In addition to stem cell biology, tissue engineering is an essential research field for regenerative medicine. In contrast to cell injection, bioengineered tissue transplantation minimizes cell loss and has the potential to repair tissue defects. A popular approach is scaffold-based tissue engineering, which utilizes a biodegradable polymer scaffold for seeding cells; however, new techniques of cell sheet-based tissue engineering have been developed. Cell sheets are harvested from temperature-responsive culture dishes by simply lowering the temperature. Monolayer or stacked cell sheets are transplantable directly onto damaged tissues and cell sheet transplantation has already been clinically applied. Cardiac cell sheet stacking produces pulsatile heart tissue; however, lack of vasculature limits the viable tissue thickness to 3 layers. Multistep transplantation of triple-layer cardiac cell sheets cocultured with endothelial cells has been used to form thick vascularized cardiac tissue in vivo. Furthermore, in vitro functional blood vessel formation within 3-dimensional (3D) tissues has been realized by successfully imitating in vivo conditions. Triple-layer cardiac cell sheets containing endothelial cells were layered on vascular beds and the constructs were media-perfused using novel bioreactor systems. Interestingly, cocultured endothelial cells migrate into the vascular beds and form perfusable blood vessels. An in vitro multistep procedure has also enabled the fabrication of thick, vascularized heart tissues. Cell sheet-based tissue engineering has revealed great potential to fabricate 3D cardiac tissues and should contribute to future treatment of severe heart diseases and human tissue model production. (Circ J 2014; 78: 2594–2603)

Key Words: Cardiac disease; Stem cells; Tissue; Transplantation; Vasculature

Regenerative medicine has progressed rapidly and is expected to offer treatment for severe diseases that have not been able to be cured by conventional therapies. Stem cell biology and tissue engineering research are absolutely essential for the future development of regenerative medicine. Stem cell biology has revealed various types of stem cells in the body and also produced pluripotent stem cells, including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Autologous and allogenic stem cells have been already used clinically in regenerative therapy. The differentiation potential of adult stem cells is limited, so ES and iPS cells may become the preferred cell sources in the future. The usage of adult stem cells has accelerated because it avoids the difficult ethical immunoreaction problems associated with other stem cells. The most common delivery methodology has been cell suspension injection and is used widely around the world. However, the initial retention rate of injected cells is very low because of wash out into the venous system or outside of the organ; moreover, cell death occurs because of central necrosis and/or anoikis. For example, Hofmann et al demonstrated significant cell loss and low engraftment (<5%) in the heart when autologous unselected bone marrow cells radiolabeled with $^{18}$F-FDG were injected into the coronary arteries of patients suffering from ischemic heart diseases. Moreover, in cell injection therapy, it is not possible to control graft size or shape, and it is difficult to repair tissue defects after surgery or in a malformation. To overcome these problems, many researchers have tried to make surgically transplantable tissues by tissue engineering technologies. Tissue engineering is a multidisciplinary research field and various biomaterials and engineering technologies have been developed to create functional tissues. Several types of bioengineered tissues have already been clinically applied and some of them, including skin and cartilage tissues, are now commercially available. Thus tissue engineering has become an indispensable field of research for effective regenerative therapy.

In this review, tissue engineering technologies, especially the challenges for fabricating vascularized 3-dimensional (3D) tissues, are described.

Tissue Engineering Strategies

Attempts to make tissue equivalents from cells started in the late 1970s. Several researchers demonstrated skin substitutes consisting of only cells or cells on collagen gel. In 1993, after
Cell Sheet-Based Myocardial Tissue Engineering (2013 Sato Award)

more than 10 years, Langer and Vacanti sensationally proposed “tissue engineering” by showing an engineered human ear on the back of a mouse. They seeded human chondrocytes onto an ear-shaped porous biodegradable scaffold composed of a synthetic polymer and cultured them in appropriate conditions with several growth factors, resulting in viable 3D ear tissues after transplantation into an athymic mouse. This concept of using 3D biodegradable scaffolds as an alternative to an extracellular matrix (ECM) has been applied to fabricating various types of tissues and is the most popular approach used in tissue engineering. For biodegradable scaffolds, both natural and synthetic polymers are used and their porosity is critical for cell seeding and media perfusion. Decellularization is achieved when tissues are enzymatically digested (with nuclease, trypsin, or dispase), chemically treated (with detergent, acid, or alkaline) or physically processed (using freeze and thaw, or mechanical pressure). The advantage of using decellularized tissue is its structural similarity to the natural tissue. In contrast to using prefabricated scaffolds, a polymer solution and cells are mixed and gelled to form 3D tissue. The method is preferable for engineering relatively homogeneous and soft tissues. Collagen, gelatin, fibrin and alginate gels have been used as hydrogel scaffolds. Eshenhagen and Zimmermann’s group have developed collagen gel-based functional heart tissues and the technique is the most commonly used for myocardial tissue engineering. As another tissue engineering strategy, “bioprinting” has now become popular. Several researchers have tried to make 3D tissues by printing cells, one by one, using 3D printing technologies. Each cell is dispensed with a small amount of polymer solution, such as alginate and gelled into its targeted position under computer-aided spatial control. The technology enables cell patterning and may realize the formation of complex 3D tissues, including several different cell types, similar to color printing. Fiber-based tissue engineering is also a novel approach to fabricating tissues. Using a microfluidics device, hydrogel microfibers encapsulating cells can be engineered and bundles of these fibers may mimic some types of tissues, including muscle tissue.

3D scaffold-based tissue engineering is a top-down technique, whereas bioprinting and cell fiber-based tissue engineering are bottom-up techniques. Each approach has advantages and disadvantages, so a combination of these techniques may lead to more useful and functional tissue fabrication.

Cell Sheet-Based Tissue Engineering

Tissue engineering technologies commonly utilize a scaffold material such as ECM alternatives to form 3D structures, as just described. These materials often cause an inflammatory reaction during biodegradation in the body and the resultant constructs exhibit low cell density. By contrast, “cell sheet-based tissue engineering” utilizing “cell sheets”, which are 2-dimensional (2D) confluent cells without any added materials (Figure 1D), has been developed by my group.

Figure 1. TE strategies. (A) Prefabricated materials are mostly used as 3D scaffolds, but decellularized tissues are also used as prefabricated scaffolds. A hydrogel and cell mixture is used for soft TE. (B) Bioprinting is a technology for printing cells with matrix one by one. (C) Cell and hydrogel are formed into fibers (fiber-based TE). (D) Cell sheets are harvested from temperature-responsive culture dishes and stacked for 3D tissue fabrication. 3D, 3-dimensional; TE, tissue engineering.
sheets can be harvested from intelligent culture dishes only by lowering the temperature, because the dish surfaces are covalently grafted with a temperature-responsive polymer, poly(N-isopropylacrylamide). The surface is slightly hydrophobic at the standard culture temperature of 37°C, so the cells adhere to the surface via ECM proteins and cell membrane proteins. Below the lower critical solution temperature of 32°C, the surface rapidly changes to become very hydrophilic and protein non-adhesive, leading to spontaneous cell detachment with intact ECM proteins. When cells are cultured to be confluent, they attach to the surrounding cells via cell-to-cell junction proteins. Conventional cell harvest using enzymatic digestion (eg, trypsinization) disrupts all cell-to-cell junctions in addition to the adhesive proteins between cells and culture surface, resulting in isolated cell detachment (Figure 2A). Using temperature-responsive culture dishes, on the other hand, a temperature decrease evokes a surface property change from cell adhesive to cell non-adhesive, but all cell-to-cell junctions are preserved, and so the confluent cells detach from the dish as a contiguous cell sheet (Figure 2B). It should be especially mentioned that cell sheets do not include the temperature-responsive polymer, which remains on the culture dishes via covalent bonding. Another critical point is that the ECM proteins underneath the cell sheets are also preserved and act as adhesive agents when the cell sheets are transferred onto target tissues or other material surfaces. Furthermore, these proteins accelerate the adhesion between cell sheets when they are stacked to fabricate 3D tissues. Because cell sheets do not include any ECM alternatives, cell sheet stacking succeeds at fabricating cell-dense 3D tissue.

Applying the technology of using temperature-responsive culture dishes, several other research groups have developed different technologies for harvesting cell sheets. For example, cells labeled with magnetite nanoparticles are seeded onto a low cell adhesive culture dish with a magnet positioned underneath. After cultivation to confluence, removing the magnet causes cell sheet detachment from the surface. This technology has also been applied to iPSC cell sheet and mesenchymal stem cell sheet fabrication. Although the technology is unique, magnetite nanoparticles may be problematic for clinical application.

The significant features of cell sheets are that they contain no scaffolds and they preserve the underlying adhesive proteins, considered preferable for transplantation into the body and for fabrication of 3D cell-dense tissue.

**Regeneration Therapy Using Cell Sheets**

**Clinical Application of Cell Sheet Transplantation**

Various types of cell sheets have already been harvested from temperature culture dishes and transplanted to cure damaged tissues/organisms in animal models. Moreover, cell sheet transplantation already has been clinically applied to treatment in 6 cases of tissue/organ diseases (Figure 3). The first clinical application of cell sheets was corneal surface regeneration. Autologous corneal epithelial cell sheets, which were originated from cornea limbal cells of the opposite healthy eye, were transplanted onto the ocular surface after resection of the diseased corneal epithelium and the visual acuity of the patient improved. Furthermore, to avoid damaging the healthy cornea or in treating bilateral corneal disease, oral mucosal epithelial cell sheets have been used as an alternative and efficacy has confirmed. Oral mucosal epithelial cell sheets have also been used clinically to prohibit esophagus constriction after endoscopic submucosal dissection (ESD) in early esophageal cancer patients. Most patients did not need additional esophageal dilatation procedures after this treatment. Transplanted cell sheets are considered to accelerate post-ESD wound healing. Recently,
cytokine mixtures that include factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor, and stromal-derived factor 1. Of those cell sheets, Sawa’s group has focused on myoblast sheets and clinically used autologous myoblast sheets for treatment of severe heart diseases.

A patient who had been supported with a left ventricular assist system for dilated cardiomyopathy was discharged from hospital without the device after autologous myoblast sheet transplantation.

Clinical studies of ischemic heart disease and pediatric patients are still ongoing.

Preclinical Studies of Cell Sheet Transplantation
In addition to the clinical applications described, many studies have demonstrated the efficacy of cell sheet-based regenerative therapy in animal models. Fibroblast cell sheets can seal lung air leaks and this procedure will be applied clinically during lung cancer operations in the near future.

Thyroid cell sheets supported recovery from hypothyroidism in rats undergoing total thyroidectomy, and islet cell sheets were able to control glucose concentration via insulin secretion in diabetic rats. Thus cell sheets may be applicable for hormone-secreting tissue regeneration.

Cell Sheet Transplantation for Heart Regeneration
In myocardial tissue regeneration, previous studies have confirmed heart function improvement after cell sheet transplantation onto damaged hearts using various types of cells, including cardiomyocytes, myoblasts, bone marrow cells, adipose tissue-derived stem cells, and cardiac stem cells. Better cell survival was reported for cell sheet transplantation than for cell injection, and continuous cytokine secretion from the surviving cells seemed to have a positive effect on heart regeneration. It is considered that cell sheets have multiple potential for neovascularization, anti-fibrosis and stem cell recruitment via secreted cytokine mixtures that include factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor, and stromal-derived factor 1. Of those cell sheets, Sawa’s group has focused on myoblast sheets and clinically used autologous myoblast sheets for treatment of severe heart diseases.

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3D Cardiac Tissue Fabrication by Stacking Cell Sheets
For the fabrication of functional 3D cardiac tissues, neonatal rat cardiac cell sheets have been stacked. The layered cardiac cell sheets communicated between themselves both morphologically and electrically within an hour, resulting in macroscopically beating 3D tissues. The ECM underneath the cell sheets is considered to contribute to this rapid electrical com-
distance in the heart is less than 25 µm, narrower than the distance in other tissues/organs. Previous reports have shown that the diffusion limit of engineered heart tissues is approximately 50–100 µm, and my group has also demonstrated that there is a limit of 3 layers, which is approximately 80 µm, when stacking rat cardiac cell sheets. These reports indicate that functional vascularization is even more critical when fabricating 3D heart tissue than it is for other tissues.

For overcoming this vascularization obstacle, a variety of methods have been developed worldwide, as described next.

**Host-Originated Vascularization (Figure 4A)**

The conventional simple strategy for neovascularization in 3D engineered tissues is to wait for host blood vessels to extend into the transplanted constructs. This strategy relies on in vivo regenerative power and is sufficient when fabricating thin tissues (eg, epithelium, cell sheet constructs ≤3 sheets) or cell-sparse tissues (eg, bone, cartilage). On the other hand, primary ischemia prior to host-originated blood vessel network formation limits transplanted construct survival when fabricating thicker, cell-dense tissues such as heart, liver, and kidney.

**Growth Factor Administration (Figure 4B)**

To accelerate blood vessel network formation in vivo, several growth factors have been administrated to engineered tissues. VEGF, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are known to promote neovascularization, including vasculogenesis and angiogenesis. VEGF and bFGF contribute to the migration, proliferation and tube formation of endothelial cells. On the other hand, PDGF con-
controls the behavior of pericytes and smooth muscle cells in blood vessel formation. Although those growth factors were expected to accelerate blood vessel formation, they degrade easily and their effectiveness lasts only for a short period, ultimately leading to insufficient vascularization. Therefore, controlled release of growth factors has been investigated to sustain their effects for blood vessel formation. In scaffold-based tissue engineering, many researchers have incorporated growth factors in the polymer scaffolds. Growth factors are simply mixed within both hard porous materials, as well as soft gel materials, and biodegradation of the scaffold causes the slow release of the incorporated growth factors. Heparin, which binds growth factors and ECM, is often loaded into the scaffolds to control growth factor release. Controlled release of growth factors, VEGF and PDGF-BB, has also been reported. In that system, VEGF is held by the base polymer scaffold and PDGF-BB is held by polymer microspheres within the scaffold, whereby VEGF is released faster than PDGF. Appropriate control of multiple growth factors using bioengineering techniques may improve neovascularization in engineered 3D tissues.

**Endothelial Cell Coculture (Figure 4C)**

A recent popular approach to introducing blood vessels in engineered tissues is to coculture the target cells with vasculature cells. Cocultured endothelial cells spontaneously form network structures in engineered tissues in vitro and these networks connect to host blood vessels after transplantation. A 2D monoculture of endothelial cells usually expresses a cobblestone appearance, whereas in coculture with other types of cells they show capillary-like endothelial cell networks in vitro. Endothelial cells seem to recognize surrounding cells and ECM, and then form networks in vitro similar to those found in vivo. However, the critical point is that in vitro endothelial cell networks form an immature prevascular structure, which means there is insufficient tubular formation in the network. Moreover, long-term static culture causes deformation of the networks, probably from cell apoptosis. Therefore the timing of transplantation of in vitro prevascularized tissues must be optimized for the in vivo formation of functional blood vessels.

In cell sheet-based myocardial tissue engineering, rat cardiac cell sheets containing endothelial cell networks were stacked in vitro and transplanted in vivo without any deformation of the prevascular structure, because of the non-invasive harvesting technique using temperature-responsive culture dishes. A study using green fluorescent protein (GFP)-positive endothelial cells clearly demonstrated that cocultured endothelial cells form continuous tubular blood vessel networks in the construct. Furthermore, GFP-positive endothelial cells migrate into host tissues and connect with host blood vessels. Blood was able to reach and perfuse the construct via the newly developed blood vessels within 1 day. Cardiac cell sheets with cocultured endothelial cells form thicker tissues in vivo than do those without endothelial cells.

In addition to endothelial cells, fibroblasts and mural cells, including pericytes and smooth muscle cells, comprise blood vessels. Therefore, a coculture system of endothelial cells and fibroblasts was used to make prevascularized skin, skeletal muscle and adipose tissues. A tri-culture of cardiomyocytes, endothelial cells and mural cells was also reported to lead to more stable vascularization. Mural cells contribute to vessel maturation and remodeling by mediating cell-cell communication and releasing growth factors.

**Problem of Primary Ischemia**

These coculture methods have been helpful for fabricating vascularized 3D tissues, but they are inadequate for the breakthroughs needed to scale-up engineered tissues. In the most rapid case, using neonatal rat cell coculture, transplanted tissues are sufficiently supplied with blood after about half a day. In vitro prevascularization does not provide a continuous tubular structure and blood is not immediately supplied to the construct. Blood vessel connections and functional tubular formation depends on in vivo regeneration, which seems to require a particular period of time and thus still limits the final thickness of in vivo engineered tissues.

**MultiStep Transplantation of Cell Sheets (Figure 4D)**

To overcome this problem, my group performed a step-by-step procedure based on cell sheet stacking technology for fabricating viable thick cell-dense tissues with perfusable blood vessels. Triple-layered cardiac cell sheets, which are at the diffusion limit without supporting vasculature, were repeatedly implanted in the dorsal subcutaneous tissues of a rat with 1- or 2-day intervals of waiting for sufficient neovascularization of the previously transplanted constructs. The resultant tissues were synchronously beating, thick cardiac tissues with abundant microcapillaries and the thickest tissue was approximately 1 mm after 10 transplantations were repeated to make a 30-layer cardiac construct.

**Ectopic 3D Tissue Fabrication Using Host-Connectable Artery and Vein (Figure 4E)**

The next obstacle in the formation of functionally vascularized tissue within the subcutaneous space is the lack of thick blood vessels for reconnecting to target tissue/organs. Several reports have demonstrated ectopic tissue fabrication using a preexisting surgically connectable artery and vein. By ectopically transplanting cardiac constructs onto omentum, the constructs were successfully blood-supplied from the omentum and then re-transplanted directly onto a heart. Another innovative approach is to place target cells, matrix gel, and a connectable artery and vein in a polycarbonate chamber and incubate all within subcutaneous tissues. Functionally vascularized tissues with connectable blood vessels were engineered and ectopically transplantable with vessel anastomoses. This intrinsic vascularization technology using a vascular chamber system has been used to engineer heart, liver, muscle and pancreas tissues. My group has also combined multistep transplantation of cell sheet constructs with usage of preexisting blood vessels. Triple-layer constructs were transplanted repeatedly over a connectable artery and vein in the femoral area. The multilayer cardiac constructs were successfully blood-supplied from the host artery and vein. Subsequently, the constructs were resected with the vessels and ectopically transplanted in the neck with direct vessel anastomoses. The tissues maintained spontaneous beating after the ectopic transplantation. This ectopic multistep procedure over surgically connectable native blood vessels is a possible solution to fabricating large-scale transplantable tissues.

**Microfabrication Technologies for Fabricating In Vitro Vascularized Tissue (Figure 4F)**

In contrast to using the power of in vivo regeneration, in vitro fabrication of functional blood vessels within 3D tissues is one of the most challenging and critical issues in tissue engineering research. Several engineers have tried to create a micro-channel network imitating native microcapillaries within 3D biodegradable scaffolds by using 3D microfabrication tech-
Thin polymer layers with a microfluidic structure were integrated with hydrogel-containing cells, which produced an artificial capillary-like network of lumens within cell-dense tissues.\textsuperscript{53} 3D printing technology offers the ability to control 3D scaffold micro-structures and introduce capillary-like lumens into the construct.\textsuperscript{54} To fabricate cell-dense tissues with sufficient microcapillaries, uniform endothelial cell seeding and perfusion through the lumens are still problematic. On the other hand, 3D bioprinting has developed as a tissue engineering strategy and has realized control of vascular cell positioning in 3D constructs.\textsuperscript{55} Although bioprinting is unique as an application of a mechanical device to biology, rapid cell movement is uncontrollable and may cancel any artificially programmed cell positioning.

**Decellularized Whole Organ as Scaffold (Figure 4G)**

These microfabrication techniques lack the native vascular structure and transition. Moreover, control of anastomoses with in vivo blood vessels has not yet been solved. A new strategy has recently shown that decellularized native tissues/organs with connectable vessels can be used as cell scaffolds to mimic the complex structure of native tissues, including luminal vascular structure. For example, decellularized whole rat heart with a complete vascular structure was used as a scaffold for heart regeneration.\textsuperscript{56} Rat cardiomyocytes were re-seeded into the original muscle areas and endothelial cells were perfused through coronary vessels. Finally, the recellularized heart started to beat again, indicating the potential of decellularized organs for in vitro 3D vascularized tissue fabrication. The same technique has been applied to kidney, liver and lung tissue engineering, but homogeneous and dense cell-seeding still seems to be the critical issue that needs to be solved.\textsuperscript{57}

**In Vitro Perfusable Blood Vessel Formation in 3D Beating Cardiac Tissues**

My group has also attempted to introduce perfusable blood vessels and scale-up 3D cardiac tissues in vitro. The basic concept is to imitate the in vivo conditions of multistep transplantation. In the first step, rat femoral tissue was resected with a connectable artery and vein as a vascular bed and the bed was continuously perfused with culture media in an original bioreactor system (Figure 5). Next, triple-layer cardiac cell sheets including endothelial cells were overlaid. As with in vivo experiments, functional blood vessel communications between the...
promise new in vitro tissue models containing perfusable blood vessels.

Many attempts have been made using a variety of strategies to create vascularized tissues both in vivo and in vitro. Recent studies have revealed clues to functional microcapillary formation in engineered 3D tissues, so further investigation should provide the techniques necessary for large-scale functional cellular-dense tissues to advanced regenerative therapy.

**Fabrication of Pulsatile Myocardial Tube**

Future organ engineering has targeted cardiac constructs with a pumping function. Zimmermann et al demonstrated that pouch-like rat cardiac constructs can be fabricated by collagen gel-based tissue engineering.60 My group has also wrapped neonatal rat cardiac cell sheets into engineered myocardial tubes. In vitro, the cell sheets were wrapped around fibrin tubes and the constructs beat and evoked a very small, but significant inner pressure gradient of approximately 1 mmHg.61 On the other hand, an adult rat thoracic aorta was resected and wrapped with neonatal rat cardiac cell sheets, then replaced with an abdominal aorta of an athymic rat. One month later, the engineered myocardial tube presented independent beating from the host heart and evoked an independent inner pressure gradient of approximately 6 mmHg, which is much higher than the result for in vitro fibrin-based myocardial tubes.62 In comparison with myocardial tubes simply transplanted into the abdominal cavity, those transplanted in place of an abdominal aorta revealed significant increases in tissue thickness, as well as expression of brain natriuretic peptide, myosin heavy chain-α, and myosin heavy chain-β. These data indicated that...
host-heart originated blood pulsation might contribute to myocardial tube growth and the hypertrophic response. Functional myocardial tube fabrication using human iPS-derived cardiomyocytes is ongoing both in vivo and in vitro, so further developments may realize a transplantable bioengineered heart-assist pump.

**Human 3D Heart Tissues Fabrication by Stacking iPS Cell-Derived Cardiac Cell Sheets**

For future clinical application of engineered cardiac tissues, it is inevitable that human cardiac cells must be sourced. It is very difficult to use autologous cardiac stem cells because of their rarity and low capacity to differentiate into beating cardiomyocytes. Currently, iPS cells are the most hopeful cell source and cardiac differentiation methodologies have been widely established. Obstacles to iPS cell usage for myocardial tissue engineering are stable cell expansion and differentiation into cardiomyocytes. My group has already developed a novel bioreactor system enabling large-scale cultivation of iPS cell-derived cardiomyocytes up to approximately $8 \times 10^7$ cells/100-ml vessel containing approximately 80% cardiomyocytes. By using these expanded cells, human cardiac cell sheets have been successfully harvested from temperature-responsive culture dishes and beating human cardiac tissues have been engineered by stacking cell sheets as was done with rat cardiac tissues. To fabricate vasascularized human myocardial tissues, human cardiac cell sheets are now being applied in vivo multistep transplantation, and in vitro perfusion culture using a collagen-based vascular bed.

**Conclusions**

We can see that stem cell biology and tissue engineering are inseparable research fields and have actually just fused to create human implantable 3D heart tissues. Cell sheet-based technology has enormous potential in myocardial tissue engineering and will contribute to future regenerative medicine rescuing many patients suffering from severe heart failure, and as well as providing novel human heart tissue models for more efficient drug screening and basic research.

**Disclosures**

Conflict of Interest: The author served as a consultant to CellSeed Inc until December in 2013 and is a stake holder of CellSeed Inc. The author received institutional research funds from Hitachi Ltd, Dai Nippon Printing Co Ltd, CellSeed Inc, Olympus Corporation, Terumo Corporation, Nikon Koden Corporation, Asahi Kasei Corporation, Panasonic Corporation, Nikon Corporation and Kowa Company Ltd.

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