Review Article

Recent Advances in Maturation of Induced Pluripotent Stem Cells-derived Cardiomyocytes

Junting Zhao[1], Richard Qiang Hao[2]

[1]Sage Hill School, 20402 Newport Coast Dr, Newport Coast, CA 92657, USA;
[2]Troy High School, 2200 E Dorothy Ln, Fullerton, CA 92831, USA

ABSTRACT

Induced pluripotent stem cells -derived cardiomyocytes (iPSC-CMs) increasingly become an important tool for studying genetic heart diseases, drug screening and heart cell therapy. However, iPSC-CMs differentiated by the traditional ways are immature and the challenge of producing fully matured iPSC-CMs that functionally resemble adult cardiomyocytes persists. Immature iPSC-CMs exhibit characteristics typical of fetal-stage cells, including less developed contractility, electrophysiological properties, and metabolic processes. These limitations restrict their clinical applications, especially in regenerative medicine. Over the past decade, numerous strategies have been developed to enhance the maturation of iPSC-CMs. These include prolonged culture periods, biochemical induction using specific hormones and energy substrates, and the application of biophysical stimuli such as electrical and mechanical stimulation. In addition, advances in biomaterials, such as extracellular matrices and hydrogels, have been crucial in replicating the physiological environment needed to support iPSC-CM development. Together, these methods aim to produce cardiomyocytes that more closely mimic adult heart cells in structure and function. This review explores recent progress in optimizing the maturation of iPSC-CMs and highlights their potential impact on future applications in cell therapy and heart disease modeling.

Keywords: Induced pluripotent stem cells; Cardiomyocytes; Maturation; iPSC-CMs; Disease Modeling; Immature

INTRODUCTION

Induced pluripotent stem cells (iPSCs) have revolutionized the field of regenerative medicine since their discovery by Yamanaka and colleagues in 2006 (1). iPSCs are reprogrammed by the introduction of four specific transcription factors including Oct4, Sox2, c-Myc and Klf4 into somatic cells (2, 3). Under proper culture conditions, iPSCs can differentiate into all cell types found in the body, and they could be used for disease modeling, cell therapy and drug screening.

iPSCs-derived cardiomyocytes (iPSC-CMs) play critical roles in studying cardiac diseases, heart cell therapy and cardiac drug tests. Many congenital cardiac diseases arise from genetic mutations in sarcomeric proteins. For instance, myosin-binding protein C mutations account for approximately ~35% of hypertrophic cardiomyopathy

Corresponding author: Richard Qiang Hao, E-mail: rhao2468@gmail.com.
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(HCM) cases, a genetic cardiac disease with prevalence of 1:500 in the general population and an annual mortality rate of 1-5% (4, 5). Despite extensive research efforts over past decades, our understanding of the pathogenic mechanism underlying HCM has been remains limited, largely due to challenges in obtaining human cardiac samples, difficulties in culturing human heart tissue and the limitations of murine HCM models that may not accurately replicate human HCM (6, 7, 8, 9). iPSC-CMs from HCM patients and health individuals, respectively, can be used to model this genetic heart disease by studying phenotypic differences observed in the iPSCs-CMS of the patients and health individuals. Additionally, ischemic heart injury which damages cardiomyocytes from the blocked coronary blood vessels, is a leading cause of cardiovascular disease and contributes to millions of deaths yearly worldwide (10). iPSC-CMs hold potential to replace the damaged cardiomyocytes in future cell therapy to restore heart function (11, 12). Moreover, iPSC-CMs are also increasingly used by pharmaceutical companies to test the toxicities and efficacies of potential drugs, which facilitates drug development (13, 14).

Although iPSC-CMs are an important tool in studying cardiac diseases, cell therapy and drug screening, the traditional methods of generating those cells often result in immature cardiomyocytes resembling the fetal stage cells. These immature iPSC-CMs are less developed in cardiac contractility, conduction velocity, and action potential duration in comparison to adult mature cardiac cells (15) (Figure 1). Therefore, it is critical to generate mature iPSCs-CMs that function similar to the adult cardiac cells. Over the past decade, significant efforts have been made to achieve this goal through different approaches including prolonged in-vitro culture, biomaterials, biochemical induction, biophysical simulation. In the article, we review the recent advance in the maturation of iPSC-CMs.

DIFFERENCE BETWEEN IMMATURE IPSC-CMS AND MATURE-CMS

In comparison to mature CMs, iPCS-CMs are generally immature cells mimicking the fetal stage cardiomyocytes, displaying different characteristics in morphology / structure, electrophysiology, Ca^{2+} handling, metabolism and transcriptional signatures (16) (Table 1).

Different Characteristics in Morphology and Structure

Immature iPSC-CMs are smaller, rounder, and lack the distinct striations seen in mature cardiomyocytes.

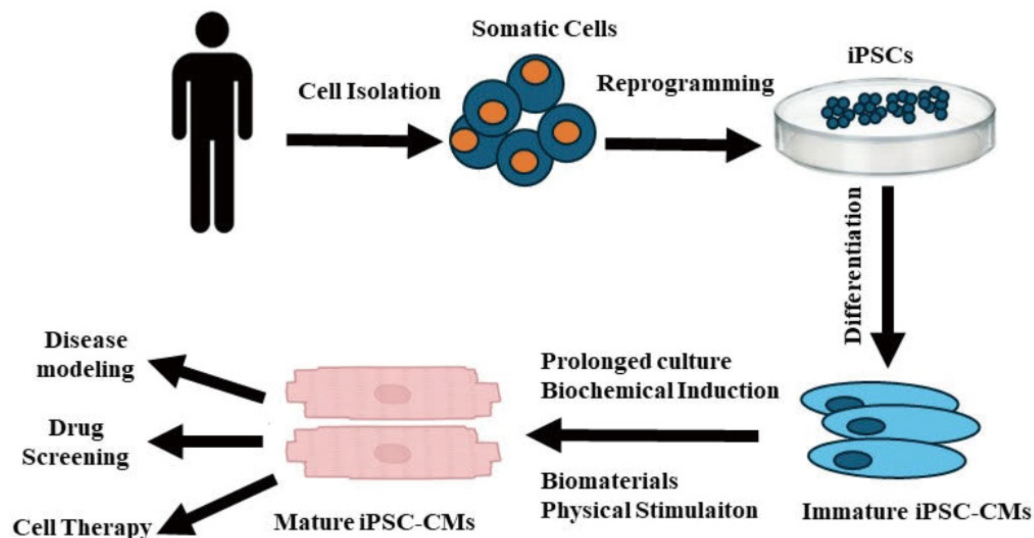

Figure 1. Somatic cells are first isolated from an individual and then reprogrammed into iPSCs. These iPSCs are subsequently differentiated into immature iPSC-CMs. Through various maturation techniques, including prolonged culture, biomaterials, biochemical induction and physical stimulation, these immature cells are transformed into fully mature iPSC-CMs. The mature iPSC-CMs may be utilized for critical applications such as disease modeling, drug screening, and cell therapy.

They also have fewer mitochondria and are typically mononucleated, indicative of their early developmental stage (16). In contrast, mature CMs are rod-shaped, longer in size with 20-30% of binucleated (2). These cells possess a high density of mitochondria. Adult mature cardiomyocytes have highly organized and well-defined sarcomeres, which contributes to the striated appearance of these cells (17). In addition, adult mature cardiomyocytes possess intercalated discs and T-tubule systems that are essential for facilitating mechanical and electrical coupling and efficient excitation-contraction coupling and rapid transmission of electrical signals (18, 19). Whereas the immature iPSC-CMs exhibit less organized sarcomeres, resulting in a less distinct striated appearance. They lack a well-developed T-tubule system, leading to less efficient excitation-contraction coupling, and decrease in synchronous contraction and response speed to electrical stimuli (19).

Different Characteristics in Electrophysiology and Ca^{2+} handling

Mature cardiomyocytes demonstrate a stable and robust action potential with a well-defined plateau phase, essential for proper cardiac function (20). Their action potentials are characterized by faster upstroke velocities and longer durations, facilitated by the presence of fully developed ion channels. Immature cardiomyocytes, however, display action potentials with less pronounced plateaus and shorter durations. The ion channel expression in these cells is incomplete, leading to slower upstroke velocities and reduced overall electrical stability (20). Calcium handling in mature cardiomyocytes is highly efficient, with well-coordinated calcium-induced calcium release (CICR) mechanisms involving the sarcoplasmic reticulum (SR) (21, 22). This ensures rapid and synchronized contraction and relaxation cycles. Immature cardiomyocytes have less developed SR and T-tubule systems, resulting in slower and less coordinated calcium transients. Consequently, their contraction and relaxation processes are less efficient compared to mature cells (22).

Different Characteristics in Metabolism and Transcriptional Signatures

In the iPSC-CMs, glycolysis is the primary pathway for ATP production. Glucose is metabolized to pyruvate, which can then enter the mitochondria for further oxidation in the tricarboxylic acid (TCA) cycle. Glycolysis is less oxygen-dependent compared to fatty acid oxidation, making it suitable for the hypoxic environment of the developing heart (23, 24). In contrast, fatty acids become the predominant energy source in adult cardiomyocytes. Fatty acids are taken up by the cell and transported into the mitochondria, where they undergo β-oxidation to produce acetyl-CoA. Acetyl-CoA then enters the TCA cycle, leading to the production of ATP through oxidative phosphorylation (23). Fatty acid oxidation provides a more efficient and higher yield of ATP compared to glycolysis, which is crucial for the high-energy demands of the adult heart (23).

The transcriptional landscape of mature cardiomyocytes is characterized by the expression of genes involved in contractile function, oxidative metabolism, and structural integrity. For instance, the myosin heavy chain (MHC) gene has two isoforms, *α-MHC* (also known as MYH6) and β-MHC (MYH7). The immature iPSC-CMs express high levels of *α-MHC* and low levels of β-MHC isoform whereas β-MHC is the predominant isoform in adult

Table 1. Comparison of immature and mature iPSC-derived cardiomyocytes (iPSC-CMs)

Features	iPSC-CMs	Adult CMs
Surface Area	1000-1300 μm^2	10,000-14,000 μm^2
Shape	Circular	Rod-Shaped
Myofibril Density	Low	High
Upstroke Velocity	Slower	Faster
Substrate Preference	Glucose	Fatty Acids
Contractions	Asynchronous	Synchronous
Sarcomere Alignment	Random	Anisotropic
Nucleation	Single Nucleated	25-30% Binucleated

cardiomyocytes (2, 15). Additionally, troponin I (TnI) has three isoforms, slow skeletal (ssTnI), fast skeletal (fsTnI) and cardiac (cTnI), and cTnI is highly expressed in adult cardiomyocytes while ssTnI is the primary isoform in immature iPSC-CMs (25). Similarly, N2B and N2BA are isoforms of titin (TTN), and N2B is mainly expressed in adult cardiomyocytes while N2BA predominates in iPSC-CMs (2, 25). Furthermore, iPSC-CMs exhibit lower levels of critical cardiac genes such as SERCA2 (sarcoplasmic reticulum ATPase), CAV3 (caveolin 3), and KCNH2 (potassium voltage-gated channel) compared to adult cardiomyocytes (25, 26).This divergence in gene expression reflects the functional specialization of mature cells and the developmental potential of immature ones.

These differences highlight the challenges in using iPSCs-CMs as a direct replacement for adult cardiomyocytes and underscore the need for further maturation of iPSCs-CMs to fully mimic the properties of adult cardiomyocytes.

METHODS OF INDUCING MATURATION OF IPSC-CMS

Prolonged in vitro culture

Prolonged in vitro culture has been shown to promote the maturation of iPSC-CMs. For instance, Lundy et al. cultured human iPSC-CMs up to 120 days and showed that the late-stage iPSC-CMs (80-120 days) exhibited significant improvements in cell size, morphology, myofibril density, sarcomere development, and multinucleation compared to the early-stage iPSC-CMs (20-40 days) (27). Further functional assessments revealed enhanced contractility, improved calcium handling, and better electrophysiological properties in the late-stage iPSC-CMs (27). Gene expression analysis confirmed the upregulation of matured cardiac marker genes such as β-MHC and connexin-43, indicating that these cells can gradually mature to resemble adult cardiomyocytes and potentially regenerate lost heart tissue (27).In consistence, Kamakura et al. extended the in-vitro culture of iPSC-CMs over a 1-year culture period. Their study indicated that the early-stage iPSC-CMs (14 days) exhibited poorly aligned myofibrils with immature Z-bands. By 180 days, myofibrils became more organized and displayed mature sarcomeric bands, but M-bands remained absent until 360 days. Despite these structural improvements, M-band-specific gene expression in iPSC-CMs was still lower than in adult hearts. Immunocytochemistry showed a shift toward more mature ventricular-type cells.

Biomaterials

Biomaterials like Extracellular matrices (ECMs) and Matrigel play critical roles in the maturation of the human iPSC-CM as they closely mimic the natural environment of cells in the body. ECMs consist of proteins like collagen, fibronectin, and laminin, which support cell adhesion, signaling, and structural integrity. Matrigel, derived from the basement membrane, is rich in growth factors and ECM proteins, promoting cellular growth and differentiation. Both ECMs and Matrigel create a more physiologically relevant environment that enhances the maturation, structural organization, and functional performance of iPSC-CMs, enabling more accurate modeling of cardiac tissue. Herron et al reported an optimal extracellular matrix that significantly enhances the electrophysiological and structural maturation of human iPSC-CMs (28). Cardiomyocytes cultured on this matrix exhibit 2× faster impulse propagation, mature action potential profiles with hyperpolarized diastolic potential, and rapid upstroke velocity (28). The matrix also promotes hypertrophic growth and increased expression of mature sarcolemmal and myofilament markers. Additionally, Feaster and colleagues reported an innovative method of enhancing the maturation of human iPSC-CMs by culturing them on a thicker Matrigel substrate called the "Matrigel mattress" (0.4-0.8 mm thick) (29). After 5-7 days of culture on this substrate, iPSC-CMs demonstrated significantly improved maturation compared to cells grown on conventional thinner Matrigel layers. These "mattress iPSC-CMs" exhibited a more rod-shaped morphology, increased sarcomere length, and contractile properties similar to those of adult rabbit ventricular myocytes (29). Additionally, they showed enhanced sensitivity to inotropic agents and upregulated key molecular maturation markers, such as cardiac troponin I and sodium current (29). Taken together, ECMs and Matrigel significantly promote the maturation of iPSC-CMs, making them invaluable tools for disease modeling, drug screening and clinical studies.

Hydrogels are crosslinked polymer networks with three-dimensional structures that can absorb substantial amounts of fluid (30). Their high-water content, soft consistency, and porous structure make them closely resemble living tissues. Hydrogel stiffness can be adjusted and the effect of hydrogel properties on the maturity of iPSC-CMs has been examined, Lee et al reported that hydrogels in medium stiffness (9 kPa) increased iPSC-CM maturation as evidenced by the expression of α-actin and Cx43, and enhanced contraction speed and exhibiting organized sarcomere structures, compared with high stiffness (16 kPa) (31).

Biochemical Induction

Energy substrates. As mature cardiomyocytes (CMs) primarily rely on lipid metabolism for energy, transitioning human iPSC-CMs from glucose-based media to media containing fatty acids can significantly advance their maturation. Yang et al. showed that the fatty acid-containing medium increased cardiomyocyte sizes and force production, improved calcium transient kinetics, and action potential upstroke velocity, along with enhanced mitochondrial oxidative capacity (32). Their findings suggest that fatty acid supplementation enhances human iPSC-CM maturation, facilitating their application in cell therapy, disease modeling, and drug testing (32). In consistence, Correia et al., 2017 demonstrated that shifting glucose-based medium to galactose- and fatty acid-enriched media accelerates human iPSCs-CM maturation into adult-like cardiomyocytes (33). These matured cells exhibit enhanced oxidative metabolism, transcriptional profiles resembling adult ventricular tissue, improved myofibril density and alignment, superior calcium handling, stronger contractility, and more physiological action potential kinetics (33). Their further analyses revealed that the addition of galactose improves overall oxidative capacity and reduces fatty acid-induced lipotoxicity. Further, Feyen et al. developed a well-balanced maturation medium containing physiologically appropriate levels of glucose and calcium (Ca^{2+}) supplemented with a complex mixture of albumin-bound fatty acids, creatine, L-carnitine, and taurine to support cardiomyocyte energetics (34). Over a 3- to 5-week directed metabolic maturation period, this medium significantly increased fatty acid oxidation while promoting electrophysiological, structural, sarcoplasmic reticulum and mechanical maturation (34). This medium improved the accuracy of disease modeling for two cardiac disorders: long QT syndrome type 3 (LQT3), and RBM20 mutant dilated cardiomyopathy (DCM) (34). These studies highlight the importance of substrate utilization in the functional maturation of human iPSC-CMs, advancing their potential for clinical and preclinical applications.

Hormones. Hormones play a crucial role in heart maturation during development. Yang et al were the first to investigate the effects of Tri-iodo-l-thyronine (T3), a key growth hormone essential for heart growth, in the maturation of iPSC-CMs (35). After the iPSC-CMs were treated with T3 for one week, the iPSC-CMs increased cardiomyocyte size, anisotropy (the structural property of non-uniformity in different directions), and sarcomere length. T3 decreased DNA synthesis and increased expression of the cyclin-dependent kinase inhibitor p21, leading to slow iPSC-CMs cell cycle activity. In addition, T3 treatment increases the contractile force of the iPSC-CMS by approximately two-fold in comparison to the control. Moreover, T3 treatment also significantly increases the maximal mitochondrial respiratory capacity. All these results indicate that T3 hormone effectively promotes human iPSC-CM maturation. Treatment with combination of T3 and Dexamethasone induced an extensive T-tubule network formation in the iPSC-CMs, Ca^{2+} release, and functional coupling between L-type Ca^{2+} channels and RYR2 were enhanced (36).

Biophysical Method

Electrical stimulation. Mimicking the electrical environment of the heart through electrical stimulation has been shown to promote the electrophysiological maturation of iPSC-CMs. Ma et al. demonstrated that applying electrical stimulation during the differentiation of human iPSCs into cardiomyocytes significantly enhanced cardiac differentiation, with spontaneously beating iPSC-CMs observed 2 days early (37). The iPSC-CMs exposed to electrical stimulation exhibited greater functional maturity. When these electrical stimulated iPSC-CMs were transplanted into mouse models of myocardial infarction, they improved cardiac function, reduced infarct size, and integrated effectively with the host heart tissue (37). Additionally, Nunes et al combined three-dimensional cell cultivation with electrical stimulation to enhance the maturation of human iPSC-CMs, and their result showed that this system generated more mature cardiac tissues with improved structural, molecular, and electrophysiological properties (38). Electrical stimulation led to enhanced myofibril organization, increased conduction velocity, and better electrophysiological and calcium-handling functions compared to non-stimulated controls (38). Through the application of a continuous stream of electrical stimulation, a human iPSC-CMs with a more rod-like structure appears, with advanced cellular alignment and better organized sarcomeres (39). Adding mechanical stimulation to the electrical stimulation will result in human iPSC-CMs having enhanced localization of N-cadherin toward the cellular membrane, sarcomeres becoming shortened. Geometric constraints can induce a uniaxial cardiomyocyte stretch, while culturing cells on elastic moduli display physiological matrix stiffness that's optimal for CM maturation parameters, including sarcomere organization and contractility.

Mechanical stimulation. Mechanical stretch has been found to enhance the structural and functional

maturation of iPSC-CMs. Abilez et al. studied different mechanic stretch conditions (5 mm, 7 mm, or 9 mm) to human iPSC-CMs cast into custom-made 12-mm long polydimethylsiloxane reservoirs, and showed that a 7 mm passive stretch induced the most consistent formation of optimal cell alignment, coordinated calcium waves, and enhanced expression of mature cardiomyocyte markers human iPSC-CMs (40). Moreover, Ruan et al. applied static mechanical stress to human iPSC-CMs for two weeks, and the human iPSC-CMs showed enhanced contractility, increased tensile stiffness, improved alignment, larger cell size, and elevated SERCA2 expression, correlating with better force-frequency response (41).

When electrical stimulation was combined with mechanical stress conditioning, force production of the iPSC-CMs further increased, indicating improved excitation-contraction coupling and maturation of the tissue. The study concludes that combining mechanical and electrical stimulation promotes the structural and functional maturation of human iPSC-CMs.

CONCLUSION

iPSC-CMs offer great potential for regenerative medicine, drug testing, and the study of heart diseases. Although current methods often result in immature iPSC-CMs, significant efforts have been made in promoting the maturation of iPSC-CMs to resemble adult heart cells. The maturation strategies, such as the prolonged in vitro culture, biomaterial application, biochemical and biophysical induction, have been demonstrated to effectively promote iPSC-CM maturation. Future research will likely focus on refining these strategies and combining some of these strategies to generate mature cardiomyocytes with the necessary attributes for clinical applications, including heart tissue regeneration. As our understanding of iPSC-CM maturation deepens, the possibility of creating fully functional, transplantable cardiac tissue becomes more attainable, offering hope for addressing heart failure and other cardiovascular diseases.

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