



Cell Sheets for Cardiac Tissue Engineering

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Abstract

Recent studies have reported that the injection of isolated cells can improve cardiac function in models of myocardial infarction. However, the loss of transplanted cells from the target site due to local hypoxia and cell washout remains a major problem. To overcome these limitations, we have developed cell sheet-based tissue engineering that allows the generation of confluent cultured cells, stacked cell sheets, and three-dimensional (3D) cell-dense tissues. Cell sheet-based patches can improve the function of damaged hearts in animal models. Stacked cardiac cell sheets beat synchronously both *in vitro* and *in vivo* and have the characteristic structure of native heart tissue. Upscaling of this technology through multistep transplantation of triple-layered cell sheets allowed the construction of functional cardiac tissue about 1 mm thick. Furthermore, we

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succeeded in bioengineering 3D cardiac tissue containing a vascular network by *in vitro* perfusion culture of cell sheets stacked sequentially on a vascular bed obtained from resected tissue. Since the vascular bed was excised with its artery and vein intact, the bioengineered tissue could be transplanted by anastomosis of its vessels with those of the host animal. Following the creation of cardiac patches for direct implantation onto a damaged heart, the next challenge will be to engineer organs with tubular or spherical structures that can function as pumps to provide circulatory support. The goal for the future is to develop this technology to create functional organ-like tissues with vascular networks that can be used in patients as an alternative to conventional organ transplantation.

1 Introduction

Regenerative medicine is receiving great attention as a potential new therapy for illnesses that cannot be completely cured by pharmacological or surgical methods. Regenerative medicine also is a promising technique for creating transplantable tissues that could substitute for conventional organ transplants in the future. To date, the clinical application of regenerative medicine has mainly involved the injection of autologous or allogeneic cell suspensions in order to regenerate defective tissue. However, ongoing research is further developing bioengineering techniques with the aim of creating transplantable tissues using biodegradable scaffolds made from synthetic or natural polymers. Tissue engineering technology has already been applied clinically to tissues with a low cell density and low vascular requirement such as bone, cartilage, and skin.

Conventional tissue engineering techniques are limited with regard to the thickness and function of the tissue that can be constructed because they rely on diffusion for the supply of oxygen and nutrients and the removal of waste products. The construction of complex and cell-dense tissues such as the heart, liver, and kidney will require innovative technologies to realize a functional vascular network within the engineered tissue.

In this chapter, we introduce three related technologies that have the potential to be developed into novel regenerative therapies: cardiac cell sheet-based tissue engineering for myocardial regeneration, creation of three-dimensional tissue with a functional vascular network, and fabrication of a bioartificial pump for circulatory support.

2 Myocardial Regenerative Therapy

Research on myocardial cell transplantation was initiated by Soonpaa et al. in the early 1990s, who demonstrated that mouse fetal cardiomyocytes successfully engrafted onto host myocardium after transplantation (Soonpaa et al. 1994). Subsequently it was reported that the transplantation of suspensions of various cell

types, including cardiomyocytes, was able to help restore cardiac function (Laflamme and Murry 2005). Skeletal muscle myoblasts are considered relatively resistant to ischemia and have been used in place of cardiac cells for myocardial regeneration. The MAGIC II trial in 2003 reported that cardiac function was improved by the injection of myoblasts collected from the patient's own skeletal muscle in combination with coronary artery bypass surgery (Menasche et al. 2003, 2008). However, because there were some cases of arrhythmia and death, the combined use of antiarrhythmic drugs and implantable defibrillators was considered essential when this technique was utilized.

In recent years, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have been actively studied as a source of cells with a high ability to proliferate and differentiate into cardiomyocytes. A method for inducing the differentiation of human stem cells into cardiomyocytes has also been established. Differentiated cardiomyocytes have been shown to engraft onto a host heart and recover cardiac function in animal models of heart failure (Caspi et al. 2007; Nelson et al. 2009; Shiba et al. 2016). Since iPS cells circumvent the moral and ethical issues associated with the use of ES cells, including the destruction of human embryos, it is anticipated that allogeneic transplantation based on cell banks will be realized in the future (Wilmot et al. 2015).

3 Scaffold-Based Engineering of Cardiac Tissue

The concept of tissue engineering was first proposed by Prof. Robert Langer at the Massachusetts Institute of Technology and Prof. Joseph Vacanti at Harvard University in the late 1980s. Tissue engineering is an interdisciplinary study born from the fusion of medicine and engineering that aims to reproduce the tissue structures of the body *in vivo* or *in vitro*. The generation of tissue requires an extracellular matrix (ECM) that acts a scaffold for the cells and the growth factors that promote cell differentiation and proliferation. In this method, cells are seeded on a three-dimensional biodegradable scaffold, cultured and then transplanted into the body. The scaffold is typically a biodegradable material composed of polyglycolic acid (PGA) or poly-L-lactic acid (PLLA) and its copolymer. Since the scaffold is slowly degraded and absorbed in the body and replaced with ECM produced by the cells, tissue structures similar to native tissues can be regenerated (Langer and Vacanti 1993). A major advantage of tissue engineering techniques is the ability to overcome the loss of cells due to the washout and necrosis that inevitably occur with cell infusion-based therapy. In addition, tissue engineering potentially allows the treatment of defective structures, such as those associated with congenital heart disease, which cannot be corrected by cell infusion- or cytokine-based therapy (Zandonella 2003). Gelatin, alginate, and PGA porous sponges have all been used as a scaffold for cell seeding (Fig. 1a) (Bursac et al. 1999; Li et al. 1999; Leor et al. 2000). In addition, three-dimensional myocardial tissue has been constructed by mixing a collagen solution with cardiac cells and culturing in a mold (Fig. 1b) (Zimmermann et al. 2006). Moreover, the imposition of a stretching load *in vitro* was

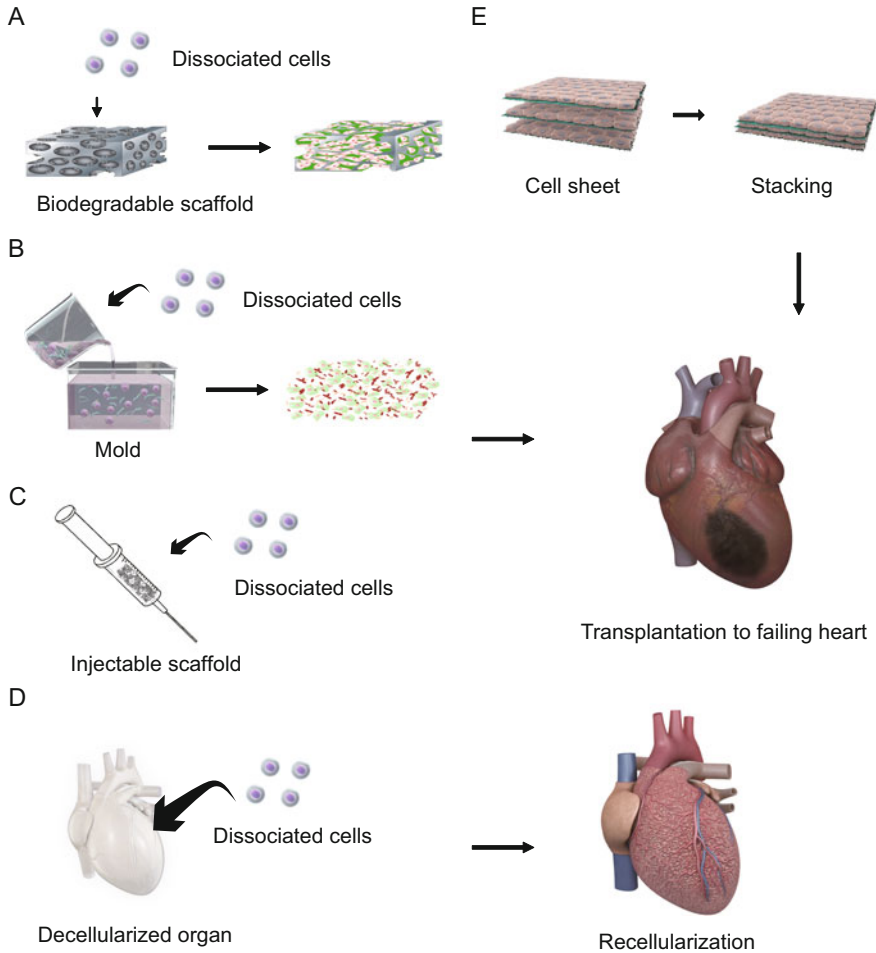


Fig. 1 Various type of tissue engineering approach for myocardial tissue reconstruction

shown to impart orientation to the myocardial tissue and enlarge the cardiomyocytes (Zimmermann et al. 2006). An intermediate approach between cell injection-based therapy and tissue engineering, which reduced the problem of cell loss, was also reported in which cells were mixed with fibrin glue or a collagen-containing solution and then injected into failing myocardium (Fig. 1c) (Christman and Lee 2006).

Various studies have investigated the transplantation of bioengineered cardiac tissue in animal models of heart failure. Li et al. created a graft by seeding rat fetal cardiac cells on a degradable gelatin mesh, and transplantation of this graft onto scar tissue in a cryoinjured rat heart resulted in an improvement in left ventricular systolic pressure when compared with the transplantation of gelatin mesh without cardiac cells (Li et al. 1999). Leor et al. seeded rat fetal cardiac cells onto a porous alginate scaffold and transplanted the resulting construct onto myocardial scar tissue in rats

with experimental myocardial infarction. Although there was no improvement in left ventricular contractility, transplantation was found to suppress left ventricular dilatation due to myocardial remodeling (Leor et al. 2000). Zimmermann et al. generated three-dimensional myocardial tissue by mixing rat neonatal cardiomyocytes with collagen solution and culturing in a silicone mold. After transplantation into rats with experimentally induced myocardial infarction, the engineered heart tissue was observed to couple electrically with the host heart, improve left ventricular contractility, and inhibit left ventricular dilatation (Zimmermann et al. 2006).

4 Cell Sheet-Based Tissue Engineering for the Generation of Cardiac Tissue

One of the limitations of using a scaffold as a cellular foothold during tissue construction is that it can be difficult to seed a sufficient number of cells within the scaffold, resulting in a construct with a small cellular component and a large amount of connective tissue. Although scaffold-based techniques are suitable for the production of tissues that are sparsely populated by cells, such as heart valves and cartilage, alternative methods are needed to create cell-dense tissues with complex structures and functions such as the heart, kidney, and liver. Therefore, we developed an original technology known as cell sheet-based tissue engineering that permits the generation of tissue without the use of a scaffold. Cell sheet-based tissue engineering relies on the controlled use of temperature changes to regulate the attachment and detachment of cells from the surface of a culture dish. Poly(*N*-isopropylacrylamide) (PIPAAm) is a thermoresponsive polymer with a critical solution temperature of 32 °C in water. When this polymer is immobilized covalently by an electron beam, it becomes hydrophobic at 37 °C, which enables cells to adhere to it. However, the polymer surface becomes hydrophilic when the temperature is lowered to 32 °C, resulting in the detachment of cells from it (Yamada et al. 1990; Okano et al. 1993). Conventionally, a protease such as trypsin is used to harvest cultured cells, but this method degrades not only the proteins that adhere the cells to the surface of the culture dish but also other proteins on the cell membrane. Thus, a major advantage of using a temperature-responsive culture dish is that a simple lowering of the temperature allows cells to be recovered as a sheet without any disruption of cell-cell adhesion or the structure and function of the ECM (Fig. 2) (Kushida et al. 1999). Furthermore, three-dimensional tissues can be constructed by laminating multiple cell sheets (Fig. 1d). Since a tissue constructed by lamination consists only of cells and the small amount of ECM that they produce, this technique avoids the problems associated with the use of scaffolds (Yang et al. 2005).

When two neonatal rat cardiac cell sheets prepared using a temperature-responsive culture dish were stacked together *in vitro*, morphological and electrical connections were established between the pair of cardiac cell sheets within several tens of minutes. Furthermore, synchronously beating myocardial tissue was created successfully by the lamination of cardiac cell sheets (Shimizu et al. 2002; Haraguchi et al. 2006). When layered cardiac cell sheets were transplanted onto the dorsal

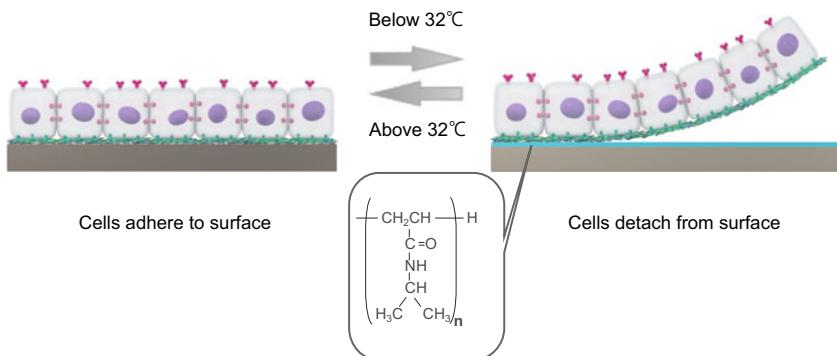


Fig. 2 Cell sheet technology using temperature-responsive culture dish

subcutaneous tissue of a rat, the transplanted myocardial graft exhibited pulsations visible to the naked eye and generated unique electrical potentials that were detected by electrography. Notably, the transplanted cardiac tissue was observed to contain newly formed capillary networks and structures characteristic of native myocardial tissue such as sarcomeres, gap junctions, and desmosomes (Shimizu et al. 2002). It was also shown that this transplanted myocardial tissue continued to engraft for a further 2 years while maintaining its spontaneous beating (Shimizu et al. 2006a).

5 Transplantation of Cardiac Patches onto Ischemic Hearts

When engineered myocardial tissue is transplanted onto an ischemic heart, it is essential that the graft connects electrically and synchronizes with the host heart to enable it to support cardiac function. To evaluate whether our bioengineered construct would possess this ability, we transplanted a stratified cardiac cell sheet onto the heart of an animal with ectopically produced myocardial infarction and evaluated whether electrical connections were formed between the host heart and graft. Morphological analysis demonstrated cell-cell junctions between the cardiomyocytes of the graft and those of the host non-infarcted tissue 1 week after graft transplantation. Immunohistochemistry experiments and transmission electron microscopy (TEM) confirmed the expression of connexin-43 and the presence of intervening plate between host cells and graft cells. In addition, it was shown that a low molecular weight fluorescent dye was able to migrate between connected cells. Immunohistochemistry also revealed the dedifferentiation of epicardial mesothelial cells that were sandwiched between the host and graft. Based on the above results, it was concluded that the transplantation of a multilayered cardiac cell sheet onto the ischemic region of a host heart resulted in the loss of mesothelial cell function and the formation of gap junctions between the cells of the graft and those of the non-infarcted myocardial tissue. This raised the possibility that the function of an

ischemic heart could be improved by a graft beating synchronously with normal myocardium (Sekine et al. 2006a).

The implantation of various cell sheets has been shown to restore cardiac function in small animal models of severe heart failure. The main mechanisms underlying the improvement in cardiac function are thought to be the promotion of angiogenesis by various cytokines secreted from the transplanted cell sheets as well as the suppression of fibrosis, apoptosis, and left ventricular remodeling (Memon et al. 2005; Miyagawa et al. 2005, 2010; Hata et al. 2006; Kondoh et al. 2006; Miyahara et al. 2006). Nevertheless, echocardiography has demonstrated a reduction in left ventricular end systolic dimension and an increase in fractional shortening ratio after the transplantation of cardiac cell sheets, implying that the improvement in host heart function by cardiac cell sheets involves not only the effects of cytokines but also an enhancement of cardiomyocyte contractility (Miyagawa et al. 2005; Sekine et al. 2011). This latter effect is thought to be related to the formation of gap junctions between graft and host cells and the synchronization of contractile function between the transplanted cardiac cell sheet and host myocardium (Sekine et al. 2006a). When we evaluated our cardiac cell sheet in an animal model of cardiac injury, the survival of transplanted cells (evaluated by *in vivo* luminescence imaging and immunohistochemistry) and the improvement in host cardiac function (assessed by echocardiography) were significantly greater following the transplantation of cardiac cell sheets than after the injection of a cell suspension (Sekine et al. 2011). This suggests that the enhancement of cardiac performance is dependent on the survival of the transplanted cells. Treatments based on the injection of cell suspensions are limited by the loss of cells that occurs due to necrosis and outflow from the transplantation site. Zhang et al. utilized quantitative TUNEL analysis to determine the rate of necrosis and showed that most cardiomyocytes become necrotic several days after their transplantation as a cell suspension (Zhang et al. 2001). Studies have also examined the technical issues involved in the outflow of cells from the transplantation site. Terrovitis et al. found that only 17% of cardiac-derived stem cells were retained 1 h after their transplantation into a normal rat heart, but the engraftment rate was improved if the host heart was temporarily stopped while the cells were injected (Terrovitis et al. 2009). Hudson et al. performed intramyocardial injections of fluorescent microspheres rather than cells in a porcine cardiopulmonary bypass model and determined the retention rate to be only 10% irrespective of whether the heart was temporarily arrested (Hudson et al. 2007). The above findings indicate that cell transplantation by injection is associated with a low retention rate. Hofmann et al. carried out transcatheter grafting of a suspension of bone marrow-derived cells, and analysis by positron emission tomography revealed that only 1–3% of cells engrafted onto the heart, with many cells flowing to the liver and pancreas (Hofmann et al. 2005). Subsequently, Kutschka et al. demonstrated that the efflux of cells from the transplantation site was lower for cells mixed with collagen than for a cell suspension (Kutschka et al. 2007). This showed that it might be possible to reduce cell loss following the transplantation of a cell suspension. Nonetheless, cell survival is better for transplanted cell sheets than for injected cell suspensions

(Sekine et al. 2011), suggesting that the use of cell sheets can maximize the potential of transplanted cells.

The layering of cardiac cell sheets generates three-dimensional myocardial tissue with a high density of cells because the tissue consists only of cells and the ECM that they produce. An additional advantage of this construct is that it can be readily engrafted onto host tissue because ECM is present on the lower aspect of the cell sheet. For adherent cells like cardiomyocytes, adhesion to the ECM is an essential function for survival and proliferation. Consistent with this, many TUNEL-positive apoptotic cells are detected 24 h after the transplantation of a cell suspension. Apoptosis caused by defective cell adhesion is referred to as anoikis (Michel 2003). Adherent cells bind to a certain substrate via integrins, but when the adhesion is broken, the cells become suspended and initiate a signal to induce anoikis. The apoptotic signal is transmitted to the nucleus through several kinases (intracellular signaling molecules) such as focal adhesion kinase (FAK). Cells transplanted as a suspension are usually harvested from a culture dish using a protease to degrade proteins on the surface of the ECM or cell membrane. Therefore, the cells in a suspension are in a state in which their adhesion to the ECM has been disrupted. By contrast, a cell sheet used for transplantation is collected from a temperature-responsive culture dish as an intact sheet without any disruption of cell adhesion to the ECM. Since anoikis would be induced more easily in cell suspensions than in cell sheets, this would explain why significantly more TUNEL-positive apoptotic cells are evident in transplanted cell suspensions than in transplanted cell sheets.

Previous studies have demonstrated that cardiac cell sheets co-cultured with endothelial cells form a network of vascular endothelial cells that promote angiogenesis after transplantation (Sekiya et al. 2006). Twenty-four hours after transplantation onto an ischemic heart, cell sheets co-cultured with endothelial cells exhibit mature blood vessels surrounded by pericytes and luminalized endothelial cells, whereas cell suspensions show no evidence of mature blood vessels (Sekine et al. 2011). It is thought that the presence of a network of endothelial cells within the cell sheet facilitates the early maturation of blood vessels after transplantation. Furthermore, transplanted cell sheets show a dense arrangement of capillaries, whereas transplanted cell suspensions contain only a small number of sparsely distributed microvessels. Interestingly, new blood vessels in the graft are remodeled from both graft- and host-derived endothelial cells, and the graft-derived endothelial cells migrate to ischemic regions in the host myocardium to contribute to the development of blood vessel walls. The above results show that the inclusion of vascular cells within a cell sheet before transplantation can help to engineer tissue with a strong ability to promote angiogenesis and improve cardiac function (Sekine et al. 2008).

Based on these findings, in 2007 we set up a collaborative clinical research project with the Department of Cardiovascular and Respiratory Surgery at Osaka University to evaluate whether the transplantation of autologous skeletal myoblast sheets could be used as a therapy for severe dilated cardiomyopathy. Patients who had been fitted with a left ventricular assist device prior to treatment showed sufficient improvement in cardiac function 3 months after cell sheet transplantation

to allow removal of the assist device and discharge from hospital (Sawa et al. 2012). In view of the promising results obtained in the above study, Terumo Co., Ltd. initiated a clinical trial in 2012 to evaluate the potential use of cell sheet transplantation in the treatment of ischemic heart disease, and regenerative therapy has been carried out in more than 35 cases to date.

6 Effects of Cell Sheet Transplantation in Infant Ischemic Hearts

Cell sheet-based therapy for severe heart failure in adults has achieved a certain degree of success in clinical trials. However, there have been no reports regarding the effects of cell sheet-based treatment on myocardial damage in infants. Therefore, we utilized a rat model of myocardial infarction to compare the therapeutic effects of cell sheet transplantation between infant and adult animals (Homma et al. 2017). Briefly, experimental myocardial infarction was induced in infant rats (2 weeks old) and adult rats (12 weeks old), triple-layered rat myoblast sheets were transplanted 1 week later, and the response to therapy was evaluated 2 weeks after transplantation. Cardiac catheterization studies demonstrated better improvement of cardiac function after cell sheet transplantation in infant rats than in adult rats. Moreover, histological evaluation showed that the hearts of infant rats exhibited greater wall thickness, less fibrosis, a higher number of dividing cardiomyocytes, and more neovascularization of the infarcted region than those of adult rats. The above results indicate that infant hearts have a greater regenerative ability than adult hearts and that this regenerative ability is stimulated by the implantation of cell sheets. It is thought that cardiomyocyte mitogenesis is one of the mechanisms contributing to the effects of cell sheet transplantation on infarcted hearts in infants, whereas this has not been reported in adult hearts. This implies that myocardial regeneration therapy might be particularly effective during infancy.

7 Vascularization of Engineered Myocardial Tissue for Scaling-Up of Tissue Size

Tissues constructed by conventional bioengineering techniques lack functional vascular networks, which results in oxygen and nutrient deficiency and accumulation of waste products. The development of technologies to generate a blood vessel network within a bioengineered construct has become an important focus of tissue engineering research, and various approaches have been attempted. A widely used method is to administer an angiogenesis-promoting growth factor either at the time of transplantation or more gradually by elution from a scaffold (Richardson et al. 2001). Although this technique can promote angiogenesis at the initial stage of transplantation, it generally does not sustain angiogenesis for a sufficiently long period of time to prevent necrosis of the inner region of the transplanted tissue due to ischemia. As a result, such use of angiogenesis-promoting growth factors is

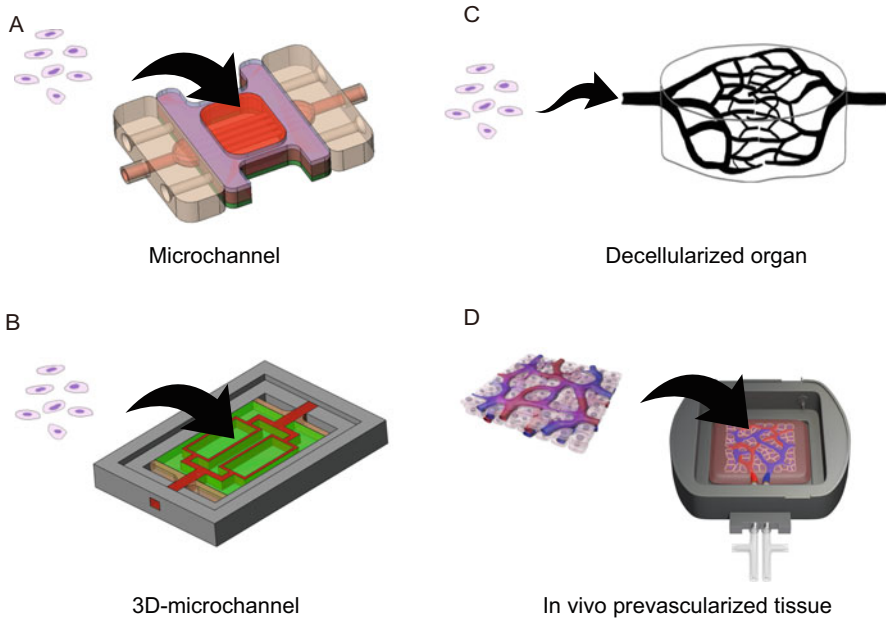


Fig. 3 Bioreactor system for application of vascular network fabrication

not suitable for the generation of thick tissues. Co-culturing with a cell type that is a constituent of blood vessels has received increasing attention in recent years as a method to promote the formation of a vascular network. As described above, we have shown that an endothelial cell network can be generated within a cell sheet containing co-cultured vascular endothelial cells and that this speeds up the development of a vascular network after transplantation (Sekiya et al. 2006). Although the co-cultured vascular cells directly contribute to blood vessel formation in the tissue after transplantation, they do not form continuous structures with lumens before transplantation. As a result, the thickness of the bioengineered three-dimensional tissue is still initially limited by ischemia after transplantation. Several methods have been advocated to overcome this limitation. We have developed a bioreactor system that initiates the formation of capillaries through the artificial construction of blood vessel-like structures in vitro that are perfused via their lumens. This technique involves the advance preparation of minute flow channels within a scaffold using microfabrication technology, which enables culture medium to be perfused through these channels during tissue culture (Fig. 3a) (Chouinard et al. 2009). An alternative approach utilizes a scaffold that imitates the three-dimensional structure of the microcirculation (“angiochip”), which is seeded with vascular endothelial cells from the luminal side to enable the construction of a vascular network during perfusion culture (Fig. 3b) (Zhang et al. 2016). Other studies have attempted to create vascularized tissues by decellularizing a living tissue or organ, seeding vascular endothelial cells within the original blood vessels, and performing perfusion culture (Fig. 3c) (Ott et al. 2008; Song et al. 2013; Ren et al. 2015).

An important aim of our research into regenerative therapy is to upscale the stacking of cell sheets to generate thick tissues. In order to achieve high-performance, the bioengineered tissue needs to be provided with a vascular network to improve the delivery of oxygen and nutrients and the removal of waste products. As a way of realizing this aim, we have devised a procedure that allows cardiac cell sheets to be sequentially transplanted every 24 h, which provides enough time for sufficient angiogenesis to occur from the host side of the layered cardiac cell sheets. Using this method, it was possible to create myocardial tissue with a thickness of about 1 mm that generated contractile force *in vivo* (Shimizu et al. 2006a). Following the success of the above experiments, we then tried to generate thick myocardial tissue by adding a vascular network to the construct *in vitro* (Fig. 4) (Sekine et al. 2013). First, we developed a vascular bed that would promote vascularization of the bioengineered tissue and a bioreactor for tissue perfusion, and we evaluated the formation of capillaries within cardiac cell sheets that were stacked on the vascular bed (Fig. 3d). The vascular bed was made from rat femoral muscle: the femoral muscle tissue was partially resected, reset to the original position, and then incubated in the host body for 1 week before complete resection with the femoral artery and vein. Cell sheets comprising cardiomyocytes co-cultured with endothelial cells were

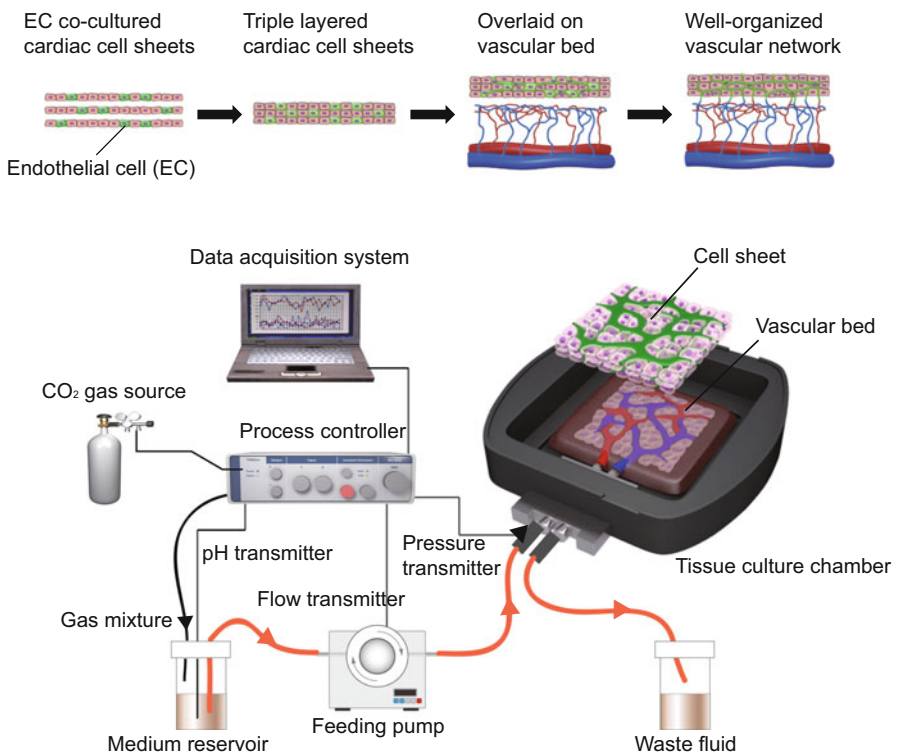


Fig. 4 Tissue perfusion using a bioreactor and stacking of cell sheets on a vascular bed

layered on the vascular bed, and tissue perfusion culture was performed using a solution supplemented with basic fibroblast growth factor (b-FGF), with inflow via the vascular bed artery and outflow via the vein. Connection of capillaries within the myocardial tissue to blood vessels of the vascular bed was confirmed after 3 days of perfusion culture, and the sequential stacking of triple-layered cell sheets on four occasions enabled the construction of myocardial tissue with a thickness of about 200 μm . Furthermore, when vascularized myocardial tissue prepared by the stepwise layering of cell sheets was transplanted into a rat and its vessels anastomosed to the cervical artery and vein of the host, the transplanted tissue was shown to engraft and retain its cellular function 2 weeks after transplantation.

In another study, cell sheets composed of cardiomyocytes co-cultured with endothelial cells were perfused via an artificial vascular bed consisting of a collagen gel containing microchannels. It was found that capillaries were constructed by the proliferation and migration of vascular endothelial cells within the microchannels and cell sheets (Sakaguchi et al. 2013).

8 Organ-Like Tissue Fabrication

Heart transplantation remains the best treatment for patients with severe cardiac failure due to ischemic heart disease. However, the shortage of donor organs worldwide substantially limits the number of heart transplants that can be performed. In addition, artificial cardiac systems such as temporary mechanical circulatory support or left ventricular assist devices have specific problems associated with thromboembolism, infection, gastrointestinal bleeding, and limited endurance. Therefore, regenerative therapy is being pursued as an alternative approach that offers new possibilities for the repair of damaged myocardium. In myocardial tissue engineering, the ultimate goal is the creation of a functional myocardial compartment that can generate significant independent pressure from its own spontaneous contraction. Following on from the creation of cardiac patches for direct implantation into a failing heart, the next challenge is to design organ-like tissues such as tubular or spherical structures that can function as pumps. In the following section, we introduce the bioengineering of organ-like tissues that can function as cardiac pumps and potentially provide circulatory support.

9 Scaffold-Based Cardiac Pumps

Several research groups have attempted to create organ-like structures using cultured cardiomyocytes. Evans et al. developed tubular heart tissue using rat embryonic heart cells containing type I collagen. A tube of aligned collagen fibers was formed using two counter-rotating cones and a polymerization chamber. Tube-cultured cardiomyocytes exhibited a level of differentiation that was higher than that of planar cultured cells and similar to that of neonatal ventricular myocytes *in vivo* (Evans et al. 2003). Yost et al. created a three-dimensional tubular collagen scaffold

with a varying fibril angle from the inside to the outside and seeded it with neonatal rat cardiac cells on both the luminal and outer surfaces at weekly intervals (Yost et al. 2004; Franchini et al. 2007). Mechanical studies demonstrated that stiffness and viscosity were significantly increased in collagen tubes seeded with cardiac cells (Yost et al. 2004; Franchini et al. 2007). Birla et al. used a biodegradable hydrogel to produce a cell-based tubular cardiac structure that was capable of generating pressure. The constructs were made by culturing neonatal rat heart cells in a fibrin gel and then wrapping this around a silicon tube. The tubular structure was composed of layers of aligned cells and exhibited contractile function that generated an internal pressure of approximately 0.08 mmHg (Birla et al. 2008). Lee et al. described a cardiac organoid chamber prepared by mixing neonatal rat heart cells with a mixture of type I collagen and matrigel. The chamber construct exhibited structural and mechanical properties similar to those of a cardiac ventricle and generated an internal pressure of about 2 mmH₂O. The cardiac organoid also showed local variations in contractile function following cryoinjury, suggesting that it could be used as an *in vitro* model of myocardial infarction (Lee et al. 2008). Yildirim et al. developed bag-like heart tissue by mixing neonatal rat heart cells with type I collagen and matrigel in a globular mold. The sac-shaped heart tissue had structural and contractile properties comparable to those of natural myocardium. Two weeks after transplantation, an artificial graft covering the entire surface of the heart was shown to be in functional communication with the host myocardium (Yildirim et al. 2007). Ott et al. attempted to create a working heart through a process of decellularization followed by recellularization. First, detergent was perfused through the coronary arteries of a rat heart in order to decellularize it while preserving its extracellular matrix and vascular architecture. Then, this construct was recellularized with neonatal rat cardiac cells or rat aortic endothelial cells. The resulting bioengineered heart was capable of contracting and generating a left ventricular pressure of up to 2.4 mmHg after 8 days of culture. This use of such an approach could potentially solve the challenge of providing a blood supply to engrafted cells by maintaining the natural structure of the heart and inducing the formation of blood vessels (Ott et al. 2008).

10 Cell Sheet-Based Cardiac Pump

As an alternative to scaffold-based heart pumps, we have utilized a temperature-responsive culture dish to create beating cardiac tubes *in vitro* that could be used to provide circulatory support (Fig. 5). Three neonatal rat cardiac cell sheets were harvested from temperature-responsive culture surfaces, layered and then wrapped around a fibrin tube. The cardiac tube created *in vitro* exhibited spontaneous and synchronized pulsations at the macroscopic level and generated a measurable change in internal pressure in response to spontaneous contraction, with a mean internal pressure gradient of 0.11 mmHg. We also confirmed an inotropic effect of increased elevated extracellular Ca²⁺ concentration in the cardiac tube (Kubo et al. 2007).

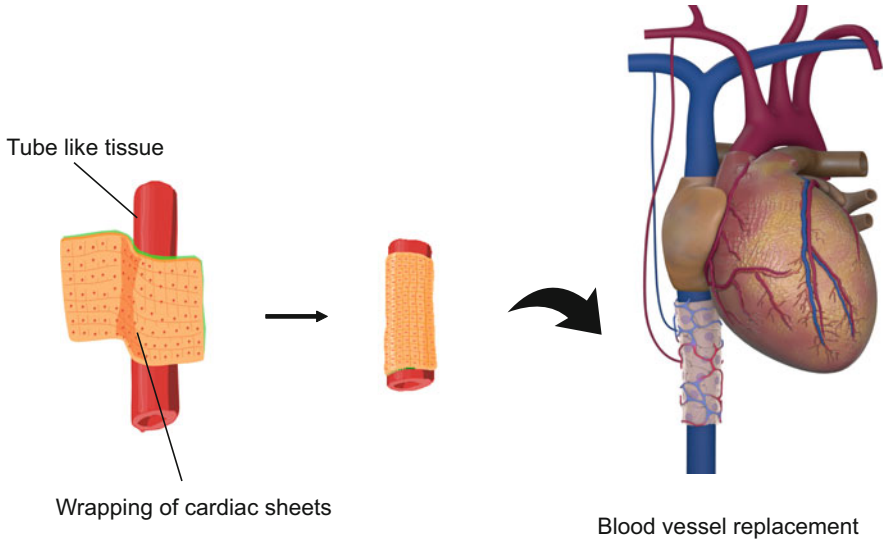


Fig. 5 Cell sheet-based myocardial tube for circulatory support

Subsequently, we created an implantable tubular myocardial structure by wrapping a neonatal rat cardiac cell sheet around a segment of thoracic aorta resected from a rat (Sekine et al. 2006a). Four weeks after transplantation of this construct into the abdominal aorta of an athymic rat, the artificial myocardial tube exhibited spontaneous and synchronous pulsations. The spontaneous contractile activity was confirmed by electrography to be independent of the host's heartbeat and generated a pressure within the graft of 5.9 mmHg. Histological examination and TEM showed that the myocardial tube was composed of tissue resembling that of the normal heart. The artificial tube was densely populated with stratified cells that stained positively for troponin T, indicating that they were cardiac cells. In addition, diffuse localization of connexin-43 throughout the graft tissue suggested that gap junctions had formed between the cells. TEM identified well-differentiated myocardial tissue with numerous mitochondria as well as myofilaments with sarcomeres. TEM also confirmed that functional microvessels containing red blood cells were present throughout the graft. When compared with a graft that was transplanted into the abdominal cavity of an animal, the myocardial tube used for aortic replacement contained significantly thicker tissue and higher expression levels of brain natriuretic peptide, alpha-myosin heavy chain, and beta-myosin heavy chain. These findings suggest that pulsatile blood flow through the lumen of the myocardial tube had stimulated the growth and hypertrophy of its cardiomyocytes. Previous studies have demonstrated that mechanical stress has the ability to induce myocardial hypertrophy during development and under pathological conditions. This phenomenon has been exploited in myocardial tissue engineering through the use of mechanical stretching to promote cardiomyocyte hypertrophy. Therefore, the application of mechanical loading, either *in vitro* or *in vivo*, appears to be an essential element in the generation of functional cardiac tissue.

We have also established that human tubular cardiac structures can improve hemodynamics after implantation in the rat inferior vena cava (Seta et al. 2017). Specifically, a triple-layered human cardiac cell sheet was wound around the rat inferior vena cava to form a tubular cardiac structure. Ultrasonography 4 weeks after transplantation demonstrated that the fabricated tubular cardiac structure was beating. Furthermore, catheterization and measurement of internal pressure revealed that the tubular myocardium generated a pulse pressure of 6.3 ± 1.6 mmHg at 4 weeks and 9.1 ± 3.2 mmHg at 8 weeks after transplantation. In addition, histological analysis confirmed that the transplanted human cardiac cell sheet had developed mature myofibrils and a rich vascular network. The creation of this cardiac tube represents the next step in myocardial tissue construction and a transition stage toward the production of an independently functioning structure with the potential to act as a bioengineered cardiac assist device.

11 Future Perspectives

The next exciting challenge in the field of regenerative therapy will be the design of organ-like tissues. Although the creation of organ-like tissues has only been implemented on a small scale, future solutions to current problems concerning cell sources and upscaling will facilitate the development of tissue-engineered cardiac assist devices or even replacement organs. The major obstacle in myocardial tissue engineering remains the poor delivery of oxygen to a three-dimensional structure, which limits the thickness of a construct to about 100 μm . Therefore, the production of thicker and more functional cardiac pumps will require new technologies to control blood vessel growth. Future attempts to promote vascular network growth and formation, using techniques such as growth factor administration, gene transfer, and co-culture with vascular progenitor cells, may contribute to the production of thicker tissues. Overcoming the limitations of passive diffusion should make it possible to create a powerful cardiac pump.

One possible technique for improving the contractile force of artificial cardiac pumps is to ensure the correct orientation of cardiomyocytes. Therefore, controlling cell orientation is also considered to be an essential element in the generation of a tissue-engineered pump with improved cardiac-like properties.

12 Conclusions

This chapter has described the advances made in regenerative medicine using cell sheets and outlined further possibilities. Regenerative medicine is expected to become a new therapeutic option for refractory diseases that are currently difficult to treat, and various approaches have been adopted in the development of potential therapeutic solutions. Cell sheets created using temperature-responsive culture dishes have several advantages over other currently available methods, including more efficient transplantation, the construction of tissues that are impossible to make with preexisting

technology, and realization of cell stratification. In addition, cell sheet-based therapies have achieved highly promising results in human clinical trials.

To construct tissues and organs that can regulate the blood circulatory system, it will be necessary to overcome numerous challenges such as the development of cardiomyocytes that can be transplanted into humans and the upscaling of constructed tissues. It is anticipated that bioengineered cardiac structures will become an important treatment for diseases that cause severe heart failure, including congenital heart disease in children. We believe that this can be achieved through future research efforts and an interdisciplinary approach to technological development.

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