Tissue Engineering Based on Cell Sheet Technology

By Noriaki Matsuda, Tatsuya Shimizu, Masayuki Yamato, and Teruo Okano*

Cell sheet technology enables novel approaches to tissue engineering without the use of biodegradable scaffolds. Cell sheet technology consists of a temperature-responsive culture dish, which enables reversible cell adhesion to and detachment from the dish surface by controllable hydrophobicity of the surface. This allows for a non-invasive harvest of cultured cells as an intact monolayer cell sheet including deposited extra cellular matrices. The monolayer cell sheet can be transplanted to host tissues without using biodegradable scaffolds and sutures. Thick tissue constructs and patterned cell sheets using two or more kinds of cell source are also developed by means of layered cell sheets in vitro. This Progress Report summarizes temperature-controlled cell adhesion-detachment behavior and applications of the cell sheet technology to regeneration of cornea, periodontal ligament, bladder epithelia, oesophageal epithelia, myocardium, and liver.

1. Introduction

Recently, cell-based therapies, regenerative medicine, and tissue engineering have been progressing rapidly. With the injection of single cell suspensions, the injected cells are expected to remain around the damaged host tissues for the maintenance and recovery of native functions. Injections of bone marrow cells have been applied in many ways, and achieved some good clinical results.\cite{1-7} However, in most cases, the injected cells cannot be retained around the target tissue, causing difficulties to control the size, shape and location of the injected cells. To overcome these problems, biodegradable polymer scaffolds have been seeded with cells to be used to fabricate three-dimensional tissue-like grafts. Tissue engineering has been applied to epidermis\cite{8-10}, bone\cite{11}, cartilage,\cite{12} blood vessels,\cite{13,14} and heart valves.\cite{15} Various carrier materials such as collagen gels,\cite{16} fibrin gels,\cite{17-20} and amniotic membrane\cite{21-24} have been used to create epithelial grafts for the regeneration of epidermis,\cite{19,20} oral mucosal epithelium,\cite{25} and corneal epithelium.\cite{26} Upon polymer degradation, cells are believed to proliferate and migrate to replace the polymer scaffold. However, the places previously occupied by the polymer scaffolds are filled with large amount of extracellular matrix (ECM). Thus, cell-sparse tissues like cartilage or bone can be fabricated successfully. On the other hand, fabricated structures with biodegradable scaffolds cannot resemble cell-dense tissues such as heart and liver, and also cause pathological cases of fibrosis. For larger constructs, cells in the center of the constructs become necrotic though the cells on the periphery are unimpaired. This is due to the limits on passive diffusion, that is, insufficient delivery of oxygen and nutrients and removal of metabolic waste. Furthermore, strong inflammatory responses are often observed upon biodegradation of the scaffolds. Macrophages and neutrophils with collagenase and elastase activities migrate to the implant site during the early wound healing response.\cite{27} This host inflammatory response can damage the seeded cells even though the biodegradable scaffolds are non-toxic and mechanically non-invasive, resulting the failure of the engineered tissues.\cite{28,29} Therefore, a new method to avoid the use of bio-

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* Prof. T. Okano, Dr. N. Matsuda, Prof. T. Shimizu, Prof. M. Yamato
Institute of Advanced Biomedical Engineering and Science
Tokyo Women’s Medical University
8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666 (Japan)
E-mail: tokano@abmes.twmu.ac.jp

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degradable scaffolds is strongly expected for the further development of tissue engineering.

We have adopted an innovative approach to tissue engineering based on “cell sheet technology".[30,31] Cell sheet technology consists mainly of a “thermo-responsive culture dish” which enables reversible cell adhesion to and detachment from the dish surface by controllable hydrophobicity of the surface.[32–34] This allows for a non-invasive harvest of cultured cells as an intact monolayer cell sheet including deposited ECM. The monolayer cell sheet can be collected simply by reducing culture temperature lower than 32 °C for less than 30 minutes, without any enzymes nor chelating agents. By this technology, we can transplant cell sheets to host tissues without using biodegradable scaffolds.[35–37] We have also developed in vitro thick tissue constructs by means of layered cell sheets, and combinatorial or patterned cell sheets using two or more kinds of cell sources. The cell sheet technology has been applied to skin,[38] corneal epithelia,[39,40] periodontal ligament cells,[41,42] bladder epithelia,[43,44] kidney glomeruli,[45–47] oesophageal epithelia,[48] myocardial cells,[49–53] hepatocytes[54] mesenchymal stem cells,[55] and so on. In this Progress Report, we summarize the thermo-responsive culture dish, temperature-controlled cell adhesion-detachment behavior, and applications of the cell sheet technology to tissue engineering.

2. Thermo-responsive Tissue Culture Dish For Reversible Cellular Adhesion and Detachment

It is well-known that poly(N-isopropylacrylamide) (PIPAAm) exhibits thermo-responsive hydrophobicity changes in aqueous solutions.[56] We have prepared PIPAAm-grafted cell culture dishes, and the surfaces are proved to show the thermo-responsive characteristics. By irradiation of electron beam onto IPAAm monomer on commercial polystyrene dishes, the monomer is polymerized and covalently bonded with the dish surface. At temperatures below 32 °C PIPAAm molecules are highly hydrated, so the PIPAAm-grafted surfaces are hydrophilic. At temperatures above 32 °C the surfaces suddenly change to hydrophobic due to extensive dehydration of PIPAAm molecules. This change is completely reversible with temperature. In general, cells can adhere hydrophobic surfaces better than hydrophilic surfaces. Thus we have applied the characteristics of PIPAAm-grafted surfaces to cell culture dish, that is cell adhesion and detachment were controlled by external temperature changes.[57,58] The “cell sheet technology" brings recovery of viable and intact cell monolayer to fruition.

Cellular interactions with PIPAAm grafted surfaces have been investigated by changing its grafting density. Table 1 shows the dependences, for surfaces prepared by changing its grafting density, of thickness of the grafted layer on surface wettability measured by captive bubble method in pure water and ability for endothelial cell adhesion.[59] Surface wettability changes are observed for all grafting densities between 20 and 37 °C, yet temperature-dependent cell adhesion and detachment are only observed on PIPAAm grafted surfaces with 15–20 nm thick. When the grafted layer is thicker than 30 nm, no cell adhesions occur. The contact angle is small at 37 °C, and less decrease in contact angles is observed by changing temperature. These results indicate that dehydration of PIPAAm chains is not complete, inducing less interaction between PIPAAm layer and cells. The contact angles for the highest grafting density are much smaller than the others. PIPAAm layer is still highly hydrated even at 37 °C, and inhibits cell adhesion. On the other hand, when the grafted layer thickness is less than 15 nm, cells adhere and proliferate on the surface at 37 °C but never detach from the surface when reducing the

| Density of grafted PIPAAm (µg cm⁻²) [a] | 1.4±0.1 | 2.9±0.1 | 1,080 |
| Thickness of the grafted PIPAAm (nm) [b] | 15.5±7.2 | 29.3±8.4 | 5,000 |
| Contact angle (degrees) [c] | 37°C | 7.7±0.6 | 69.5±1.2 | 49.6 (40°C) |
| 20°C | 6.2±1.2 | 60.0±0.1 | 11.5 (10°C) |
| Cell adhesion 37°C | Yes | No adhesion | No adhesion |
| Cell detachment 20°C | Yes | – [d] | – [d] |


Teruo Okano, Ph.D., is a Professor and a Director of the Institute of Advanced Biomedical Engineering and Science at Tokyo Women’s Medical University. He is also an Adjunct Professor at the Center for Controlled Chemical Delivery at the University of Utah (USA). He has been a Fellow of the American Institute of Medical and Biological Engineering since 1997, and also a Fellow of the International Union of Societies for Biomaterials Science and Engineering since 2000. He serves as an Associate Editor for the Journal of Biomedical Materials Research, Bioconjugate Chemistry, and Tissue Engineering. He is also a member of the editorial boards for the Journal of Controlled Release, Drug Targeting, and Advanced Drug Delivery Reviews. His research interests involve the use of intelligent biomaterials for research applications in various fields such as tissue engineering, drug and gene delivery, microfluidics, and cell-based on-chip assays.Recently, he has been awarded the Leona Esaki prize (2005) and a Nagai Innovation Award from the Controlled Release Society (2006). He received his Ph.D. in polymer chemistry from the Department of Applied Chemistry, Waseda University (Japan).
temperature to around 20 °C. Because the surface is proved to be fully covered with grafted PIPAAm molecules as determined by angular-dependent X-ray photoelectron spectroscopy (XPS) analyses,[60,61] influence between ungrafted polystyrene surface is not the case for the retention of cell adhesion. Therefore, interactions among adhered cells and PIPAAm chains are the nature of no-detach phenomenon. Thus the grafting density is the dominant cause for these cell adhesion-detachment phenomena.

The grafting density affects chain mobility of PIPAAm layer, which has a significant effect on the observed surface wettabilities of PIPAAm grafted layer with temperature.[57,58,62] Single-point attached, freely mobile grafted PIPAAm surfaces show the largest change in wettability, whereas multi-point grafted PIPAAm surfaces show limited wettability changes with temperature. Swelling ratio of single-side fixed, cross-linked PIPAAm hydrogel is much less than that of freely cross-linked hydrogel. Hydrophobized PIPAAm hydrogels copolymerized with n-butyl methacrylate (BMA) show sudden expansion when the dry network core is swollen from both sides of the network.[63] Fixation of the PIPAAm on a surface limits polymer chain mobility near the interface, producing extensive hydrophobic aggregates and limited hydration of the polymer chain. Since the polymer chains nearly connect through multiple three-dimensional cross-linking points, hindrance of hydration on one end hinders general network response of polymer chains. Thus, general network response to external stimuli is affected by the limited molecular mobility, and the outermost PIPAAm chains have more degree of freedom, ability to respond to stimuli and hydration than inward.[64] From above discussions, Figure 1 schematically illustrates possible causes for different chain mobility of grafted chains of PIPAAm layers. At the interface of the PIPAAm chain and polystyrene surface, grafted PIPAAm chains are aggregated and immobilized with the hydrophobic surface due to strong hydrophobic interaction at 37 °C. This strong hydrophobicity of the grafted PIPAAm chains contacted with polystyrene surface affects the “restricted layer” of PIPAAm graft chains,[64] and thus hydration of water molecules is limited. PIPAAm grafted surfaces with such restricted chain mobility and limited hydration capability have the ability for temperature-regulated cell adhesion and detachment properties. The maximum thickness to appear the restricted layer on the surface can be considered in between 15 and 20 nm. When PIPAAm graft is thicker than 30 nm, cells cannot interact strongly to the PIPAAm chains to adhere. It is known that cells do not adhere even at 37 °C on a PIPAAm gel ungrafted on polystyrene surface. The PIPAAm chains in the “relaxed layer” are still dehydrated but less restricted like a free gel.[64] Therefore, controlling PIPAAm surface graft density is a critical issue to reliably produce temperature-responsive cell adhesion-detachment behavior.

3. Temperature-controlled Cell Adhesion-Detachment and Cell Sheet Manipulation

For contact between cells and material surfaces, cells physically adhere onto hydrophobic surfaces better than hydrophilic surfaces. After a period of time of such a physical contact (passive adhesion), metabolic processes using ATP causes a change in cell morphology to spread. On the hydrophobic surface of thermo-responsive culture dish, adhered cells can proliferate normally to confluency, and express normal phenotypic markers for each cell type as well as a normal polystyrene culture dish. Spread cells rarely detach from the hydrophobic surface without reversing events associated with morphology changes from spread to round cell shapes and release of surface-engaged receptors. Simply by reducing temperature below 32 °C (typically 20 °C for 30 minutes), spread and adhered cells can be spontaneously detached from the thermo-responsive culture dish surface due to hydration of PIPAAm surface. This detachment process does not damage cells since the temperature change is physically mild conditioned, and no enzymes like trypsin or chelator such as EDTA are required. Enzymes destroy cell-to-cell junctions, producing single cell suspensions, while confluent cells can be recovered as contiguous intact cell monolayer retaining cell-to-cell junctions[65] by using thermo-responsive culture surface. Furthermore, these monolayers of cells maintain basal surface ECM proteins after detachment (Fig. 2).[36] This cell adhesion-detachment modulation on the PIPAAm grafted surface is a novel concept (Fig. 3). Various kinds of cells have been harvested on the PIPAAm grafted culture dish under general incubation conditions of 5% CO₂ and 37 °C.

During cell detachment from hydrated PIPAAm grafted surfaces, cell metabolic changes occur.[52,66] Optimum temperatures for maximum cell recovery efficiency from PIPAAm grafted surfaces are different for cell species, e.g., 20 °C for cultured endothelial cells and 10 °C for hepatocytes. Cells treated with sodium azide, an ATP synthesis inhibitor, or genistein, a
tyrosine kinase inhibitor, did not change their morphologies and remained adherent on the PIPAAm grafted surface even at low temperature. Treatment with phalloidin, an actin filament stabilizer, inhibited cell detachment. These results suggest that cell detachment from hydrated PIPAAm grafted surface is governed by active energy-consuming metabolic processes, that is, intracellular signal transductions and reorganization of the cytoskeleton.\cite{66} Morphological transitions in cell sheet during detachment process observed at 20 °C are as follows; cells begin to detach from the periphery of the dish surfaces, maintaining cell-to-cell connections and producing some sheet contractility. Supporting membranes such as poly(ethylene terephthalate) membranes,\cite{36,67} poly(vinylidene difluoride) membranes,\cite{56,67} chitin membranes,\cite{37} or parchment paper sheets have been used to manipulate the cell sheets without contraction. The cell sheets can be manipulated with the supporting membranes by physicochemical interaction such as interfacial tension, then directly transferred onto native tissues or other cell sheets with adhesive effects by retained ECM on the basal surface of the recovered cell sheets.\cite{68}

In this way, cell sheet technology has several advantages over direct cell injection or tissue reconstruction with biodegradable scaffolds. Only a mild low temperature treatment without any enzymes for cell detachment allows differentiated cellular form and complete functions.\cite{69} Cell sheets can be attached to host tissues and even wound sites via their deposited ECM on the basal surface of the recovered cell sheets.

There are fewer inflammatory responses that are often observed upon biodegradation of scaffolds.

4. Patterned Surfaces for Heterotypic Cell Co-culture

Heterotypic cell-to-cell interactions are essential to regenerate novel tissue architectures with characteristic physiological cellular functions in vitro. Patterned surface modification have been utilized for co-culture of different cell types, achieving controlled cellular functions, including growth, differentiation and apoptosis.\cite{70–74} We have proposed a patterned surface modification technique using area-selective electron beam polymerization of PIPAAm into domains on polystyrene dishes. Cells are plated and cultured on the pattern-grafted surfaces at 37 °C, and then the temperature is reduced to 20 °C. Cells on the PIPAAm surface are detached by gentle pipetting, and only on the polystyrene regions remain adhered. Other cell types are then seeded selectively on PIPAAm patterned surface by reascending temperature to 37 °C. Therefore, two cell types can be co-cultured in determined spatial arrangements, resulting improved cellular functions.\cite{75,76} We have also prepared patterned surfaces by localized ablation of UV ArF excimer laser. Excess amount of PIPAAm (> 3 μg/cm²) is grafted onto polystyrene surface using electron beam irradiation, which inhibits cell adhesion and migration even at 37 °C. Then localized ablation of UV ArF excimer laser is carried out for photodecomposition of only the surface region to conveniently attain micro patterned surfaces. Time-of-flight secondary ion mass spectrometry analysis revealed that the ablated domains exposed basal
polystyrene and were surrounded with PIPAAm grafted chemistry. We have also successfully prepared micro patterned surfaces with grafting polyacrylamide (PAAm), a hydrophilic polymer, followed by UV ArF excimer laser ablation. Hepatocytes[77] and endothelial cells[78] adhere only to the ablated domains adsorbed with fibronectin at 37 °C. Furthermore, we have also prepared patterned dual thermo-responsive surfaces for co-culture of heterotypic cells and then recovered as an intact cell sheet. Hydrophobic-hydrophilic transition temperature of thermo-responsive surface decreases by introducing n-butyl methacrylate (BMA), a hydrophobic monomer, into PIPAAm grafted dish using electron beam-irradiated copolymerization.[79] Then, BMA is polymerized in patterns on PIPAAm grafted surfaces to form patterned dual thermo-responsive surfaces. Co-cultured monolayer sheet of hepatocytes and endothelial cells are obtained as contiguous sheet by changing culture temperatures to 27, 37, and 20 °C.[80]

5. Applications of Cell Sheet Technology

“Cell sheet technology” with thermo-responsive culture dish allows non-invasive harvest of cultured cells as an intact monolayer cell sheet including deposited ECM. This technology enables direct transplantation of cell sheets to host tissues without scaffolds, fixation, or sutures. The direct transplantation have been applied to corneal epithelia, mucosal epithelia, periodontal ligament cells, bladder epithelium and oesophageal epithelia. The cell sheets can be layered on top of one another, creating three-dimensional constructs such as thick cardiac muscle. We have attempted to create not only simple layered structure but also functional organ-like structures. In vitro micropumps and potential in vivo tubular circulatory support device have been investigated by using myocardial sheets. Heterotypic cell sheets with hepatocytes and endothelial cells have been created by patterned co-culture or heterotypic stratification. In the following sections, specific applications of cell sheet technology are described.

5.1. Corneal Surface Reconstruction

Although corneal transplantation using donated eyes is the most common method of treatment, the high risk of graft rejection and a shortage of donor corneas remain significant drawbacks. Cultured corneal epithelial cells treated with dispase[26] or fibrin gel[18] have been used for ocular surface reconstruction. These methods require carrier substrates to be surgically placed on host corneal stroma. Besides, infection, inflammation, and micro-trauma due to incomplete biodegradation remain controversial issues with regards the use of fibrin gel for tissue engineering. On the other hand, limbal epithelial stem cells can be isolated and cultured on the thermo-responsive culture surfaces. The corneal epithelial cell sheet, which is intact and contains both cell-to-cell junction and ECM proteins, can be transplanted without the need for any carrier substrate or sutures.[96] Clinical results have shown that the corneal surface remains clear with significantly improved visual acuity more than one year after the corneal epithelial cell sheet transplantation. Transplantation of corneal epithelial cell sheets also prevent the development of corneal haze after excimer laser keratectomy.[81]

We have also shown that autologous oral mucosal epithelial cell sheets can be used as an alternative to corneal epithelial cell sheets.[40,82] Oral mucosal epithelium cell sheets can be harvested on thermo-responsive culture dishes and transplanted in the same manner as corneal epithelial sheets. The oral mucosal epithelial cell sheets more closely resemble native corneal epithelium.[27,40] Furthermore, transplanted oral mucosal epithelial cell sheets show modulation in their keratin expression profiles towards a corneal phenotype in a rabbit model.[40,82] The direct interaction between the transplanted epithelial sheet and host stroma, and the maximum corneal transparency are the greatest benefit of excluding the use of scaffolds or carrier substrates.

5.2. Periodontal Ligament Cell Sheets

The clinical features of periodontitis are inflammation from gingival pocket areas, and subsequent destruction of periodontal ligament. Many surgical procedures have been applied to induce tissue regeneration, however epithelial tissues always proliferate into the defect at a faster rate than the underlying mesenchymal tissues.[83] Thus, long junctional epithelium attachment to the dentin root surface is established and the original form and functions of periodontal tissues cannot be restored. Guided tissue regeneration (GTR) is a concept to regenerate periodontal tissues by deriving cells from periodontal ligament and excluding epithelial and gingival connective tissue cells from the healing area.[84–86] Some growth or differentiation factors[87,88] or various grafting materials[89,90] have been applied to create an environment suitable for selective cell repopulation, but are still insufficient to induce periodontal regeneration because repopulation of cells into the defect area does not occur completely.[42,91] Application of cultured periodontal ligament cells in suspension or applied in various scaffolds induces a new periodontal tissue on root dentin surfaces, however also invokes unpredictable adverse effects, such as ankylosis.[92]

Periodontal ligament cells are harvested on thermo-responsive culture dishes in the presence of ascorbic acid, producing intact periodontal cell sheets with thick extracellular matrix (ECM).[42] Fibronectin is a major protein incorporated into the ECM, which functions as a natural adhesive to attach cell sheet to other surfaces. We have transplanted periodontal cell sheets onto denuded root dentin surfaces of athymic rat[42] and beagle dog.[43] At 4 weeks postsurgery of athymic rat with periodontal cell sheets transplantation, newly formed immature fibers are obliquely anchored on dentin surfaces like native periodontal ligament fibers (Fig. 4).[42] Cementum and
5.4. Endoscopic Oesophageal Epithelial Transplantation

Endoscopic mucosal resection (EMR) has enabled the non-invasive removal of gastrointestinal tract cancers[47] without the need for oesophageal reconstruction.[98] Although EMR is a widely accepted technique, respectable size is limited[99] and retrieval of multiple specimens which can damage the tissues is needed. A new method of endoscopic submucosal dissection (ESD) permits large en bloc resections without the size limitation[100] enabling removal of large cancers using a single procedure.[100,101] However, severe inflammation causes oesophageal scarring and stenosis[102,103] without any method to enhance wound healing. We have developed a method combining ESD with the endoscopic transplantation of autologous oral mucosal epithelial cell sheets.[48] After ESD, the cell sheets are attached directly to the esophageal ulcer beds using endoscopic forceps without suturing. The presence of an epithelial cell layer dramatically enhances post-ESD wound healing and reduces host inflammatory responses.[48]

5.5. Myocardial Cell Sheet-3D Tissue Reconstruction

Cardiomyocyte sheets can be harvested as intact monolayers along with their deposited ECM by using thermo-responsive culture dishes. Neonatal rat cardiomyocyte sheets show synchronized pulsation, suggesting complete morphological and electrical cell-to-cell connections.[104] We have attempted to reconstruct three-dimensional myocardial tissues by layering cardiomyocyte sheets.[105,106] It is a crucial point whether electrical and morphological communications are established between bilayer cell sheets. We examined electrical communication of two partially overlapped monolayer cardiomyocytes by using microelectrodes.[65] Electrical potentials of the two cardiomyocyte sheets were initially dissociated, but began to synchronize with slight delays after 30 min. Once some gap junctions were established between the two cell sheets, electrical impulses reached the monitoring electrode via both direct conduction from the pace maker cell sheet and roundabout conduction through the established gap junctions. These delays diminished in a time-dependent manner, and electrical potentials became completely synchronized after 45 minutes. These results suggest that additional gap junctions were randomly formed between the layered cell sheets and then were established throughout the interface in 45minutes. Connexin 43, the gap junction precursors, were detected on the free cell membrane as well as at cell-to-cell interfaces. Connexin 43 was maintained during the harvest of confluent cardiomyocytes from thermo-responsive culture dishes, and may allow for rapid electrical coupling between layered cell sheets. In myocardial tissue engineering, electrical structural cell-to-cell tight junctions are critical to synchronized, functional beating. The cell sheet technology has advantages in the rapid formation of gap junctions and functional communication between layered cardiomyocyte sheets. The transplantation of these constructs to impaired hearts also promotes

alveolar bone exist on either side of the periodontal ligament. On the other hand, extensive new bone formation leading to ankylosis with partial root resorption is observed without periodontal cell sheets transplantation. These results suggest that periodontal ligament cell sheet technology can be useful for periodontal tissue regeneration.

5.3. Urothelial Cell Sheets

Gastrointestinal flaps are generally used as augmentation methods of urinary bladder. However, stone formation, urinary tract infection, mucous production and carcinoma, due to the intestinal mucosa, and severe fibrosis due to frequent contact of urine are major complications of this procedure.[43,93,94] Application of prefabricated seromuscular intestinal flaps, of which surface is partially grafted with urothelium, has resulted in good performance.[83] However, this method has a complicated two-stage operation process to apply the urothelium onto the flap. We have created in vivo. This versatile method should prove useful in polarized intestinal flaps, of which surface is partially grafted with urothelial cell sheets.[43] The urothelial cell sheets are grafted on demucosalized gastric flaps, resulting five of eight flaps showed urothelial regeneration in canine model.[44] The cell sheets are attached spontaneously to the flap without any suture or fixation, generating a multilayered urothelium in vivo. This versatile method should prove useful in polarized renal tubule reconstruction[45] and urinary tract tissue engineering.[44]
electrical communication and coupling between graft and host, due to the presence of initial gap junction precursors and direct cell-to-cell communications.\[65\]

Triple layer cardiac cell sheet grafts, which are the co-culture of cardiomyocytes and endothelial cells, were transplanted into dorsal subcutaneous tissues of nude rats. At three weeks after the transplants, surface electrogroms were detected independently from host electrocardiograms,\[52\] and the graft has survived at least up to one year.\[50\] Neovascularizations within the grafts occurred promptly after implantation and that vascular network was organized after a few days in vivo. The observed blood vessel reconstruction was completely due to endothelial cell networks that originated from within the grafts.\[100\] Interestingly, these newly formed graft-derived blood vessels also sprouted into the underlying host tissues, to form functional connections with the host vasculature. Furthermore, endothelial cell network in vitro form functional micro vessels and sprout into the host tissues after transplantaion. With cardiac cell sheets harvested from thermo-responsive dishes, their own deposited ECM is preserved, and the cell sheets are also able to retain intact, undisrupted endothelial networks, as well as the expression of angiogenesis promoters such as VEGF and Cox-2. It is known that the co-culture of endothelial cells, fibroblasts, and skeletal myoblasts has also shown to play a significant role in the vascularization of engineered skeletal muscle,\[106\] demonstrating the importance of cell-to-cell interactions in creating endothelial cell networks. In cardiac cell sheets, the observation that cardiomyocyte clusters surround areas enriched with endothelial cells also suggests that the endothelial networks are activated by both direct cell-to-cell interactions, as well as secreted proteins from cardiomyocytes. Therefore, a mutually beneficial relationship between cardiomyocytes and endothelial cells seems to contribute to the formation of endothelial cell networks in vitro.\[105\] By controlling the endothelial cell population within the cardiac cell sheets, the potential for vascularization can also be regulated, which will likely overcome the limits of mass transport to create thick and functional tissues. However, not only biological factors but also physical stimuli such as flow and shear stress are required to form mature vascular networks under in vitro conditions.\[107,108\]

From monolayer to five-layer neonatal rat cardiomyocyte sheets were stratified in vitro, and then were transplanted into the dorsal subcutaneous tissue of nude rats. One month after the procedure, the sheet constructs were resected.\[50\] The constructs survived without necrosis for less than three-layer sheets, but the four- and five-layer constructs were replaced with disordered vasculature and connective tissue, indicative of necrosis. A critical obstacle opposing cardiac tissue fabrication is the inability to constantly and rapidly supply sufficient oxygen and essential nutrients while removing metabolic wastes via reliable vascular networks. The lack of sufficient vascularization induces necrosis and prevents unlimited stacking of additional cell sheets. The thickness limit for layered cardiomyocyte sheets in vivo subcutaneous tissue appears to be ~80 μm. We attempted to overcome the thickness limitation of layered cell sheets by “polysurgery”, a repeated transplantation of layered cell sheets after intervals that produce sufficient neovascularization within the previously implanted grafts.\[50\] If two triple-layer cell sheets were stratified and transplanted by single-step procedure, significant necrosis occurred in the tissue graft (Fig. 5A).\[50\] On the other hand, when triple-layer cell sheets were repeatedly transplanted at a one-day interval, the stacked grafts were completely synchro-
Triple layer cardiomyocyte sheets were also repeatedly transplanted over an exposed superficial caudal epigastric artery and vein in the leg of nude rats with the one day interval procedure.[50] After two weeks, the transplanted grafts, which showed strong and synchronous pulsation over the vessels, were resected together with the femoral artery and vein. Indian ink was then infused from the inlet of the femoral artery, resulting the ink diffused throughout the graft and discharged from the femoral vein. This indicates vasculogenesis induced from the host and blood supply was indeed from the selected artery. Then, the multilayer cardiomyocyte graft was resected with the connectable host artery and vein, and then transplanted into the neck of another nude rat. The femoral artery and vein of the graft were connected to the carotid artery and the jugular vein respectively, in the new host. The graft began to pulsate spontaneously just after reperfusion,[50] and maintained the pulsation even two weeks after the procedure. These results support the clinical potential to transplant multilayer grafts ectopically fabricated with a surgically connectable artery and vein.

In addition to the application of cardiac patches, the next challenge is the creation of functional organ-like structures with the ability to act as independent cardiac assist devices. We have created cellular micropumps on-chip using cardiomyocyte sheets with intrinsic pulsatile mechanical functions.[109,110] These cardiomyocyte micropumps have achieved greater than 3.5 μN of contractile forces and 2 nL/min of directional flow rates. We also have developed a micro-spherical heart-like pump by wrapping a cardiomyocyte sheet around a fabricated hollow elastomeric sphere of 5-mm diameter. The device has generated fluid oscillations in a capillary connected to the pump for at least five days in vitro.[111] These micropumps work only with chemical energy input to produce reliable mechanical force, thus, these devices would be applicable to various microfluidic components used where electricity cannot be supplied. Furthermore, we have shown the construction of pulsatile myocardial tubes with the potential for in vivo circulatory support (Fig. 7).[112] Six-layer cardiomyocyte sheets constructs are wrapped around 1.5-cm portion of resected thoracic aorta from adult rats in vitro. Then, the myocardial tubes are transplanted into abdomen of other nude rats by connecting abdominal aorta by the cuff method[113] with sutures. The implanted myocardial tubes demonstrate spontaneous and synchronized pulsation four weeks after transplantation. The myocardial tubes produce circulatory supportive blood pressures of 5.9±1.7 mmHg independent from the host hearts. It is also suggested that hypertrophy of the functional myocardial tube is induced by mechanical stretching due to the host blood flow pulsation within the lumen of the tube. From these findings, we assert that the layering of cardiomyocyte sheets presents a significant advantage over scaffold-based tissue engineering in the creation of cell-dense, functional myocardial tissues.[112]

5.6. Co-cultured Sheet of Hepatocyte and Endothelial Cells

Monocultured hepatocytes in vitro result significant loss in cell numbers and functional disappearance, thus can be maintained less than ten days.[114] We have approached this problem by preparing co-cultured cell sheet of hepatocytes and endothelial cells using patterned dual thermo-responsive surfaces. Copolymerization of n-butyl methacrylate (BMA) and PIPAAm decreases hydrophobic-hydrophilic transition
temperature of thermo-responsive surface. Therefore, patterned copolymerization of BMA on PIPAAm grafted surfaces realizes patterned dual thermo-responsive surfaces. Hepatocytes are seeded onto the patterned surfaces and cultured at 27 °C for two days. Hepatocytes adhere only onto relatively hydrophobic P(IPAAm-BMA) co-grafted domains, since BMA units restrict PIPAAm chain mobility, concomitantly increase chain hydrophobicity. Hepatocytes slowly proliferate within the P(IPAAm-BMA) co-grafted domains after an additional two days culture by raising temperature to 37 °C to promote seeded hepatocytes spreading. Endothelial cells were then seeded onto hydrophobized PIPAAm grafted regions at 37 °C, achieving successful co-culture of hepatocytes and endothelial cells in monolayer patterns (Fig. 8). Co-cultured cell monolayers completely detach from the patterned dual thermo-responsive dishes as contiguous cell sheets by decreasing temperature to 20 °C. Co-cultured hepatocytes and endothelial cells on the patterned surfaces remain connected with each other with intact extracellular matrices secreted below them. Hepatic cellular life and functions such as albumin secretion and ammonium metabolism are proved to be enhanced by the hepatocyte-endothelial cell interactions. The smaller the patterns, higher hepatic functions are observed, indicating the heterotypic cell-to-cell communications act in a paracrine manner. Therefore, increasing the boundary between hepatocytes and endothelial cells is important for the reconstruction of liver tissue. We have also attempted double-layered co-culture overlaying endothelial cell sheet onto hepatocyte monolayer. The double-layered structure of endothelial cells and hepatocytes remained in tight contact, and maintained the differentiated cell shape and the albumin expression for over 40 days of culture. This new co-culture method is valuable for constructing highly functioning stratified tissues, such as liver lobule.

6. Future Prospects

Lack of sources for human cells is a crucial problem for the common application of tissue engineering. Autologous cell source should be used to avoid immunological rejection. Further advance in embryonic stem cell technology may allow for the provision of various cell types, but ethical considerations still arise. Our application of autologous oral mucosal epithelium for the treatment of corneal disease is a novel concept because an alternative autologous cell source is prepared from small, relatively non-invasive biopsies. We have also applied skeletal myoblasts for the treatment of damaged hearts. The use of allogeneic feeder cells and fetal bovine serum may also be excluded for the preparations of cell sheets. Our new temperature-responsive culture inserts having submicron-scale pores successfully avoided the use of feeder layer and fetal bovine serum. If absolute procedures for the preparation of cell source are developed, some obstacles must be overcome for the creation of three-dimensional functional organ-like structures. “Cell sheet technology” has many advantages over conventional tissue engineering approaches. When thick and large tissue graft is constructed, generation of necrotic cores due to limited passive diffusion of oxygen, nutrients and metabolic wastes is a critical problem. Thus, effective vascular reconstruction is the key concept to make thicker tissue grafts with improved cell viability especially for large oxygen consuming cells such as cardiomyocytes. Myocardial tissue grafts engineered with cell sheet technology have their own inherent potential for the neovascularization in vivo. Polysurgery allows creating of thick, viable and functional myocardial tissue with well-organized microvessels. Moreover, heterogeneous layering of endothelial cell sheets between cardiomyocyte sheets or patterned co-culture sheet of cardiomyocytes and endothelial cells may promote neovascularization. Interactions between endothelial and cardiomyocyte cells also promote cell survival and proliferation. Mixing of human embryonic stem cell-derived cardio-

![Figure 8. Schematic representation of a method for patterning cell co-culture and harvesting of co-cultured cell sheets using a dually patterned surface. First cell type, hepatocytes is seeded and cultured at 27 °C, resulting in localization of hepatocytes onto P(IPAAm-BMA) co-grafted islands showing hydrophobic nature (A). Second cell type, endothelial cells seeded and cultured at 37 °C, results in generation of patterned co-cultures (B). Decreasing temperature to 20 °C induces detachment of co-cultured cell sheet (C, D). Reproduced with permission from [115]. Copyright 2006 Elsevier Ltd.](image-url)
myocytes and embryonic fibroblast enhanced generation of significant capillary network of the engineered cardiac tissue. An encapsulated vascularized tissue graft has been generated in vivo by placement of an arteriovenous blood vessel loop inside a semi-sealed polycarbonate chamber that is implanted into the groin of a rat. The encapsulated tissue graft is transplantable to other parts of the body, or possibly to an extracorporeal circulation in vitro. Although neonatal rat cardiomyocytes were seeded into the chamber with Matrigel at the time the chamber is implanted, such an approach may cause problems commonly found when biodegradable scaffolds are used. Our technology of layering cell sheets without the use of biodegradable scaffolds can more closely resemble the cell-dense architecture of native tissues.

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