Cell sheet engineering

by Masayuki Yamato and Teruo Okano

We have developed 'cell sheet engineering' in order to avoid the limitations of tissue reconstruction using biodegradable scaffolds or single cell suspension injection. Our concept is tissue reconstruction, not from single cells, but from cell sheets. Cell sheets are prepared using temperature-responsive culture dishes. Temperature-responsive polymers are covalently grafted onto the dishes, allowing various types of cells to adhere and proliferate at 37°C. The cells spontaneously detach when the temperature is reduced below 32°C without the need for proteolytic enzymes. The confluent cells are noninvasively harvested as single, contiguous cell sheets with intact cell-cell junctions and deposited extracellular matrix (ECM). We have used these harvested cell sheets for various tissue reconstructions, including ocular surfaces, periodontal ligaments, cardiac patches, and bladder augmentation.

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In the 21st century, novel therapeutics will be established. Controlled drug delivery has already been commercialized for limited uses. Gene therapy in human patients is being investigated experimentally. Furthermore, robotic surgery systems, as well as computer-aided surgery navigation systems, are commercially available. Tissue engineering was first proposed in the 1980s by a chemist, R. Langer, and a surgeon, J. P. Vacanti[1]. The key technology is the use of biodegradable polymer scaffolds, preformed in the target tissue shape, for cell seeding[2], as demonstrated in the well-publicized reconstruction of cartilage tissues for the growth of human ears on mice. By combining preformed biodegradable polymer scaffolds and specific cell types, various tissues including cartilage, bone, and blood vessels have been reconstructed, although, so far, therapeutic use has been very limited.

After transplantation of tissue-engineered constructs into hosts, the scaffolds degrade over weeks or months. The space formerly occupied by the scaffold is filled with proliferated cells and/or deposited ECM, such as collagen. However, ECM deposition in tissues often results in fibrosis, a pathological state. Furthermore, various properties of the scaffold can be undesirable in some tissues. For example, the opacity and inflexibility of polymer scaffolds inevitably impair

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ophthalmological and cardiac tissue reconstruction, respectively.

With the rapid progress in the understanding of stem cell biology during the 1990s, the clinical use of stem cells seems to be promising. Recently, novel therapeutics using tissue engineering and stem cell technology, termed regenerative medicine, have been investigated. In experimental transplantation into small animals, such as rats and mice, the injection of single cell suspensions sometimes works. However, single cell suspension injection does not seem to be suitable for large tissue reconstruction, since only a few percent of the injected cells are integrated into host tissues. It is apparent that we currently lack successful methods of tissue reconstruction. This is the reason why we have proposed 'cell sheet engineering'. As biodegradable polymers are the key technology in first-generation tissue engineering, we are developing temperature-responsive culture dishes for the next generation.

Temperature-responsive culture dishes

Histologically, parenchyma (tissue characteristic of an organ) comprise intimately associated cell sheets. For example, the liver comprises sheets of hepatocytes and endothelial cells that are interconnected to form a continuous three-dimensional tissue lattice. Cooperation between several types of cell sheets has been revealed to be essential in tissue functions that are damaged in pathological conditions. Such observations are also supported by developmental biology, since every organ originates from three different cell sheets called the endoderm, mesoderm, and ectoderm. The cell sheets interact with each other in the development of organs. These considerations have encouraged us to initiate cell-sheet-based tissue reconstruction.

Conventionally, cells are harvested using proteolytic enzymes such as trypsin and dispase. These enzymes degrade cell adhesion molecules and the deposited ECM to detach cultured cells. But at the same time, cell-cell junction proteins, as well as receptor proteins expressed on the cell membrane, are often damaged. Harvest of cultured cell sheets, therefore, is only achieved with exceptional cell types whose cell-cell junctions are less susceptible to such enzymes. In order to solve this problem, we first developed temperature-responsive culture dishes[3]. A temperature-responsive polymer, poly(*N*-isopropylacrylamide), is covalently grafted onto a culture dish surface. A grafted polymer layer of ~20 nm thickness allows control of temperature-responsive cell adhesion/detachment (Fig. 1). At 37°C, the surface is relatively hydrophobic, similar to commercially available tissue culture dishes, but becomes hydrophilic below 32°C. Various cell types adhere, spread, and proliferate on the surface at 37°C. On reducing the temperature to below 32°C, cells spontaneously lift up from the surface without the need for trypsin. Highly trypsin-susceptible cells, such as hepatocytes[4] and glial cells[5] retain their differentiated native cell functions after this noninvasive cell harvest.

Confluent cells are recovered as a single contiguous cell sheet with intact cell-cell junctions and deposited

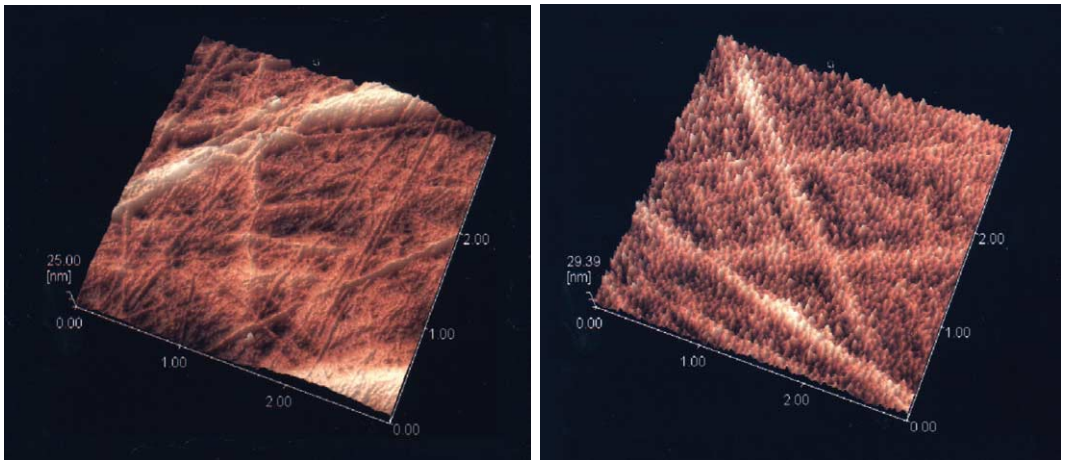

Fig. 1 Atomic force microscope images of temperature-responsive culture dish surfaces. Nongrafted, polystyrene culture dish surfaces (left) and poly(N-isopropylacrylamide)-grafted culture dish surfaces (right) were examined in air. Defects and scratches were observed on both surfaces. Small prickles can be seen on the grafted surface. Image size is 3 μm x 3 μm. Height is 25 nm (left) and 30 nm (right).

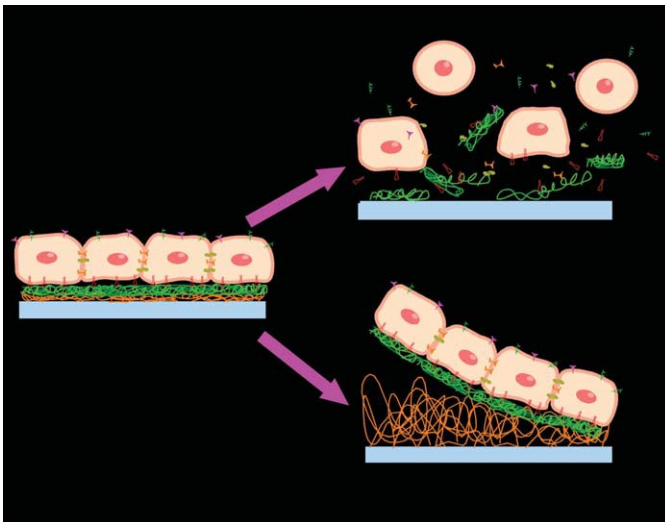

Fig. 2 Cell sheet harvest. Trypsin degrades deposited ECM (green), as well as membrane proteins, so that confluent, monolayer cells are harvested as single cells (upper right). The temperature-responsive polymer (orange) covalently immobilized on the dish surface hydrates when the temperature is reduced, 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

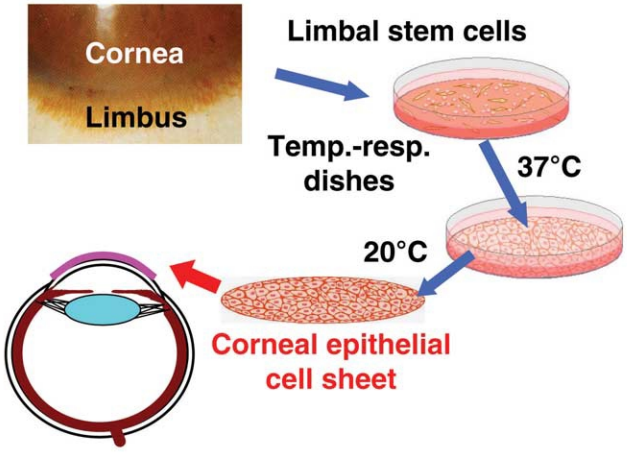

Fig. 3 Corneal epithelial cell sheet transplantation. Limbal stem cells are isolated from a small limbal tissue biopsy and cultured on temperature-responsive culture dishes at 37°C. Transplantable corneal epithelial cell sheets are harvested by reducing the temperature to 20°C and grafted onto a damaged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

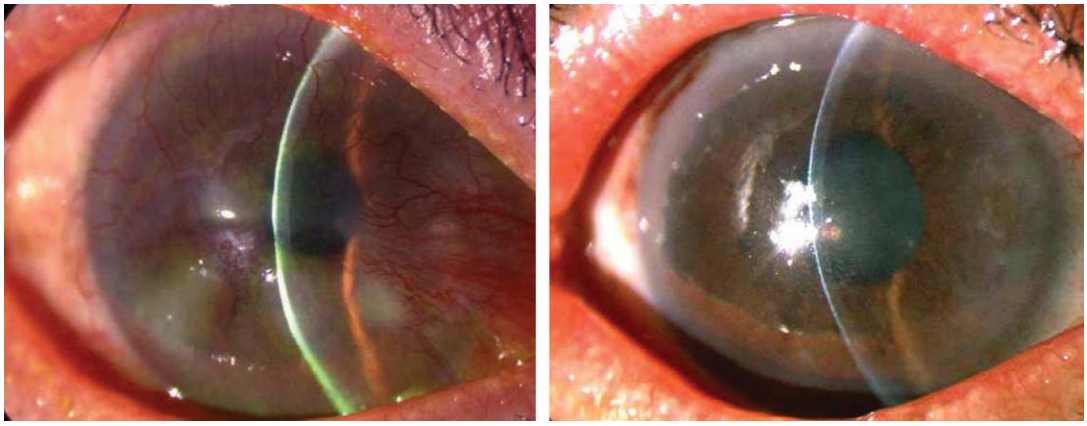

Fig. 4 Corneal regeneration. A patient suffering from Saltzman syndrome was transplanted with a corneal epithelial cell sheet. Photographs were taken before (left) and after (right) the surgical operation.

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.

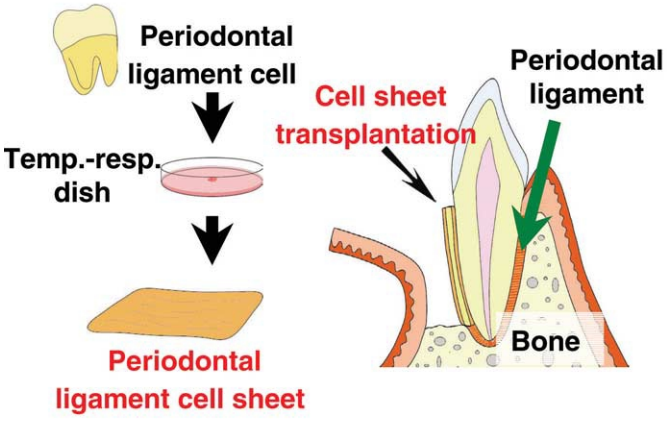

Fig. 5 Periodontal ligament cell sheet transplantation. Human periodontal ligament cells are isolated from an extracted tooth and cultured on temperature-responsive culture dishes at 37°C. Transplantable cell sheets are harvested by reducing temperature to 20°C, and grafted onto an athymic rat periodontitis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

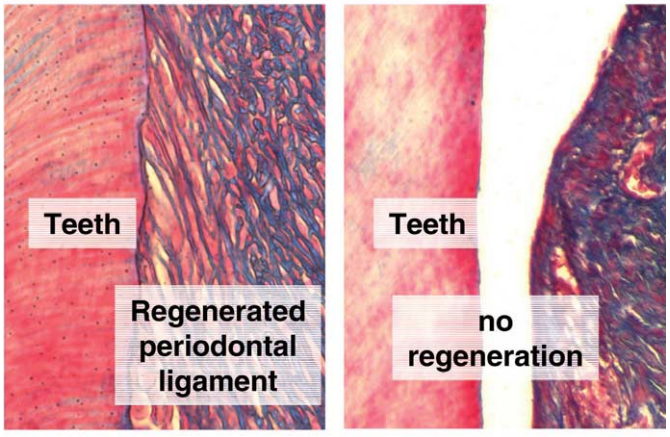

Fig. 6 Periodontal ligament regeneration. Four weeks after transplantation, periodontal ligament tissues have regenerated (left). No regeneration was observed in the control without cell sheet transplantation (right).

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.

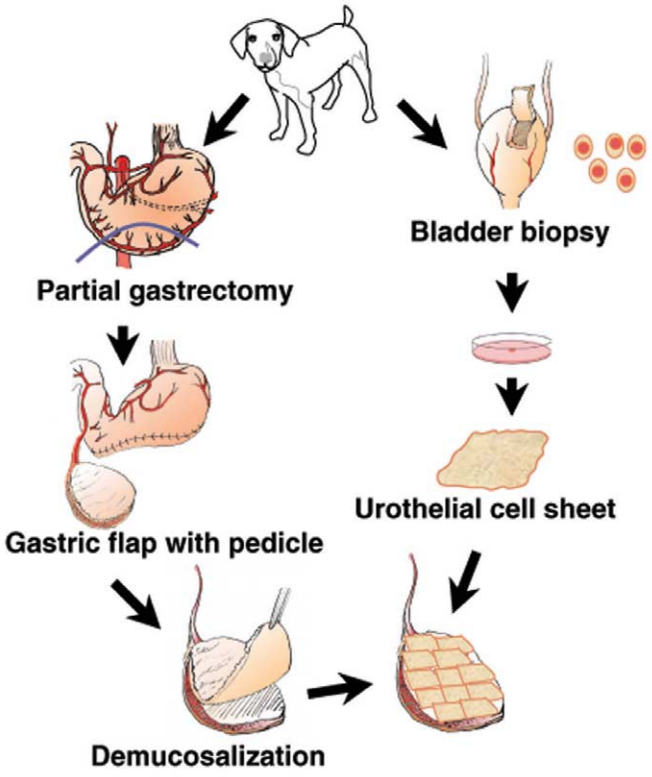

Fig. 7 Bladder augmentation with urothelial cell sheets. Conventional bladder augmentation procedure is modified with the use of urothelial cell sheets. Gastric flaps are demucosalized and harvested urothelial cell sheets are grafted onto the bare smooth muscle layers. These constructs are then used 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

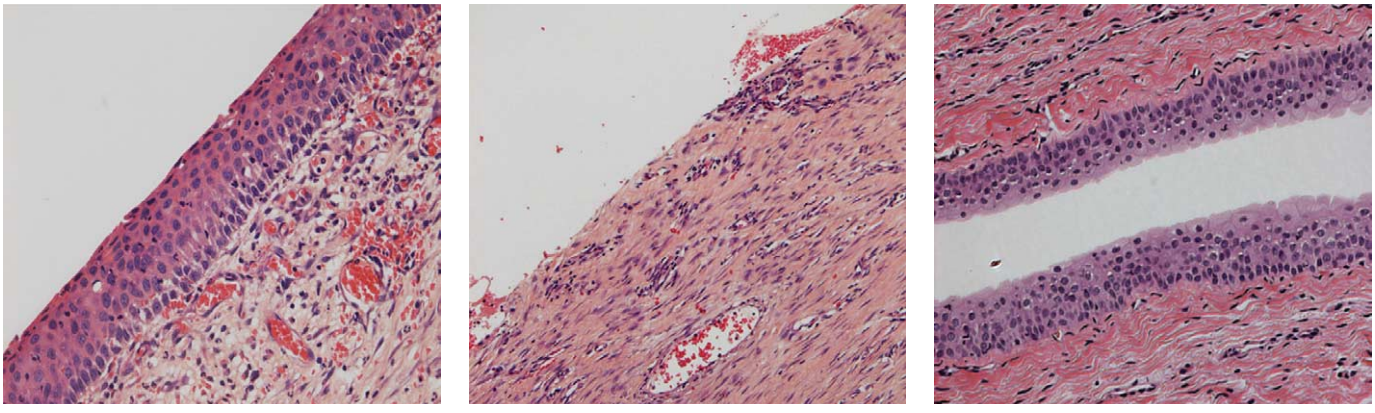

Fig. 8 Urothelium regeneration. Four weeks after surgery, urothelium has regenerated in the urothelial cell sheet-grafted group (left). No urothelial regeneration is observed in the control without cell sheet graft (center). The regenerated urothelium is similar to native ureter (right). (Reprinted with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

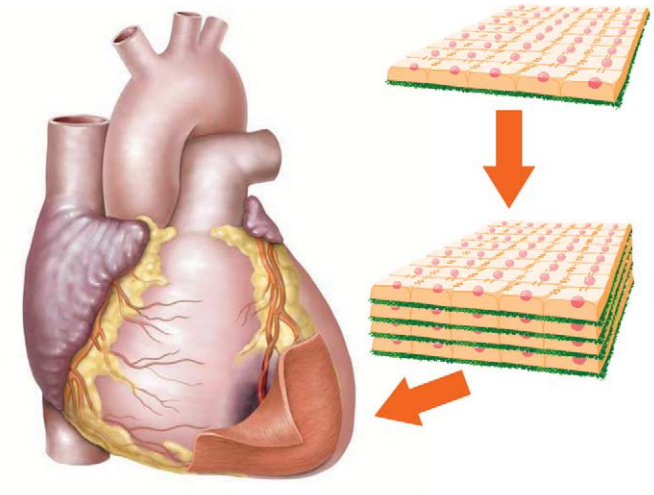

Fig. 9 Cardiac patch cell sheet engineering. Cardiac myocyte sheets are harvested from temperature-responsive culture dishes. Four cell sheets are then stratified and transplanted to ischemic hearts as cardiac patches.

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

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