HIPSC, hypoimmune pluripotent stem cell. MI, myocardial infarction. PCT, PSC-CM transplantation. PSC-CM, pluripotent stem cell-derived cardiomyocyte.

all compose the possibility of complications (Fang et al., 2024; Kozlov, 2024). Transplantation of homologous iPSC-CMs, while unable to replace the entire diseased organ, can avoid the long-term use of anti-rejection medication, as well as the need for a major operation (Marchiano et al., 2023; Nakamura et al., 2021). Therefore, xenotransplantation and iPSC-CM transplantation should serve patients under different conditions and stages, as xenotransplantation offers patients with end-stage HF and waiting for heart transplant more chances, whereas iPSC-CM transplantation would serve those who can still benefit from partial regeneration of cardiac muscles, preventing them from progressing into end-stage HF. Future clinical trials may provide more evidence for the comparison and indications of these treatment options.

In the future, the indications of PSC-CMs may broaden from MI to cardiomyopathy, HF, CHDs (such as septal defect), and arrhythmias. More importantly, the cell sources for PSC-CMs ought to be more stable, with minimal immunogenicity. Finally, with PSC-CMs of multiple modalities transplanted through minimally invasive techniques, patients with CVDs may experience improved cardiac function, based on robust host-graft integration (Figure 5). To summarize, PCT continues to represent a cutting-edge and promising strategy for cardiac regeneration, bringing hope to patients struggling with cardiovascular diseases. Despite prevailing challenges and ongoing preclinical research and clinical trials, the authors are cautiously optimistic regarding the therapeutic prospects of PCT.

Compliance and ethics

The authors declare that they have no conflict of interest. All figures in the text are generated using biorender.com.

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