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Cell sheet engineering for heart tissue repair ☆

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Abstract

Recently, myocardial tissue engineering has emerged as one of the most promising therapies for patients suffering from severe heart failure. Nevertheless, conventional methods in tissue engineering involving the seeding of cells into biodegradable scaffolds have intrinsic shortcomings, such as inflammatory reactions and fibrous tissue formation caused by scaffold degradation. On the other hand, we have developed cell sheet engineering as scaffoldless tissue engineering, and applied it for myocardial tissue engineering. Using temperature-responsive culture surfaces, cells can be harvested as intact sheets and cell-dense thick tissues are constructed by layering these cell sheets. Myocardial cell sheets non-invasively harvested from temperature-responsive culture surfaces are successfully layered, resulting in electrically communicative 3-dimensional (3-D) cardiac constructs. Transplantation of cell sheets onto damaged hearts improved heart function in several animal models. In this review, we summarize the development of myocardial tissue engineering using cell sheets harvested from temperature-responsive culture surfaces and discuss about future views.

Keywords: Regenerative medicine; Tissue engineering; Temperature-responsive cell culture surface; Myocardial tissue engineering

Contents

1. Introduction

Recently, cell-based therapies have emerged as alternative treatments to cardiac transplantation for the repair of damaged heart tissue, since the benefits of heart transplantation are restricted by donor shortages [1]. Although cell suspension transplantation is one of the promising treatments for impaired heart tissue, it is often difficult to control shape, size and location of the

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injected cells. Additionally, isolated cell transplantation may not be applicable in treating myocardial tissue defects. Therefore, research on methods of transplanting tissue-engineered functional heart grafts has been established over the past decade.

Tissue engineering has been developed as a basic technology for regenerative medicine. The popular components used in tissue engineering approaches have generally included isolated cells or cell substitutes, appropriate signaling molecules such as cytokines or growth factors, and extracellular matrix (ECM) proteins [2]. As alternatives for the extracellular matrix, 3-dimensional (3-D) biodegradable scaffolds have been used for the reconstruction of various tissues and organs including cartilage, bone, skin, blood vessels and heart valves.

By using the methods based on biodegradable polymers, the spaces occupied by the biodegradable polymers often become filled with large amounts of deposited ECM, with the number of cell-to-cell connections becoming reduced in the resultant tissues. In addition, scaffold biodegradation can result in inflammatory responses and pathological fibrotic states. To overcome these problems, we have developed a novel tissue engineering methodology termed "cell sheet engineering", that constructs 3-D functional tissues by layering two-dimensional (2-D) confluent cell sheets without the use of any biodegradable ECM alternatives.

2. Cell sheet harvest using temperature-responsive culture dishes

Cell sheets are obtained by using specialized cell culture surfaces that are covalently grafted with the temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PIPAAm) [3,4]. The surfaces are slightly hydrophobic and cells adhere and proliferate under normal culture conditions at 37 °C. By lowering the temperature below 32 °C, the surfaces become highly hydrophilic and therefore non-adhesive to cells due to rapid hydration and swelling of the grafted PIPAAm. This unique surface change allows cultured cells to detach spontaneously from these grafted surfaces simply by reducing the culture temperature (Fig. 1). When cells are confluent in culture, they connect to each other via cell-to-cell junction proteins and deposited ECM. Typical cell harvests using enzymatic digestion therefore result in the disruption of both adhesive proteins and membrane receptors. With the temperature-responsive culture surfaces, cells can be non-invasively

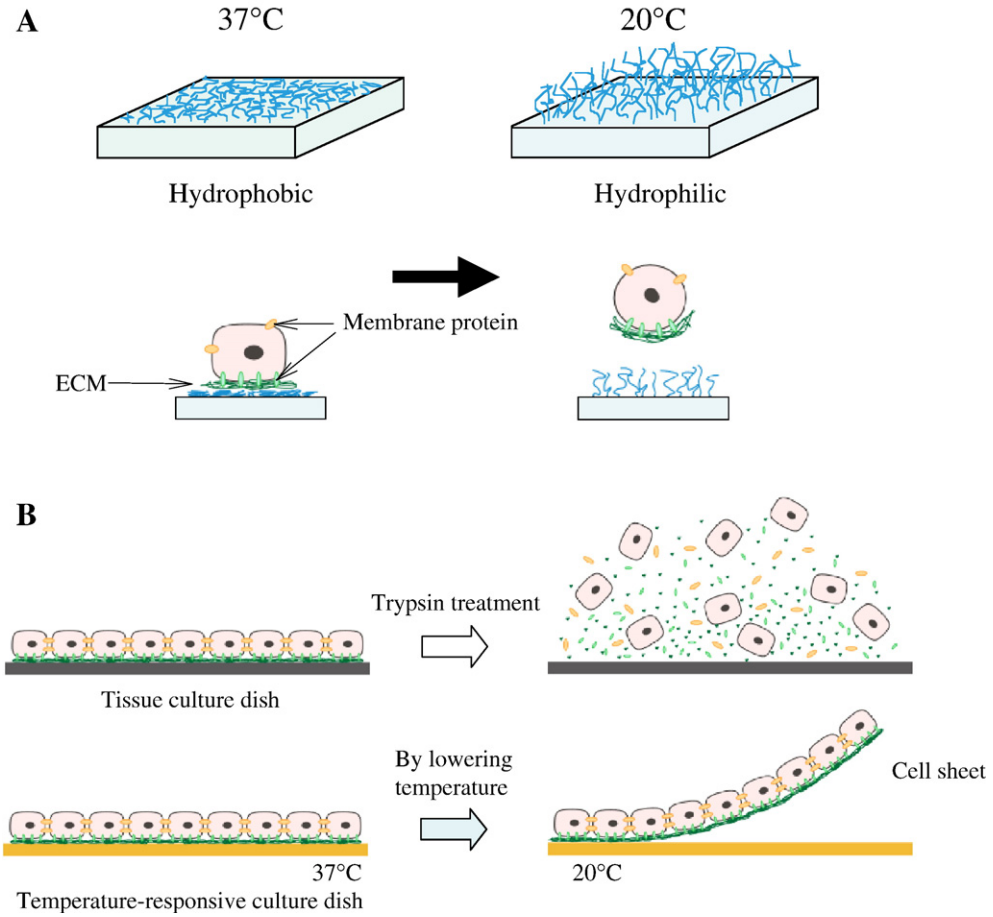

Fig. 1. Cell harvest from temperature-responsive culture surfaces. A. Cells can attach and proliferate onto hydrophobic temperature-responsive surfaces at 37 °C. By lowering the temperature below 32 °C, the surfaces change to hydrophilic and become non-adhesive to cells due to rapid hydration and swelling of the grafted PIPAAm. B. When cells are confluent in culture, they connect to each other via cell-to-cell junction proteins and ECM. Typical cell harvest using enzymatic digestion results in the disruption of both adhesive proteins and membrane receptors. With the temperature-responsive culture surfaces, cell-to-cell connections and deposited ECM are preserved and the cells are released as a contiguous cell sheet.

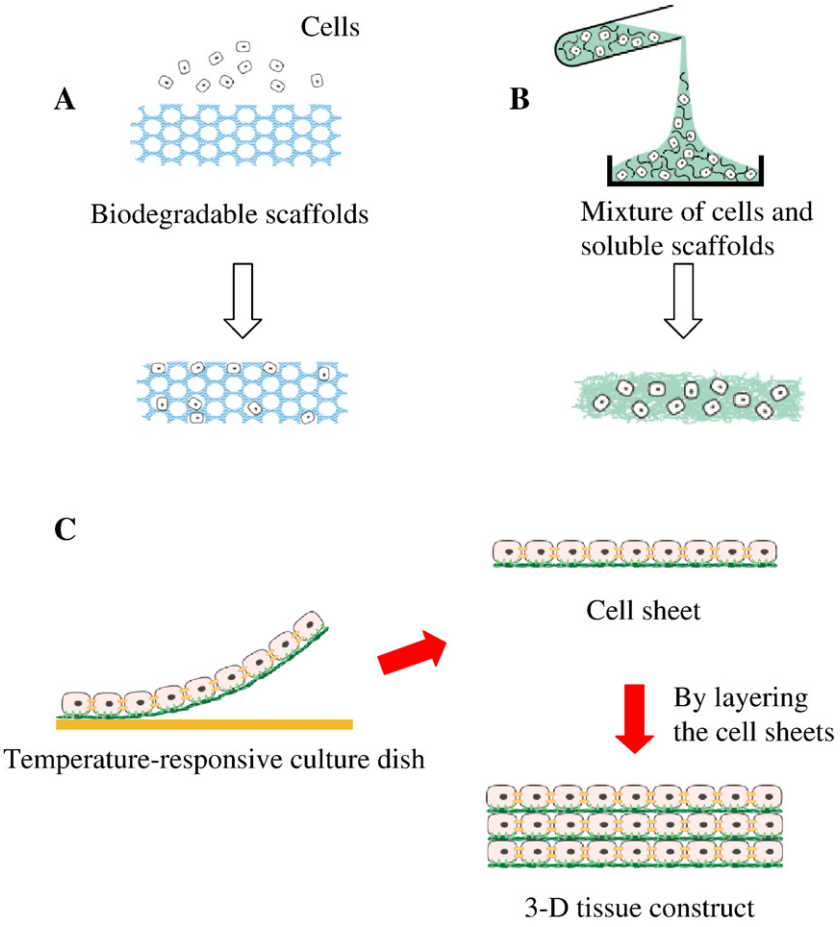

Fig. 2. Myocardial tissue engineering methods. A. Isolated cells can be seeded into prefabricated, highly porous scaffolds, such as PGA, gelatin and alginates. B. 3-D constructs can also be generated by gelling the mixture of cells and ECM solutions. C. Cell sheets harvested from temperature-responsive culture surfaces can be layered to reconstruct 3-D tissues.

harvested as intact cell sheets along with their deposited ECM [5]. Due to the presence of deposited ECM on the basal sheet surface, cell sheets harvested from temperature-responsive culture surfaces can be directly attached to host tissue without the use of any mediators such as fibrin glue or sutures. Various types of transplantable cell sheets have been fabricated including keratinocytes [6], retinal pigment epithelial cells [7], corneal epithelial cells [8], oral mucosal epithelial cells [9,10], urothelial cells [11,12], periodontal ligament cells [13,14], aortic endothelial cells [5,15], corneal endothelial cells [16,17], cardiac myocytes [18,19], and kidney epithelial cells [20].

3. Myocardial tissue engineering by layering cell sheets

In myocardial tissue engineering, various biomaterials such as poly (glycolic acid) (PGA), gelatin, alginate and collagen have been used as prefabricated biodegradable scaffolds [21]. For the repair of damaged cardiac muscle, two strategies have been applied to incorporate cells into the scaffolding materials. One method is to seed cells into prefabricated, highly porous scaffolds (Fig. 2A). The group of Papadaki and Vunjak-Novakovic reported the cultivation of neonatal rat cardiomyocytes on PGA scaffolds using a rotating bioreactor [22,23]. Li et al. have demonstrated that the transplantation of tissue-engineered cardiac grafts using gelatin scaffolds could replace myocardial scars and right ventricular outflow track defects [24,25]. Similarly, Leor et al. reported that cardiac grafts using alginate scaffolds attenuated left ventricular dilatation and heart function deterioration in animal models of myocardial infarction [26]. In contrast to seeding cells into prefabricated scaffolds, Zimmermann and Eschenhagen's group has developed a method of mixing cells with ECM alternatives (Fig. 2B). They have engineered 3-D heart tissues by gelling mixtures of cardiomyocytes and collagen solution, and shown that these tissue constructs could improve damaged heart function [27,28].

Nevertheless, in contrast to cell-sparse tissues such as cartilage or bone that have been successfully engineered with biodegradable scaffolds, the regeneration of cell-dense tissues such as heart tissue may not be sufficiently achieved by using the methods based on biodegradable polymers. Since biodegradable scaffolds often become superseded by large amounts of deposited ECM, cell-to-cell connections are often reduced in the resultant tissues. Scaffold biodegradation results in inflammation and fibrous tissues, which are also observed in pathological states including ischemic heart disease and dilated cardiomyopathy. Cell sheet engineering makes it possible to

avoid these shortcomings associated with biodegradable polymers. When cells are cultured to confluence on the temperature-responsive surfaces, cells can be non-invasively harvested as intact sheets without disruption of cell-to-cell connections. By layering cell sheets harvested from the temperature-responsive culture surfaces, rapid cell-to-cell connections are also established between the layered cell sheets due to the deposited ECM on the basal sheet surface (Fig. 2C). As mentioned previously, cell sheet grafts do not contain any exogenous materials such as scaffolds or carrier substrates, so that potential inflammatory reactions can theoretically be minimized. These methods are particularly suited for generating dense, thicker tissues by layering cell sheets, so cell sheet engineering has also been applied for myocardial tissue reconstruction.

Neonatal rat cardiomyocytes can be non-invasively harvested as contiguous cell sheets from temperature-responsive culture dishes simply by reducing the culture temperature (Fig. 3A) [18,29]. When two cell sheets were layered, the cardiomyocytes began to pulsate spontaneously and simultaneously. To determine the time course of electrical communication, electrical potential of layered cardiomyocyte sheets were monitored using a multiple-electrode extracellular recording system [30]. While the layered cardiomyocyte sheets initially showed independent pulsations, electrical potentials of these two cardiomyocyte sheets rapidly synchronized within 1 h. Immunohistological analyses and dye transfer assay also showed that the formation of the gap junction occurred rapidly between the layered cardiomyocyte sheets. The presence of gap junction precursor proteins, such as connexin 43, on the cell surface of cardiomyocyte sheets may allow for the rapid electrical coupling between the layered sheets. Furthermore, deposited ECM on the basal surface of the cell sheets can be used as adhesive components to promote intimate attachment between the layered cell sheets and accelerate the docking of the gap junction precursor proteins. These results demonstrate that electrically communicative 3-D cardiac constructs can be achieved by layering monolayer cardiomyocyte sheets. Indeed, when four cardiomyocyte sheets were layered, macroscopic pulsations could be observed. *In vivo*, layered cardiomyocyte sheets were transplanted into dorsal subcutaneous tissues of nude rats and after 3 weeks, surface electrograms originating from the grafts were detected independently from host electrocardiograms. Additionally, this spontaneous beating could be clearly observed within the transplanted grafts. Histological studies showed characteristic structures of heart tissue including elongated sarcomeres, desmosomes, and gap junctions within the transplanted grafts. We also demonstrated that pulsatile layered cardiomyocyte sheets survived and grew for a prolonged period of time [31]. After layered cardiomyocyte sheets were transplanted into the subcutaneous tissues of athymic rats,

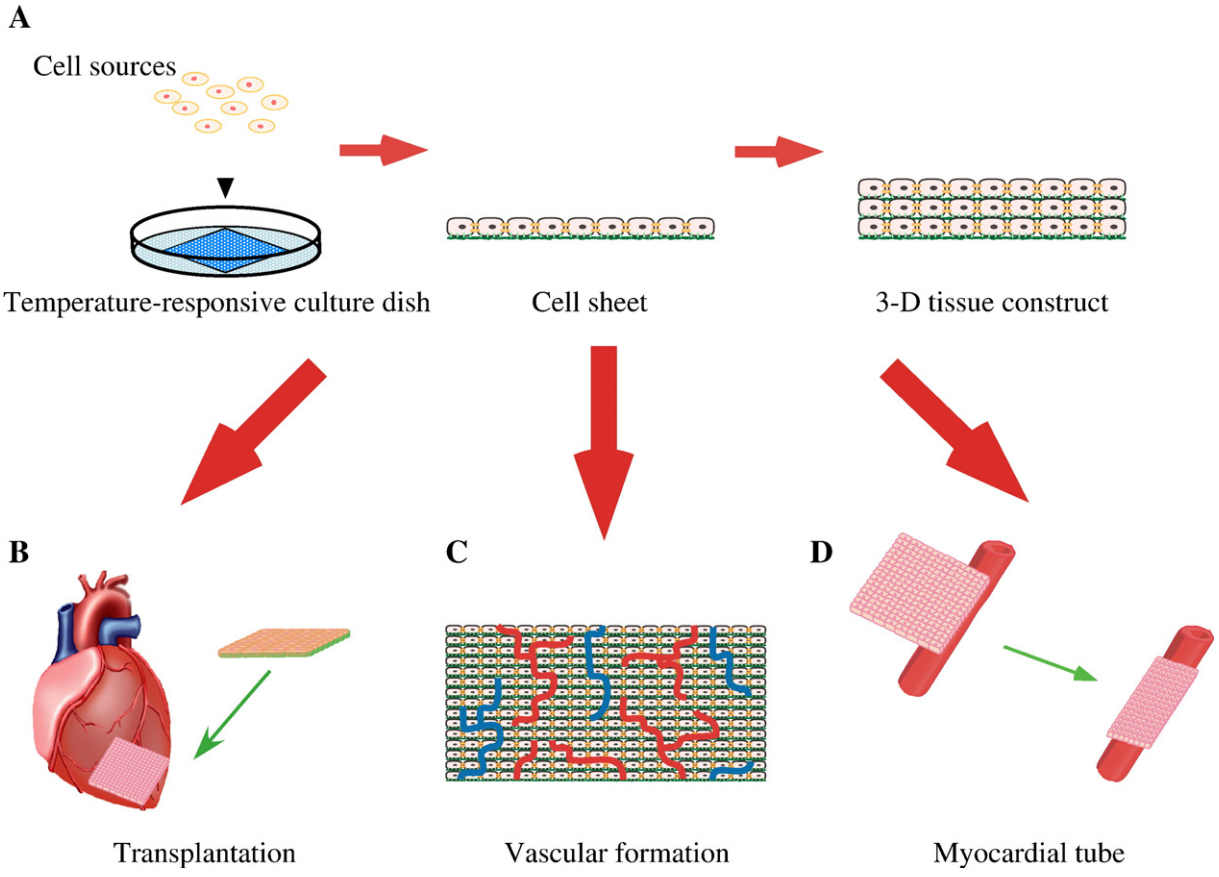

Fig. 3. Future views of myocardial tissue reconstruction by using cell sheet engineering. A. 3-D myocardial tissue generated by using cell sheets. As candidates for the source of cardiomyocytes, various kinds of stem cells have been studied. B. Transplantation of the layered cell sheet constructs directly to impaired hearts. C. Thick cardiomyocyte grafts with well-organized microvascular networks. D. Myocardial tubes as independent cardiac assist devices.

the microvasculature within the myocardial tissues became rapidly organized within a few days. Three days after transplantation, the beatings of the transplanted grafts could be observed macroscopically and continued for up to 1 year. The size, conduction velocity, and contractile force of transplanted grafts also increased in proportion to host rat growth. To improve impaired heart function with myocardial tissue grafts, synchronized beating between the grafts and host heart is likely required. After transplantation of layered cardiomyocytes sheets onto infarcted rat hearts, cardiomyocytes bridged between the grafts and hearts in intact areas [32]. Morphological analyses demonstrated the existence of gap junctions and intercalated disks between the bridging cardiomyocytes. Additionally, dye transfer assay revealed small molecules passing through functional gap junctions. Finally, transplantation of layered cardiomyocyte sheets was also able to repair damaged hearts, with improvement in host ejection fraction, and inhibition of left ventricular dilatation [33].

Thus far however, clinical applicable cardiomyocyte sources have yet to be established. Therefore, various types of cell sheets have been applied to repair the impaired heart. Memon et al. demonstrated that layered skeletal myoblast sheets transplanted to infarcted rat hearts were able to enhance left ventricular contraction, reduce fibrosis, and prevent left ventricular dilatation. This improved cardiac function was observed in association with the recruitment of hematopoietic stem cells through the release of various growth factors including VEGF, FGF and SDF-1 [34]. Kondoh et al. revealed that myoblast sheet graft implantation improved cardiac performance and prolonged life expectancy, in association with a reduction in myocardial fibrosis and re-organization of cytoskeletal proteins in dilated cardiomyopathic hamsters [35]. Miyahara et al. showed that adipose tissue-derived mesenchymal stem cell sheets improved cardiac function in damaged hearts, with reversal of cardiac wall thinning and prolonged survival after myocardial infarction. This recovery after myocardial infarction suggested that the improvement in cardiac function may be primarily due to growth factor-mediated paracrine effects and/or a decrease in left ventricle wall stress by the relatively thick mesenchymal cell sheets [36]. Overall, these transplantation studies have shown that cell sheet transplantation may be effective in improving cardiac function of impaired hearts.

4. Problems and future perspective of myocardial tissue engineering

4.1. Cell sources

Although it has been possible to reconstruct myocardial tissues using tissue engineering methods, several crucial problems remain unresolved for future clinical applications. One of the critical problems is the potential source of cardiac cells. Thus far, primary cells derived from fetal or neonatal hearts have been applied to regenerate myocardial tissues in animal studies, it is difficult to obtain human fetal or neonatal cardiomyocytes with regard to ethical and moral concepts. From these points of views, numerous classes of stem cells have been examined as candidate cell sources. In cell-based cardiac repair, a number of studies have been performed with both adult and embryonic stem cells.

An advantage of applying adult stem cells from autologous sources is the avoidance of immunologic reactions. On this basis, bone marrow stem cells, skeletal myoblasts, adipose tissue stromal cells, and umbilical cord stem cells have been applied [37–41]. Orlic et al. showed that injection of Lin− c-kit+ bone marrow cells in the peri-infarcted left ventricle of mice resulted in myocardial regeneration [38]. In contrast, several groups have reported that Lin− c-kit+ bone marrow cells do not transdifferentiate into cardiomyocytes in similar condition as the original study, if any, at a low frequency through cell fusion [42–44]. The possibility of bone marrow stem cells in regenerating heart tissue is therefore controversial and more detailed studies are needed.

Recently, progenitor cells in the adult heart, which have the potency to differentiate into mature cardiomyocytes, have been reported. Beltrami et al. showed that Lin− c-kit+ cells from the adult myocardium could be expanded, and when transplanted into rat infarcted heart, these cells differentiated into cardiomyocytes [45]. Subsequently, Messina et al. reported that c-kit+ cell populations grow as self-adherent clusters from subcultures of postnatal atrial or ventricular human biopsy specimens and from adult murine hearts [46]. Moreover, Linke et al. demonstrated that c-kit+ cell populations in the dog heart could be recruited and activated by HGF and IGF-1 after myocardial infarction to invade into the damaged tissue and promote the formation of new myocardium [47]. Similarly, Oh et al. reported that Sca-1+ cells isolated from adult mouse heart possessed the cardiac phenotype after treatment with 5′-azacytidine, and that these cells home to the injured myocardium when injected intravenously into a model system of ischemia/reperfusion injury [48]. Matsuura et al. also showed that Sca-1+ cells from adult murine heart could be proliferated *in vitro* and differentiated into beating cardiomyocytes with oxytocin treatment [49]. Lawgwitz et al. reported that Isl-1+ cells could be identified as cardiac progenitors in postnatal rat, mouse and human myocardium [50]. However, Pfister et al. showed that the potential for cardiomyogenic differentiation is restricted to CD31−/Sca-1+ cells [51]. While various populations of cardiac progenitors have been established, it remains unclear at present whether c-kit+ cells, Sca-1+ cells, or Isl-1+ cells represent heterogeneous or homogeneous cell populations, or whether these cells possess the same potential for differentiation in response to various signals. From the point of view of avoiding immune responses, it is attractive to apply autologous adult stem cells for cell-based cardiac repair. Nevertheless, because of the difficulties in obtaining and amplifying the progenitors into sufficient numbers of transplantable cells, myocardial tissue engineering from adult stem cells has yet to be established.

In contrast, the methods to quickly and efficiently amplify mouse embryonic stem cells into various types of cells *in vitro* have been developed although there is some variability between cell lines. Over the past several years, a number of research groups have demonstrated the ability to differentiate mouse embryonic

stem cells into cardiomyocytes. Fijnvandraat et al. reported that cardiomyocytes derived from mouse embryonic stem cells showed a variety of electrophysiological phenotypes [52,53]. Klug et al. demonstrated that genetically selected cardiomyocytes from mouse embryonic stem cells could produce spontaneous and rhythmic contractile activity, and stable differentiated cells were observed as long as 7 weeks after direct injection into the heart [54]. In 1998, the first human embryonic stem cells were established [55] and subsequently, hundreds of human embryonic stem cell lines have been derived worldwide, with numerous reports about the characterization of human embryonic stem cells. Human embryonic stem cells have the potency to differentiate into cells representing all three embryonic germ layers [56], including neurons [57], cardiomyocytes [58], hematopoietic cells [59], and endothelial cells [60], making them an extremely intriguing cell source. Kehat et al. demonstrated that human embryonic stem cell-derived cardiomyocytes display functional and structural properties consistent with early-stage cardiomyocytes [58]. Subsequently, they reported that excitable human embryonic stem cell-derived cardiomyocytes integrated structurally and functionally over the long term with rat cardiomyocyte cultures, and could be successfully paced in swine ventricles under complete heart block [61]. Despite their attractive potential to differentiate into various cell types, several issues remain unresolved for the application of embryonic stem cells to human trials. In regard to ethical and moral issues, the experimental and clinical usage of human embryonic stem cells remains controversial due to the need for fertilized embryos for cell derivation. Besides ethical problems, another risk related to embryonic stem cells is the possibility of teratoma formation. Transplantation of embryonic stem cell-derived grafts into adult tissues may cause the formation of teratomas, which are a specific type of tumor consisting of derivatives of all three germ layers. In addition, since the processes of differentiation and proliferation of embryonic stem cells are currently not well understood, the methods to control the fates of embryonic stem cells are not well developed. Finally, human embryonic stem cells proliferate more slowly and methods to maintain human embryonic stem cells *in vitro* culture are far more complex than their mouse counterparts. Considering these difficulties, it currently remains a challenge to obtain large quantities of human embryonic stem cells for potential clinical applications. Nevertheless, Watanabe et al. have recently succeeded in increasing the efficiency of cell expansion in human embryonic stem cells [62], which may contribute to the future use of human embryonic stem cells for clinical trials.

Although they possess great potential, embryonic stem cells are nevertheless allogenic, and present a risk of immune rejection. Establishing cell banks containing human embryonic stem cell lines that perfectly match major histocompatibility complex antigen to hosts may be able to overcome this limitation in the future. By using somatic cell nuclear transfer or therapeutic cloning methods, the resultant embryonic stem cells may be able to act as autologous cells [63]. Recently, interesting studies have also shown that pluripotent stem cells can be generated from mouse embryonic or adult fibroblast culture by introducing four factors, Oct3/4, Sox2, Klf4, c-myc [64,65]. These methods of nuclear reprogramming may be helpful for creating embryonic stem cell-like autologous cells from adult tissues. Overall these studies provide an avenue to avoid the immune rejection for tissue-engineered grafts, although further investigation must be needed.

In either case, for constructing transplantable myocardial tissues, the development of a readily available source of cardiomyocytes that can be controlled and transplanted in a safe manner, still has to be established. More detailed basic studies are required in order to solve these problems before entering the clinical trials.

4.2. Vascular formation within the bioengineered 3-D myocardial tissues

Recently, poor vascularization has limited the viability of constructs due to hypoxia, nutrient insufficiency, and waste accumulation, causing difficulties in bioengineering cell-dense thick tissues, despite rapid progress in tissue engineering. Therefore, we require new methods for fabricating functional tissues with a well-organized vasculature.

In cell sheet engineering, the thickness limit for layered cardiomyocyte cell sheets in subcutaneous tissue was approximately 80 μm (3 layers). Recently, we have made some attempts both *in vivo* and *in vitro* to overcome this thickness barrier.

We demonstrated that approximately 1 mm thick cardiac tissue grafts with well-organized microvascular networks could be fabricated by multi-step transplantations [19] (Fig. 3C). Repeated transplantation of triple-layer grafts was performed. The two overlaid layered grafts were completely synchronized with whole tissue survival. Moreover, complete graft perfusion via the vessels was observed when a functional multilayer graft was fabricated over a surgically connectable artery and vein, then the graft with these thick artery and vein was successfully transplanted ectopically. Long-standing barriers to produce thick, vascularized tissues can be overcome using these *in vivo* cell sheet integration methods.

We have also attempted to control the vascularization processes *in vitro* to create thicker functional tissues. When endothelial cells were co-cultured within cardiac cell sheets, angiogenesis-related genes expression and the formation of the endothelial cell networks were observed *in vitro* [66]. The endothelial cell networks were maintained within the cell sheets after harvest from temperature-responsive culture dishes, and matured to form tubularized vascular networks after *in vivo* transplantation. Moreover, all of the graft vessels originated from the grafts themselves, and migrated to connect with the host blood vessels. Finally, by changing the ratio of endothelial cells, the formation of blood vessels within the grafts could be controlled. Myocardial tissue grafts engineered with cell sheet engineering therefore have the potential for the *in vivo* neovascularization that can be regulated *in vitro*.

4.3. Future challenges in myocardial tissue engineering

As a further advanced therapy for severe heart failure, we have attempted to create functional organ-like structures with the ability to act as independent cardiac assist devices (Fig. 3D).

We demonstrated the *in vitro* fabrication of pulsatile myocardial tubes using a novel cell sheet-wrapping device [67]. The tubular constructs, which consisted of three neonatal rat cardiomyocyte sheets wrapped around fibrin tubes, demonstrated spontaneous, synchronized pulsation. Moreover, inner pressure changes evoked by tube contraction were observed in the tubular constructs. The mean inner pressure gradient of each beat was 0.11±0.01 mmHg. Histological studies also showed both well-differentiated sarcomeres and diffuse gap junctions within the tubular constructs that resembled native cardiac muscle. These data indicated that tissue-engineered myocardial tubes might possess native heart-like structures and functions. We also showed pulsatile myocardial tubes created from cardiomyocyte sheets *in vivo* [68]. The abdominal aorta of athymic rats were replaced by a resected adult rat thoracic aorta sequentially wrapped with six neonatal rat cardiomyocyte sheets. Spontaneous and synchronous pulsation independent of the host heartbeat was observed after transplantation. The graft pressures evoked by myocardial tube pulsation were observed independently from that of host. In this case, inner pressure gradients originating from the aortic replacement myocardial tubes were 5.9±1.7 mmHg, which was much greater than the values generated by the *in vitro* myocardial tubes. Histological analysis and transmission electron microscopy studies revealed the myocardial tubes consisted of heart-like tissues. Compared with grafts implanted in the abdominal cavity, the thickness of the cardiac tissues of the myocardial tubes in place of abdominal aorta were significantly increased, as well as expression of brain natriuretic peptide, myosin heavy chain-alpha, and myosin heavy chain-beta. Functional myocardial tubes that have the potential for circulatory support could therefore be created with cell sheet engineering. These new myocardial structures present a possible core technology for the creation of engineered tissues capable of acting as independent cardiac assist devices.

5. Conclusions

Myocardial tissue engineering based on cell sheet engineering can provide new *in vitro* heart models and might be useful for cardiovascular tissue repair.

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