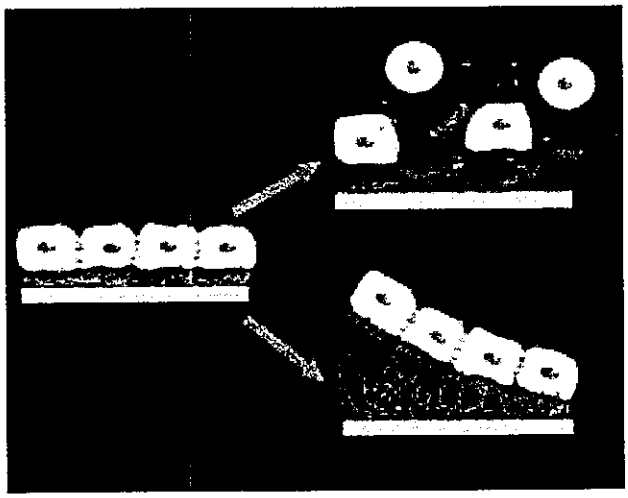

Fig. 2 Cell sheet harvest. Trypsin degrades deposited ECM (green), as well as membrane proteins, so that confluent, monolayer cells are harvested as single cells (upper right). The temperature-responsive polymer (orange) covalently immobilized on the dish surface hydrates when the temperature is reduced, decreasing the interaction with deposited ECM. All the cells connected via cell-cell junction proteins are harvested as a single, contiguous cell sheet without the need for proteolytic enzymes (lower right).

ECM[6] (Fig. 2). Harvested, viable cell sheets can be transferred to other culture dishes *in vitro*[7] or to tissue surfaces *in vivo*. We call this two-dimensional cell sheet manipulation. Since the ECM associated with the basal side of the cell sheets shows adhesion, the harvested cell sheets can be stratified to reconstruct thicker or more complex tissue architectures, such as cardiac muscle, liver lobule, and kidney glomeruli (three-dimensional cell sheet manipulation). In the following sections, we demonstrate how these cell sheets can be used in regenerative medicine.

Ocular surface regeneration

Our first clinical application of cell sheets harvested from temperature-responsive culture dishes used epidermal cell sheets. Human epidermal cell sheets prepared using temperature-responsive culture dishes are less fragile and show better adhesion to wound beds than similar cell sheets harvested by conventional dispase treatment. Immunoblotting reveals that dispase degrades cell-cell junction proteins and the ECM, but these proteins remain intact in our harvesting method[8].

Noninvasive cell sheet harvest and transplantation using temperature-responsive culture surfaces has also been applied to ocular surface regeneration[9,10]. Corneal epithelial stem cells are known to localize in the limbus, the border area between the cornea and conjunctiva. Ocular trauma, such as alkali burns and severe ocular diseases including Stevens-Johnson Syndrome and ocular pemphigoid, cause corneal opacification and visual loss because of limbal stem cell deficiency. Although corneal transplantation is required in these cases, the number of donor corneas is very limited in Japan and some European countries. Limbal stem cells were isolated and expanded on temperature-responsive culture dishes at 37°C in order to treat these patients (Fig. 3).

Multilayered corneal epithelial cell sheets are harvested intact simply by reducing the temperature to 20°C without the use of proteases. Cell-cell junctions and the ECM on the basal side of the sheet, critical to sheet integrity and function, remain intact. A viable population of corneal progenitor cells, close in number to that originally seeded, is found in the sheets by colony-forming assay. Harvested sheets are easily manipulated, less fragile, transplantable without any carriers, and readily adhere to corneal stroma so that suturing is not required.

In all cases, significant improvement of visual acuity can be observed (Fig. 4). In our experience, 2 mm x 2 mm of limbal tissue biopsy is sufficient for a single recipient, suggesting more than 20 patients can be transplanted with corneal epithelial cell sheets using stem cells prepared from a single donor eye. We are also working with corneal endothelial cell sheet transplantation, as well as retina pigmented epithelial cell sheet transplantation, in animal models. The use of biodegradable polymer scaffolds should be

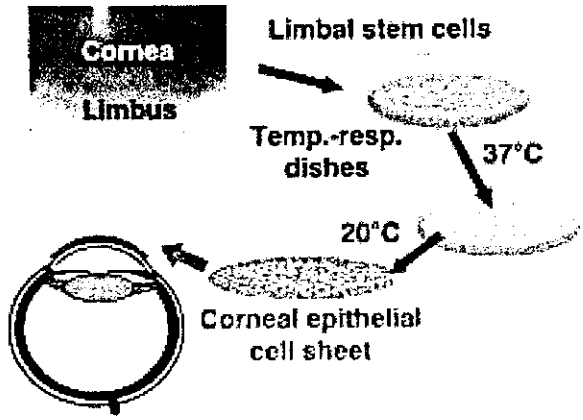

Fig. 3 Corneal epithelial cell sheet transplantation. Limbal stem cells are isolated from a small limbal tissue biopsy and cultured on temperature-responsive culture dishes at 37°C. Transplantable corneal epithelial cell sheets are harvested by reducing the temperature to 20°C and grafted onto a damaged cornea.

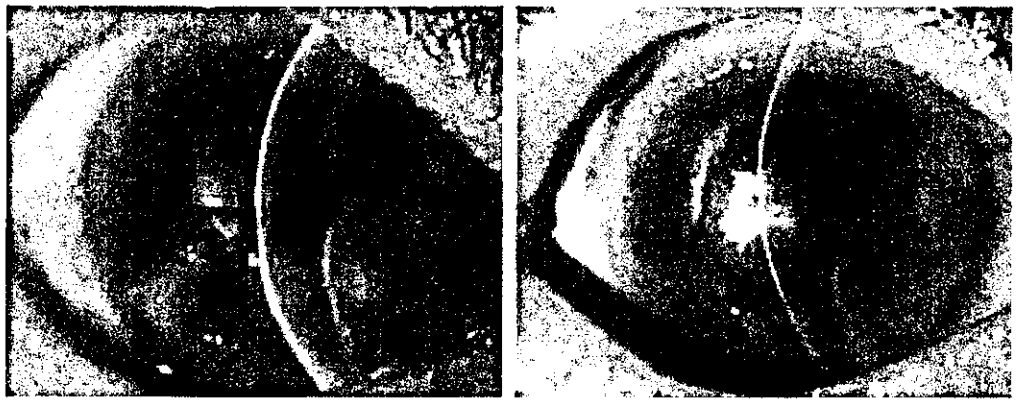

Fig. 4 Corneal regeneration. A patient suffering from Saltzman syndrome was transplanted with a corneal epithelial cell sheet. Photographs were taken before (left) and after (right) the surgical operation.

avoided in these cases because of the optical transparency often required in ocular tissue regeneration.

Periodontal regeneration

Periodontal diseases are very common in the elderly. Since conventional methods are insufficient to attain complete and reliable clinical regeneration of periodontal tissues, patients suffer from periodontitis, halitosis, and tooth loss. We have applied cell sheet engineering to this problem (Fig. 5). Human periodontal ligament cell sheets harvested from temperature-responsive culture dishes were transplanted into a mesial dehiscence model (where the gum has pulled away from the front of the tooth) in athymic rats (where the cell sheets will not be rejected) to examine whether these cell sheets can regenerate periodontal tissues. In this study, periodontal ligament-like tissues, which include an acellular cementum-like layer and fibrils anchoring into this layer, were identified (Fig. 6). The fibril anchoring resembles native periodontal ligament fibers. Such regeneration was not observed in nontransplanted controls. These results suggest that this technique could be useful in periodontal tissue regeneration.

Bladder augmentation

In bladder augmentation cystoplasty using gastrointestinal flaps, severe complications such as lithiasis, urinary tract infection, and electrolyte imbalance are often induced.

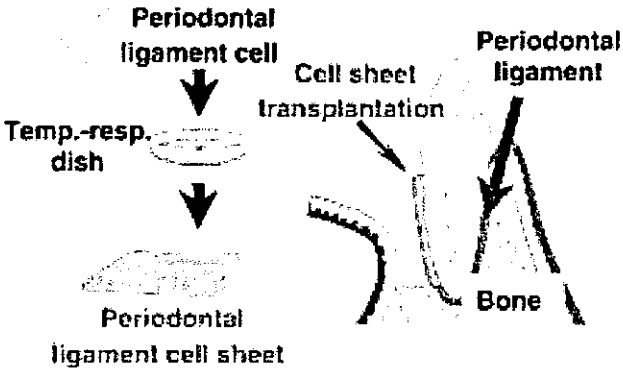

Fig. 5 Periodontal ligament cell sheet transplantation. Human periodontal ligament cells are isolated from an extracted tooth and cultured on temperature-responsive culture dishes at 37°C. Transplantable cell sheets are harvested by reducing temperature to 20°C, and grafted onto an athymic rat periodontitis model.

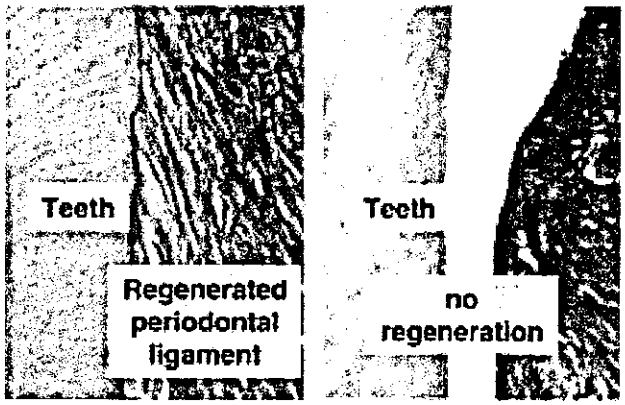

Fig. 6 Periodontal ligament regeneration. Four weeks after transplantation, periodontal ligament tissues have regenerated (left). No regeneration was observed in the control without cell sheet transplantation (right).

All these complications result from gastrointestinal mucosa in the flaps. We have developed a novel augmentation cystoplasty using gastrointestinal flaps and cultured urothelial cell sheets (Fig. 7). Gastrointestinal mucosa in the flaps is replaced with urothelial cell sheets, which have been expanded on temperature-responsive culture dishes from an autologous small biopsy. We are now working with a canine model[11,12]. Stratified urothelial cell sheets are cultured and then harvested intact from these dishes on reducing the temperature. Electron microscopy and immunoblotting reveal well-developed microridge, microvilli, and cell-junction complexes. The intact urothelial cell sheets are then autografted onto demucosalized gastric flaps. Urothelial cell sheets spontaneously attach to the demucosalized tissue surfaces completely, without any suturing or fixing. Three weeks after autografting, dogs were sacrificed and the gastric flaps with urothelial cell sheets were examined. Viable urothelial regeneration was observed in the development of a stratified viable epithelium similar to native urothelium (Fig. 8). As shown in this urological study, cell sheets harvested from temperature-responsive culture dishes can be a powerful tool in reconstructive surgery. This versatile technology should prove useful in various surgical reconstructions.

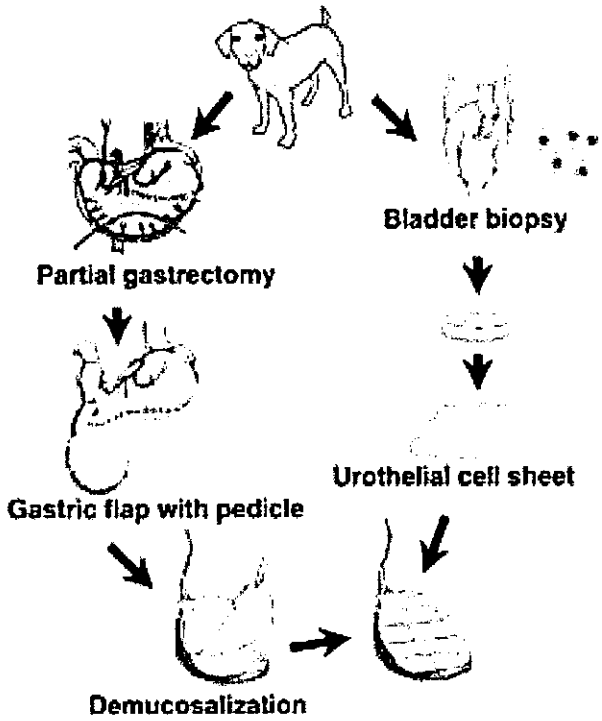

Fig. 7 Bladder augmentation with urothelial cell sheets. Conventional bladder augmentation procedure is modified with the use of urothelial cell sheets. Gastric flaps are demucosalized and harvested urothelial cell sheets are grafted onto the bare smooth muscle layers. These constructs are then used in bladder augmentation. (Reprinted with permission from[12]. © 2004 Blackwell Publishing Ltd.)

Cardiac patches

In addition to two-dimensional cell sheet manipulation, three-dimensional cell sheet manipulation has been used in cardiac tissue engineering. Recent progress in cell transplantation therapy to repair impaired hearts has encouraged further attempts to bioengineer three-dimensional heart tissue from cultured cardiac myocytes. Cardiac tissue engineering has also been pursued using conventional technology with biodegradable polymer scaffolds as a temporary ECM. However, the inflexible and

Fig. 8 Urothelium regeneration. Four weeks after surgery, urothelium has regenerated in the urothelial cell sheet-grafted group (left). No urothelial regeneration is observed in the control without cell sheet graft (center). The regenerated urothelium is similar to native ureter (right). (Reprinted with permission from[12]. © 2004 Blackwell Publishing Ltd.)

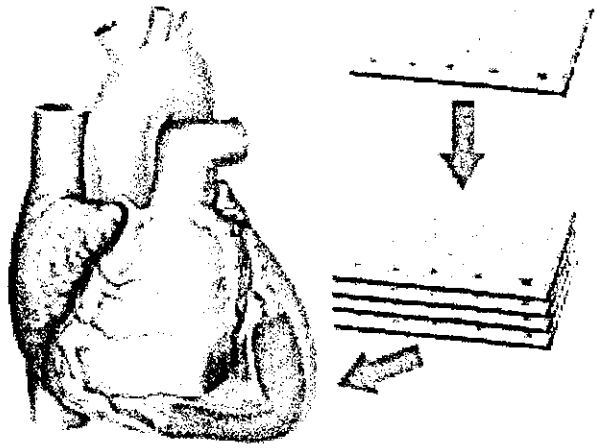

Fig. 9 Cardiac patch cell sheet engineering. Cardiac myocyte sheets are harvested from temperature-responsive culture dishes. Four cell sheets are then stratified and transplanted to ischemic hearts as cardiac patches.

bulky properties of the scaffolds significantly hamper the dynamic pulsation of cardiac myocytes. We have developed a new method to fabricate pulsatile cardiac patches by cell sheet engineering, layering several cell sheets three-dimensionally (Fig. 9). Neonatal rat cardiac myocyte sheets are harvested from temperature-responsive culture dishes by reducing the temperature and then overlaid to construct cardiac grafts[13-15]. Layered cell sheets begin to pulse simultaneously and morphological communication via connexin 43 is established between the sheets. When four sheets are layered, the engineered construct can be seen to pulse spontaneously with the naked eye. These cardiac patches were transplanted into subcutaneous tissues of nude rats. Three weeks after transplantation, surface electrograms originating from transplanted grafts were detected and spontaneous beating was macroscopically observed. Histological studies show characteristic structures of heart tissue and multiple neovascularization within contractile tissues. Long-term survival of pulsatile cardiac grafts has been confirmed more than one year later. These results demonstrate that electrically communicative, pulsatile three-dimensional cardiac constructs can be achieved, both *in vitro* and *in vivo*, by layering cardiomyocyte sheets. We are now working with cell sheet patches fabricated with autologous skeletal myoblasts in large animal models. Cardiac tissue engineering based on this technology may prove useful for heart model fabrication and cardiovascular tissue repair.

Conclusion

The cell sheet manipulation techniques described here can be applied to many types of cell and tissue structures, including tubes, bags, and solid masses. We believe that two- and three-dimensional cell sheet manipulation – cell sheet engineering – should prove useful as a fundamental, generalized technique in next-generation tissue engineering and regenerative medicine. MT

Acknowledgments

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Cell-Sheet Engineering Using Intelligent Surfaces

Joseph Yang, Masayuki Yamato, and Teruo Okano

Abstract

The possibility of recreating various tissues and organs for the purpose of regenerative medicine has received much interest. However, the field of tissue engineering has been restricted by the limitations of conventional approaches. A method to circumvent the need for traditional scaffold-based technologies is cell-sheet engineering, which uses temperature-responsive culture dishes. These surfaces, which are created by grafting the temperature-responsive polymer poly(*N*-isopropylacrylamide) onto ordinary culture dishes, enable the non-invasive harvesting of cells as intact sheets by simple temperature reduction. This article reviews current research on the applications of cell-sheet engineering for the reconstruction of various tissues, as well as the intelligent surfaces used by this novel technology.

Keywords: cell-sheet engineering, intelligent surfaces, regenerative medicine, temperature-responsive polymer, tissue engineering.

Introduction

At the beginning of the 21st century, it is almost certain that tissue engineering will soon be able to reconstruct various tissues and organs for the purpose of regenerative medicine. During the past 20 or so years since Joseph P. Vacanti first proposed the concept,[1,2] the field of tissue engineering has progressed significantly. The current paradigm is to construct scaffolds from biodegradable polymers, into which cells can be seeded. It is thought that seeded cells can proliferate and deposit extracellular matrix (ECM) molecules such as collagen and fibronectin, eventually regaining their native structure and tissue morphology upon scaffold degradation.[3]

Using these methods, there has been some success in developing cell-sparse tissues (having large amounts of ECM and relatively few cells) such as heart valves,[4] bone,[5] and cartilage,[6] as evidenced by Vacanti's group, who were able to reconstruct human ears on the backs of mice[7] (see the article by C.A. Vacanti in the October 2001 issue of *MRS Bulletin*).

However, the use of these polymer scaffolds can have undesirable consequences. First, inflammatory reactions often occur because of the implantation of a non-natural device or the incomplete degradation of the polymer scaffold. Second, with scaffold degradation, the space formerly occupied by the polymer is usually filled by cells and large amounts of deposited ECM, which can lead to a pathological state of fibrosis that poorly resembles the native tissue structure. In addition, it is commonly seen that cells on the periphery of the scaffolds are maintained and more closely resemble native tissues, whereas there is significant necrosis of cells at the interior, because restrictions on passive diffusion limit both the delivery of nutrients and the removal of metabolic wastes. Therefore, current technologies are still severely lacking in terms of achieving successful tissue reconstruction.

Cell-Sheet Engineering: A Novel Method

Generally speaking, the architecture of many tissues, such as the heart or liver, consists of closely associated cells with comparatively little associated ECM. To recreate these tissues, it is necessary to engineer cell-dense structures that mimic normal structure and function. For this purpose, we have developed a new approach: cell-sheet engineering.

At the heart of this technology is the use of temperature-responsive culture dishes (Figure 1).[8,9] To create these surfaces, the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) is covalently grafted onto normal tissue-culture polystyrene (TCPS) dishes, with radical polymerization initiated by electron-beam irradiation. PIPAAm-grafted culture surfaces enable the control of cell adhesion with simple temperature changes, by exploiting the significant property changes of the polymer across its lower critical solution temperature (LCST) of 32°C. In culture conditions at 37°C, the culture surface is slightly hydrophobic, allowing cells to adhere and grow similarly to normal TCPS dishes. After the necessary culture period, the temperature is lowered to 20°C, causing the polymer to become hydrophilic. Upon this transition, the polymer swells, and a hydration layer is created at the cell–surface interface so that cells detach spontaneously, enabling them to be harvested as intact sheets.[10] During cell harvesting, PIPAAm remains on the culture dish surface because the temperature-responsive polymer is covalently bonded to the dish. Normally in cell-based therapies, including tissue engineering, cells are grown on TCPS dishes and harvested using proteolytic enzymes such as trypsin or dispase (to degrade the adhesive molecules and ECM that attach cells to the culture surface). However, treatment with these enzymes also degrades cell–surface molecules, including growth factor receptors, ion channels, and cell-to-cell junction proteins that are vital for the differentiated functions of many cell types. Using temperature-responsive surfaces, we avoid the need for such proteolytic enzymes; thus, crucial cell-to-cell and cell-to-ECM interactions are preserved.[10]

Using this non-invasive cell harvesting method, intact cell sheets can be recovered along with their deposited ECM on the basal surface. Cells harvested in this fashion can then be transferred to other surfaces, such as new culture dishes,[11,12] other cell sheets,[13] and even directly to host tissues.[14–17] The deposited ECM acts as a "molecular glue," providing direct contact and adhesion to these surfaces without the need for additional mediators such as carrier substrates or sutures.

Tissue reconstruction using cell-sheet engineering can be accomplished in three ways. First, we can transplant single cell sheets, for use as corneal surfaces[15,16] and periodontal ligament tissue,[18] as well as in the reconstruction of the bladder[17] and skin,[19] using

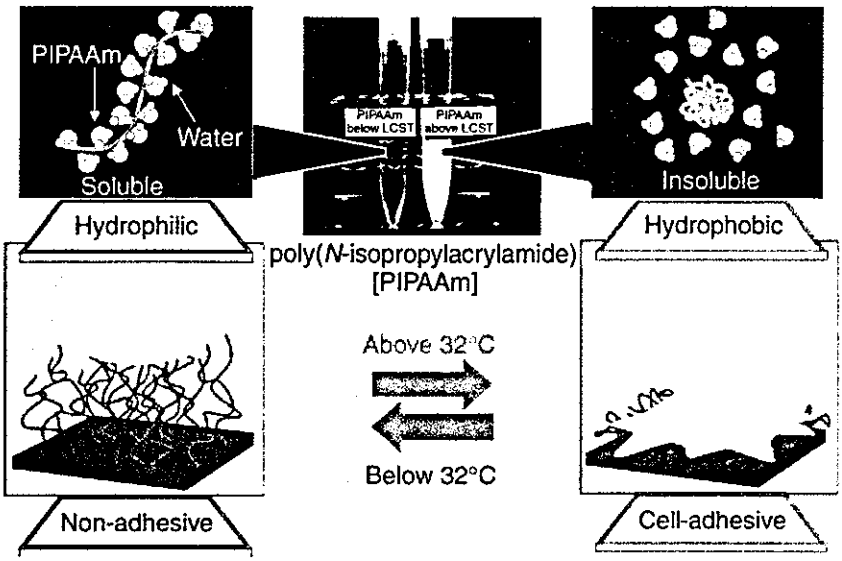

Figure 1. Temperature-responsive culture dishes. The temperature-responsive polymer poly(N-isopropylacrylamide) (PIPAAm) exhibits a transition from hydrophobic to hydrophilic across its lower-critical solution temperature (LCST) of 32°C. After electron-beam polymerization and grafting to normal tissue-culture polystyrene (TCPS) dishes, temperature-responsive culture surfaces can be produced. The non-invasive harvest of various cell types as intact sheets, along with deposited extracellular matrix, can be achieved by reducing the culture temperature.

two-dimensional manipulation. Second, we can recreate three-dimensional structures by homotypic layering of cell sheets, as in the case of cardiac muscle.[14] Third, using heterotypic stratification of different cell sheets, we can produce laminar structures to recreate liver lobules[13] or kidney glomeruli.

Using cell sheets, we are thus able to engineer various cell-dense tissues that demonstrate differentiated functions while altogether avoiding the need for biodegradable scaffolds, whose applicability is strictly limited.

Applications for Tissue Regeneration

Corneal Tissue

In the clinical setting, ocular trauma due to chemical or thermal burns, or severe disease, can lead to corneal opacification with accompanying loss of vision. In these cases, loss of corneal epithelial stem cells occurs; these stem cells are located in the limbus, the border between the cornea and neighboring conjunctiva. Corneal transplantation is an option, but because of a high risk of graft rejection and the limited supply of donor materials, treating these patients is still very difficult. To overcome these obstacles, small sections of limbal tissue are harvested, and the isolated stem cells are expanded *ex vivo* (Figure 2). Using this method, fabricated multilayered corneal epithelial cell sheets that resemble native corneal epithelium can be harvested by simple temperature reduction.[15] These cell sheets and their deposited ECM can be easily manipulated and adhere to the host cornea without sutures.

In contrast to cells harvested by treatment with dispase (an enzyme used in cell isolation and tissue dissociation), these engineered cell sheets are less fragile and contain cell-to-cell junction and ECM proteins that can be damaged by dispase. Additionally, this method enables the transplantation of corneal epithelial stem cells without the need for carrier substrates, such as amniotic membrane or fibrin gel, which can impair visual acuity. The subsequent degradation of carrier substrates may also invoke inflammatory responses that can cause microtrauma to the patient's ocular surface.

A biopsy of 2 mm × 2 mm is sufficient to create each limbal stem cell sheet, meaning that numerous patients could be treated from a single donor eye with general immunosuppressive drug treatment. We have also shown that cell sheets composed of oral mucosa epithelia from the patient's own mouth can be used, demonstrating a novel alternative cell source and eliminating the need for immunosuppression in treating patients who have damage to both corneas.[16] Oral mucosa epithelial cell sheets fabricated on the temperature-responsive dishes can be harvested and transplanted in the same manner as corneal epithelial sheets. Interestingly, oral mucosa epithelial sheets fabricated by this method more closely resemble native cornea epithelia than native oral mucosa epithelia. Results

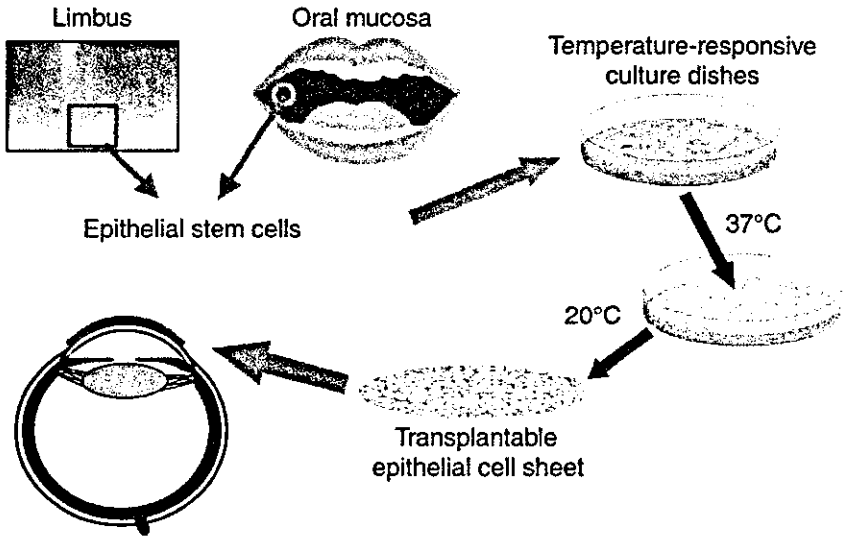

Figure 2. Corneal surface reconstruction. Small biopsies from the limbus (the border between the cornea and neighboring conjunctiva) or from oral mucosa provide for the isolation of epithelial stem cells. Cell sheets fabricated on temperature-responsive culture dishes can be harvested and transplanted directly to the ocular surface without the need for carrier substrates or sutures.

from all cases in human trials have demonstrated improved visual acuity based on eye chart tests, with all corneas maintaining a clear and smooth surface.[16]

Additionally, we are currently studying animal models for both corneal endothelial cell sheets and retinal pigment epithelial cell sheets. The use of carrier-free cell sheets is favorable for ocular applications, because it eliminates the need for additional substrates, scaffolds, or sutures that may impair vision.

Periodontal Tissue

Periodontal tissue attaches teeth to the jaw bones. Periodontal disease, frequently seen in older patients, can result in complications such as halitosis and tooth loss. Current methods generally result in incomplete regeneration of periodontal tissue because of the reliance on neighboring host cells to migrate into the defect to regenerate native tissue. To overcome these obstacles, human periodontal ligament cell sheets were fabricated on temperature-responsive dishes and grafted into an immunodeficient rat model in which the gum was pulled away from the tooth. Cell-sheet transplantation was able to recreate a tissue, with collegen fibrils anchored to an acellular cementum layer, that closely resembled native periodontal tissue.[18] In control experiments without transplantation, the periodontal tissue did not regenerate and the gap remained. From these promising results, human trials for periodontal regeneration are being initiated.

Bladder Reconstruction

Bladder augmentation is often necessary to treat patients suffering from bladder cancer or congenital defects. In these cases, current methods generally use segments of the gastrointestinal tract to reconstruct a portion of the bladder (or in some cases, the entire bladder). However, these methods have complications attributed to the presence of gastrointestinal mucosa, such as changes in the patient's physiological pH, stone formation, and urinary tract infection. Some researchers have therefore attempted to cover a demucosalized surface with urothelial grafts from the bladder mucosa,[20,21] but these methods have had mixed results and have not yet been clinically applied.

In a canine model, urothelial cells from a small bladder biopsy were cultured on temperature-responsive dishes.[17,22] Autologous urothelial sheets were then harvested and autografted onto demucosalized gastric flaps. Cell sheets harvested by low-temperature treatment attached to the host tissue without the need for sutures. After three weeks, the gastric flaps were examined, and results showed the development of a stratified epithelial layer similar to native urothelium. Proton pump (for the production of gastric acid), which is seen in native gastric mucosa, was not observed in the reconstructed epithelial cell layers. We believe that this approach, with improved reconstruction of functional urothelium tissue using cell sheets and demucosalized gastric flaps, will improve treatments for those patients suffering from bladder defects.

Heart Tissue

It is estimated that every year 20,000–40,000 patients in the United States alone could benefit from heart transplants. It is thought that these patients, along with a significant number of additional patients with diminished cardiac function, could benefit from tissue-engineered constructs.

Current cardiac tissue engineering methods focus either on injection of cell suspensions or scaffold-based constructs. However, both methods have significant drawbacks.

With direct injection of cell suspensions, the size, shape, and location of the cells are difficult to control, because of migration of the injected suspension. Additionally, in seriously damaged myocardium, the local structure generally cannot support the seeding of the cell suspension, resulting in fewer cells present at the injured site.

In native heart muscle, cells are significantly dense, with less ECM than tissues such as cartilage, which have been successfully recreated with scaffolds. Therefore, cardiomyocytes (the functional cells of heart muscle) on the periphery of the scaffold resemble native cells, while there is significant necrosis at the center of the scaffold attributed to diffusion limits.

Additionally, cells are generally not intimately connected throughout the construct, but are separated by large amounts of ECM, leading to less compact tissue with significantly lower contractile force as compared with native tissues.

In cardiac tissue, a major function is electrical transmission, so that cardiomyocytes pulsate synchronously. Creation and maintenance of cell-to-cell communication via gap junctions is integral to the development of functional tissue. The enzymatic digestion of cultured cells, necessary for the creation of cell suspensions for direct injection and scaffold seeding, severely disrupts these cell-to-cell junctions critical to cell communication and adherence.

Confluent cell monolayers can be harvested from temperature-responsive dishes without the need for enzymatic digestion, thus maintaining cell-to-cell communication. Harvested cardiomyocyte sheets can adhere to one another and beat spontaneously and simultaneously. Morphological studies showed that gap junctions are formed between cell sheets, and membrane bilayers attached to form homogeneous 3D tissue. When four cell sheets were layered, the simultaneous beating could be seen with the naked eye.[14] Additionally, when these four-layer constructs were implanted into the backs of immunodeficient rats, spontaneous, macroscopic beatings were seen and could be maintained for more than one year. Resected grafts revealed tissue with microstructures characteristic of native cardiomyocytes, as well as blood vessel formation with microvascular networks present.

The presence of adhesive proteins on the basal side of the cell sheets not only provides connection to other cell sheets, but also to host tissues. We believe that through the layering of multiple cardiomyocyte sheets, a sort of 3D "heart bandage" can be fabricated (Figure 3). Applying these electrically connected cell sheets to damaged heart tissue may soon enable surgeons to more successfully treat patients with diminished cardiac function due to myocardial infarction or congenital defects. However, as promising as results have been, the issue of cell source remains, as adult cardiomyocytes do not proliferate under current culture conditions.

Liver Tissue

In liver lobules, hepatocytes and sinusoidal endothelial cells are connected in a complex 3D architecture, and interactions between the different cell types are necessary for the differentiated functions of the liver. Tissue-engineered constructs containing only hepatocytes lose their differentiated cell shape and function after a very short period in culture. Other researchers have attempted co-culture techniques, but it remains difficult to control the patterning of the various cell types, especially in recreating a layered structure similar to native liver lobules.

To examine the role of endothelial cells in maintaining differentiated functions, endothelial cell sheets cultured on temperature-responsive dishes were overlaid on monolayer hepatocytes to mimic the *in vivo* liver architecture.[13] This co-culture system revealed hepatocytes that maintained differentiated cell shape and expression of albumin (a marker of hepatocyte function) for more than 40 days, whereas hepatocyte monolayers without interactions with endothelial cells lost albumin expression after only 10 days. We believe that this heterotypic stratification of cell sheets is not limited to the liver and could be applied to other organs with highly organized structures.

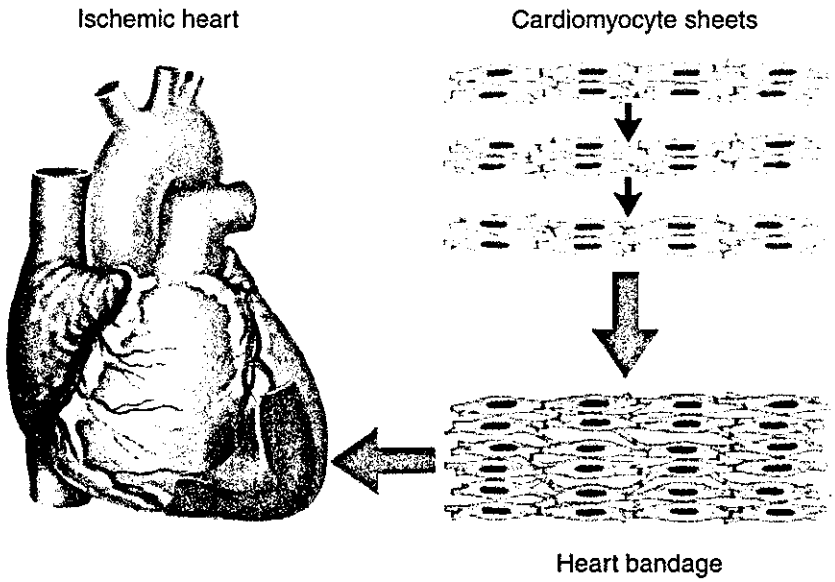

Figure 3. Myocardial cell-sheet engineering. Cardiomyocyte sheets harvested from temperature-responsive culture surfaces can be layered to form three-dimensional tissues that beat synchronously and simultaneously. We believe that layered cardiomyocyte sheets can act as a "heart bandage" for the recovery of ischemic cardiac tissue.

Intelligent Surfaces

Using the intelligent surface properties of temperature-responsive culture dishes, cell-sheet engineering has been used to reconstruct various tissues without the need for biodegradable scaffolds. These temperature-responsive surfaces are being modified to further expand their applicability.

Immobilization of Cell-Adhesive Peptides

Current methods for cell culture generally use animal-derived products such as fetal bovine serum or mouse 3T3 feeder cells to enhance cell attachment and growth. However, due to safety issues attributed to the risk of pathogen transmission, the use of these products is best avoided. Additionally, the U.S. Food and Drug Administration classifies tissue-engineered constructs co-cultured with animal cells such as mouse 3T3 cells as xenografts, thus delaying their clinical application.

To overcome the need for serum, we immobilized the synthetic adhesive peptide Arg-Gly-Asp-Ser (RGDS) onto temperature-responsive dishes. The temperature-responsive PIPAAm surfaces were functionalized by copolymerization with a reactive comonomer containing a free carboxyl group, after which the synthetic RGDS peptide could be immobilized on the temperature-responsive surface.[23,24] Cells attach, spread, and grow to confluency at 37°C, even in serum-free conditions supplemented with recombinant growth factors. After reaching confluency, cells can be harvested as intact sheets by simple temperature changes, in the same manner as normal PIPAAm dishes. Cells cultured on these dishes in serum-free conditions resemble cells cultured in a medium containing 10% fetal bovine serum. In addition, cell harvest from these surfaces is achieved by controlling the binding affinity between the RGDS ligand and cell–surface integrin proteins, with the RGDS ligand remaining on the culture surface after cell sheet removal (Figure 4).

Furthermore, work is under way on immobilizing other growth factors to temperature-responsive surfaces. Copolymerization with other functionalized comonomers containing amino or hydroxyl groups will enable the attachment of various ligands (Figure 5). We believe this will eliminate the need for other animal-derived products such as feeder cells and will increase cell growth such that the length of culture can be dramatically decreased.

Micropatterned Surfaces

To mimic normal function, it is necessary to recreate a 3D tissue architecture with the integration of multiple cell types. As shown with liver reconstruction, interactions between various cell types are needed to preserve differentiated cell functions. Layering of various patterned cell sheets is thought to be able to create structures that resemble normal tissues. However, the creation of micropatterned surfaces is generally very complex and must exploit differences in cell adhesion for co-culture, thus significantly limiting applicability. Therefore, we created a novel method for micropatterned cell seeding.[25,26]

Using metal micropatterned masks, we can selectively graft PIPAAm onto TCPS dishes and create surfaces with controlled, localized temperature-responsive regions. Hepatocytes seeded at 20°C, a temperature at which PIPAAm-grafted portions are hydrophilic, attached only to ungrafted TCPS portions and conformed to the pattern. After five hours, the culture temperature was raised to 37°C, and fibroblasts seeded at this time attached only to the PIPAAm-grafted regions, creating and maintaining a micropatterned co-culture system. This technique conceivably enables the facile design and creation of micropatterned cell sheets that can be manipulated to create 3D structures that mimic normal tissues, if the surfaces can be grafted with different temperature-responsive polymers.

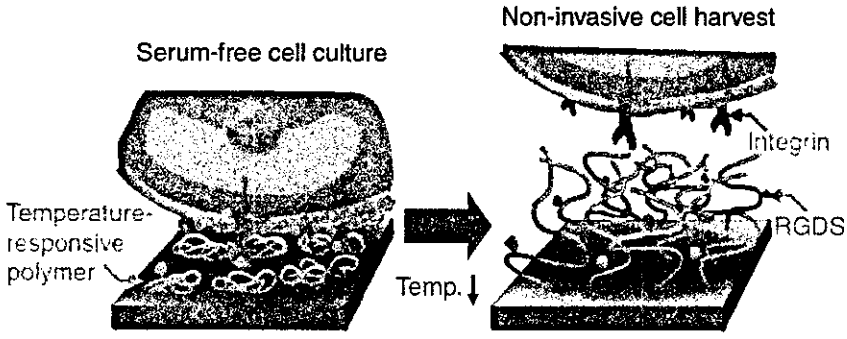

Figure 4. Immobilization of Arg-Gly-Asp-Ser (RGDS) peptides to temperature-responsive surfaces. Cells can be cultured in serum-free conditions by immobilizing the synthetic cell-adhesive RGDS peptide to temperature-responsive culture dishes. By decreasing the culture temperature, cells can still be non-invasively harvested, while the RGDS peptides remain attached to the temperature-responsive polymer surface.

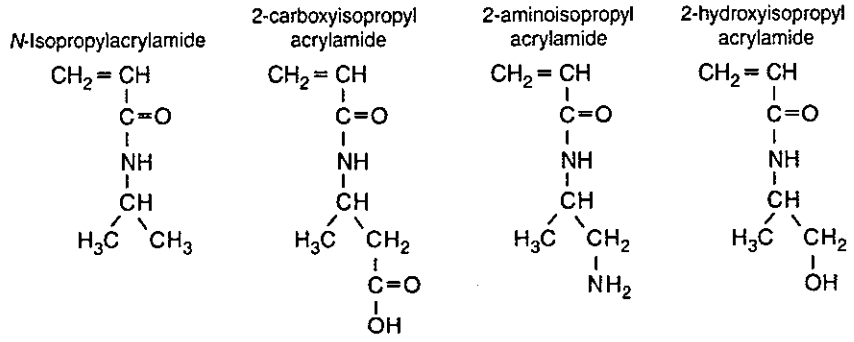

Figure 5. Synthesis of functionalized temperature-responsive copolymers. Various functionalized comonomers, such as 2-carboxyisopropyl acrylamide, 2-aminoisopropyl acrylamide, and 2-hydroxyisopropyl acrylamide, can be synthesized. These comonomers contain the isopropylacrylamide backbone chain of poly(N-isopropylacrylamide) (PIPAAm) and reactive pendant groups. These functionalized PIPAAm derivatives show sensitive phase transitions in response to temperature change similar to PIPAAm, enabling the immobilization of various ligands to the temperature-responsive surfaces.

We have recently shown that by incorporating a hydrophobic monomer, *n*-butyl methacrylate, into the PIPAAm feed, we can systematically lower the LCST of the copolymers.[27] Using this technique, it is possible to create micropatterned surfaces with different temperature-responsive domains across the entire culture surface, providing the intact harvest of cell sheets with various cell types. We are currently using this approach to create cell sheets consisting of cardiomyocytes or hepatocytes co-cultured with endothelial cells to mimic microvascular networks within the tissues.

Conclusions

Although the field of tissue engineering has made significant advances during the past 20 years, there still remains considerable difficulty in recreating many tissues and organs because of the limitations of traditional scaffold-based methods. We believe that cell-sheet engineering, which utilizes temperature-responsive intelligent surfaces, will overcome the problems that have limited conventional approaches in the past and establish a new basis for regenerative medicine.

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