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Building A New Treatment For Heart Failure-Transplantation of Induced Pluripotent Stem Cell-derived Cells into the Heart

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Abstract: Advanced cardiac failure is a progressive intractable disease and is the main cause of mortality and morbidity worldwide. Since this pathology is represented by a definite decrease in cardiomyocyte number, supplementation of functional cardiomyocytes into the heart would hypothetically be an ideal therapeutic option. Recently, unlimited *in vitro* production of human functional cardiomyocytes was established by using induced pluripotent stem cell (iPSC) technology, which avoids the use of human embryos. A number of basic studies including ours have shown that transplantation of iPSC-derived cardiomyocytes (iPSC-CMs) into the damaged heart leads to recovery of cardiac function, thereby establishing "proof-of-concept" of this iPSC-transplantation therapy. However, considering clinical application of this therapy, its feasibility, safety, and therapeutic efficacy need to be further investigated in the pre-clinical stage. This review summarizes up-to-date important topics related to safety and efficacy of iPSC-CMs transplantation therapy for cardiac disease and discusses the prospects for this treatment in clinical studies.

Keywords: iPSC, Cardiomyocytes, Transplantation, Heart failure, Cardiomyoplasty, Immunogenicity.

INTRODUCTION

Advanced cardiac failure is a progressive intractable disease, yielding the major cause of mortality and morbidity worldwide, despite intensive medical and/or interventional treatments [1]. Cardiac transplantation is the only established treatment that has been shown to improve prognosis of this pathology, although transplantation therapy is persistently hampered by the short supply of donor hearts [2]. Implantation of mechanical-assist devices or artificial hearts as a destination therapy is promising; however, it inevitably involves a number of device-related complications and marked economic/social burdens [3]. It has been suggested that myocardial reversibility is enhanced by some treatments, such as somatic stem/progenitor cell transplantation, gene therapy, new medications, or mechanical unloading [4]; however, our laboratory has reported that therapeutic efficacy of these treatments is dependent upon the myocardial viability prior to the treatment [5]. The critically ill heart that is represented by a decrease in cardiomyocyte number and thereby poor myocardial viability is unlikely to gain substantial therapeutic benefits from treatments that enhance myocardial reversibility, but hypothetically would recover *via* supplementation of functional cardiomyocytes into the heart [6].

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are the sole established cell source for generating self-beating cardiomyocytes *in vitro* [7]. In particular, iPSCs are a promising treatment in regenerative therapy, since these unique cells can be generated from any individual [8]. Our laboratory has been studying iPSCs primarily for the purpose of regenerative therapy as well as drug discovery in the last 10 years with intense collaboration with Professor Shinya Yamanaka, one of the founders of the iPSC field [9-12]. Transplantation of iPSC-derived cardiomyocyte (iPSC-CM) sheets over the left ventricular (LV) surface has been shown to improve cardiac function in murine and porcine models of chronic myocardial infarction (MI) [9, 12]. Importantly, mechanisms underlying this functional improvement included mechanical contribution of the graft [12], thus establishing the "proof-of-concept" of this treatment. However, cardiac treatment using iPSC-CMs is substantially limited by intrinsic properties of iPSCs and their derivatives and target pathology of the heart.

Firstly, the efficiency of cardiomyogenic differentiation of iPSCs has been shown to be variable in *in vitro* culture protocols, depending upon the cell line [8]. In addition, a large number of new cardiomyocytes are required for cardiac treatment, despite that the protocol was established in small culture dishes [6]. Moreover, an optimal transplantation method has not been fully established, depending upon the target cardiac pathology [13]. Furthermore, the potential tumorigenicity or immunogenicity of iPSC derivatives is not fully resolved [14]. This review summarizes multiple important topics related to the safety and efficacy of iPSC-CM transplantation therapy for cardiac disease and discusses the prospects for this treatment for a first-in-human study.

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CARDIOMYOGENIC DIFFERENTIATION OF iPSCs

iPSCs are generated from multiple cell sources, such as fibroblasts, T cells, adipose tissue, and cord blood, by using factor-based reprogramming including Oct-3/4, Sox2, c-Myc, and Klf4 [15]. Cardiomyogenic differentiation is reportedly inducible from any iPSC, regardless of the cell source or the reprogramming factors, by multiple *in vitro* cell culture protocols as described below. In addition, it has been reported that reprogramming using certain factors directly induces cardiomyogenic differentiation. In this section, generation of cardiomyocytes from reprogrammed somatic cells is summarized.

Cytokine-Based Cardiomyogenic Differentiation of iPSCs

The standard *in vitro* protocol to induce cardiomyogenic differentiation of pluripotent stem cells was reported by Keller *et al.* in 2008 [16]. They sequentially added multiple factors to ESCs to generate self-beating cardiomyocytes. At stage 1, activin A and BMP4 were added to upregulate WNT signaling proteins that induce mesodermal differentiation, and then, at stage 2 the WNT inhibitor DKK1 was added to specify cardiac mesoderm with addition of VEGF or bFGF that promotes expansion and maturation of cells of cardiac lineage [16]. As a result, self-beating embryoid bodies emerged in a consistent manner, expressing cardiac specific markers, such as cardiac troponin T, cardiac myosin light/heavy chains or connexin 43 [10]. However, the efficiency of cardiomyogenic differentiation or the degree of iPSC maturation was variable, depending upon the cell lines or the culture conditions [8, 11]. Additional protocols, such as mechanical stretching or factor addition, were reported to enhance the efficiency and/or uniformity of generating mature cardiomyocytes *in vitro* [11, 17].

Small Molecule-Based Cardiomyogenic Differentiation of iPSCs

Cytokine-based generation of iPSC-CMs for regenerative therapy purposes would be limited by its cost effectiveness as a treatment for cardiac disease, which requires a large number of functional cardiomyocytes for transplantation. On the other hand, synthetic small molecules could potentially resolve this issue. It has been reported that some small molecules that modulate WNT signaling were able to induce cardiomyogenic differentiation of iPSCs [18, 19] or to enhance cytokine-based cardiomyogenic differentiation of iPSCs [17], whereas the efficiency, consistency, and maturity of these iPSC-CMs have not been fully established.

Direct Reprogramming Toward Cardiomyocyte Generation

It has been reported that direct reprogramming of cells using certain transcription factors or microRNA, not *via* iPSCs, induces cardiomyogenic differentiation of multiple cell sources, such as dermal fibroblasts, cardiac fibroblasts, or skeletal myoblasts *in vitro* [20]. Since tumorigenicity of the reprogrammed cardiomyocytes might be reduced compared to that of iPSC-CMs, this direct reprogramming technique is promising for the purpose of cardiac regeneration therapy. However, the efficiency of cardiomyogenic differentiation or robustness of the generated cardiomyocytes are not fully established at present [21].

In summary, cytokine-based cardiomyogenic differentiation has been established on a relatively small scale, although further modification of the protocol is needed to optimize the iPSC-CMs for transplantation purposes. Small molecules are worthy of further investigation as inducers of cardiomyogenic differentiation of iPSCs for clinical application of regenerative therapy. Direct reprogramming toward cardiomyocytes might be clinically useful in the future, although further studies are needed.

LARGE-SCALE CELL CULTURE FOR CELL TRANSPLANTATION THERAPY

Introduction of iPSC-derived functional cells to the diseased area is a promising treatment to compensate lost organ function. For the development and implementation of cell transplantation therapy using iPSC-derived cells, obtaining sufficient numbers of cells should be a primary goal. The amount of iPSC-derived cells to be prepared depends on the purpose. For example, in treating myocardial infarction, hepatic disorders, and diabetes, approximately one hundred million to one billion iPSC-derived cells per patient are estimated to be required [22, 23]. Furthermore, in the case of low purity of target cells after differentiation, it is assumed that two or three times the amount of cells may be needed to purify sufficient numbers of target cells.

In the conventionally and widely applied methods for generating ESCs/iPSCs, because they are mainly cultured using cell culture dishes, it is challenging to prepare sufficient numbers of iPSC derivatives for clinical purposes. To prepare one billion iPSC-CMs, more than one hundred 100mm diameter dishes would be needed [9]. For the preparation of cells used in transplantation therapy, limitations of space and labor possibly hamper the 2-D cell culture because the cell preparation should be conducted in the highly regulated area such as cell processing center or cell isolator. Furthermore, handling large numbers of dishes incurs a high risk of contamination. In addition, from the standpoint of quality control, fewer culture vessels would reduce cell variation. In this section, the latest information regarding large-scale culture of iPSCs and their derivatives are summarized and discussed.

Stem Cell Maintenance By 2-dimensional and 3-dimentional Cell Culture

Instead of one hundred dishes, culture equipment having a large surface area has been developed. For example, a 5-layer-format multiflask provides 875cm^2 of culture area, which is equivalent to approximately sixteen 100-mm dishes [24]. Although this method is not technically demanding, the use of a cell scraper, which is commonly used in passaging iPSCs (Protocols on iPS Cells, CiRA; http://www.cira.kyoto-u.ac.jp/e/research/protocol.html), is not feasible. Microcarrier beads also provide larger growing areas for cells and conserve culture media and reagents. Cells cultured on the beads are suspended in growth media and stirred continuously in a bottle, resulting in proliferation comparable to that of cells cultured in dishes and with stemness maintained for at least 6 weeks [23, 25]. The difficulty of passaging cells on the beads limits continuous scale-up culture. In addition,

complete removal of the beads from cell suspension is not easy; thus, microcarrier bead cultures are likely inappropriate for clinical-grade usage. An alternative is to culture iPSC aggregates in stirring-culture flasks. Unlike bioreactors using microcarrier beads, floating cell aggregates without scaffolds can also be cultured in vessels with stirring. Many reports have shown that ESC/iPSC aggregates successfully proliferate while maintaining pluripotency [26-28]. To protect cells from shear stress that is caused by stirring, Amit *et al.* used ESC spheroids as the starting material. The culture volume can be from 25 mL to over a liter, depending on the cell dose required. In addition, Otsuji *et al.* recently developed a new shear stress-free suspension culture system [29]. By adding a low concentration of methylcellulose to the culture medium, the increased viscosity of the medium allows cell aggregates to remain suspended without stirring.

Bioreactor Systems For Generating Donor Cells For Cell Transplantation Therapy

To prepare cells for transplantation in the clinical arena, bioreactors appear to be a more realistic and favorable option, as they support biological environments for cells. In a bioreactor system, cells are cultured in bottles and are protected from open air, reducing the possibility of contamination. In culture media, parameters such as pH, oxygen, and temperature are monitored and tightly regulated. Furthermore, bioreactors can save not only space and labor, but also the cost for reagents and culture media. As discussed above, many reports have shown that culturing ESCs and iPSCs using bioreactors is feasible and effective to expand iPSCs. As for the induction of differentiation, iPSCs are typically cultured in a 3-D state referred to as embryoid bodies, which are relatively easy to transfer from static culture to suspension culture. Furthermore, cell aggregates can differentiate into cardiomyocytes in the reactor, which are harvested and dispersed as single cells. Cardiomyocytes grown in bioreactors form sheet-shaped grafts that exhibit synchronized beating *in vitro* [30]. The bioreactor system would thus be an ideal application for clinical preparation of cells that display iPSC characteristics such as stemness maintenance, expansion, and differentiation.

CELLULAR CARDIOMYOPLASTY USING iPSC-CMs IN THE FAILING HEART

Although cell therapy using myoblasts, bone marrow cells, or other stem cells are considered to have some impact on functional recovery of failing heart mainly by cytokine paracrine effects [31], its effectiveness may be limited in severely damaged myocardium because of a lack of residual myocytes that can undergo cytokine therapy-induced angiogenesis. Cardiogenic stem cells such as c-kit-positive cells were recently reported to display cardiomyogenic lineage, and some studies have shown that these cells can differentiate into cardiomyocytes to improve heart function [32]. However, because these cells display relatively low potential for differentiating into cardiomyocytes, this functional recovery may also depend on cytokine paracrine effects. In addition to paracrine effects, iPSC-CMs are considered to have some potential for supplying myocytes that can work synchronously with recipient myocardium as "mechanically working cells" to replace severely damaged myocardium [31], and offer the potential for "true myocardial regeneration therapy" that can regenerate severely damaged myocardium.

Although iPSC-CMs are expected to open a new era of cell therapy as mentioned above, there are several drawbacks that should be overcome prior to clinical application as follows.

1. Can iPSC-CMs improve cardiac function in the failing heart by directly contributing to contractile force *in vivo*, as evidenced by electrical and histological integration with recipient myocardium without arrhythmogenicity?
2. Can iPSCs survive as working cells for long time after implantation? And what is the best way to prolong survival of implanted cardiomyocytes and show better functional improvement?
3. What type of cardiomyocyte, such as mature or ventricular, is ideal for improvement of cardiac function?
4. Is it true that iPSC-CMs are better compared with other somatic cells such as myoblasts or bone marrow cells in terms of functional recovery and do they promise true regenerative therapy in severely damaged myocardium?

Several studies have demonstrated functional recovery after iPSC-CM transplantation *via* cell sheets [9, 10, 33, 34] or needle injection [35] in porcine or rat heart failure models. In addition, some studies have reported that a large number of implanted iPSC-CMs cannot be histologically detected *in vivo*, and that this functional recovery may be induced mainly by angiogenesis, not by cardiomyogenesis [33, 34].

Do iPSC-CMs have the ability to induce cardiomyogenesis in damaged myocardium after implantation? Some discussion of the *in vivo* behavior of cardiomyocytes after implantation is warranted. We previously reported that X-ray diffraction identified myosin and actin-myosin lattice planes typical of organized cardiac muscle fibers within the transplanted cell sheets, which supported synchronized movement of implanted myocytes in a rat infarction model [12]. Other studies reported that ESCs, not iPSC-CMs, showed electrical coupling between implanted cardiomyocytes [36] and recipient myocardium, and that cardiomyocyte implantation reduced arrhythmogenicity in a porcine infarction model [37], and quality of electrical integration improved long term after implantation in mouse heart [38]. As mentioned above, it may be evident that implanted cardiomyocytes are contractile and can form electrical and histological syncytia with recipient myocardium. However, realizing their full potential requires that a large number of cardiomyocytes survive and contribute to functional recovery mainly *via* direct mechanical effects on the heart, not *via* cytokine effects, and it may be crucial to develop new methods to prolong myocyte survival [33] or cardiac tissue with appropriate extracellular matrix or well-developed vasculature [39].

Cell delivery route may have tremendous impact on cell survival after implantation [40]. There is a lot of iPS cells delivery methods including direct injection, transcatheter application, and implantation of tissue engineered devices containing iPS-CM. One of candidates as cell delivery root may be implantation of scaffolds containing iPS-CM and this method could also provide functional recovery in heart fail-

ure animal model, but it may be not clear that implanted scaffolds containing cardiomyocytes could electrically connect with recipient myocardium *via* Connexin 43 because implanted scaffolds avoid electrical connection between implanted cardiac tissue with recipient myocardium.

Some studies have shown that the cell sheet technique may be superior to other cell delivery routes in terms of cell survival and functional recovery in somatic cells [41]. However, it has not been determined which cell delivery route is best for promoting the maximal function of iPSC-CMs *in vivo*, and introduction of cardiomyocytes into recipient myocardium by the cell sheet method resulted in poor iPSC-CM survival in a porcine infarction model [9]. However, some trials have been conducted to improve myocyte survival [42, 43] and function as myocardial tissue [44]. *In Vivo* incubation techniques may be the first choice to prolong cell survival in clinical situations. Cell sheet implantation combined with omentum induced mature vasculature, which included endothelial cells and smooth muscle cells both in implanted cell sheets and implanted sites in recipient myocardium [39], leading to better cell survival in damaged myocardium [45]. This method has already been introduced to iPSC-CM sheet implantation and can form cardiac tissue with a rich vascular network that can be detected at least 1 year after implantation on the epicardium [33]. Although some studies have reported new methods that are aimed at activating cardiomyocytes [46] or prolonging survival of somatic cells or cardiomyocytes combined with other non-cardiogenic cells such as endothelial cells [34], further study may be needed to determine optimal methods for prolonging cardiomyocyte survival *in vivo*. In addition to poor blood perfusion, there are many issues such as immunogenicity and poor cell-ECM attachment that may have remarkable effects on cell survival, although no studies have addressed these problems. These issues should be resolved to improve the efficacy of iPSC-CMs.

Another concern may be which type of myocyte, such as mature *vs* immature and ventricular *vs* atrial, has the greatest impact on cardiac performance. We can speculate that differentiated cardiomyocytes, which have a character of early progenitor cells, have a potential of cell growth and might be able to show longer survival after *in vivo* implantation, whereas those cells have a possibility of tumor formation due to massive cell growth and weaker contractile force compared with mature cardiomyocytes. On the other hand, matured cardiomyocytes might have a better contractile force compared with immature cardiac cells because of more developed sarcomeres in cytosol, though they have a less potential of cell growth which lead to shorter survival *in vivo*.

Some studies have reported that atrial-type cardiomyocytes can improve cardiac performance in a rat infarction model possibly *via* paracrine effects [47], but their overall superiority has yet to be confirmed. Further study may be needed to elucidate which cell type will be appropriate for the improvement of *in vivo* performance, and suitable selection or differentiation methods should be developed to enhance its effectiveness. Some studies have reported that immature cardiomyocytes derived from iPSCs could be induced to differentiate to a mature phenotype by mechanical stretching *ex vivo* [11], and implanted immature cardiomyocytes differentiate to mature cardiomyocytes by *in vivo* incubation possibly by exposure to several neuro-hormonal factors, mechanical loading, and electrical stimulation [48].

ELIMINATION OF UNDIFFERENTIATED CELLS AMONG iPSC-CMs

As a therapy associated with regenerative medicine, clinical trials using human pluripotent stem cells (hPSCs), such as human embryonic stem cells (hESCs) [49-51] and human induced pluripotent stem cells (hiPSCs) [52], have been conducted. Retinal pigment epithelial (RPE) cells derived from hPSCs were transplanted into patients with eye diseases such as age-related macular degeneration or Stargardt's macular dystrophy. Notably, in 2014, using hiPSC-derived RPE cells, a first-in-human clinical trial was performed by Dr. Masayo Takahashi and colleagues at RIKEN CDB, Kobe, Japan [52], and there have been no serious adverse events such as tumor formation to date. One of the most critical issues to be addressed is safety, including prevention of tumorigenesis within hiPSCs [14]. For safer clinical application of hiPSCs, it would be essential to efficiently remove residual undifferentiated cells with tumorigenicity. Recent advances in novel techniques for eliminating hiPSCs are presented below (Table **1**).

Survivin is known to be highly expressed in various cancers, and is shown to confer a survival advantage to tumor cells; therefore, survivin inhibitors have been developed as molecular-targeted drugs for cancers. Regarding hESCs, survivin was suggested to contribute to teratoma formation [53]. Lee *et al.* [54] have demonstrated that survivin and BCL10 are preferentially expressed in hPSCs, and that survivin inhibitors were completely cytotoxic to hPSCs *in vitro*. Even when hPSCs were treated only once with a survivin inhibitor *in vitro*, teratoma formation was completely prevented after transplanting these cells into immunodeficient mice. It is important that cell viability and functionality were maintained even if cells that differentiated from hPSCs were treated with survivin inhibitors [54]. For example, the survivin inhibitor QC has long been used as a nutritional supplement, resulting in no serious side effects, and it would be suggested that safety regarding QC has been established. If survivin is expressed on differentiated tumors as well as undifferentiated cells, it is possible that survivin inhibitors may be used not only in *ex vivo* purging, but also through *in vivo* administration. On the other hand, regarding BCL10, a selective inhibitor for BCL10 must be newly developed.

Benvenisty *et al.* [55] have revealed that survival of hPSCs is dependent on oleate metabolism, suggesting a unique role for lipid metabolism in hPSCs. By high-throughput screening of 52,000 small molecules, they discovered that an oleate synthesis inhibitor, designated as PluriSIn #1, is potent in selective elimination of hPSCs. PluriSIn #1 was demonstrated to induce endoplasmic reticulum (ER) stress and apoptosis in hPSCs, thereby preventing teratoma formation from undifferentiated cells [55]. Although PluriSIn #1 has been reported to be used only in *ex vivo* purging, it should be beneficial in the future to develop a clinical-grade compound and/or a compound that can be administered *in vivo*. The authors also attempted to establish Plur-

Table 1. Accumulated evidence for elimination of undifferentiated cells.

Reference	Chemical or Antibody *etc.*	Mode of Action	Drug
Lee *et al.* [54]	Chemical inhibitor	Survivin inhibition	QC YM155
Ben-David *et al.* [55]	Chemical inhibitor	Oleate synthesis inhibition	PluriSIn #1
Ben-David *et al.* [57]	Antibody	Claudin-6 purging	Anti-Claudin-6 Ab
	Toxin-conjugated Ab	Binding to Claudin-6	Saporin-conjugated Ab
	Toxin	Binding to Claudin-6	Clostridium Perfringens Enterotoxin
Vazquez-Martin *et al.* [58]	Chemical	AMPK activation	Metformin
Tang *et al.* [59]	Antibody	SSEA-5 purging	Anti-SSEA-5 mAb
Richards *et al.* [60]	Chemical	ER stress	JC011
Tateno *et al.* [62]	Lectin-toxin fusion protein	Lectin-binding to iPSCs Cargo of toxin	rBC2LCN-PE23
Huskey *et al.* [63]	Chemical inhibitor	CDK1 inhibition	Purvalanol A Dinaciclib
Wu *et al.* [64]	Chemical inhibitor	CDK9 inhibition	Flavopiridol

Abbreviations: AMPK, AMP-activated protein kinase; SSEA-5, stage-specific embryonic antigen-5; ER, endoplasmic reticulum; PluriSIn, pluripotent cell-specific inhibitor; CDK, cyclin-dependent kinase.

iSIn#1 as therapy against undifferentiated cancer cells , which would be highly dependent on oleate [56].

Claudin-6 is a tight-junction protein, and has been identified as a specific surface marker of hPSCs by Benvenisty *et al.* [57]. The authors indicated that claudin-6 is uniquely expressed in hPSCs, and that it is useful for selectively removing undifferentiated cells. They developed three methods for depleting claudin-6-positive cells: (1) cell sorting using an anti-claudin-6 antibody; (2) saporin toxin-conjugated antibody targeting claudin-6; (3) *Clostridium perfringens* enterotoxin, which binds several claudins including claudin-6. These approaches were shown to eliminate the tumorigenic potential of hPSCs [57].

Metformin, a widely used drug for diabetes mellitus, has been reported to have versatile functions, including antitumor effects. Metformin is an agonist for AMP-activated protein kinase, and has been shown to repress the expression of Oct4 and survivin. Vazquez-Martin *et al.* [58] demonstrated that metformin limits the tumorigenicity of mouse iPSCs. Since the side effects of metformin in humans are well known, it would be great progress for clinical application if it is also effective in hiPSCs.

Tang *et al.* [59] reported that stage-specific embryonic antigen-5 (SSEA-5) is a newly discovered antigen preferentially expressed on hPSCs. They produced a monoclonal antibody against SSEA-5, and demonstrated that it is possible to efficiently remove undifferentiated cells through fluorescence-activated cell sorting [59]. If a magnetic sorting system using a clinical-grade anti-SSEA-5 antibody were to become established, it would be a promising method for purging.

ER stress is known to be a response to unfolded proteins deposited in the ER, in situations where proteins are not correctly folded. If the unfolded protein response is sufficiently robust, apoptosis is induced within cells. Richards *et al.* [60] have identified the ER stress inducer JC011 as a potent and specific inducer of the unfolded protein response that is toxic to hPSCs. However, further studies are needed to clarify its precise mechanism.

Lectins are proteins that bind to glycan structures. Tateno *et al.* [61] reported that one lectin, called rBC2LCN, specifically binds to hPSCs. Based on this report, in order to eliminate hiPSCs they generated a lectin-toxin fusion protein (rBC2LCN-PE23), which could bind to and be internalized by hiPSCs and demonstrated that the lectin-toxin fusion protein was selectively toxic to hiPSCs *in vitro*. [62].

Huskey *et al.* [63] have shown that cyclin-dependent kinase 1 (CDK1) is essential for cell viability in mouse and human ESCs, indicating a unique dependency of ESCs on CDK1 activity. The CDK1 inhibitors purvalanol A and dinaciclib have been shown to be potent inducers of cell death, preferentially in ESCs [63]. In another report, Wu *et al.* [64] demonstrated that the CDK9 inhibitor flavopiridol has a suppressive effect on Nanog and c-Myc expression in mouse ESCs. Since there is no evidence for prevention of teratoma formation through CDK9 inhibition, further elucidation of this mechanism will be needed. In addition, there are some studies showing selective elimination of hPSCs by etoposide [65, 66] and by a natural marine product derivative [67].

Collectively, since the above-mentioned strategies have been shown to deplete undifferentiated cells, these methods could hypothetically be applied to various types of cells differentiated from hPSCs. On the other hand, there are other

strategies where cardiomyocytes are specifically collected either based on metabolic characteristics or surface markers. Tohyama *et al.* [68] found that only cardiomyocytes could survive under culture with non-glucose (lactate-rich) medium. Furthermore, there are several cardiac markers; signal receptor protein α (SIRPA) (CD172) [69, 70] vascular cell adhesion molecule 1 (VCAM1)[69], and stage-specific embryonic antigen-1 (SSEA-1) [71-73]. Using these markers, cardiomyocytes are able to be sorted by flow cytometry or immunomagnetic bead; clinical limitation is that flow cytometry is time-consuming. Although immunomagnetic sorting is useful for clinical trial, it is expensive and its yield after sorting might be relatively low.

The potential limitations of these methods include possible difficulty in cell sorting with a 3-D tissue culture system, or possible drug permeability issues. Teratomas are differentiated tumors derived from undifferentiated cells. Once teratoma formation is achieved *in vivo*, the above-mentioned strategies would be unable to eradicate the established teratoma (*i.e.* the differentiated tumor). *Ex vivo* purging of tumorigenic cells prior to transplantation would be indispensable in such cases.

IMMUNE REACTION AGAINST iPSCs

Host immune reaction against the transplanted iPSCs is a potential problem that would limit the therapeutic efficacy of cell transplantation therapy using iPSCs. Immune reactions against the iPSCs would include both acquired and innate immunity. Much evidence from clinically performed organ transplantation or cell transplantation suggests that the immune reaction against the transplanted iPSCs depends on the nature of the transplant, *i.e.* whether it is autologous or allogeneic.

Autologous Immunogenicity of iPSCs

Autologous iPSC transplantation can in principle escape the acquired immune reaction because they are of the same origin as the immunocompetent cells, and are therefore expected to be recognized as "self" by the acquired immune system that has matured to establish self-tolerance. Though Zhao *et al.* reported immunogenicity of murine iPSCs as compared to ESCs due to the abnormal expression of antigens based on the epigenetic differences between iPSCs and ESCs or mutations in the coding sequences of iPSCs [74], other studies have found only negligible immunogenicity of *in vitro*-differentiated iPSCs [75-77]. Thus far, the majority of studies have not demonstrated acquired immune reactions against autologous iPSCs and their derivatives.

On the other hand, it was reported that iPSCs or their derivatives were highly susceptible to natural killer (NK) cells because they expressed low levels of major histocompatibility complex (MHC) class I molecules [78, 79]. MHC class I molecules are expressed on almost all of the cells in the body and bind to their receptor on NK cells to inhibit their cytotoxic activity [80]. Therefore, low expression of MHC class I molecules in the iPSCs or their derivatives could cause an innate immune reaction by NK cells, being recognized as "non-self" even in the case of autologous transplants. Regulating NK cell-related immune reactions might be the key to overcoming immune rejections of autologously transplanted iPSCs.

Allogeneic Immunogenicity of iPSCs

In the allogeneic transplantation of iPSCs, acquired immune reactions, in addition to the innate immune reactions observed in autologous transplantation, are hypothetically caused by the allogeneic antigens. Allogeneic antigens mainly consist of MHC molecules, which directly activate the recipient T cells *via* T-cell receptors as the "direct pathway", and minor antigens in addition to peptides derived from MHC molecules, which are presented after being phagocytosed and digested by antigen-presenting cells of the recipient to activate recipient T cells as the "indirect pathway". In the transplantation of iPSCs, the main pathway of the host immune reaction would begin with the indirect pathway, because the expression of MHC molecules in the iPSCs or their derivatives is relatively low [78, 81]. However, it has not been concluded whether MHC molecules or minor antigens have greater influence on the immune reaction after antigen presentation in the allogeneic transplantation of iPSCs.

MHC genes are the most polymorphic genes, reaching more than tens of thousands of haplotypes constituted from the combination of more than a dozen alleles in each locus in humans, which affects antigen recognition by T cells by influencing both peptide binding and the contacts between T-cell receptors and MHC molecules. Such MHC-restricted T-cell recognition can distinguish allogeneic organs and tissues and induce their rejection [80]. However, although it is estimated that roughly 1–10% of naïve T cells recognize non-self MHC molecules [80], and mixed lymphocyte reactions *in vitro* revealed proliferative reactions of $CD4^+$ or $CD8^+$ T cells against the allogeneic iPSCs [76], it remains unclear how severe rejection could be accompanied by the very low expression of MHC class I molecules in iPSCs *in vivo*.

On the other hand, a relatively small number, albeit more than 49 minor antigens, has been reported in bone marrow transplantation thus far [82]. Almost all of these minor antigens are based on non-synonymous single-nucleotide polymorphisms, which are estimated at 10,000-11,000 between non blood-relative donor and recipient [83]; therefore, the number of minor antigens in iPSCs could be in the thousands. Other possible minor antigens include cell-surface glycans. It was reported that some special types of *N*-glycans were expressed on the iPSC-CMs but not on cardiomyocytes in the heart [84, 85]. The immunogenicity of such minor antigens in iPSCs, reported in the other cells and including cell-surface glycans, needs to be further explored.

Preventing Immune Rejection Against iPSCs

Cell transplantation therapy using iPSCs theoretically enables autologous transplantation. However, the clinical application of this approach is limited by safety concerns and high costs. To overcome this limitation, banked iPS cells, in which safety is established in advance, are under development with the aim of transplanting iPSC derivatives in an allogeneic fashion. Based on much evidence form organ transplantation, there are several approaches to prevent im-

mune rejection of allogeneic iPSCs. One is immunosuppressive therapy using combinations of several different types of immunosuppressants, for example a lymphocyte signal transduction inhibitor such as cyclosporine or tacrolimus, an antimetabolite such as mycophenolate mofetil or methotrexate, and a glucocorticoid such as prednisolone. The other is reduction of immunogenicity of donor cells by matching the types of antigens such as MHC in donor and recipient. An alternative approach is to regulate the host immune reaction by using cells reported to have immunosuppressive functions such as mesenchymal stem cells (MSCs) or regulatory T cells [81].

Immune-suppressive therapy using immunosuppressants would be based on the organ transplantation or xenotransplantation of iPSCs [9, 33], suggesting that at least three types of immunosuppressants are needed: lymphocyte signal transduction inhibitor, antimetabolite, and glucocorticoid. Another choice for preventing rejection of iPSCs, matching of MHC between the donor and recipient, could possibly decrease the initial population of T cells recognizing the non-self MHC molecules on the transplanted cells and decrease the rate of rejection in organ transplantation [86], and therefore might be applicable to iPSC transplantation, though little evidence regarding immunosuppressive therapy needed for the transplantation of allogeneic iPSCs in such an expected clinical situation as MHC-matched allogeneic iPSC transplantation would require further detailed study about it. For the MHC-matched approach, establishment of iPSC lines from healthy donors with homozygous MHC genotypes is useful in minimizing the banked iPSC line number [87-90]. A project for banking iPSCs with homozygous MHC haplotypes, especially focused on the loci of MHC-A, MHC-B and MHC-DR, has already been launched in Japan.

Regulating host immune reactions using immunosuppressive cells is another promising approach. One such immunosuppressive cell could be the MSC [91]. Most studies have shown that immunosuppressive properties of MSCs are broad: suppression for proliferation of T cells, B cells [92-94], and NK cells [95], or for maturation of dendritic cell [96, 97]. In addition, transplantation of allogeneic and autologous MSCs can be effective for immune regulation [98]. These broad mechanisms for immune regulation and their versatility for clinical application might be quite useful to regulate immune reactions related to iPSC transplantation. Further studies concerning the transplantation method for MSCs to fully exert their effect in the transplantation of iPSCs would be required for clinical application.

SUMMARY

In summary, *in vitro* unlimited production of human functional cardiomyocytes was established by using iPSC technology. Transplantation of iPSC-CMs into the damaged heart was shown to recover cardiac function *via* direct mechanical effect of transplanted cells, indicating that this treatment would be promising therapy for advanced cardiac failure in which definite loss of native cardiomyocytes is the primary and fundamental pathology. However, considering clinical application of this therapy, there are many hurdles to be resolved, such as culture protocol, transplantation method, or safety assurance for tumorgenicity and immunogenicity, which are now under intensive investigations to promptly realize the first-in-human study.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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