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Cardiac Differentiation of Embryonic Stem Cells by Patterning Culture

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1. Introduction

Although heart transplantation has been established as an ultimate therapy for severe heart failure, it is not a universal therapy because of the lack of donor hearts. As the alternative to heart transplantation, regenerative medicine based on cell transplantation attracts increasing attention. Recent studies revealed that bone marrow-derived cells (Messina et al., 2004; Wollert et al., 2004), endothelial progenitor cells (Kawamoto et al., 2001), adipose-derived cells (Wang et al., 2009), and myoblasts (Memon et al., 2005; Hata et al., 2006; Kondoh et al., 2006) have the potential to improve cardiac function when they are transplanted into a failing heart. Because these somatic cells can be harvested from the patients themselves, their clinical applications have already started. It is considered that the therapeutic potential of these cells depends on the paracrine effects such as the promotion of angiogenesis, suppression of fibrosis, suppression of apoptosis, and attraction of stem cells, and/or the direct contribution to angiogenesis by the differentiation into vascular cells. It was also reported that bone marrow-derived cells and adipose-derived cells have the potential to differentiate into cardiomyocytes (Makino et al., 1999; Planat-Benard et al., 2004). However, the efficiency is too low to directly contribute to the cardiac contractility. Therefore, although the above cells are significantly useful for cardiac regeneration, they cannot replace the dysfunctional cardiomyocytes in a failing heart, and thus their therapeutic potential is limited. Cardiac contractility mainly depends on the cardiomyocytes which account for one third of the total cell content of a heart (Brutsaert, 2003). Therefore, for the better recovery of cardiac contractility, supplementation of functional cardiomyocytes must be necessary.

The representative diseases requiring heart transplantation are dilated cardiomyopathy (DCM) and dilated form of hypertrophic cardiomyopathy (D-HCM). Actually, in Japan, approximately 90% of the patients undergoing heart transplantation so far were DCM or D-HCM. In both DCM and D-HCM, cardiac contractility is decreased by the dysfunction and loss of cardiomyocytes. In approximately 20% of DCM and 60% of HCM, the responsible gene mutations were identified. One of the destinations in cardiac regenerative therapy is to transplant enough amounts of functional cardiomyocytes to mechanically support the cardiac contractility, replacing the dysfunctional and lost cardiomyocytes in a failing heart of such cardiomyopathies. A human left ventricle contains several billions of cardiomyocytes (Olivetti et al., 1991). Therefore, more than a billion is the desired number of cardiomyocytes to be transplanted for the recovery of cardiac contractility. It is impossible

to prepare such a large number of cardiomyocytes from somatic stem cells, such as bone marrow-derived cells and adipose-derived cells, in the present technological situation. On the other hand, embryonic stem cells (ESCs) have substantial potential to generate such a large number of cardiomyocytes, because of their unlimited self-renewal capacity and pluripotency (Murry & Keller, 2008; Passier et al., 2008). Additionally, induced pluripotent stem cells (iPSCs), the equivalents of ESCs artificially created from somatic cells (Takahashi et al., 2007; Yu et al., 2007), are also candidates. At present, ESCs and iPSCs are the only and promising cell sources to generate enough amounts of cardiomyocytes for the direct compensation of lost contractility of a failing heart.

Here the characteristics of iPSCs are briefly mentioned. The major difference of iPSCs compared to ESCs is that they can be created from somatic cells of adults. Therefore, the ethical issue in the case of ESCs which must be extracted from embryos have nothing to do with iPSCs. Additionally, if the iPSCs created from the cells of patients themselves are used as the cell sources for regenerative therapies, immunological rejection after transplantation does not occur. On the other hand, as the drawback of iPSCs, it is considered that their tumorigenicity may be relatively higher than that of ESCs because of the artificial manipulation for the creation of iPSCs. It should be also discussed whether the iPSCs from patients themselves should be used or not when the diseases may be caused by gene mutation. Other biological properties of iPSCs can be considered basically equivalent to those of ESCs. Therefore, the research subjects in preparing differentiated cells for transplantation from iPSCs are almost same as those from ESCs described below.

When undifferentiated ESCs are transplanted into a body, they generate teratomas with high probability. Transplantation of unidentified ESC-derived cells also has the possibility to cause lethal problems. Therefore, for the clinical uses, ESCs must be differentiated into identified objective types of cells and the cells must be purified before transplantation. Accordingly, the major two subjects in ESC research have been, firstly, to enhance the differentiation efficiency into the objective cells such as cardiomyocytes, and secondly, to purify the differentiated objective ESC-derived cells. At an early stage of ESC research, the culture procedures for the induction of cardiac differentiation, such as the hanging drop method, were developed (Hescheler et al., 1997). Thereafter, a lot of biochemical substances, such as retinoic acid (Wobus et al., 1997), ascorbic acid (Takahashi et al., 2003), nitric oxide (Kanno et al., 2004), BMP inhibitors (Yuasa et al., 2005), Wnt inhibitors (Naito et al., 2006), and activin A (Laflamme et al., 2007), were found to promote cardiac differentiation of ESCs. Additionally, the coculture methods with specific cells, such as END2 cells (Mummery et al., 2003) and OP9 cells (Yamashita et al., 2005), were found to promote cardiac differentiation of ESCs. With respect to the purification of ESC-derived cardiomyocytes, Klug et al. developed a genetic purification method using antibiotic-resistant gene which was designed to express in differentiated cardiomyocytes, at an early stage of the research (Klug et al., 1996). Similar genetic purification methods were developed thereafter by the use of reporter genes such as EGFP (Anderson et al., 2007; Hidaka et al., 2003; Muller et al., 2000). Although these methods are significantly useful for the basic research of ESC-derived cardiomyocytes, the genetic modification of ESCs is a big hurdle for clinical uses. Therefore, nongenetic purification methods also started to be developed. For example, some research groups enriched ESC-derived cardiomyocytes by density gradient centrifugation methods (Laflamme et al., 2007; Xu et al., 2006), and other groups purified cardiac progenitor cells by labelling specific surface antigens (Hidaka et al., 2009; Hirata et al., 2007; Yamashita et al., 2005; Yang et al., 2008). Hattori et al. very recently reported a novel nongenetic purification

method using mitochondria as the marker of cardiomyocytes, and achieved more than 99% purity of ESC-derived cardiomyocyte (Hattori et al., 2010). As the nongenetic purification methods will be improved more and more hereafter, the subject of how the requisite number of cardiomyocytes can be prepared easily and at a low cost, that is, “cost-effectiveness” must become more important than mere the efficiency of cardiac differentiation. Because, even if high efficiency of cardiac differentiation is achieved, the culture methods requiring considerable labor and an extremely high cost are not suitable for the preparation of a large number of cardiomyocytes. In this chapter, we will introduce our newly developed culture method which achieves high cost-effectiveness in preparing ESC-derived cardiomyocytes.

2. Culture methods for cardiac differentiation of ESCs

It has been elucidated that the formation of ESC aggregates induces multicellular interactions and thus promotes their differentiation into the derivatives of all three germ layers (Wobus & Boheler, 2005). This property of ESC is utilized also for the induction of cardiac differentiation. ESC aggregates are generally prepared via the formation of suspended spherical ESC aggregates called embryoid bodies (EB). The prepared EBs are then usually plated on the cell-adhesive dishes and cultured for additional days for further differentiation. There are two major conventional methods for the preparation of EBs. One is a hanging drop method, and the other is a suspension culture method (Fig. 1). In the case of hanging drop method, the droplets of ESC suspension are hanged from the lid of culture dishes for several days, and then an EB is formed in each droplet. In this method, the size of EBs can be controlled by ESC concentration in the suspension. It has been elucidated that the direction and efficiency of ESC differentiation significantly depend on the size of EBs (Ng et al., 2005; Wobus et al., 1991). Therefore, the hanging drop method is advantageous to prepare size-controlled EBs for the efficient and reproducible ESC differentiation into a specific lineage. On the other hand, however, the hanging drop method is disadvantageous in the mass preparation of EBs, because of its time-consuming and labor-intensive procedure. In the case of suspension culture method, ESCs are cultured in non-cell-adhesive culture dishes for several days, and then EBs are formed randomly and spontaneously. In this method, a large number of EBs can be easily prepared. On the other hand, however, the size of EBs is not controlled, and thus the differentiation efficiency and reproducibility become much lower than the case of hanging drop method. As described above, a large number of cardiomyocytes, preferably more than a billion, are needed to be transplanted for the direct compensation of cardiac contractility of a failing heart. For this purpose, more cost-effective culture methods for the preparation of ESC-derived cardiomyocytes are needed. Driven by such a necessity, several research groups started to develop scalable culture methods for the mass preparation of size-controlled EBs, utilizing such as multiwell plates (Kim et al., 2007; Ng, et al., 2005), microwell substrates (Khademhosseini et al., 2006; Mohr et al., 2006; Ungrin et al., 2008), patterning culture (Bauwens et al., 2008), and rotary suspension culture (Carpenedo et al., 2007). These novel culture methods are certainly useful for the mass preparation of size-controlled EBs. Especially, the microwell substrates have been commercially available already (AggreWell™, STEMCELL TECHNOLOGIES INC). It should be noted, however, that the EBs prepared by these culture methods must be subsequently collected and plated onto cell-adhesive culture dishes for further promotion of cardiac differentiation. After plating EBs, ESCs proliferate and expand out of the EBs on the

surfaces of culture dishes. Accordingly, the size of resultant EB outgrowths cannot be regulated, which affect and possibly decrease the eventual efficiency and reproducibility of cardiac differentiation. To avoid this problem, EBs are often plated one by one into individual wells of multiwell plates. However, this procedure is of course time-consuming and labor-intensive, and thus not suitable for the mass preparation of ESC-derived cardiomyocytes.

Fig. 1. Concept of the mass preparation of size-controlled EBs by patterning culture.

Here, we would like to introduce our newly developed culture method that enables easy mass preparation of size-controlled ESC aggregates, using cell-patterning technique (Fig. 1.). We fabricated the culture substrates, the surfaces of which comprised arrays of cell-adhesive circular micro-domains and the rest of non-cell-adhesive domains. Mouse ESCs seeded on this “cell-patterning substrates” successfully formed size-controlled aggregates on the circular micro-domains and differentiated into cardiomyocytes. In this method, ESC aggregates are prepared directly on the surface of culture substrates. Therefore, the preparation of suspended EBs is not necessary, and the overall procedure of preparing ESC-derived cardiomyocytes is further simplified than the other methods mentioned above. Moreover, the size of ESC aggregates is controlled throughout the culture period for differentiation, which certainly contributes to the reproducibility of final differentiation efficiency. The details of this “cell-patterning method” are described below.

3. Cardiac differentiation of ESCs by cell-patterning method

3.1 Fabrication of cell-patterning substrates

We fabricated the cell patterning substrates using photolithography-based technique. The details of fabrication process are described elsewhere (Sasaki et al., 2009). Figure 2 shows the schema of fabrication process. Glass coverslips were cleaned by oxygen plasma treatment. The

Fig. 2. Schema of the fabrication process of cell-patterning substrates.

surfaces of the coverslips were coupled with 3-methacryloxypropyltrimethoxysilane as described elsewhere (Okusa et al., 1994). The silanized coverslips were spin-coated with g-line positive photoresist (OFPR-800 LB, 34cP; Tokyo Ohka Kogyo) and pre-baked. The photoresist-coated surfaces were exposed with patterned visible light by a maskless photolithography device (Itoga et al., 2006) or through photomasks. The coverslips were washed with developer solvent so that the light-exposed portions of photoresist were removed. The coverslips were post-baked and immersed in acrylamide solution with *N,N'*-methylenebis(acrylamide), ammonium peroxodisulfate, and *N,N,N',N'*-tetramethylethylenediamine, so that the acrylamide was polymerized and grafted onto the silanized glass surface by redox reaction. The coverslips were thoroughly washed with water to remove the ungrafted polyacrylamide, and then washed with acetone to remove the remaining photoresist on the surface. The resultant surfaces comprised non-cell-adhesive hydrophilic polyacrylamide domains and cell-adhesive silanized glass domains. The cell-patterning substrates were thus fabricated. We fabricated the cell-patterning substrates, on the surfaces of which cell-adhesive circular domains with the diameter of 100 μm , 200 μm , 300 μm , 400 μm , or 1 mm were arrayed at constant intervals. The substrates were cut to appropriate size, put into culture dishes, sterilized with ethylene oxide gas, and used for the patterning culture of ESCs. Before ESCs were seeded, the substrates were coated with fibronectin to promote cell attachment onto the silanized glass surfaces.

3.2 Formation of size-controlled ESC aggregates on the cell-patterning substrates and cardiac differentiation

We used a mouse ESC line EMG7 in which α -cardiac myosin heavy chain promoter-driven EGFP gene was introduced (Yamashita et al., 2005). EMG7 cells express EGFP when they differentiate into cardiomyocytes. Undifferentiated EMG7 cells were maintained on gelatine-coated dishes in the presence of leukaemia inhibitory factor as described elsewhere (Hirai et

al., 2003). For the induction of cardiac differentiation, we used α -MEM (Product # M0644; Sigma-Aldrich) supplemented with fetal bovine serum (FBS), and in some cases with L-ascorbic acid 2-phosphate. Undifferentiated EMG7 cells were seeded onto the cell-patterning substrates at the density of $2-3 \times 10^4$ cells/cm² and cultured for differentiation at 37 °C in a humidified atmosphere with 5% CO₂. The medium was changed every one or two days.

Figure 3 shows the time series of microscopic images of EMG7 cells cultured on the cell-patterning substrate with 200- μ m-diameter cell-adhesive domains, in the presence of 5% FBS. EMG7 cells seeded on the substrate proliferated within the circular domains and reached confluency within the circular domains on day 3, forming circular cell colonies. As the cells further proliferated, the colonies gradually formed three-dimensional cell aggregates. Around day 9, EGFP fluorescence became obvious and some of the EGFP-positive aggregates were beating, which indicated the cardiac differentiation of EMG7 cells. We confirmed that over 50% of cell aggregates were EGFP-positive on day 10. Figure 4 shows the microscopic image of EMG7 cells cultured on the cell-patterning substrate with 1-mm-diameter cell-adhesive domains in the presence of 10% FBS, on day 13. This microscopic image reveals that the cell aggregate formation and EGFP expression (i.e., cardiac differentiation) occurs preferably near the edge of circular domain. Such a structure strongly indicates the mechanism of cell aggregate formation, as described below. Generally, when cultured cells become confluent on a culture surface and contact with each other, their proliferation is suppressed by the phenomenon which is known as “contact inhibition” (Takai et al., 2008). Consequently, the cells form monolayer structure in the case of ordinary adhesion culture. On the other hand, in the case of patterning culture, when the cells become confluent on the cell-adhesive circular domain, the cells at the edge of circular domain are still not surrounded by the cells. Therefore, their proliferation is not suppressed by contact inhibition. Because the proliferated cells at the edge of the circular domain cannot attach to the non-cell-adhesive surface outside the circular domain, they must fold toward the inside of the domain and form the aggregate. When the diameter of cell-adhesive circular domain was 200 μ m, EMG7 cells formed aggregate over the whole region of the circular domain until day 6 (Fig. 3). Accordingly, eventual EGFP-expressing regions included not only the vicinity of edge but also the center part of the circular domain (Fig. 3). We investigated the dependency of cardiac differentiation efficiency on the diameters of cell-adhesive circular domains. Undifferentiated EMG7 cells were cultured for differentiation on the cell-patterning substrates with the cell-adhesive circular domains with a diameter of 100 μ m, 200 μ m, 300 μ m, or 400 μ m, in the presence of 5% FBS. Figure 5 shows

Fig. 3. Microscopic images of EMG7 cells cultured on the cell-patterning substrate with 200- μ m-diameter cell-adhesive domains on day 0, 3, 6, and 9. EGFP fluorescence images are superimposed on phase contrast images. Scale bars represent 200 μ m. This figure is taken from Sasaki et al., 2009 with modification.

Fig. 4. Microscopic image of EMG7 cells cultured on the cell-patterning substrate with 1-mm-diameter cell-adhesive domains on day 13. EGFP fluorescence image is superimposed on phase contrast image. Scale bar represents 200 μm

Fig. 5. Microscopic images of EMG7 cells cultured on the cell-patterning substrate with 100- μm -diameter domains (a), 200- μm -diameter domains (b), 300- μm -diameter domains (c), and 400- μm -diameter domains (d), on day 10. EGFP fluorescence images are superimposed on phase contrast images. Scale bars represent 300 μm . This figure is taken from Sasaki et al., 2009 with modification.

the microscopic images of cultured EMG7 cells on day 10. In all cases, the formation of cell aggregate on the circular domains and EGFP expression (i.e., cardiac differentiation) were confirmed. However, when the diameter of circular domains was 100 μm , the aggregates were easily detached from the substrates by medium changes, so that a number of the aggregates were lost. We prepared single cell suspension from these samples, and analyzed the percentages of EGFP-positive cells by flow cytometry. The details of single cell preparation are described elsewhere (Sasaki et al., 2009). Figure 6 shows the result of flow cytometry. The percentage of EGFP-positive cells was maximal when the diameter was 100 μm and 200 μm , reaching approximately 1.5%. We consider the reason of this result as below. Because the aggregate formation occurs from the edge of circular domain, the aggregate formation on the center part of 300- and 400- μm -diameter domains might be insufficient at the initial stage of differentiation, which resulted in the lower efficiency of cardiac differentiation. When the diameter was 100 μm , a number of cell aggregates were detached, so that the yield of resultant differentiated cells was significantly reduced. From these results, we concluded that the optimal diameter of circular domains is 200 μm under this experimental condition.

As the next step of research, we are now investigating culture conditions to acquire ESC-derived cardiomyocytes more efficiently. We fabricated the cell-patterning substrates, on the

surface of which 200- μm -diameter cell-adhesive circular domains were arrayed triangularly at 100 μm intervals. Undifferentiated EMG7 cells were seeded onto this cell-patterning substrate and cultured for differentiation in the presence of 20% FBS and 0.5 mM L-ascorbic acid 2-phosphate for 7 days. The medium was changed everyday in this period. Consequently, cell aggregates were formed on the circular domains. Thereafter, the cells were cultured for further differentiation in the presence of 1% FBS and 0.5 mM L-ascorbic acid 2-phosphate for additional days. In this period, the medium was changed every other day. Figure 7 shows the microscopic image of differentiated EMG7 cells on day 14. We confirmed that over 80% of the cell aggregates were EGFP-positive. On day 15 the percentage of EGFP-positive cells was analyzed by flow cytometry (Fig. 8). The percentage reached over 5%, which is comparable to or rather higher than the reported values in the case of traditional hanging drop method.

Fig. 6. Percentages of EGFP-positive cells in the differentiated EMG7 cells cultured on the cell-patterning substrates on day 10. Error bars represent the SD of 5 independent experiments.

Fig. 7. Microscopic image of EMG7 cells cultured on the cell-patterning substrate with 200- μm -diameter cell-adhesive domains on day 14. EGFP fluorescence image is superimposed on phase contrast image. Scale bar represents 300 μm .

Fig. 8. Scatter diagram of differentiated EMG7 cells on day 15, obtained by flow cytometry

3.3 Future research in cell-patterning method

As described above, we have demonstrated that the cell-patterning method enables easy mass preparation of size-controlled mouse ESC aggregates and subsequent cardiac differentiation. As a next step of research, it is of course important to investigate more preferable culture conditions for efficient cardiac differentiation. However, the most important subject that should be done is to apply the cell-patterning method not only to mouse ESC differentiation but also to human ESC differentiation. As is well known, the difference between the properties of mouse ESCs and human ESCs is not so small. For example, undifferentiated human ESCs survive poorly when they are dissociated into single cells (Amit et al., 2000). Therefore, some kind of contrivance is needed, such as the use of Rho-associated kinase inhibitor (Watanabe et al., 2007), when human ESCs are seeded onto the cell-patterning substrates. It should be also investigated whether the cell-patterning method can be applied to the differentiation into the cells other than cardiomyocytes, such as neurons and hepatocytes. And finally, the cell-patterning substrates for ESC differentiation should be commercialized to promote the research of ESC-based regenerative therapies in the world.

4. Methods of cell transplantation

4.1 Cell Injection

For the establishment of cardiac regenerative therapy based on cell transplantation, the subject of how to transplant the cells is exceedingly important as well as how to create the cells for transplantation. The simplest method of cell transplantation is to inject cell suspension into the cardiac tissue of failing heart. A number of clinical studies and trials have been done by the cell injection method, and achieved some therapeutic gain (Mathur & Martin, 2004; Menasche et al., 2001; Wollert et al., 2004). However, it was reported that the engraftment of injected cells at the targeted region is very poor because of the outflow and death of the cells (Hofmann et al., 2005; Zhang et al., 2001). Moreover, it is pointed out that the injury of cardiac tissue due to the cell injection itself may disturb the intercellular electric connection and cause lethal arrhythmia. Therefore, if a large number of cells are injected into cardiac tissue to compensate the poor engraftment of cells, the probability of the occurrence of lethal arrhythmia must increase.

4.2 Tissue engineering

In order to improve the engraftment of transplanted cells, “tissue engineering” has been developed. Tissue engineering is a technology of creating three-dimensional tissues from cells *in vitro*. The engineered tissues generally consist of cells, signalling molecules such as growth factors, and scaffolds, which are the three major elements in tissue engineering (Langer & Vacanti, 1993). As the scaffolds of cells, biodegradable polymers such as collagen, gelatine, alginate, polylactic acid, and polyglycolic acid are generally used. After the transplantation of engineered tissues, these scaffolds are gradually degraded and finally replaced by native extracellular matrix. In the field of cardiovascular medicine, blood vessels and heart valves have been created by tissue engineering and clinically used (Poh et al., 2005; Shin'oka et al., 2001). The application of tissue engineering to the myocardial regeneration has been also investigated by a number of groups (Leor et al., 2000; Li et al., 1999; Radisic et al., 2004; Zimmermann et al., 2002). However, the engineered tissues with biodegradable scaffolds eventually form cell-sparse and fibrotic tissues, unlike the native cardiac tissues which are highly dense with cells for the close electrical connection and organized contraction. Additionally, inflammatory responses due to the degradation of scaffolds may result in the failure of engineered tissues (Mikos et al., 1998). Therefore, the engineered tissues without the scaffolds must be favorable for the regeneration of cell-dense tissues such as myocardium. Driven by such a necessity, we have developed a novel technology called “cell-sheet engineering” to create scaffold-free tissues. The summary of cell-sheet engineering and its application to cardiac regenerative therapies are described below.

4.3 Cell-sheet engineering

We developed temperature-responsive culture surfaces, on which the temperature-responsive polymer poly(N-isopropylacrylamide) is grafted at nanometer-level thickness (Okano et al., 1993). This surface is cell-adhesive at 37 °C, and becomes non-cell-adhesive below 32 °C. The cells cultured at 37 °C on this surface can proliferate and become confluent on the surface. By the decrease of temperature below 32 °C thereafter, the cells can be harvested noninvasively as an intact cell sheet. Now the culture dishes with the temperature-responsive surfaces are commercially available (UpCell™ Surface; Thermo Fisher Scientific). When the cultured cells are harvested from ordinary culture dishes by the treatment with an enzyme such as trypsin, extracellular matrix and membrane proteins on the surfaces of cells are disrupted, which certainly decreases the engraftment of these cells to the host tissue on transplantation (Fig. 9). On the other hand, when the cell sheet is harvested from the temperature-responsive surface by decreasing temperature, extracellular matrix and membrane proteins are retained on the surfaces of cells (Fig. 9). Accordingly, the cell sheet can engraft to the host tissue rapidly and stably on transplantation. It is also possible to create a thicker tissue by layering cell sheets *in vitro*.

Cell-sheet engineering enabled the creation of cell-dense tissues without noncellular scaffolds. As mentioned above, scaffold-free engineered tissues are favorable to be transplanted for myocardial regeneration. We have demonstrated the therapeutic effectiveness of the cell sheets created from cardiomyocytes (Miyagawa et al., 2005; Sekine et al., 2006), myoblasts (Hata et al., 2006; Kondoh et al., 2006; Memon et al., 2005), and cardiac progenitor cells (Matsuura et al., 2009). We also started the clinical study of myoblast-sheet transplantation for DCM patients. Although cell-sheet engineering is extremely useful to create scaffold-free tissues, the number of cell sheets which can be layered *in vitro* is limited to 3-4, because the deficiency of nutrient supply into the thick tissue causes necrosis. To

create

Fig. 9. Comparison of cell harvest between enzymatic digestion and decreasing temperature of temperature-responsive surface.

Fig. 10. Strategy for the establishment of cardiac regenerative therapy based on cell-sheet engineering, as the alternative to heart transplantation.

thicker tissues from cell sheets, vascularization in the engineered tissue to supply nutrients is necessary. We overcame this subject by utilizing the vascularization capacity of a living body. When the triple-layer cardiomyocyte sheet is transplanted into subcutaneous tissues, vascularization occurs within a day. Therefore the next triple-layer cardiomyocyte sheet can be transplanted on there without necrosis. By such a multistep transplantation, we succeeded to create 1-mm thick myocardium with a well organized microvascular network (Shimizu et al., 2006). We are now trying to simulate this *in vivo* situation *in vitro*. We also succeeded to create tubular pulsatile tissues by wrapping cardiomyocyte sheets around a blood vessel or tubular scaffold (Kubo et al., 2007; Sekine et al., 2006). We confirmed that the beating of thus prepared myocardial tube can contribute to blood pressure independently of intrinsic heartbeat. Our final goal is to create thick and powerfully contractile cardiac tissues from human ESCs or iPSCs, which can mechanically support the blood circulation of patient with heart failure. The schema of our strategy for the establishment of cardiac regenerative therapies as the alternative to heart transplantation is illustrated in Fig. 10.

5. References

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