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Review Article

In vitro maturation of human pluripotent stem cell-derived cardiomyocyte: A promising approach for cell therapy

Yun-Gwi Park[1,#], Yeo-Jin Son[1,#], Sung-Hwan Moon[1,2,*] and Soon-Jung Park[1,*]

[1]Stem Cell Research Institute, T&R Biofab Co. Ltd., Seongnam 13487, Korea
[2]Department of Animal Science, Sangji University, Wonju 26339, Korea

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***Correspondence**
Sung-Hwan Moon
E-mail: moonsh22@sangji.ac.kr
***Correspondence**
Soon-Jung Park
E-mail: parksoonjung@gmail.com

[#]These authors contributed to this work as first authors.

Author's Position and Orcid no.
Park Y-G, Researcher,
https://orcid.org/0000-0002-7139-7637
Son Y-J, Researcher,
https://orcid.org/0000-0002-4034-1462
Moon S-H, Assistant professor,
https://orcid.org/0000-0002-6642-5699
Park S-J, Senior researcher,
https://orcid.org/0000-0002-9384-6034

ABSTRACT Currently, there is no treatment to reverse or cure heart failure caused by ischemic heart disease and myocardial infarction despite the remarkable advances in modern medicine. In addition, there is a lack of evidence regarding the existence of stem cells involved in the proliferation and regeneration of cardiomyocytes in adult hearts. As an alternative solution to overcome this problem, protocols for differentiating human pluripotent stem cell (hPSC) into cardiomyocyte have been established, which further led to the development of cell therapy in major leading countries in this field. Recently, clinical studies have confirmed the safety of hPSC-derived cardiac progenitor cells (CPCs). Although several institutions have shown progress in their research on cell therapy using hPSC-derived cardiomyocytes, the functions of cardiomyocytes used for transplantation remain to be those of immature cardiomyocytes, which poses a risk of graft-induced arrhythmias in the early stage of transplantation. Over the last decade, research aimed at achieving maturation of immature cardiomyocytes, showing same characteristics as those of mature cardiomyocytes, has been actively conducted using various approaches at leading research institutes worldwide. However, challenges remain in technological development for effective generation of mature cardiomyocytes with the same properties as those present in the adult hearts. Therefore, in this review, we provide an overview of the technological development status for maturation methods of hPSC-derived cardiomyocytes and present a direction for future development of maturation techniques.

Keywords: cardiomyocyte, cell therapy, human pluripotent stem cell, maturation, safety

INTRODUCTION

According to a report by the World Health Organization, cardiovascular diseases (CVDs) are the leading cause of death worldwide, with 8.9 million estimated deaths in 2019. In addition, it has been reported that those who had SARS-CoV-2 infection, which had a sweeping impact worldwide, had sequelae with long-term cardiovascular consequences (Abbasi, 2022). Since human cardiomyocytes cease to proliferate and the cardiac regenerative potential is lost shortly after birth (Hirose et al., 2019), treatments for any damage to the heart require medical interventions such as mechanical devices (e.g., a left ventricular assist device) or a cardiac transplant, allowing only limited available options. To overcome these limitations, the use of adult stem cells and pluripotent stem

cells (PSCs) as cell therapy has garnered attention. In particular, differentiation of cardiomyocytes using PSCs capable of differentiating into various somatic cell types has drawn increased interest as alternative approach for cell therapy (Yu et al., 2019; de Lange et al., 2021). Consequently, various methods for the differentiation of cardiomyocytes have been developed and actively utilized to explore cell therapy, offering therapeutic strategies against cardiac repair or drug-induced cardiotoxicity (Burridge et al., 2015; Park et al., 2019; Choi et al., 2020).

Recently, a research team in France reported the completion of a phase 1 clinical trial involving human embryonic stem cells (hESCs)-derived CPCs to demonstrate the safety and effect of cardiac function improvement (Menasché et al., 2018). In Japan, based on the results of preclinical studies with heart failure (HF) animal models (Kashiyama et al., 2019; Kawaguchi et al., 2021), an ongoing clinical trial has been utilizing the transplantation of human induced pluripotent stem cells (hiPSCs)-derived cardiomyocyte sheets or spheroids. As can be seen from the above examples, research on the investigation of cell therapy using hPSC-derived cardiomyocytes for the treatment of heart diseases with limited alternative treatment options has developed into an area of global competition among world-class research groups. However, regarding the applications of hPSC-derived cardiomyocytes (CMs) in clinical settings, it is necessary to overcome the issue of graft-related arrhythmias. In several papers, as a result of transplanting hPSC-derived cardiomyocytes into heart disease model animals, arrhythmias (e.g., ventricular tachycardia) occurred in the early stage of transplantation. This arrhythmia showed time-dependent decreasing (Shiba et al., 2016; Romagnuolo et al., 2019). The induction of arrhythmia occurs due to the fact that the transplanted CMs in the early postoperative period are still immature compared to *in vivo* CMs. After transplantation, over the course of *in vivo* maturation of CMs, the incidence of arrhythmia gradually dissipated. Based on this finding, it is expected that graft-related arrhythmias occurring in the early postoperative period can be overcome if maturation of hPSC-derived CMs can be achieved prior to transplantation. Therefore, in this review, 1) an overview of the current progress of clinical studies using human PSC-derived CPCs or CMs is presented, 2) characteristics of immature CMs and mature CMs are comparatively analyzed, and 3) indicators for enhancement of maturation for key techniques of CM maturation are identified. Based on the findings of this review, we aim to present an effective strategy for the development of an optimal maturation technology for CMs.

An overview of clinical trials for cell therapy using hPSC-derived CPCs or CMs

Following the pioneering step of Mesoblast Ltd in 2005 involving a clinical trial using mesenchymal stem cells (MSCs), active research has been ongoing for the development of cell therapy for the treatment of HF and other heart conditions (Hare et al., 2009). However, after Mesoblast Ltd.'s reported failure in meeting the primary endpoint in phase 3 clinical trial, cell therapies using hPSC-derived CMs have drawn increasing interest for research and clinical applications. Unlike MSCs, CMs can directly participate in actual heart functions following engraftment at the transplant site, indicating a higher applicability and significance. Based on this perspective, the present review aims to investigate the current status of clinical trials using PSC-derived CMs (Table 1).

1) A clinical study with hESC-derived CPCs

Regarding cell therapy using hESC-derived cardiovascular progenitors, a phase 1 trial conducted by a research team in France has been reported (Menasché et al., 2018). In the trial, from 2013 to 2018, ten patients with ischemic heart disease received hESC-derived CPCs by epicardial delivery of the cell-laden fibrin patch, and the clinical outcomes of the treatment were monitored. A 20 cm^2 fibrin patch with a dose of 5×10^6 to 1×10^7 (Median: 8.2×10^6) hESC-derived CPCs was delivered to the epicardium of the infarct area. A year of follow-up monitoring revealed that one patient died because of treatment-unrelated comorbidities, but all others had uneventful recoveries. In all patients apart from the deceased patient, symptomatic improvement with an increased systolic motion of the cell-treated segments was confirmed, and there were no arrhythmias, complications of the immunosuppressive treatment, or intracardiac or off-target tumors. From these results, it can be interpreted that various factors released from the grafted CPCs promote myocardial and vascular regeneration, and such regeneration contributed to improving the function of infarcted hearts. However, considering that one patient died of heart failure 22 months post-transplantation, it is speculated that

Table 1. Clinical trials with hPSC-derived CPCs or cardiomyocytes

Institution (Nation)	Assistance Publique (France)	Osaka University (Japan)	Heartseed (Japan)
Status (Term)	Phase 1 completed (2013.05.27–2018.03.22)	Phase 1 undergo (2019.12.02–2023.05.30)	Phase 1 undergo (2022.04.19–2024.03.31)
Target disease	Ischemic Heart Disease	Myocardial Ischemia	Heart Failure
Patient	10 patients for 5 years	10 patients for 3 years	10 patients for 5 years
Cell source	ESC	Allogeneic iPSC	Allogeneic iPSC
Cell type	CPC ($SSEA1^+$ $Isl-1^+$)	Cardiomyocyte	Cardiomyocyte
Transplantation	20 cm^2 Fibrin patch embedding hESC-derived CPCs	hiPSC-derived cardiomyocyte sheets	hiPSC-derived cardiomyocyte spheroids
Clinical mechanism	Improves heart function by releasing factors that promote myocardial and/or vascular regeneration	1) Helps heart function through self-beating 2) Improves heart function by releasing factors that promote myocardial and/or vascular regeneration	1) Helps heart function through self-beating 2) Improves heart function by releasing factors that promote myocardial and/or vascular regeneration
Outcome measures	1 year follow-up after surgery: 1) Feasibility of patch's generation and its efficacy on cardiac functions 2) Evidence for new clinical/biological abnormalities, occurrence of arrhythmias or development of a cardiac or extra-cardiac tumor	1 year follow-up after surgery: 1) Left ventricular (LV) ejection fraction, contractile and remodeling of the LV, New York Heart Association functional classification 2) Serious adverse events, abnormal blood biochemical or tumor marker tests and cardiac function clinical events 3) Minnesota Living with Heart Failure Questionnaire, 36-Item Short Form Survey etc.	1 year follow-up after surgery: 1) LV ejection fraction, myocardial wall motion evaluation, myocardial blood flow, myocardial viability 2) Safety and Tolerability 3) Kansas City Cardiomyopathy Questionnaire etc.
Result	1. Cardiac function improvement effect 2. No arrhythmia, teratoma and immune-suppressant complications	Ongoing	Ongoing
Pre/Clinical publication	Menasché et al., 2018	Kashiyama et al., 2019	Kawaguchi et al., 2021

there is a limitation to achieving cardiac repair to the level of healthy adults by transplantation of CPCs.

2) A clinical study with hiPSC-derived cardiomyocyte sheets

Clinical trials on cell therapy using hiPSC-derived CMs have been conducted by two Japanese research teams. One of the research teams at Osaka university generated iPSC-CM sheets using cynomolgus macaque to induce cardiomyogenic differentiation in a preclinical study. The generated CMs were in the form of cell sheets (3.6×10^6 cells/sheet), and four pieces of the iPSC-cardiac sheets were transplanted in a cynomolgus macaque MI model. The effect of transplantation was evaluated through a 6-month follow-up monitoring. As a result, it was confirmed that cardiac function improved regardless of the matching/mismatching of the major histocompatibility complex (Kashiyama et al., 2019). Based on this preceding study, the research team is conducting a phase 1 trial to investigate the therapeutic effect of CM sheets derived from allogeneic iPSCs for 10 patients with myocardial ischemia for 3 years since 2019. Through 1-year follow-up post-transplantation, the treatment effect including functional and structural recoveries of the left ventricle, as well as adverse events including abnormal findings in the blood biochemical tests or cardiac function events and tumor development, will be examined for evaluation.

3) A clinical study with hiPSC-derived cardiomyocyte aggregates

Another ongoing clinical trial using hiPSC-derived CMs is being conducted by Tokyo-based Heartseed. First, a preclinical study was conducted using a rat model (i.e. a small animal model) and a micro-miniature pig model (i.e. a large animal model). Cardiac spheroids (200 μm) consisting of approximately 1,000 hiPSC-derived CMs were prepared, and 3,000 spheroids (total cell number: 3×10^6) and 1×10^5 spheroids (total cell number: 1×10^8)

were transplanted in the rat heart failure model and the pig heart failure model, respectively, to evaluate the effect of transplantation. As a result, the cardiac spheroid transplantation group maintained the recovery of cardiac function for a longer period of time in the large animal model (Kawaguchi et al., 2021). Based on this preceding study, a phase 1 trial is underway to determine the effect of iPSC-derived cardiomyocyte spheroids on 10 heart failure (HF) patients for 5 years starting from 2022. Each patient will undergo transplantation of cardiac spheroids, and through 1-year post transplantation follow-up, adverse events in terms of safety, tolerability, and cardiac function recovery with outcome measures such as myocardial viability and myocardial blood flow will be evaluated.

Comparison of the characteristics between immature cardiomyocytes and mature cardiomyocytes

There is a general consensus among stem cell researchers on the immature state of hPSC-derived CMs, but methods for evaluating the degree of maturation remain to be determined. Therefore, in addition to developing methods for enhancing the maturation of hPSC-derived CMs, methods for evaluating the degree of maturation also need to be established. As a first step, in this review, we comparatively analyzed the characteristics of mature CMs in adult heart and immature (fetal and hPSC-derived) CMs (Table 2).

1) Morphology and cellular structure

Mature cardiomyocytes (CMs) in adult hearts have rod-like shapes, and the size of a single CM is about 150 μm in length, 20 μm in width, and 15 μm in height, with a volume of 40,000 μm^3 (Bulatovic et al., 2016). Regarding the morphology of the nucleus, mature CMs are multinucleated and have tetraploid nuclei. They are characterized by highly organized sarcomere and well-developed sarcoplasmic reticulum (SR) and transverse tubules (T-tubules) (Yang et al., 2014a). In contrast, immature hPSC-derived CMs have different shapes such as a circular or oblong geometry, and they are approximately 5-10 μm in diameter, 30 μm in length, 10 μm in width, and 5 μm in height, with a volume of 2,000 μm^3, showing a smaller size compared to that of mature CMs (Lundy et al., 2013, Bulatovic et al., 2016). Regarding the nucleus morphology, the immature CMs are mononucleated or have diploid nuclei as in the case of other somatic cells. Regarding the structure of the cytoskeleton, the sarcomere is disorganized and shows irregular distribution in the cytoplasm. Moreover, following induction of CM differentiation, no or few T-tubules are observed in hPSC-derived CMs in the early stage of beating (Yang et al., 2014a; Denning et al., 2016; Park et al.,2022) (Fig. 1A).

2) Electrophysiological properties

In general, adult CMs maintain a resting membrane potential of -90 mV until electrical impulses are transmitted from adjacent cells. When triggered by the adjacent cells, fast Na^+ channels begin to open, and Na^+ leaks into the cell. This produces rapid depolarization with the transmembrane potential rising to approximately +20 mV. The upstroke velocity at this time is measured to be approximately 150-350 V/s (Lin et al., 2017). After depolarization, an outflow of K^+ occurs due to diffusion through the cell membrane, but with a constant inward current of Ca^{2+}, the plateau phase is maintained for some time (Liu et al., 2016; Sharifi et al., 2017; Zhao et al., 2018). Next, Ca^{2+} channels are gradually inactivated, and with a persistent outflow of K^+, the transmembrane potential is brought back toward a resting potential of -90mV, thereby completing a cycle. Subsequently, depolarization newly starts when triggered by the adjacent cells (Goversen et al., 2018; Koivumäki et al., 2018). In adult CMs, the action potential duration of one cycle is measured to be ~300 ms, and the conduction velocity, i.e. the velocity of transmission of electrical impulses to adjacent cells, is known to be approximately 50 cm/s (Tveito et al., 2012). In contrast, hPSC-derived CMs showed spontaneous beating, with a resting membrane potential of approximately -60 mV, similar to that of nodal CMs. In addition, when depolarization occurs, the subunits of $Nav_{1.5}$ protein involved in the inflow of sodium ions are fetal isoforms, and the upstroke velocity is measured at approximately 10-50 V/s, slower than that of adult CMs (Veerman et al., 2017). Furthermore, since IKI, a type of channel protein in adult CMs that promotes K^+ inflow and inhibits its outflow, is present at a low concentration in hPSC-derived CMs, K^+ ion outflow occurs at a faster rate after depolarization than that in adult CMs. This causes rapid repolarization in which the transmembrane potential is brought back to the resting membrane potential after depolarization (Jeevaratnam et al., 2018).

Table 2. Clinical trials with hPSC-derived CPCs or cardiomyocytes

Characteristics	Category	Cardiomyocytes		References
		Immature (Fetal or PSC-derived)	Mature	
Morphology	Shape	Circular and oblong	Anisotropic rod-like	Lundy et al., 2013
	Size	Diameters: 5–10 μm Heights: ~5 μm Length: 30 μm Width: 10 μm Volume: 2,000 μm^3	Heights: 15 μm Length: 150 μm Width: 20 μm Volume: 40,000 μm^3	Bulatovic et al., 2016 Lundy et al., 2013
	Nucleus	Mononucleated diploid	Multinucleated tetraploid	Denning et al., 2016
	Structure (Cytoskeleton)	Sarcomere: disorganized, short SR and T-tubule: poorly developed	Sarcomere: highly organized, long SR and T-tubule: well-developed	Denning et al., 2016 Yang et al., 2014a
Electrophysiology	Beating	Spontaneously beat Force: 0.08–4 mN/mm^2 mixed action potential (nodal, atrial ventricular-like)	Start: Sinoatrial node No beat until triggered by the depolarization form adjacent cells Force: 40–80 mN/mm^2	Lin et al., 2017
	Resting membrane potential	−60 mV, nodal like	−90 mV	Koivumäki et al., 2018
	Conduction velocity	10–20 cm/s	Around 60 cm/s	Denning et al., 2016
	Upstroke velocity	Slow, 10–50 V/s	Fast, 150–350 V/s	Denning et al., 2016 Veerman et al., 2017
	Repolarization	Fast	Slow, after plateau phase	Jeevaratnam et al., 2018 Zhao et al., 2018
	Channel protein	I_{Na}, I_{Ca-L}, I_{to} Low I_{K1},	I_{Na}, I_{Ca-L}, I_{to}, I_{Kr}, I_{Ks}	Goversen et al., 2018
Calcium handling		1) Similar expression of Na^+/Ca^{2+} exchanger with adult CMs 2) Low Expression of other Ca^{2+} releasing related protein 3) Increasing of calcium stores in the SR 4) Slower calcium dynamics with delayed time to peak and slower decay of the calcium signal	Depolarization → opening of LTCC → calcium influx → calcium-induced calcium releasing → sliding of myofilament and contraction of muscle Calcium releasing: 1) SERCA2a 2) Sarcolemmal Na^+/Ca^{2+} exchanger 3) Sarcolemmal Ca^{2+}-ATPase 4) Mitochondrial Ca^{2+} uniport	Hwang et al., 2015 Karakikes et al., 2015 Youm, 2016 Eisner et al., 2017
Metabolism	Mitochondria	Location: perinuclear space Low number and small size (<5% of total cell volume) Round shape and low cristae density In mouse, opened mitochondrial permeability transition (MPT) not induce cytochrome c leakage or apoptosis	Location: between myofibrils and under the sarcolemma increase in both size and number (~30% of total cell volume) Oval shape and dense cristae → sufficient surface area	Feric and Radisic, 2016
	Energy source	Energy was generated mainly by glycolysis (Low fatty acid / High glucose or lactate) Approximately 15% of total energy consumption in fetal cardiomyocytes is supplied with β-oxidation of fatty acids	Approximately 80% of total energy consumption in adult cardiomyocytes is supplied with β-oxidation of fatty acids 60–70% ATP for contractile function, followed SERCA and of other ion transporters	Correia et al., 2017 Galdos et al., 2017 Karakikes et al., 2015 Piquereau et al., 2018

Table 2. Continued

Characteristics	Category	Cardiomyocytes		References
		Immature (Fetal or PSC-derived)	Mature	
Gene expression	Sarcomeric gene (MHC)	α-MHC	β-MHC	Katrukha, 2013 Lundy et al., 2013
	Troponin I	TNNI1	TNNI3	Katrukha, 2013
	Others	Low expression levels of SERCA2, Caveolin 3, KCNH2 etc.		Lundy et al., 2013

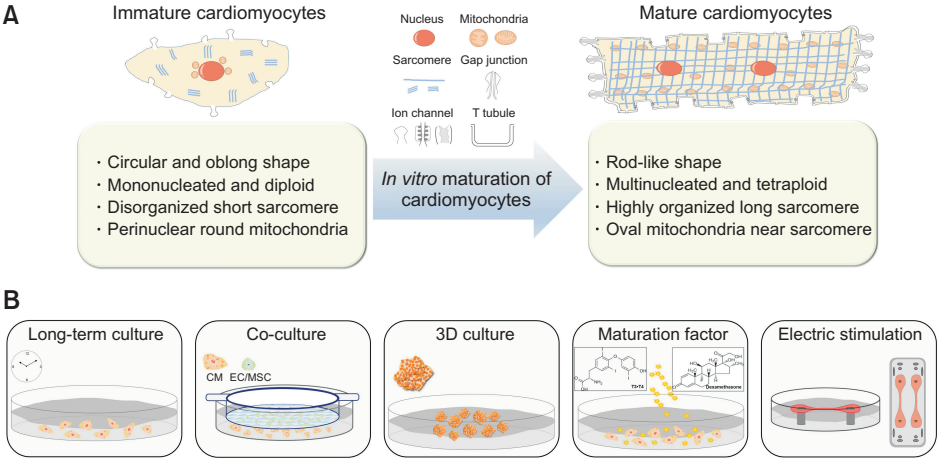

Fig. 1. Experimental methods for maturation of hPSC-derived cardiomyocytes. (A) Compared to matured cardiomyocytes, morphology and structure, calcium handling, metabolism, and electrophysiology are different in immature hPSC-CMs. (B) Various methods of maturation hPSC-CMs.

3) Calcium handling

Calcium ions (Ca^{2+}) play an important role in various signal transduction pathways, metabolism, and transcriptional regulation in cells, and in the case of myocytes, the intracellular Ca^{2+} concentration is also involved in the contraction and relaxation of the cardiac muscle. This indicates that Ca^{2+} also plays an important role in CMs, which is also a type of myocyte. In mature CMs, the concentration of Ca^{2+} is precisely controlled through various mechanisms. In the excitation-contraction coupling, when intracellular depolarization occurs, the L-type calcium channel (LTCC) opens, producing an inward current of Ca^{2+} from outside the cell membrane to the cytoplasm, and the increased cytoplasmic Ca^{2+} concentration induces the release of calcium-induced Ca^{2+} through ryanodine receptors (RYR) in the SR, thereby further increasing the cytoplasmic Ca^{2+} concentration. Ca^{2+} with increased cytoplasmic concentration binds to the troponin protein to induce muscle contraction. After the contraction is completed, cytoplasmic Ca^{2+} concentration is lowered again by removing Ca^{2+} from the cytoplasm through the following process: (1) Ca^{2+} is pumped back into SR via sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a); (2) Ca^{2+} is pumped out of the cell by the sodium-calcium exchanger; (3) Ca^{2+} uptake into mitochondria; (4) additional leak of Ca^{2+} by sarcolemmal calcium ATPase (Eisner et al., 2017). In the case of immature CMs lacking T-tubules, LTCC and RYR are separated in space, leading to a delay in the release of Ca^{2+} in the SR after depolarization, and also, the expression of Ca^{2+} handling protein is also lower than that in mature CMs, exhibiting a slower calcium dynamic overall. However, among the Ca^{2+} handling proteins, the expression of sodium-calcium exchanger is higher in immature fetal CMs than in mature CMs, and this characteristic can also be observed in the case of hPSC-derived CMs (Hwang et al., 2015).

4) Metabolism

The human heart requires a continuous supply of energy to maintain its ongoing pumping activity with systolic and diastolic movements from birth to death. Accordingly, the cardiac energy metabolism in CMs is highly effective. The typical features of fetal cardiac mitochondria include a low number and a small size, occupying <5% of the total cell volume. With the development of the fetal heart, both the number and size of the cardiac mitochondria show a

robust increase, accounting for ~30% of the cell volume (Galdos et al., 2017). The differences can also be observed in terms of the morphology and location of the individual mitochondria with cardiomyocyte maturation. For mature CMs, mitochondria take a long, oval shape and are located between myofibrils and under the sarcolemma with the formation of dense cristae (Feric and Radisic, 2016). For immature CMs, round-shaped mitochondria are mainly located in the perinuclear space, with rudimentary cristae in the inner membrane of mitochondria. In terms of energy production through cellular metabolism with mitochondria having the central role, approximately 80% of the total energy in mature CMs is generated through β-oxidation metabolism of fatty acids (Karakikes et al., 2015; Piquereau et al., 2018). In the case of immature CMs, approximately only 15% of the energy is produced through β-oxidation of fatty acids, and in many cases, energy is generated through the metabolism of glucose or lactate (Correia et al., 2017). Since hPSC-derived CMs also exist in an immature state, their metabolism largely depends on glycolysis rather than β-oxidation of fatty acids.

5) Gene expression

Mature CMs and immature CMs show differences in terms of subunits of specific genes (proteins) in CMs. Myosin heavy chains (MHCs) are motor proteins that convert chemical energy derived from the hydrolysis of ATP into mechanical force that drives diverse motile processes in cardiac and skeletal muscle. MHCs are also expressed in two different, developmentally-regulated subunits: the fetal α-isoform (α-MHC) and the adult β-isoform (β-MHC). In mature CMs, β-MHC synthesized by the expression of the MYH7 gene is predominant, and in immature CMs, α-MHC synthesized by the expression of the MYH6 gene is mainly expressed (Katrukha, 2013; Lundy et al., 2013). Troponin I (TnI) behaves as a molecular switch of sarcomere within the myocytes and typically has three isoforms (TNNI1: Slow skeletal TnI, TNNI2: Fast skeletal TnI, TNNI3: Cardiac TnI). In mature CMs, cardiac TnI3 is predominantly expressed, whereas in immature CMs, slow skeletal TnI2 is predominantly expressed (Katrukha, 2013). In addition, the expression of genes such as SERCA2, Caveolin 3, and KCNH2 was lower in immature CMs (Lundy et al., 2013).

Methods of promoting maturation of cardiomyocytes

As described above, although there have been numerous studies on the generation and utilization of PSC-derived CMs, these cells show characteristics similar to those of immature fetal CMs. Accordingly, various approaches for obtaining mature cardiomyocytes by inducing *in vitro* maturation of PSC-derived CMs have been actively investigated. The representative maturation methods in the current research trend are outlined as shown below (Fig. 1B)

1) Long-term culture of cardiomyocytes

In the course of human heart development from fetal to adult CMs, the duration of maturation for fetal immature CMs from birth is over 10 years (Bulatovic et al., 2016). Accordingly, a number of studies have been conducted by different research teams to enhance the maturation of CMs through *in-vitro* long-term culture of hPSC-derived CMs (Shinozawa et al., 2012; Kamakura et al., 2013; Lundy et al. al., 2013; Cyganek et al., 2018). Through the long-term culture process lasting from 60 days to 1 year, the number of cells expressing the cTnT gene (cTnT+), a representative marker of CMs, and multinucleated cells increase, and CMs undergo morphological changes such as the increase in the sarcomere length and cell size with an increase in the expression of associated genes. Thus, it was confirmed that the maturity of hPSC-derived CMs was enhanced with time. In particular, formation of M-bands was confirmed in CMs following a long-term culture of 360 days or longer, but the amount of sarcomeric structures in a single CM was still scarce compared with that of adult CMs (Kamakura et al., 2013). The findings that hPSC-derived CMs did not reach the full level of maturation as that of adult CMs require additional investigation on other factors such as *in vivo* paracrine and hormone signals. In a recent study, during the initial process of CM differentiation, platelet-derived growth factor receptor-α (PDGFRα)-positive cells that can respond to CM differentiation cues and are thus known as one of the markers of CM differentiation were first collected, and subsequently, long-term culture of immature CMs was conducted. Through this method, the formation of a T-tubule-like structure, which was not confirmed in previous studies, was exhibited on day 231 of the long-term culture (Fukushima et al., 2020).

2) Addition of maturation factor

From the viewpoint of evolution, the closer the relation to mammals in the phylogenetic tree, the serum thyroxine level increases and the cardiac regenerative potential decreases, showing the inverse correlation between the serum thyroxine level and cardiac regenerative potential. Inactivation of thyroid hormone signaling reduced mouse cardiomyocyte polyploidization, and the mouse CMs remain in the immature state for longer (Hirose et al., 2019). This implies that regulation of thyroid hormones may serve to limit the CM regenerative capacity and induce CM polyploidization, thereby promoting the maturation of CMs. Accordingly, recent studies have investigated the method of adding Tri-iodo-L-thyronine (T3), a thyroid hormone, during the culture of CMs to induce *in vitro* maturation of CMs.

In a number of studies, the results showed that treatment with T3-induced gene expression of hPSC-derived CMs from the fetal-like level to the adult-like pattern increased the force per contractile along with the rate of calcium release and reuptake with SERCA2α to promote further maturation of CMs (Ivashchenko et al., 2013; Yang et al., 2014b; Birket et al., 2015). In addition, T3 treatment in combination with IGF-1, Dexamethasone increased the expression of PGC-1a and PGC-1b, which are important regulators of fatty acid oxidation (FAO) and mitochondrial function in CMs, and contributed to the formation of a uniform sarcomeric structure, thereby inducing the maturation of immature CMs.

3) 3-Dimensional (3D) cell culture methods

All cells exist in 3D space *in vivo*, but standard cell culture is performed in 2D, typically in a flat petri dish. This indicates that cells are cultured under conditions different from those of *in vivo* environments, which may lead to differences in cellular responses and functions compared to those observed *in vivo*. Therefore, research has been conducted on culturing hPSC-CMs in 3D systems and not in the conventional 2D systems. On day 6 of hPSC-CM differentiation, the cardiac progenitors were recovered, and the 3D aggregation of the hPSC-derived cardiac progenitors was induced. After a 29-day culture, the maturation of 3D-cultured CMs was compared with that of 2D-cultured CMs. In the case of CMs in aggregates, contractility was more than three times faster, and improved action potential kinetics were achieved. Moreover, in terms of gene expression with 3D culture, the expression of atrial-like CM markers decreased, and the expression of ventricular-like CM markers increased (Correia et al, 2018).

Among the 3D culture methods, improved maturation of CMs was reported for a culture of engineered heart tissues (EHTs). The hPSCs were differentiated into CMs, and after 30 days, hPSC-derived CMs were cultured in the form of engineered cardiac tissue constructs, a type of EHTs. Using the EHT culture method for 3 weeks without other methods to promote maturation apart from the 3D culture, T-tubule systems similar to the ventricular CMs of the adult heart were developed, and within a relatively short time of 2 weeks, an increase in expression of various genes related to calcium-handling and contraction was confirmed (de Lange et al, 2021). In terms of cell therapy, 3D culture has advantages over 2D culture. In both the results of a preclinical study (Kawaguchi et al., 2021) published by Heartseed, based on which a phase 1 trial is underway, and a recent report published by the authors' research team, a single CM or CM aggregates were grafted on the infarcted area of an animal model with MI to evaluate the effect of transplantation. As a result, when CMs were transplanted in the form of 3D aggregates, the *in vivo* survival rate was higher than that of single CM transplantation, and the results also showed that the engraftment of mature CMs was induced, and the method was more effective in improving the symptoms of MI (Park et al., 2022).

4) Various other cell co-culture methods

As with all other organs in the human body, the heart does not consist of CMs alone but includes various other cells such as endothelial cells (Ecs), MSCs, and fibroblasts. The coexistence of different types of cells means that each cell can influence each other through intracellular interactions, and such interactions are also involved in the maturation of CMs (Zhang et al., 2012). Therefore, research has been conducted to investigate the effect of inducing CM maturation using a co-culture system in which hPSC-derived CMs are cultured with other types of cells. On comparing three different culturing methods of CMs: (1) hiPSC-CMs cultured alone, (2) hiPSC-CM co-cultured with MSC, and (3) addition of MSC-derived soluble factors, both the co-culture group and the soluble factor addition group showed higher structural

and functional maturity of CMs (e.g. cell size, sarcomere length, contractility, electrophysiology) compared to the CMs cultured alone (Yoshida et al., 2018). The results confirmed that among CM culture methods, co-culture with MSCs promoted the maturation of CMs by releasing various bioactive factors favorable for cardiac cells. In addition to MSCs, endothelial cells (Ecs), whose number is approximately three times higher than that of CMs in the human heart, are also known to play a significant role in the early-stage heart development or cardiac repair (Talman et al., 2018). Based on this, during hPSC-CM differentiation, CMs were co-cultured with Ecs and after 2 weeks of additional culture, the maturity of cultured CMs was evaluated based on the cell size, sarcomere organization, and gene expression related to mature CMs (Dunn et al., 2019). The results confirmed that the maturity of CMs was higher when co-cultured with CPCs in the early or mid-stage of CM differentiation than in the case of CPC monoculture or co-culture with EC of CMs after the completion of differentiation. The results indicate that Ecs induce maturation of CMs by delivering microRNA through paracrine factors and gap junctions in immature CMs. In addition, when microtissues were prepared using 3D co-culture with CPC, EC, and MSC in a ratio of 2:1:1, respectively, and cultured for 1 week, CMs maturation was accelerated compared to that of microtissues with CPC alone. (Varzideh et al, 2019). For transplantation of hiPSC-derived CMs to an animal MI model, 3D printing was used to prepare hMSC-loaded patch, and the patch was epicardially implanted. As a result, the hMSC-secreted factors improved the survival of hiPSC-CMs compared to the transplantation of CMs alone, and the method was also effective in enhancing vascular regeneration and promoting the maturation of CMs (Park et al., 2019).

5) Electric stimulation of cardiomyocytes

In this maturation method, cardiac cells were isolated from rats to investigate whether the morphology and functionalities of CMs changed (developed) similar to those of *in vivo* CMs of rats with or without electric stimulation under *in vitro* culture environment (Lasher et al., 2012). To compare differences in the maturation of CMs with/without electric stimulation, CMs with electric stimulation of 2 ms symmetric biphasic square pulses, 4 V/cm, and 1 Hz for 9 days using a carbon-rod of scm-spacing were compared with CMs without electric stimulation on days 3, 6, and 9. In terms of morphology, CMs applied with electric stimulation showed more similar values of length, width, and height with the age-matched myocardium compared to those of CMs without electric stimulation. In terms of functionalities, the decreased excitation threshold and increased maximum capture rate were observed in CMs with electric stimulation, which are the indicators of maturation. In a study where electric stimulation of 5 ms pulse, 5 V/cm, and 2 Hz was applied to iPSC-derived CMs to induce maturation of CMs, the result confirmed that maturation of CMs can be induced by electric stimulation in terms of cell alignment, contractility and passive stiffness, and the increase in expression of calcium-handling protein (Ruan et al., 2016).

CONCLUSION REMARKS AND FUTURE PERSPECTIVES

With CVDs reported as the leading cause of death worldwide, development of cell therapy that enables the fundamental treatment of CVDs has gained increasing attention. In particular, there is an ever-increasing importance of cell therapy based on PSC-derived CMs because the method can be applied for various diseases in the heart, a representative organ of the cardiovascular system, and the method allows unlimited (mass) generation of CMs with extremely low regenerative capacity. Therefore, in this review, the current status of clinical trials using PSC-derived CPCs and CMs as cell therapy was outlined, and the different characteristics of mature adult CMs and immature CMs were comparatively analyzed. Furthermore, since PSC-derived CMs are in an immature state, various culture methods that promote maturation of CMs were discussed. A number of studies have shown that immature PSC-derived CMs can mature into having properties similar to those of adult CMs by implementing various maturation methods. Therefore, the authors' research team aims to develop a more effective method of promoting the maturation of CMs by analysis and incorporating the results of the recent publications and applying an optimal combination of these different methods. Furthermore, with additional implementation of a co-culture system including MSC and EC using 3D printing techniques, the establishment of a future direction for CM engineering by forming surface modification using bioinks, and creation of a 3D-printed cardiac model, it

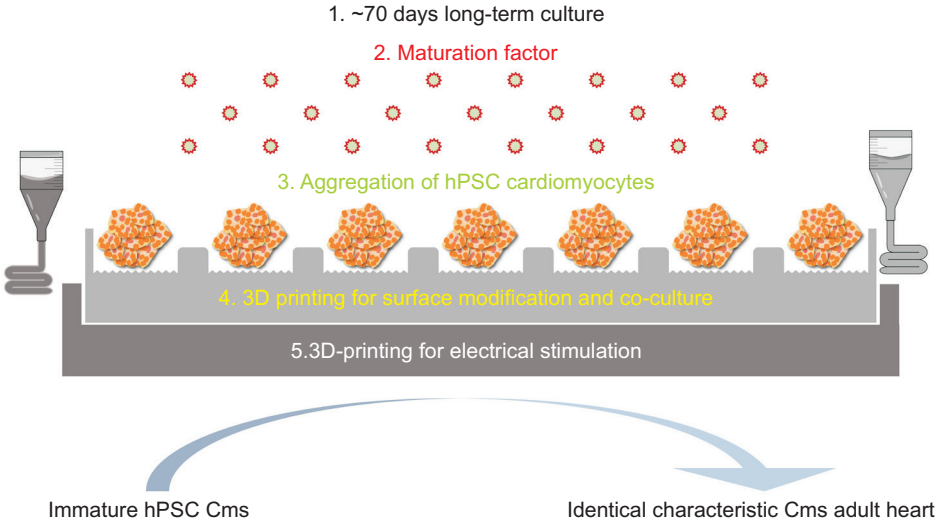

Fig. 2. The new strategy to mature hPSC-derived cardiomyocytes into the identical characteristic cardiomyocytes in adult heart through a combination of cardiomyocyte maturation methods.

is expected that a model with the same morphology and functionality as *in vivo* cardiomyocytes can be developed with more efficient intercellular signal transduction and synchronization of the maturation level of individual CMs (Fig. 2). Although challenges still remain to be overcome, continuous efforts on the technological development of maturation methods are expected to present a new opportunity for cardiovascular research, and considering the track record of the emergence of new technologies and analytical tools up until the techniques developed to date, the new technologies and analytical tools are expected to create synergistic effects. If the technology for maturation of hPSC-derived CMs into cells with the same functional and structural characteristics as those of mature CMs *in vivo* can be developed through various approaches as described above, it is expected that the effects of CM-based cell therapy can be clearly established along with extensive utilization of the developed maturation technology in areas of basic research, including the analysis of cardiac functions, and for various clinical applications, including cardiotoxicity evaluation of drug candidates.

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