Review

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# Recent progress in induced pluripotent stem cell-derived cardiac cell sheets for tissue engineering

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**Summary** **The past decade has witnessed remarkable development in tissue engineering technologies and stem cells. Our lab has developed a novel technology — "cell sheet technology" for tissue engineering. After the confluent cells are cultured on an innovative temperature-responsive culture dish, the cells can be harvested as an intact sheet by lowering temperature. We have successfully created multiple cell sheet-based tissues for therapies of a vast variety of diseases, in particular, myocardial diseases. On the other side, the discovery of human induced pluripotent stem cells (hiPSC) enables stable production of defined tissue-specific cell types and thus makes it possible to regenerate tissues or even organs for clinical application and in vitro drug screening/disease modeling. Recently, we have combined cell sheet technology and hiPSC-derived cardiac cells for fabrication of functional human cardiac tissues. This review summarizes ongoing challenges in this field and our progresses in solving issues, such as large scale culture of hiPSC-derived cardiac cells, elimination of undifferentiated iPSCs to decrease the risk of tumor formation as well as myocardial tissue fabrication technologies.**

***Keywords:*** Cell sheet engineering, Induced pluripotent stem cell-derived cardiac cells, Large-scale suspension culture systems

## 1. Introduction

Various multipotent adult stem cells have been studied for repairing cardiac tissues, such as mesenchymal stem cells (*1,2*), bone marrow stem cells (*3*), skeletal myoblasts (*4*), and cardiac stem cells (5,6). However, low cardiogenic ability, insufficient survival rate and unestablished strategy to produce a large amount of cardiac cells limit the clinical application of adult stem cells. As a landmark breakthrough in science, human induced pluripotent stem cells (hiPSC) (*7,8*), which are able to differentiate into almost any tissue specific cell type, have received tremendous concern. When compared to human embryonic stem cells (ESC), hiPSCs circumvent ethical problems and are easily accessible. Technologies to induce differentiation of hiPSCs into various human somatic cells have been established (*9-13*). In particular, hiPSCs have been demonstrated to be able to differentiate into cardiac cells at high efficiency (*14*). Thus, human cardiac cells, which have been quite difficult to obtain previously, become available for research. Therefore, hiPSC-derived cardiac cells are considered to be the most promising cell source for heart regeneration. However, there are a number of factors that have hindered breakthroughs in clinical application of hiPSC-derived cardiac cells. These include the potential risk of tumorigenicity from undifferentiated hiPSCs and the lack of large-scale culture systems for clinical treatment (*15,16*).

Besides stem cells, transplantation techniques are also important for successful cell-based therapies. For example, dissociated cell injection has been demonstrated to be difficult to survive and retain cells around the target tissue (*17,18*). To solve this problem, we have pioneered scaffold-free 'cell sheet-based tissue engineering' (*19*). Multilayered *in vivo*-like cell-dense tissues, such as heart (*20-25*), have been constructed by using this technology. When compared to scaffold-

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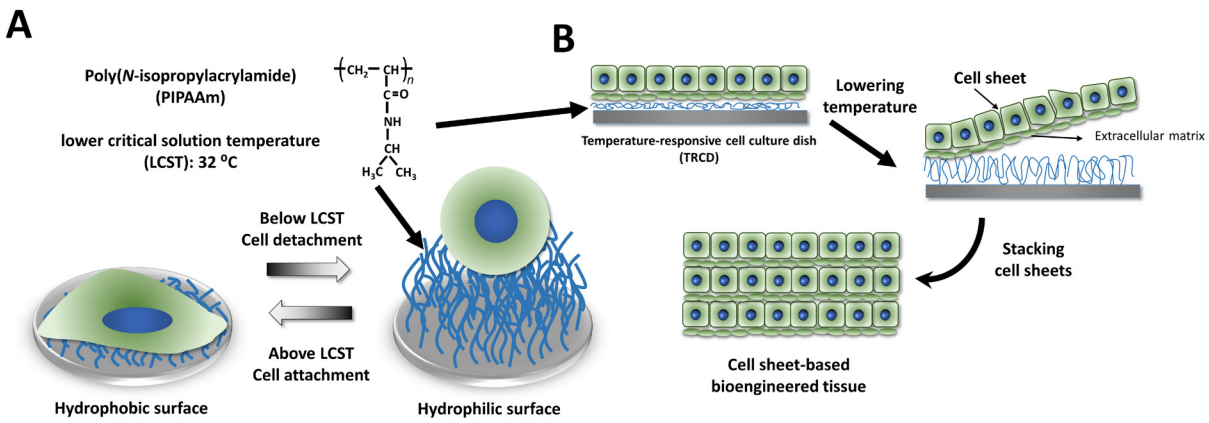


**Figure 1. (A)** Temperature-dependent changes of hydrophilicity/hydrophobicity of PIPAAm coated temperature-responsive cell culture dishes (TRCDs) allow controllable cell attachment/detachment. **(B)** Confluently cultured cells on a TRCD can be harvested as a free cell sheet, which retains the extracellular matrix and cell-cell junctions. Cell sheets can be further stacked to fabricate 3D bioengineered tissues.

based tissue engineering, scaffold free cell sheet-based tissue engineering does not have the problem of inflammatory responses, which are usually found during biodegradation of the scaffolds and lead to the failure of transplantation (*26*).

We have developed an innovative temperature-responsive cell culture dish (TRCD) to harvest cell sheets by reducing temperatures (*27,28*). The TRCDs are conventional culture dishes that are covalently immobilized with temperature-responsive polymer poly(N-isopropylacrylamide) (PIPAAm) at nanometer level thickness. As shown in Figure 1A, the TRCDs are hydrophobic when the temperature is above PIPAAm's lower critical solution temperature (LCST) of 32°C, so cells adhere to TRCDs stably. However, when the temperature is reduced below 32°C, the TRCDs become hydrophilic and enable cell detachment. If cells are confluently cultured on TRCDs, an intact sheet can be obtained by lowering the temperature (Figure 1B). A 'cell sheet' is composed of a sheet-like monolayer of cells and attached extracellular matrix (ECM) layer below the cells. The cell sheets can be stacked to form thick, multilayered tissues, mimicking stratified structure of the *in vivo* environment (Figure 1B). Without using enzymes, cell-cell junction and ECM proteins can be retained in the cell sheet, which preserve cell functions and facilitate adhesion of cell sheets onto target sites (*29,30*).

Until now, we have published many reviews to introduce our work on both basic research and clinical application of cell sheet-based tissue engineering (*31-41*). In this review, we mainly focus on our recent progresses relevant to hiPSCs.

## 2. Large-scale culture systems for cardiac differentiation from hiPSCs

When fabricating human tissues with hiPSCs for transplantation, a huge amount of cells is required. For example, 1-2 × $10^9$ cardiac cells are necessary to repair the cardiac tissue of one patient after myocardial infarction (*42*). Therefore, establishment of large-scale culture methods of hiPSCs is an indispensable technology for their clinical application. Due to limited culture space, conventional two-dimensional (2D) culture systems do not allow large-scale production of hiPSCs, whereas three-dimensional (3D) suspension culture systems have demonstrated their ability to scale up hiPSCs production (*43,44*).

We have reported that a stirred suspension culture bioreactor combined with appropriate growth factors enables effective and easy production of mouse ESC-derived cardiomyocytes (*45*). However, the number of cardiomyocytes produced by this bioreactor is relatively low due to the change in culture conditions, such as lactate accumulation and pH decreases in the medium, which limit cell proliferation and differentiation. In order to maintain suitable culture conditions, we have integrated a continuous perfusion system into the stirred suspension culture bioreactor. This novel bioreactor, which allows embryoid body (EB) formation of mouse ESCs, is capable of expanding the cell number by up to 300-fold of the initial seeding cell number and efficiently promoting cardiac differentiation as well (*46*).

Compared with mouse ESCs, hiPSCs die easier in the single cell state. EB formation is indispensable for 3D suspension culture and low shear stress agitation is the key point for high density large-scale culture. Expansion of hiPSCs in the undifferentiated state through EB formation when using a suitable commercially available spinner flask at the optimal agitation rate has been reported (*47*). However, shear stress in 3D suspension culture is a bigger hurdle for the process of differentiation. Since hiPSCs just proliferate and secrete various types of extracellular matrix in the process of the undifferentiated expansion process, it

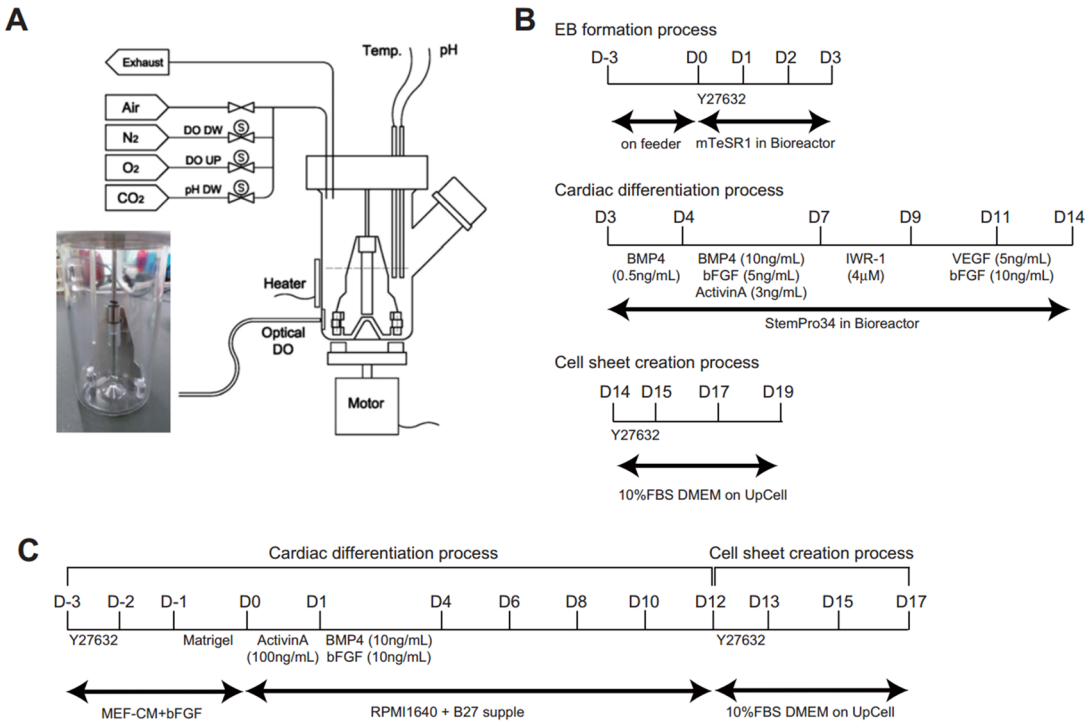


**Figure 2. Scheme of the bioreactor and culture process (A)** Schematic of the bioreactor system. The photograph shows the impeller. **(B)** Schematic of the culture process for cardiac differentiation in the bioreactor system. **(C)** Schematic of the culture process for cardiac differentiation in monolayer culture. This figure and the figure legend are reproduced with permission from ELSEVIER. (*48*)

might be easier to regulate EB formation. On the other hand, according to the changes of the characteristics of hiPSC-derived cells in the process of differentiation, EBs become fragile and a lower shear stress bioreactor is necessary. Recently we have developed a new type of bioreactor with a delta shaped impeller and it has enabled us to generate robust numbers of cardiomyocytes (> $1.0 \times 10^8$ cells/100mL bioreactor) from hiPSCs through an optimized concentration of growth factors, a small compound and dissolved oxygen concentration in 2 weeks (Figure 2) (*48*). This culture strategy has been already applied for not only cardiomyocyte production for preclinical study in a large animal model (*49*), but also other types of hiPSC-derived cells such as pancreatic progenitor cells (*50*), pancreatic exocrine cells (*51*), thyroid follicular cells (*52*) and vascular endothelial cells (*53*).

### 3. Elimination of undifferentiated hiPSC-derived cardiac cells

Any remaining undifferentiated hiPSCs may generate tumors after transplantation, but there is no differentiation protocol to promise 100% cardiac differentiation efficiency. Therefore, it is necessary to purify the differentiated cardiomyocytes. We have reported several protocols to eliminate undifferentiated hiPSCs (*54-57*).

First, we have demonstrated for the first time that methionine free culture medium is capable of efficiently eliminating undifferentiated hiPSCs without affecting viabilities of differentiated hiPSCs, such as cardiomyocytes and fibroblasts (*54*).

Second, we have demonstrated that TRPV-1 activation through transient culture at 42°C and an agonist is able to eliminate undifferentiated hiPSCs from bioengineered cardiac cell sheet tissues (*55*). TRPV-1 expression levels are significantly higher in hiPSCs than that in hiPSC-derived cardiomyocytes and apoptosis of hiPSCs at 42°C is TRPV-1-dependent. At the same time, this thermal change does not affect gene and protein expression levels of hiPSC-derived cardiomyocytes and fibroblasts. By using this approach, the final undifferentiated iPSCs in the cardiac tissues is only 0.4%.

Both of the above two methods do not bring in extra substances, so there is no worry about the effects of remaining materials in the regenerative medicine final product. However, more quantitative data about the elimination effects of these methods is needed. In addition, their impact on subsequent tumor prevention needs to be examined. In our recent study (*56*), we have demonstrated that culture at 42°C for 2 days eliminates $1 \times 10^2$ hiPSCs in fibroblast cell sheets and successfully prevents tumor formation in a nude rat model (Figure 3). Furthermore, the combination of methionine-free medium and 42°C culture sufficiently eliminates remaining robust hiPSCs ($1 \times 10^4$ hiPSCs) in fibroblast

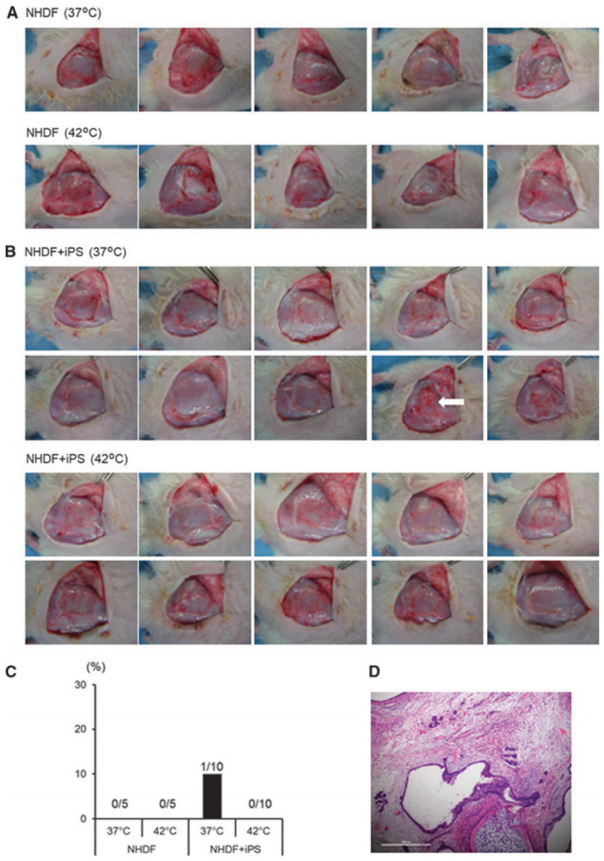


**Figure 3. Evaluation of tumorigenicity of fibroblast sheets with 1 × $10^2$ iPS cells.** One day after starting co-culture of human dermal fibroblasts (1 × $10^5$ cells) and human iPS cells [0 (A) and 1 × $10^2$ cells (B)] on 24-well temperature-responsive culture plates, cells were cultured at 37°C or 42°C for 2 days until day 3 and then cultured further at 37°C until day 5. Monolayered cell sheets were then transplanted onto subcutaneous tissues of nude rats. **(A, B)** Macroscopic images of transplanted regions at 3 months after transplantation (A, *n* = 5 for each condition; B, *n* = 10 for each condition). Arrow indicates a tumor. **(C)** Percentages of tumor formation. **(D)** Representative image of a HE-stained tumor. Scale bar, 500 mm. HE, hematoxylin and eosin. This figure and the figure legend are reproduced with permission from Mary Ann Liebert, Inc. (*56*)

cell sheets and prevents tumor formation and tumor-related death.

In another study, we demonstrated that dinaciclib, the cyclin-dependent kinase 1/9 inhibitor, selectively eliminates undifferentiated hiPSCs without affecting the viability of cardiac cells (*57*). Nanomolar concentrations of dinaciclib is enough to induce DNA damage and upregulation of p53 protein levels in undifferentiated hiPSCs but not hiPSC-derived cardiomyocytes, this is because of a difference in MCL-1 transcriptional downregulation and MCL-1 degradation between hiPSCs and hiPSC-derived cardiomyocytes through the treatment with dinaciclib. A beating hiPSC-derived cardiac cell sheet can be fabricated after treatment with dinaciclib. In addition, combining dinaciclib with methionine-free medium and 42°C may further enhance the ability to prevent tumor formation (*56*).

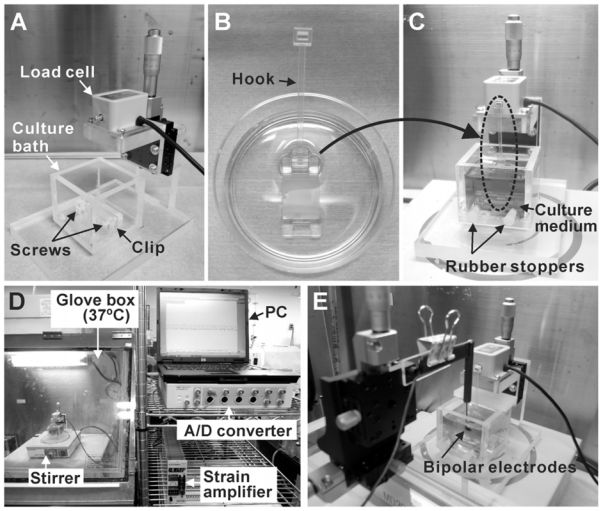


**Figure 4. Configuration of contractile force measurement system. (A)** The appearance of a contractile force measurement device. **(B)** The hook made by a 3D printer was fixed to the handle. **(C)** The cardiac cell sheet-tissue was mounted to the force measurement device vertically and fresh medium (Medium D) was poured. **(D)** The entire appearance of a contractile force measurement system. **(E)** The appearance of electrical pacing system. This figure and the figure legend are reproduced under the terms of the Creative Commons Attribution License, which permits unrestricted use. (*62*)

## 4. Cardiac tissue fabrication using hiPSC-derived cells

Previously, we have succeeded in fabrication of beating myocardial tissue *in vivo* by stacking neonatal rat cardiomyocyte sheets (*24*). Further, millimeter scale cardiac tissues are successfully fabricated *via* repeated transplantation of triple-layer cardiomyocyte sheets (*23*). By using the same polysurgery strategy, we recently have succeeded in fabrication of thick, functional human cardiac tissues from hiPSC-derived cardiac cells in a nude rat (*58*). hiPSC-derived cardiac cells are able to proliferate and become mature *in vivo*. The transplanted hiPSC-derived myocardial grafts survive, show spontaneous beating *in vivo* and demonstrate well-organized vascular networks.

In addition to sheet-like grafts, we have fabricated tubular tissues *via* wrapping neonatal rat cardiomyocyte sheets (*59,60*). The myocardial tubes are capable of generating measurable inner pressure changes through myocardial tube contraction *in vitro* (*59*). Furthermore, *in vivo* myocardial tubes have been successfully fabricated by wrapping rat cardiac cell sheets around resected adult rat thoracic aorta and transplanting the tube grafts in place of the abdominal aorta of athymic rats (*60*). The *in vivo* myocardial tubes generate inner pressure changes of about 6mm Hg according to their contraction. Recently, we reported a successful fabrication of human tubular cardiac tissues derived from hiPSCs (*61*). We wraped triple-layered cell sheets around the inferior vena cava of nude rats. Two months

later, the maximum inner pressure changes were around 9.1mm Hg under electrical stimulation. The mRNA expression of several contractile proteins in cardiac tissues at two months *in vivo* were significantly higher than that at one month. These tubular human cardiac tissues that can generate pulse pressure *in vivo* may contribute to the development of a bioengineered heart assist pump.

Besides cell-based therapies, hiPSCs can be used in development of human cell-based models for drug discovery. In addition, patient-specific hiPSCs allow customized *in vitro* models, which may be helpful in screening drugs that are effective on specific patients. Recently, we have developed a novel measuring system to dynamically detect the contractile force of hiPSC-derived cardiomyocyte sheets (Figure 4) (*62*). The cell sheets are attached to a thin fibrin gel for stable conduction of contractile force. Then the cell sheet/gel tissues are adapted to the measuring system and the real-time contractile force can be monitored clearly. The contractile force of the beating tissues are around 1 mN, and the mean force value per cross-sectional area is 3.3 mN/mm$^2$. The generated contractile forces are equal or higher than that of previous reported values. To verify whether this system can be used for drug screening, adrenaline is administered to hiPSC-derived cardiac tissues. As expected, both the contractile force and the beating rate significantly increase, reproducing the same behavior as *in vivo* cardiac tissues. Therefore, this force measurement system has great potential to be applied for *in vitro* drug testing.

In addition, we have recently reported an *in vitro* human non-alcoholic steatohepatitis (NASH) model by co-culture of primary human hepatocytes (PHH) and human fibroblasts in a cell sheet-based bioengineered tissue (*63*). As far as we know, this model is the first *in vitro* model that recapitulates hepatocellular ballooning, which is the key histological feature of NASH and used for the diagnosis of NASH. Although PHHs are the gold standard of *in vitro* human liver model, they are scarce, expensive and short-lived *in vitro*, limiting their application. Therefore, we are now considering development of hiPSC-derived hepatocytes in place of PHHs for building a cell sheet-based human NASH model.

## 5. Conclusion

We have done a lot of work in development of hiPSC technology and hiPSC-derived cardiac tissues, but still many issues need to be solved. For example, although we have succeeded in creating thick, vascularized cardiac tissues *in vitro* using neonatal rat cardiomyocytes (*64,65*), it is a big challenge to reproduce the same result using hiPSC-derived cardiac cells. Therefore, we are currently developing suitable perfusion systems for vascularization of hiPSC-derived cardiac tissues *in vitro*. Another issue is an unclear maturation mechanism of hiPSC-derived cardiac cells. When hiPSC-derived cardiac cells just finish differentiation, they are still immature and very different from cardiomyocytes *in vivo*. Study of the maturation process needs to be investigated. In addition to cardiomyocytes, functions of other cell types, such as cardiac fibroblasts, need to be cleared more. For example, we have found that cardiac fibroblasts possess anti-angiogenic properties mediated by Ly6/Plaur domain-containing 1 (LYPD1) (*66*). Thus, inhibition of LYPD1 may contribute to the fabrication of vascularized functional bioengineered tissues. We believe that unremitting efforts on development and integration of advanced technologies, such hiPSC technology, cell sheet technology, CRISPR technology and so on, will help us to achieve our final goal – regeneration of human hearts to save lives.

*Conflict of Interest*: Tatsuya Shimizu is a member of the scientific advisory board and a stakeholder of CellSeed Inc. Tatsuya Shimizu and Katsuhisa Matsuura are inventors of a bioreactor system for PSC culture, the patent of which is held by Able Co. and Tokyo Women's Medical University (US9574165B2). Tokyo Women' s Medical University receives research funds from CellSeed Inc. Able Co. and Nihon Kohden Co.

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