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Thermally-triggered fabrication of cell sheets for tissue engineering and regenerative medicine

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ABSTRACT

Cell transplantation is a promising approach for promoting tissue regeneration in the treatment of damaged tissues or organs. Although cells have conventionally been delivered by direct injection to damaged tissues, cell injection has limited efficiency to deliver therapeutic cells to the target sites. Progress in tissue engineering has moved scaffold-based cell/tissue delivery into the mainstream of tissue regeneration. A variety of scaffolds can be fabricated from natural or synthetic polymers to provide the appropriate culture conditions for cell growth and achieve in-vitro tissue formation. Tissue engineering has now become the primary approach for cell-based therapies. However, there are still serious limitations, particularly for engineering of cell-dense tissues. "Cell sheet engineering" is a scaffold-free tissue technology that holds even greater promise in the field of tissue engineering and regenerative medicine. Thermoresponsive poly(*N*-isopropylacrylamide)-grafted surfaces allow the fabrication of a tissue-like cell monolayer, a "cell sheet", and efficiently delivers this cell-dense tissue to damaged sites without the use of scaffolds. At present, this unique approach has been applied to human clinical studies in regenerative medicine. Furthermore, this thermally triggered cell manipulation system allows us to produce various types of 3D tissue models not only for regenerative medicine but also for tissue modeling, which can be used for drug discovery. Here, new cell sheet-based technologies are described including vascularization for scaled-up 3D tissue constructs, induced pluripotent stem (iPS) cell technology for human cell sheet fabrication and microfabrication for arranging tissue microstructures, all of which are expected to produce more complex tissues based on cell sheet tissue engineering.

1. Introduction

Cell/tissue-based therapy has become a promising approach to cure a number of diseases and disorders. To obtain sufficient therapeutic effects, transplanted cell populations need to first survive and then function appropriately in the transplanted site. However, the conventional method which delivers cells directly by injecting suspended cells to the damaged site in the body has limited therapeutic effect due to the poor cell retention and survival at the target site. The dream to obtain more significant therapeutic effects on the damaged tissues led to the development of tissue engineering, which was first proposed in the 1980s by R. Langer and J. P. Vacanti [1–3]. Tissue engineering enables the production of native-like tissues that can potentially be transplanted for use in regenerative medicine. In addition, human cell-based engineered tissues are expected to be used as in-vitro tissue models for biological studies and drug discovery [4–6]. Animal models have long been the main approach for drug discovery and prediction of pharmacokinetics but they have limitations for tissue modeling to understand the mechanisms in the human body. In addition, in-vitro tissue models will reduce the use of experimental animals, which has become an ethical cornerstone in the fields of pharmaceutical and cosmetic development. Therefore, human cell-based tissue models having native-like structures and functions are necessary for preclinical testing and drug discovery.

In contrast to suspended cells, therapeutic cells are typically cultured with supporting materials like "scaffolds" that provide a specific environment for cell growth and then the engineered tissues are delivered to the damaged tissue while maintaining their supporting structure. A wide range of natural and synthetic polymeric materials have reportedly been used as scaffolds to provide cells with a 3D culture environment for adhesion, proliferation, and differentiation into a specific cell phenotype [7–9]. Advances in biomaterials research have meant that collagen, fibrin and alginate can be used to organize and arrange cells three-dimensionally in vitro [10,11]. Although tissue engineering has emerged as an important field in regenerative medicine and tissue modeling, the implantation of naturally-derived polymers may trigger an immune rejection in some patients. Therefore, synthetic polymers (e. g., poly(lactic-*co*-glycolic acid) (PLGA)) have also been studied for tissue construction [12,13]. On the other hand, most of the commonly

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used scaffold materials still have limitations such as insufficient cell migration into the scaffold and poor permeability to permit the ingress of cells and nutrients. In addition, the degradation of the scaffold can cause inflammation and decrease in in-vivo stability of scaffold-based tissues after implantation. To overcome these issues, some advanced technologies have been developed. For example, decellularized tissues promise to be used as 3D scaffolds to produce regenerated tissue constructs with biomimetic contents and structures [14,15]. Technologies of 3D bioprinting have advanced dramatically and have the potential to create various kinds of tissue structures [16,17]. On the other hand, "cell sheet engineering" is a scaffold-free tissue technology that promises in the field of tissue engineering and regenerative medicine. Whereas in many cases 3D scaffolds occupy some space within the constructed tissues that prevents cell-cell interactions, this scaffold-free cell/tissue generation system is effective to produce cell-dense tissue constructs.

2. Thermally-triggered cell detachment for cell sheet fabrication

2.1. Thermoresponsive surface for regulating cell adhesion and detachment

A number of stimuli-responsive polymers have been used to produce intelligent nano—/micro-systems in the fields of diagnostics and drug delivery system, and tissue engineering [18–21]. Common triggers to change the physical properties of these polymers are a change in temperature or pH, and light irradiation or exposure to an electrical or magnetic field. In particular, thermoresponsive poly (*N*-isopropylacrylamide) (PIPAAm) is well-known as a functional polymer used in thermally-induced drug release systems, and thermally-regulated separation systems [22–25]. The physical properties of PIPAAm change across its lower critical solution temperature (LCST). Specifically, whereas it has a hydrophilic property below the LCST (32 °C in aqueous media), the polymer becomes hydrophobic above the LCST (Fig. 1A) [26]. This temperature-dependent switching behavior has been uniquely used to regulate cell adhesion and detachment. Whereas PIPAAm-grafted surfaces behave as a cell adhesive surface at the normal culture temperature (37 °C), the surface becomes highly hydrophilic and is then non-adhesive below the LCST. As a result, cultured cells on the surface detach spontaneously from the surface (Fig. 1B) [27–29]. Importantly, this thermally triggered cell detachment allows us to collect cultured cells without having to use an enzymatic treatment. Therefore, when cells are confluent on the thermoresponsive surface, a cellular monolayer can be harvested as a single continuous cell sheet by lowering the culture temperature (Fig. 1C). As alternative, polyglycerol-based copolymers can also be used to harvest cell sheets by lowering the culture temperature. For example, thiol end-functionalized polyglycerol copolymers can be simply immobilized on gold surface via thiol-gold interactions [30]. This kind of thermoresponsive surface allows detaching a fibroblast cell sheet within minutes. In contrast to conventional studies, naturally derived materials are widely used as scaffolds to provide an appropriate culture environment for transplanted cells and then deliver the therapeutic cells onto target sites. On the other hand, the thermoresponsive surface is able to release a single cell sheet with only the associated critical extracellular matrix (ECM) (Fig. 1D and E). Since the cell sheet is harvested without enzymatic treatment, important membrane proteins and cell-cell junctions remain intact within the cell monolayer. This unique tissue fabrication method is called "cell sheet engineering", and is currently being applied to regenerative medicine and tissue modeling [31,32].

2.2. Nano-scale grafting of thermoresponsive polymer for control of cell behavior

To regulate cell behaviors by changes in temperature, PIPAAm needs to be grafted precisely at a nano-scale. The amount grafted and the thickness of the PIPAAm are key factors to provide the appropriate hydrophobicity for cell adhesion at normal culture temperature, and to allow the surface to become non-adhesive surface by lowering the culture temperature. Some unique techniques have been developed to achieve the nano-scale grafting on culture substrates [33–37]. Electron-beam (EB) irradiation to a monomer (IPAAm) solution on a tissue culture polystyrene (TCPS) dish induces covalent grafting of PIPAAm at a nano-scale on the surface. The thickness of PIPAAm layer can be optimized by changing the monomer concentration and EB irradiation conditions. A previous study demonstrated that a PIPAAm layer approximately 20 nm thick is appropriate to achieve both cell adhesion

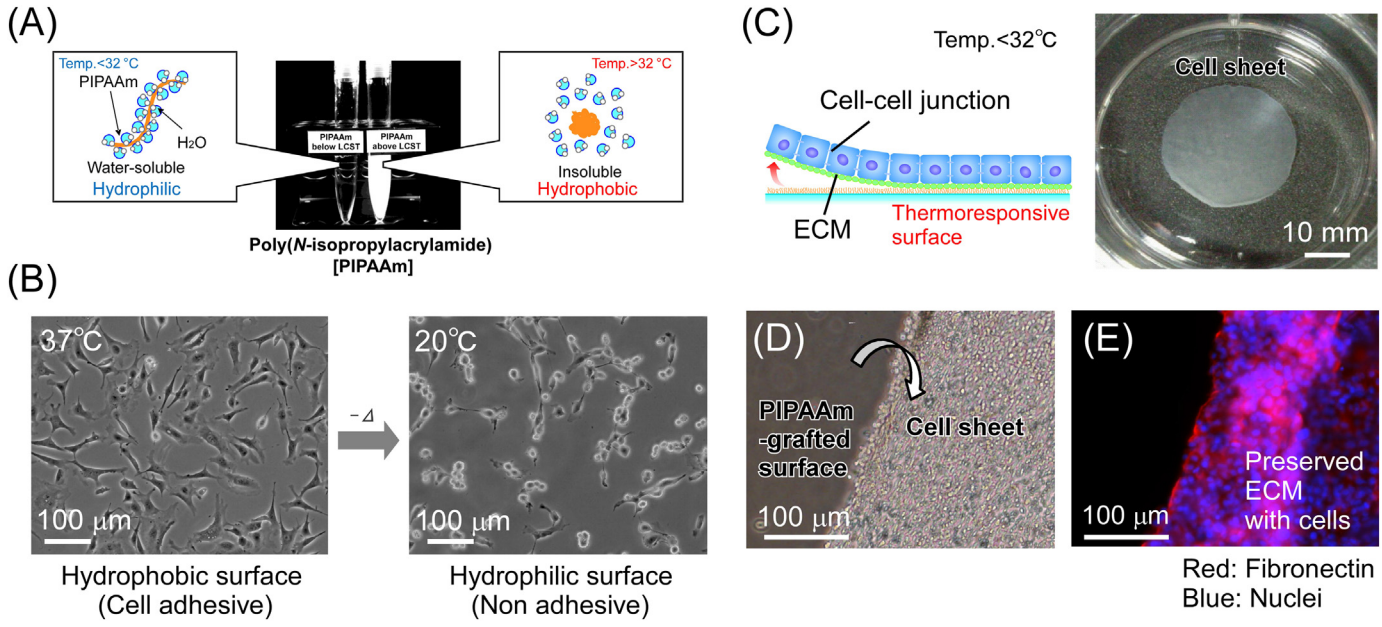

Fig. 1. (A) Temperature-dependent change of hydrophilicity/hydrophobicity of PIPAAm in aqueous solution. (B) Thermally triggered detachment of adhering cells on a thermoresponsive culture substrate by lowering culture temperature to 20 °C. (C) Fabrication of a single continuous cell sheet with preserved cell-cell junctions and ECM without the use of enzymatic treatment. Photograph shows that the cell sheet shrinks two-dimensionally when detaching from the surface. (D) Phase contrast and (E) fluorescence microscopic images of a detaching cell sheet from a thermoresponsive surface. A cell sheet was harvested with the ECM intact. Fibronectin and cell nuclei are stained with red and blue, respectively. Adapted with permission from [33].

and detachment [34]. When PIPAAm is grafted much thicker on the surface, the PIPAAm layer makes the surface too hydrophilic for most types of cells, such that cells are unable to adhere in any significant number on the surface even at normal culture temperature (Fig. 2A) [38,39]. The ideal polymer grafting technique has been achieved and thermoresponsive culture dishes prepared using EB-irradiation methods are now commercially available as functional cell culture substrates for cell sheet fabrication. Other polymer grafting techniques have also been used to produce similar thermoresponsive surfaces. PIPAAm can be grafted on normal culture dishes by γ-irradiation [40] and plasma irradiation [41].

Surface-initiated living radical polymerization (LRP) processes are widely used to synthesize polymers at precisely controlled molecular weights. In previous studies, atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization have been successfully used to fabricate PIPAAm grafting on culture surfaces [33,36,37,42]. Since the LRP processes can provide polymer-brush-type thermoresponsive surfaces, surface-initiated LRP approaches can be used to graft polymer brushes with precisely adjusted chain length and graft density of the polymer on culture surfaces [33]. For example, PIPAAm brush surfaces can be optimized for regulating cell adhesion and detachment by adjusting the graft density of polymer brushes (Fig. 2B). Through changes to the graft density of PIPAAm, an optimized polymer surface can be made for cultured cells. In addition, a block copolymer of PIPAAm domain and a hydrophobic polymer domain can be used to achieve nano-scale grafting of PIPAAm on a surface [35]. In this approach, unlike other polymer grafting, PIPAAm can first be synthesized as a free polymer and then it can be simply applied by spin-coating on the culture surface due to the presence of the anchoring hydrophobic polymer. By being blended with a silane-coupling agent, PIPAAm can also be simply coated using spin-coating method on glass substrates and silicon wafers, resulting in the fabrication of thermoresponsive films [43]. Whereas the thickness of grafted PIPAAm layer needs to be adjusted at nano-scale on these kinds of substrate, a thermoresponsive hydrogel can also be applied to harvest a cell sheet. By incorporating RGD peptides and bFGF within the hydrogel, endothelial cells can adhere significantly on the hydrogel surface and then detached from the surface by lowering the temperature [44]. With the variety of PIPAAm grafting approaches growing, a suitable type of thermoresponsive surface can be selected according to the cell types used to construct the cell sheet.

3. Cell sheet layering for production of scaffold-free 3D tissue constructs

In contrast to scaffold-based tissue constructs, cell sheet-based tissue engineering uniquely allows us to create scaffold-free 3D tissues by layering multiple cell sheets. Simply by placing one cell sheet onto another, the layered cell sheets stack tightly with each other because of the preserved ECM (Fig. 3A) [45,46]. Importantly, the cells can communicate with each other, both physically and biologically because of no use of scaffolds which inhibit the communications [47–50]. For

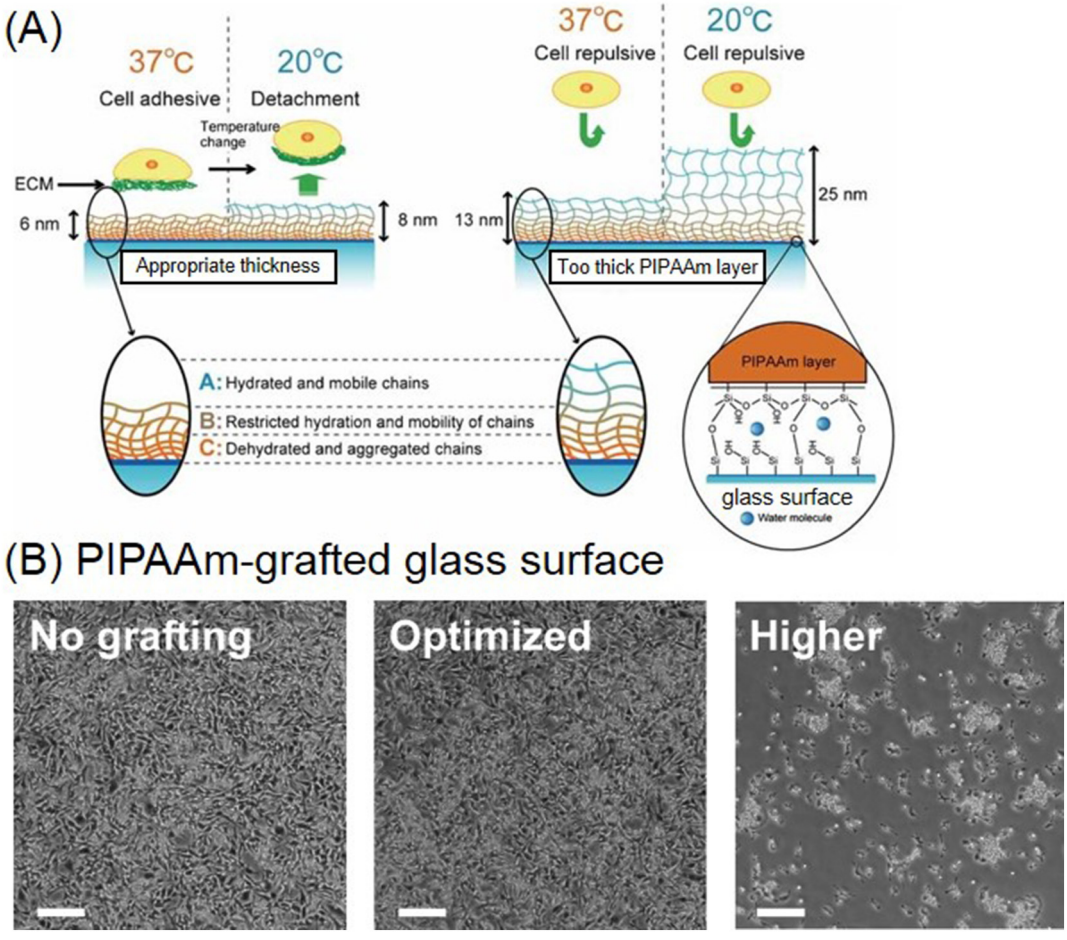

Fig. 2. (A) Schematic illustration of molecular motion of PIPAAm chains and cell adhesion/detachment behaviors on the PIPAAm-grafted surfaces at different graft thickness. PIPAAm layer at an appropriate thickness allows both cell adhesion at 37 °C and cell detachment at 20 °C. In contrast, a greater amount of grafted PIPAAm causes it to become non-adhesive for cells regardless of culture temperature. (B) Microscopic images of cell adhesion on PIPAAm-grafted glass surfaces at different graft amounts. Whereas an optimized graft amount (e.g., 0.35 ±0.02 μg/cm^2) allows cells to adhere in a similar way to a bare glass surface, and a greater graft amount (e.g., 0.50±0.04 μg/cm^2) causes a decrease in cell adhesion even at normal culture temperature. Adapted with permission from [33,38].

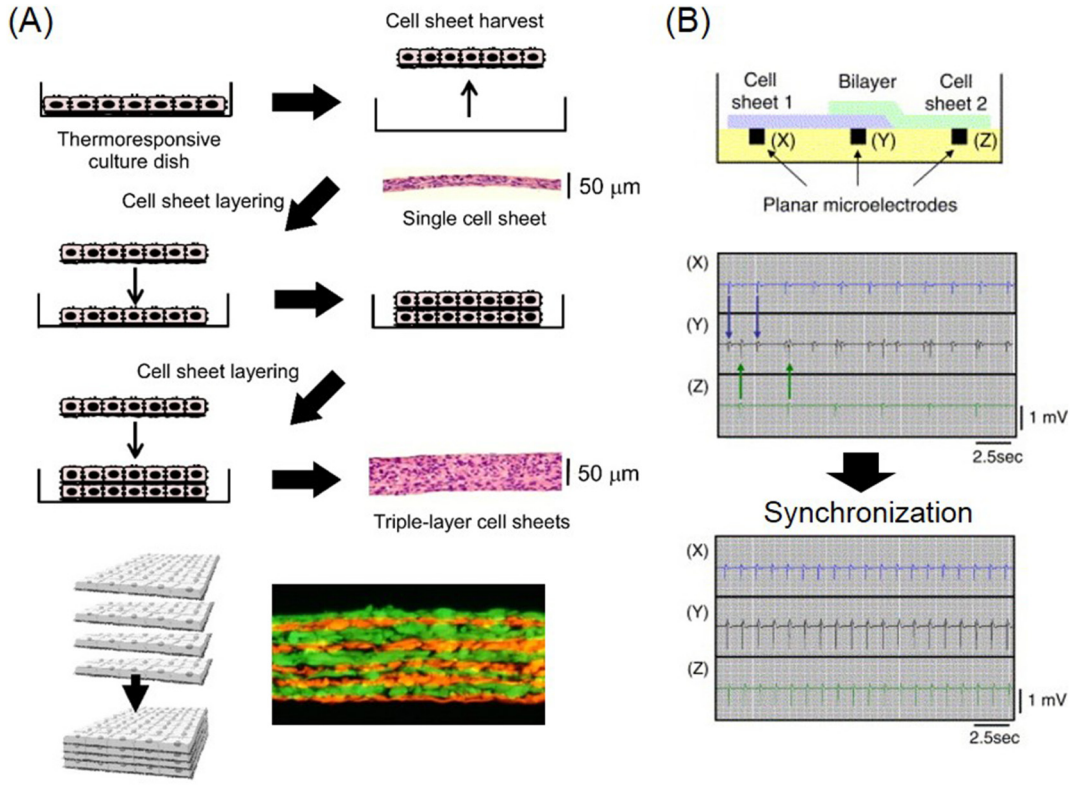

Fig. 3. (A) Cell sheet layering process for production of a multilayered cell sheet construct without the use of a 3D scaffold. Due to the preserved ECM proteins, multiple cell sheets can be stacked tightly by simple layering. (B) The different beating rates of two cell sheets synchronized after layering. Whereas the two cell sheets show individual beating rates before layering, the subsequent layered cell sheet construct shows that the beating was synchronized. Adapted with permission from [45,47].

example, when cardiomyocytes are harvested by lowering the temperature, the resulting single cardiomyocyte sheet displays synchronized beating within the cell sheet. Although multiple cell sheets usually contract at different rates, their individual rhythms synchronize when multiple cardiomyocyte sheets are layered to create a 3D myocardial tissue construct (Fig. 3B) [47,48]. This indicates that by simply layering one cell sheet on another they can interact electrochemically to synchronize their beating. Therefore, this technique offers the potential for constructing truly (not only physically) 3D tissues. In clinical applications, depending on the damaged tissue, layered cell sheets can be used to transplant a significant number of cells.

4. Clinical applications for cell sheet engineering

The advantages of cell sheet-based tissue engineering have already been demonstrated in human clinical studies of cell transplantation. The use of the functional culture surfaces allows us to harvest therapeutic cells with preservation of the important cell-cell junctions and the associated ECM [51,52]. This is advantageous to manipulate cell sheets when transplanting onto a target site. The preserved ECM layer acts as a glue to bind the cell sheets tightly to the host tissue. Therefore, cell transplantation can be completed without the use of artificial scaffolds or additional treatments such as suturing. With these advantages, human clinical studies of the cell sheet-based tissue regeneration are on-going.

4.1. Cornea regeneration

The unique features of cell sheets can be exploited to effectively treat epithelial tissues. In fact, oral mucosal epithelial cell sheets can be transplanted to severely damaged cornea to regenerate cornea. Severe trauma or eye diseases such as Stevens-Johnson syndrome result in the complete loss of corneal epithelial stem cells and severe visual loss. Epithelial cell sheets have been fabricated on the thermoresponsive surface and are transplantable by simple handling with a supporting poly(vinylidene fluoride) (PVDF) membrane and placing the sheet onto the patient's corneal stroma [53]. The transplanted cell sheet adheres rapidly and tightly onto the host corneal surface without suturing. A pre-clinical study has demonstrated that this kind of cell sheet retained the corneal epithelium-like structure. After transplantation, since the cell sheet works as a regenerated cornea, vascularization and inflammation are inhibited in the transplanted tissue, and the corneal clarity and smoothness recover significantly (Fig. 4A). Clinical studies demonstrated that transplantation of an autologous epithelial cell sheet to human patients provided good tolerability and efficacy post-transplantation.

4.2. Esophagus reconstruction after endoscopic submucosal dissection treatment

Endoscopic submucosal dissection (ESD) is a commonly used operative procedure to resect early-stage cancers in the gastrointestinal tract. Although ESD is a relatively low invasive technique, post-operative inflammation and stenosis are major complications observed on the resected area. Therefore, severe esophageal stricture after ESD requires repeated endoscopic balloon dilations or a temporary stent. In order to prevent such post-operative events, mucosal epithelial cell sheets are transplanted to the ulcer wound bed induced by ESD. Since cell sheets can easily adhere to the transplanted sites without suturing or the use of other adhesives, transplantation of autologous oral mucosal epithelial cell sheets promotes epithelialization of the resected area and significantly prevents esophageal stenosis [54–57]. Furthermore, an autologous epidermal cell sheet is also useful to prevent esophageal stenosis. The transplanted cells can contribute to promotion of tissue regeneration as a cell source, in addition to its paracrine effects. The first clinical study showed early re-epithelialization of the ulcer site and prevention of stricture (Fig. 4B) [55]. Currently, a further randomized study is being

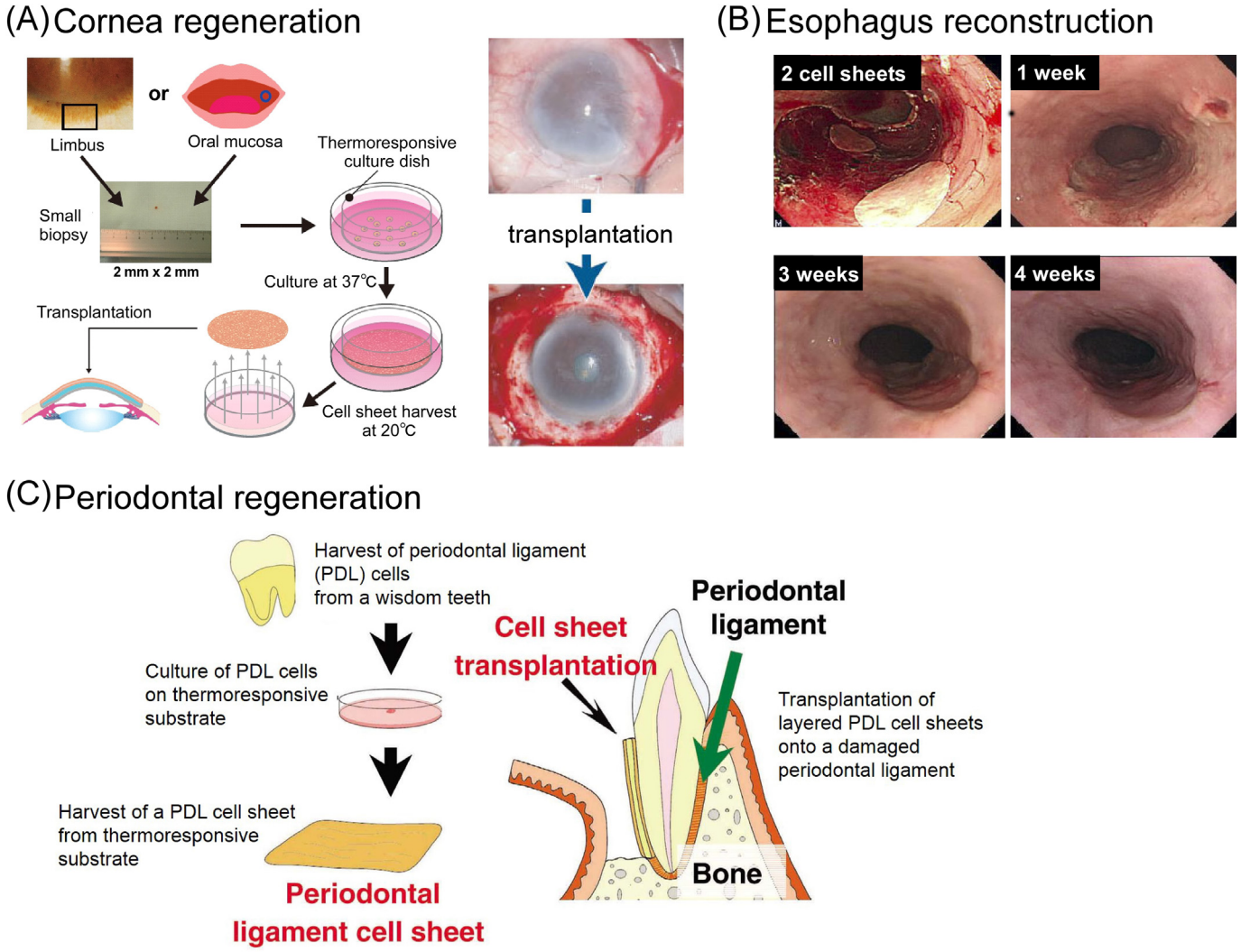

Fig. 4. (A) Schematic illustration of the preparation method of an epithelial cell sheet using the thermoresponsive surface and photographs of a transplanted cell sheet onto a diseased eye. (B) In a human clinical study case esophageal stricture was significantly prevented by the transplantation of two cell sheets. In this case, epithelialization was observed at the center of the ulcerative site at 1 week after transplantation, and was completed without any evidence of stricture at 4 weeks. (C) Schematic illustration of the preparation method of a PDL cell sheet and transplantation of layered PDL cell sheets. Adapted with permission from [32,55].

prepared to assess the potential benefits of cultured autologous cell sheets therapies in regenerative medicine.

4.3. Myocardium regeneration

Cell-based therapy is also useful to significantly treat patients with severe heart failure including dilated cardiomyopathy (DCM) and ischemic heart disease. Skeletal muscle myoblasts have commonly been used as a therapeutic cell source because of advantages including autologous origin, high obtainability, high proliferative potential [58,59]. Therefore, myoblast transplantation has been studied by many groups. However, there was a problem in that injected cells were poorly retained to the target cardiac tissue. In many cases, multiple injections were required to deliver significant numbers of cells to the target site. In addition, the needle injection itself is invasive and often induces severe cell death at the injected area, resulting in a risk of myocardial infarction [60]. Sawa and co-workers have demonstrated that autologous skeletal myoblast sheets were able to be effectively transplanted to the target site and significantly treat DCM [61,62]. Since myoblasts never differentiate into cardiomyocytes, paracrine effects of directly delivered cytokines from the myoblasts should be the major mode of action. In fact, the myoblast sheets could potentially release therapeutic cytokines including stromal-derived factor-1 (SDF-1), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) and locally deliver them to the damaged tissue [63]. In addition, the flexible properties of cell sheets allow the dynamic pulsation of the host myocardial tissue. Recent studies demonstrated that this scaffold-free cell/tissue delivery system has also been used to deliver cardiac stem cells and adipocytes within a cell sheet for the treatment of myocardial infarction in mice [64,65]. In a clinical case report, 20 autologous myoblast sheets were transplanted to the heart of a patient with DCM and resulted in dramatically improved cardiac functions.

4.4. Periodontal ligament regeneration

Periodontal ligaments (PDLs) play an important role as a supporting tissue to connect teeth with the alveolar bone. Therefore, periodontitis (periodontal disease, also known as gum disease) can cause tooth loss in adults. Since PDL is well-known to include stem cell populations and can possibly differentiate into alveolar bone and cementum, cells harvested from the PDL have the potential to regenerate periodontal tissues and cementum. In fact, layered PDL cell sheets triggered the regeneration of both bone and cementum in a canine model [66,67]. In a human clinical study, PDL cells were obtained from an unnecessary tooth (e.g., wisdom tooth) (Fig. 4C). The harvested PDL cells are cultured with media containing autologous serum until reaching confluence on

a thermoresponsive substrate. After lowering the culture temperature to 20 °C, the PDL cells can be harvested and transplanted as a cell sheet. To deliver a significant number of cells, triple-layered cell sheets are generally implanted in the proximity of the infrabony defect. The safety and efficacy of the cell sheet approach have been confirmed in nine cases [68].

4.5. Cartilage regeneration

Cell sheet transplantation might be applicable as a curative treatment for osteochondral defects. Although artificial joints are already available for the patients, repeated abrasion of articulated surface can cause immovable joints and pain. In the cell sheet approach, chondrocyte sheets can be transplanted to effectively deliver therapeutic cells to the damaged site [69]. In a preclinical study using minipig model, triple-layer chondrocyte sheets were transplanted and the treatment showed a good stained matrix and interaction with surrounding tissue, indicating sufficient cartilaginous repair and regeneration [70]. A clinical study is ongoing where autologous chondrocyte sheets are transplanted onto the lesion of early middle-stage osteoarthritis. Moreover, this cell sheet therapy is also expected to be effective treatment for full-thickness cartilage defects.

5. Cell sheet-based cardiac tissue engineering

5.1. Cardiac cell sheet fabrication

Heart regeneration will become a practical approach for treatment of patients with severe heart failure. The previous studies demonstrated that myoblast sheets could be used to treat severe heart diseases because the transplanted cell sheet construct provides paracrine effects which can be delivered continuously and locally to the damaged tissue. On the other hand, a number of researches are focusing on the construction of tissue-engineered cardiac tissues as transplantable hearts in regenerative medicine or tissue models to discovery therapeutic drugs for treating cardiac diseases [71–74]. For example, decellularized heart tissue has been effectively used to provide culture cells with a biomimetic 3D environment including its architecture and vasculature structure [72,74]. In contrast, based on the cell sheet technology, cardiac cell sheets can be created by culturing cardiomyocytes on a thermoresponsive surface, and 3D cardiomyocyte sheet constructs can be produced without the use of 3D scaffolds. In the native myocardium, electrical coupling between cardiomyocytes occurs via gap junctions, and are essential to synchronizing the beating of the whole organ. Within multilayered cardiomyocyte sheet constructs, gap junctions are rapidly established between the cell sheets, and then the pulsations of multiple cell sheets are synchronized [47]. Transplantation of cell sheet constructs onto infarcted rat hearts demonstrated the morphological and functional connections via bridging cardiomyocytes that migrated from the transplanted grafts into the host heart [75,76]. When compared to direct cell injection, the cardiac cell sheet transplantation exhibited superior cell survival and engraftment.

5.2. Vascularization in layered cardiac cell sheets

By layering multiple cardiomyocyte sheets, 3D myocardial tissues can be produced [47,48]. However, severe necrosis can be found within the engineered myocardial tissue in a long-term in-vitro culture [77]. Therefore, to produce large-scale cell sheet constructs, vascularization is required to supply sufficient oxygen and nutrients inside these thick tissues [78–81]. Typically in tissue transplantation, blood vessels originating from the recipient invade the transplanted tissue, and then oxygen and nutrients are delivered within the tissue. However, thick tissues often become necrotic before sufficient neovascularization can develop within the tissue. To promote vascular formation within engineered tissues, co-culture with endothelial cells (ECs) is a promising approach. ECs can be incorporated simply by being sandwiched between two other cell sheets. Interestingly, the ECs form vascular-like branching networks within the layered cell sheet construct, which promote vascularization and connections to the host vasculatures after transplantation [50,75,82]. However, to support the survival of long-term cultures of thick 3D tissues, a technique for promoting formation of mature blood vessels is necessary. To mimic in-vivo conditions, layered cardiomyocyte sheets co-cultured with ECs have been cultured on a vascular bed using a resected section of femoral tissue with a connectable artery and vein [83]. The vascular bed is continuously perfused with culture media in a bioreactor system (Fig. 5A). After blood vessel formation within the cell sheet construct, the new vessels connect to the vasculatures in the vascular bed, so by this method functional vasculatures can now be engineered in vitro. (Fig. 5B and C). A collagen-based vascular bed containing microchannels has also be used for vascularized tissue formation [84]. When triple-layer cardiac cell sheet constructs on a collagen-based vascular bed in a bioreactor system, co-cultured ECs migrate from the engineered tissue into the collagen gel to reach the microchannels. Finally, the ECs covered the inner surface of the microchannels to form functional vessels, and red blood cells in the medium can successfully pass through the newly formed microcapillaries in the vascular bed and perfuse throughout the cell sheet construct. Therefore, repeating the same procedure of cell sheet layering, the in vitro cell-dense tissues can be scaled up to any desired thickness (Fig. 5D). To date, triple-layered cell sheet constructs were successfully scaled up to a 12-layered tissue by repeating these procedures 4 times (Fig. 5E). It is advantageous that these tissue constructs were engineered completely in vitro. Therefore, they hold the promise to be used as in-vitro tissue models containing perfusable blood vessels. Furthermore, this technique is expected to be applied to many other tissue types required for generation of thick vascularized tissues.

5.3. iPS cell-derived cardiac cell sheet for human heart tissue fabrication

The progress in induced pluripotent stem (iPS) cell technology has opened up an entirely new era of life science research [85–87]. In the cell-based therapies, iPS cell technologies allow us to use the patient's own cells in their treatments. Moreover, patient-specific cells can be used to create a customized tissue models for a specific disease. In particular, cardiac cells are unable to be obtained in large quantities from human body for engineering cardiac tissues: as such, human iPS cells are an excellent source of cells for cardiac tissue engineering. Therefore, in a large number of studies, an efficient culture/proliferation system of human iPS cells and a technique of their differentiation into cardiomyocytes have been investigated [88–90]. A newly developed bioreactor system allows for large-scale cultivation of human iPS cells in a 3D suspension culture (Fig. 6A). It is also important that this culture system efficiently induces differentiation of the iPS cells into cardiomyocytes (Fig. 6B and C) [91,92]. As a result, a significant number of human cardiac cells are able to be collected from the suspension and cell sheets composed of cardiac troponin T (cTnT)-positive cells can be produced on a thermoresponsive surface (Fig. 6D). This differentiation process has been continuously improved to efficiently harvest human cardiac cells [93,94]. In addition, another differentiation system of human iPS cells into cardiac cells have been applied for human cardiac cell sheet preparation (Fig. 6E and F) [95]. A triple-layered cell sheet construct was transplanted into a rat myocardial infarction model and the cell sheet transplantation improved cardiac function. These iPS technologies allows us to obtain a significant cell number of human cardiac cells, so that now human cardiac cell sheets are being applied to in-vitro perfusion cultures using a collagen-based vascular bed to fabricate thicker vascularized myocardial tissues. In addition, a system to measure the contractile force of the cardiac cell sheet constructs has also been developed [96]. These human tissue constructs are expected to become a tissue model for drug discovery. This measurement system is potentially useful to evaluate the contractile functions and drug responsibility of engineered cardiac tissues.

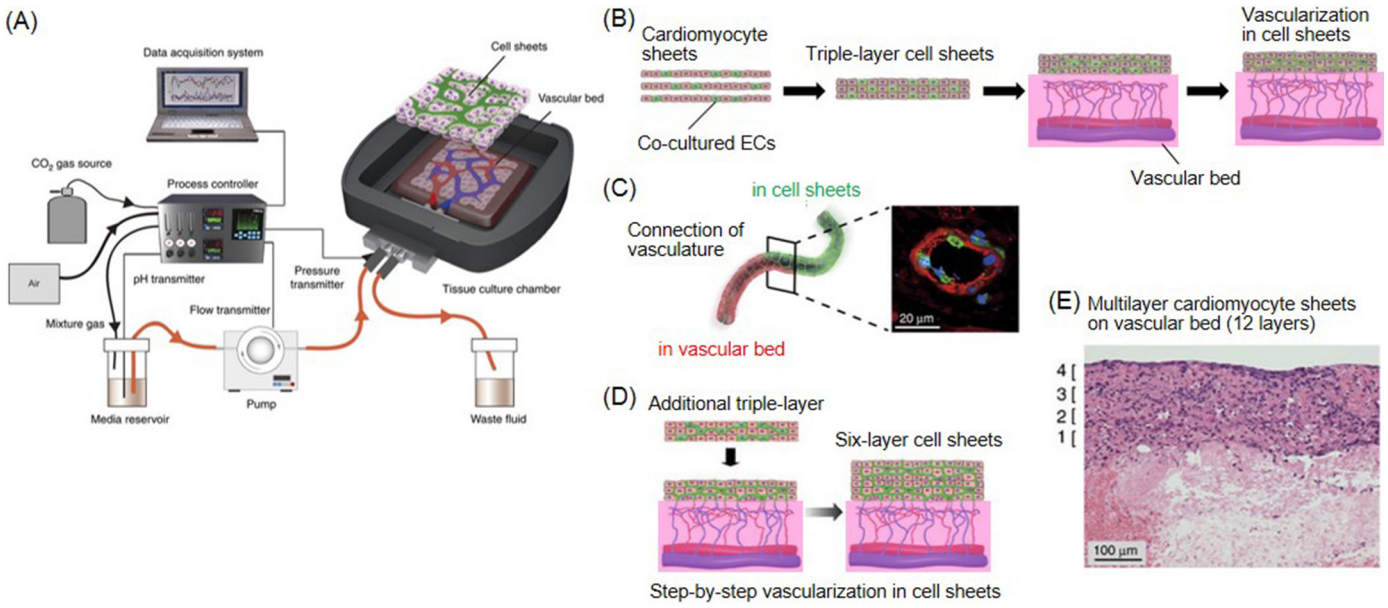

Fig. 5. (A) Schematic illustration of a custom-made bioreactor for perfusion culture of a layered cell sheet construct with a vascular bed. (B) Production of a vascularized tissue construct using a vascular bed. ECs co-cultured with cardiomyocyte sheets form a vascular-like branching network. (C) The EC network can connect to the vasculature in the vascular bed, suppling oxygen and nutrients to the multilayer cell sheet construct through the newly formed vasculature. (D) After the vascularization in the first step, repeated layering of additional triple-layer cell sheets will produce thicker cell sheet constructs complete with new vasculature formations. (E) A previous study demonstrated that a 12-layer cardiomyocyte sheet construct can be produced using this vascular bed system without severe necrosis inside the construct. Adapted with permission from [83].

6. Cell sheet-based technology for liver tissue engineering

6.1. Hepatic cell sheet transplantation

Liver regeneration is expected as a new attractive therapy for treating liver diseases, because orthotopic liver transplantation is severely restricted by a shortage of donor livers. Therefore, cell therapy using hepatocytes has been investigated by injection of freshly harvested hepatocytes into the spleen or the portal vein [97]. However, it is well known that hepatocytes rapidly lose their hepatocyte-specific functions in normal in-vitro 2D culture. Moreover, their functional survival over the long term after transplantation is poor. In liver tissue engineering therefore, the key issue in producing artificially functional hepatic tissues is the ability to maintain their important hepatic functions [98–100]. In cell sheet-based tissue engineering, hepatocytes cultured on thermoresponsive culture substrates can be harvested as a cell sheet by lowering the culture temperature to 20 °C. Importantly, the hepatocyte sheets maintain their intercellular microstructures such as desmosomes, gap junctions, and bile canaliculi [101]. This is advantageous because these microstructures are critical for preserving hepatocyte-specific functions. Furthermore, thick 3D liver tissues can be produced simply by layering multiple hepatocyte sheets onto each other. After transplantation in mice, these hepatocyte sheets form stratified liver structures made of host ECs and the donor hepatocyte sheet, and successfully maintain their functions for a long time period (e. g., >200 days). In fact, this approach significantly improved the functions of the damaged liver tissue. Recently, hepatocyte cell sheets have also been fabricated from hepatocyte-like cells that were in turn derived from human mesenchymal stem cells or iPS cells [102,103]. The transplantation of the iPS cell-derived hepatic cell sheets improved the lethal liver injury in liver-injured mice. In the near future, human liver fabrication might become an alternative option for treatment of liver diseases.

6.2. 3D co-culture system based on cell sheet layering

Living tissues are comprised of multiple cell types where cell-to-cell interactions influence and maintain the development of physiological functions and activities. In particular, since it is well known that ECs provide the appropriate environment for hepatic cells under normal culture conditions, co-culture techniques can support hepatocyte functions in-vitro [104,105]. Furthermore, the patterned hepatocytes are able to effectively maintain their functions by optimizing the size of the hepatic cell patterns with ECs [106,107]. Based on these results, co-cultured cell sheets of hepatocytes and ECs patterned at the optimal size were produced using a micropatterned thermoresponsive surface (Fig. 7A-C) [108,109]. Since the co-patterned hepatocytes maintain the ability to synthesize albumin and urea, this approach using the patterned hepatocyte assembly is expected to be useful for liver tissue engineering. Cell sheet layering technology also allows the production of unique 3D co-cultured tissue constructs. Whereas co-culture methods with ECs significantly contribute to maintaining hepatic functions, the structural configuration of hepatocytes in a 2D culture system is considerably different from that of the natural liver. By layering a hepatocyte cell sheet with EC sheets, a 3D co-patterned hepatic tissue can be uniquely fabricated (Fig. 7D) [110,111]. Specifically, by sandwiching a hepatocyte sheet between two EC sheets, in vivo-like hepatocyte polarization was organized within this 3D hepatic tissue construct (Fig. 7E). This morphological similarity between the engineered hepatic tissues and native liver is advantageous for mimicking more closely the native liver, which effectively deliver blood components to the hepatocytes. Additionally, hepatocyte-specific functions (albumin secretion, ammonia removal and the induction of cytochrome P450) was maintained in the long term (Fig. 7F) [111]. Therefore, the 3D hepatic tissue constructs could be valuable as a tissue model for drug discovery, as a transplantable tissue for cell therapies and as an efficient culture platform for bioartificial liver devices [112].

7. Epithelial cell sheet for endometrium regeneration

Uterine disorders such as intrauterine adhesions (IUA), known as Asherman's syndrome, often cause infertility. Clinical therapy for this kind of disease is mainly to prevent re-adhesion by surgical synechiotomy, administration of hormones after the operation, and the use of intrauterine devices. However, re-adhesion is often observed because the epithelial cells are severely damaged during surgery and are unable to significantly regenerate after the therapy. Recently, it was

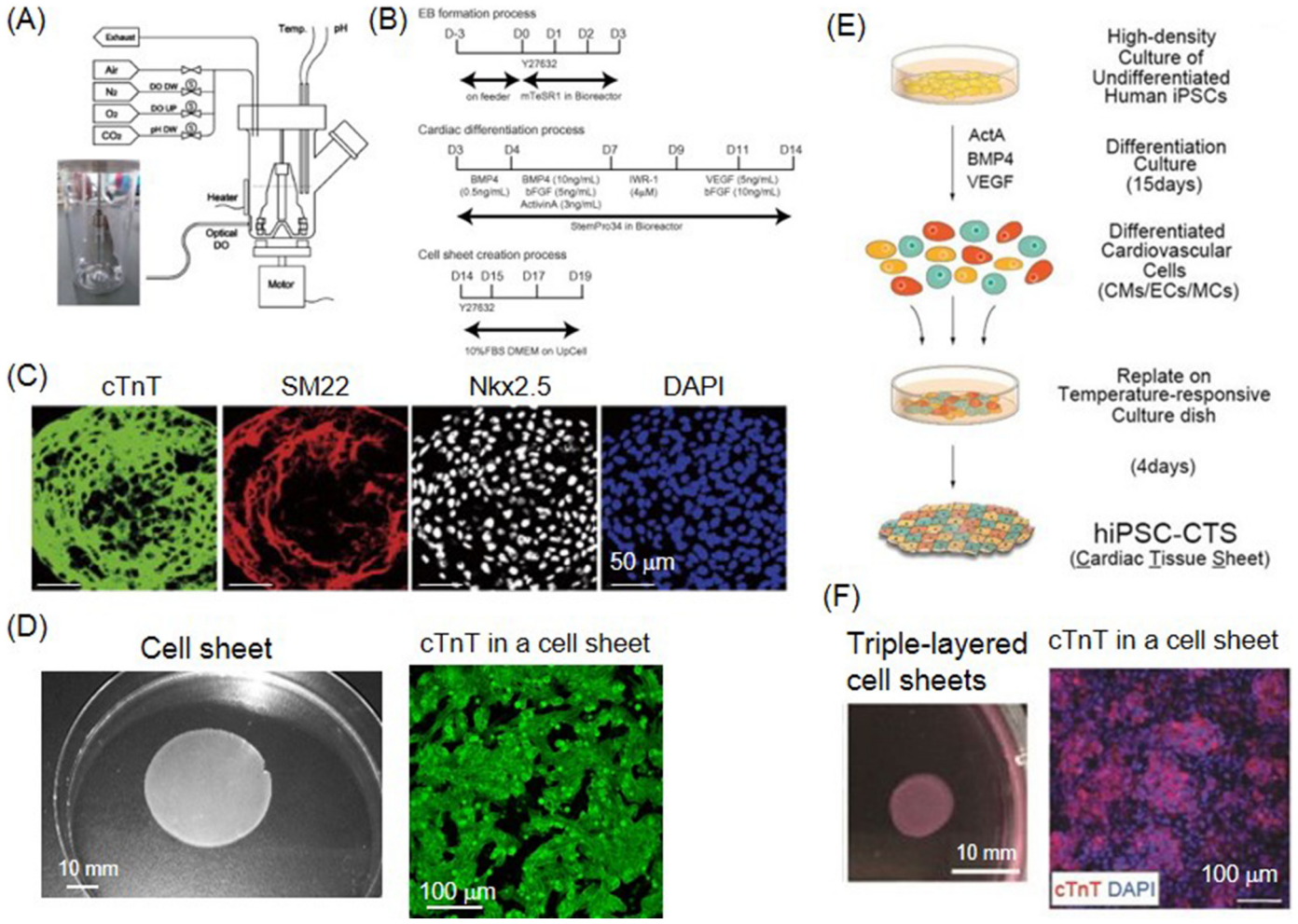

Fig. 6. (A) Schematic illustration of a bioreactor system for iPS cell expansion and cardiac differentiation. (B) Schematic of the cardiac differentiation process in the bioreactor system and cell sheet fabrication on a thermoresponsive substrate. (C) Microscopic images of an embryonic body (EB)-like cell aggregate consisting of cTnT-positive cells that co-expressed SM22 and Nkx2.5. Nuclei were stained with DAPI. (D) Photograph and fluorescence microscopic image of a cell sheet composed of human iPS cell-derived cTnT-positive cells harvested from a thermoresponsive surface. (E) Schematic diagram of fabrication of a cell sheet composed of human iPS cell-derived cardiomyocytes (CMs), endothelial cells (ECs) and vascular mural cells (MCs). (F) Photograph of triple-layered cardiac cell sheets and fluorescence microscopic image of a cell sheet composed of cTnT-positive cells harvested from a thermoresponsive surface. Adapted with permission from [92,95].

demonstrated that epithelial cell sheets can be used to regenerate the epithelial tissue of the endometrium. In an IUA rat model, oral mucosal epithelial cell sheets can be transplanted onto the endometrium defect since they preserve important cell–cell junctions and an associated ECM [113]. This cell sheet transplantation induces the formation of a luminal structure in the center of the uterus, resulting in the suppression of inflammatory reaction and the prevention of IUA caused by endometrial damage. Since the anatomy of the uterus is different between human and rat, the rat IUA model prepared in this study might not accurately mimic clinically observed human IUA. Although this difference needs to be considered carefully, this cell sheet engineering can potentially become a new therapeutic method to prevent re-adhesion after the treatment of IUA.

8. Cell sheet-based anisotropic tissue engineering

8.1. Regulating cell orientation in cell sheet engineering

Some kinds of native tissues form specific structures that are well-known key factors that produce an associated functionality [114–117]. Early in the development stage, myoblasts first form an aligned structure and then differentiate into multinucleated myotubes. The newly formed myotubes elongate unidirectionally and consequently mature muscle fibers form a bundle structure in native skeletal muscle. Since this anisotropic architecture is a crucial component to produce the mechanical function of skeletal muscle, myogenic cells need to be regulated directionally to regenerate native tissue-like functions. To achieve regeneration of these types of tissues, surface patterning techniques can be used to control the cell orientation [118,119]. For example, microgrooved polydimethylsiloxane (PDMS) substrates are widely used to control cell alignment. By simple polymer grafting techniques (e.g., EB irradiation, UV irradiation), PIPAAm can be grafted on microgrooved PDMS and TCPS substrates. The PIPAAm grafting allows the micropatterned thermoresponsive surface to function both to regulate cell orientation and release the aligned cells [120,121]. A thermoresponsive surface consisting of nanotopographic patterns (800 nm wide and 500 nm deep) is also useful to produce a cell sheet forming aligned orientation (Fig. 8A) [122]. These topographical guidance cues to align muscle cells and the thermally-triggered surface alternation induces the detachment of the aligned cells as a single continuous cell sheet. Micro-contact printing method was also used to regulate cell orientation on a thermoresponsive surface. Since cell-adhesive proteins such as fibronectin can be simply patterned on the surface, cells cultured in serum-free medium adhere only on the protein patterns and spread in the same direction as the stripe patterns (Fig. 8B) [123]. By changing to serum containing medium, the aligned cells proliferate until reaching confluence on the patterned thermoresponsive surface. In the addition to these conventional patterning methods, the LRP process is also used to fabricate

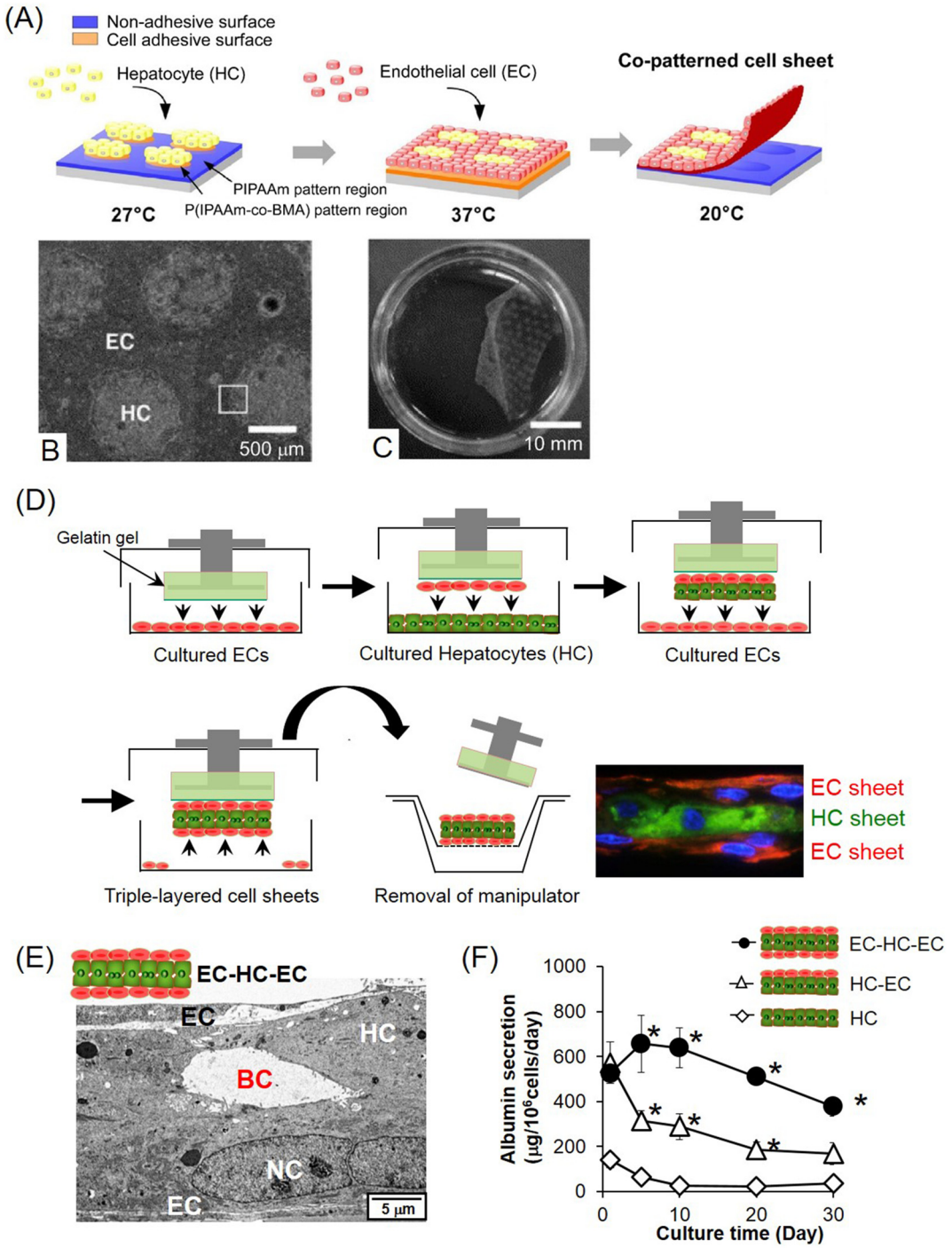

Fig. 7. (A) Fabrication process of co-patterns of rat primary hepatocytes (HCs) and endothelial cells (ECs) and harvest of a cell sheet using a micropatterned thermoresponsive surface. (B) Microscopic image of co-patterned HCs and ECs. (C) Photograph of a detaching cell sheet, composed of patterned HCs and ECs, by lowering culture temperature. (D) Preparation procedure of multilayer cell sheets composed of HCs and ECs. EC monolayer can be harvested as a single continuous cell sheet by attaching it to a gelatin gel-coated manipulator and transferring onto a hepatocyte sheet. Next, the two-layered cell sheets can be transferred onto a secondary EC sheet to complete the sandwich of a hepatocyte sheet between two EC sheets. (E) The formation of bile canaliculus (BC) can only be observed in multilayer cell sheets where a hepatocyte sheet is sandwiched between two EC sheets (EC-HC-EC). NC: Nucleus. (F) Albumin secretion is maintained in the long-term within the 3D co-cultured hepatic tissue (EC-HC-EC), compared to two-layered hepatocyte and EC sheets (HC-EC) and a single hepatocyte sheet (HC). Adapted with permission from [108,109,111].

micropatterns on a thermoresponsive polymer layer. Through a two-step polymerization, hydrophilic polymer (e.g., poly(*N*-acryloylmorpholine) (PAcMo)) can be grafted site-specifically on a PIPAAm layer, resulting in the formation of two different kinds of polymer domains (Fig. 8C) [124,125]. Cells seeded on the surface recognize the difference in cell-surface affinity between the two regions. The width of the patterns is a key factor in the regulation of cell orientation, and a 50 μm/50 μm stripe pattern was appropriate to produce a cell sheet composed of aligned cells including fibroblasts, osteoblasts and myoblasts [118,119,125]. As an alternative, polyacrylamide can also be grafted spatio-selectively using a photomask through a photo-initiated polymerization process (Fig. 8D) [126,127]. Simply by culturing cells on these surfaces, the cells are aligned, reach a confluence while maintaining the orientation, and can be harvested as a single cell sheet from the culture surfaces. In native vasculatures, smooth muscle cells (SMCs) formed an anisotropic structure which is important to generate mechanical functions. SMC sheets

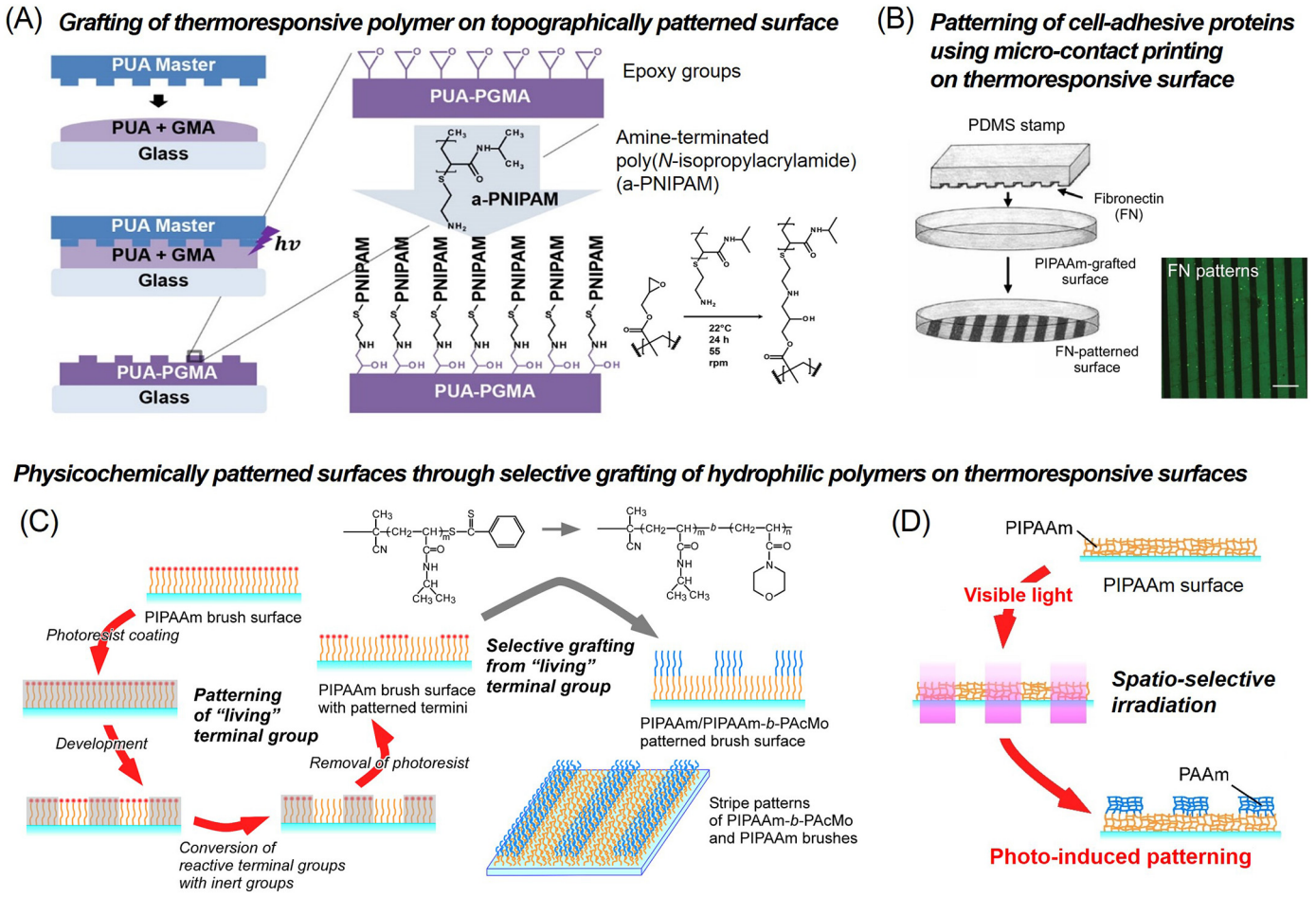

Fig. 8. (A) Schematic illustration of capillary force lithography to fabricate a topographic surface and PIPAAm grafting through epoxy amine addition reaction on the surface. (B) Micro-contact printing of fibronectin to produce cell-adhesive protein patterns on a thermoresponsive surface. (C) Schematic representation of photolithographically patterned PIPAAm brush surface through selective grafting of poly(*N*-acryloylmorpholine) (PAcMo) segments from PIPAAm blocks. The red termini indicate reactive groups. PAcMo segments shown as the blue brushes were grafted through the second-step living radical polymerization. (D) Schematic illustration of spatio-selective grafting of polyacrylamide (PAAm) through photo-induced polymerization using a photomask. Adapted with permission from [49,122–127].

harvested from patterned thermoresponsive substrates were remarkably stiffer in the aligned direction compared to the perpendicular direction [121]. This mechanical anisotropy is necessary for engineering vascular tissue constructs that mimic the complex mechanical behaviors of native vasculatures. The cell orientation influences not only the physical but also the biological properties of the cell sheets. For example, VEGF secreted by a human dermal fibroblast sheet was significantly increased by regulating their alignment within the cell sheet [125]. As described above, vascularization is one of the main issues to successfully construct large-scale tissues [83,84]. Since angiogenic growth factors, such as VEGF, are key mediators to promote vascularization, this type of biological property can be exploited to contribute to the promotion of vascularization in engineered tissues.

8.2. Cell sheet-based skeletal muscle tissue engineering

Since native skeletal muscle tissue is made of highly oriented myofibers, the orientation of muscle cells needs to be regulated to produce biomimetic muscle tissue constructs [128–130]. The micropatterned thermoresponsive surface can be used to align human skeletal muscle myoblasts in a parallel direction with striped patterns and then harvested as a single continuous cell sheet (Fig. 9A) [49,50]. Interestingly, the aligned orientation of the myoblasts can be maintained even after transferring onto a normal culture substrate. This is probably due to the unique property of myoblasts which prefer to form into an aligned structure. In addition, they can differentiate into myotubes while maintaining their aligned orientation (e. g., culture in 2% horse serum containing media) (Fig. 9B), and this aligned structure will be important to produce biomimetic muscle tissue [131]. In fact, regulating the aligned orientation stimulates the formation of longer myotubes, compared to randomly oriented myoblasts (Fig. 9C). Furthermore, since the cell sheet can be transferred with an aligned orientation, the aligned myotubes can be cultured on a fibrin-based gel to promote the maturation of the myotubes. This cell sheet-based muscle tissue can be produced by transferring the cell sheet onto a fibrin-based gel and then culture it in a differentiation medium. After 3 weeks of culture, this tissue construct shows sarcomere structures which are required for the tissue to be able to contract (Fig. 9D) [127]. As a result, this engineered tissue shows unidirectional contraction with electrical pulse stimulation (EPS). In addition, twitch and tetanic contractions can be observed depending on the EPS conditions (Fig. 9E). Since the tetanic contraction is mainly caused by the physiological temporal summation phenomena of the skeletal muscles, these contractile behaviors indicate that this cell sheet-based tissue is physiologically functional and has the potential to be applied as a skeletal muscle tissue model.

9. Tissue engineering by combination of cell sheet and scaffold-based engineering

The unique properties of cell sheets can also be used in conjunction with scaffold-based fabrication. In the field of vascular tissue engineering, the basic strategy is to mimic a native blood vessel by using a

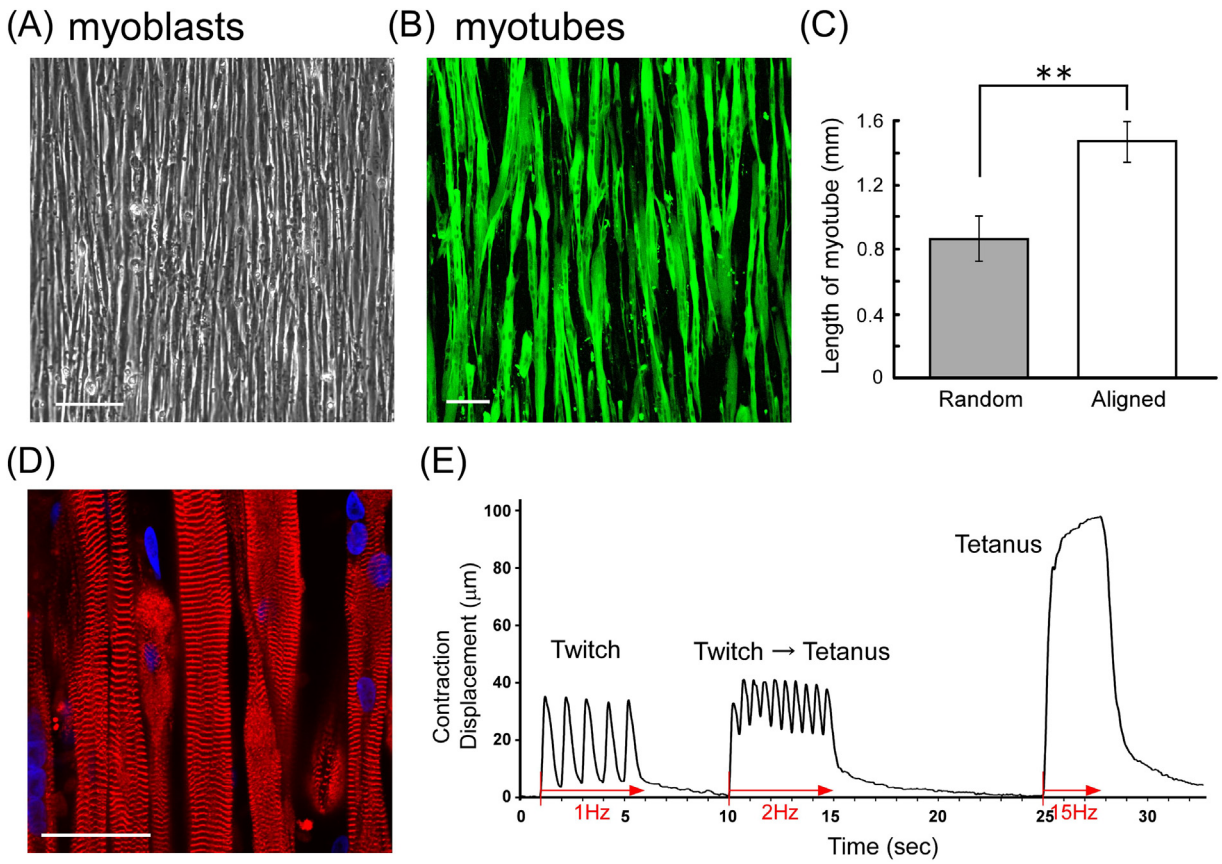

Fig. 9. (A) Phase contrast microscopic image of aligned myoblasts on the micropatterned surface. (B) Confocal microscopic image of differentiated myotubes from aligned myoblasts on a normal cell culture surface. The cell sheet was transferred onto a normal culture dish, and then the differentiation was induced by 5 day-culture in the differentiation medium. This indicates that the aligned cell orientation can be maintained after transfer on a non-patterned surface. Green: myosin heavy chain. (C) Length of myotubes differentiated from randomly oriented (Random) and aligned myoblasts (Aligned). ($^{**}p < 0.01$) (D) Confocal microscopic image of myofibers forming sarcomere structure cultured on a fibrin-based gel. The aligned cell orientation can be maintained after transfer on the gel for several weeks. Red: α-actinin. Blue: Cell nuclei. (E) Contractile behaviors of the myotubes electrically stimulated at different frequencies (1, 2, and 15 Hz). The contraction displacement was monitored by microscopy and calculated using a motion analysis tool. The arrows indicate the time range of EPS at each frequency. Twitch contractions partially fused by increasing the frequency to 2 Hz, and complete tetanic contraction was shown at 15 Hz. Scale bar: (A, B) 100 μm, (D) 50 μm. Adapted with permission from [49,127].

tubular scaffold with ECs and SMCs. Tubular scaffolds have often been produced by electrospinning of biocompatible polymers [132,133]. By seeding ECs onto a tubular scaffold composites of synthetic polycaprolactone and type I collagen, an engineered vascular tissue can be produced and maintain its structural integrity even after implantation into a rabbit aorto-iliac bypass model [134]. To produce mature blood vessels, SMCs play an important role since they contribute to contractility and the mechanical stability of mature vasculatures [135,136]. However, due to the tubular-shaped geometry of the vascular scaffolds, cell seeding onto the exterior surface of the scaffold fails to achieve efficient cell penetration into the vascular scaffolds. On the other hand, SMC sheets can be produced using a thermoresponsive substrate and the vascular graft can be effectively wrapped with a cell sheet (Fig. 10A) [137]. Cell sheet technology enables a mature smooth muscle layer to be produced before applying it to the vascular graft. Unlike the simple seeding of SMCs, SMC sheets harvested from a thermoresponsive surface preserve the important cell-cell junctions and generate contractile proteins when applied onto the vascular scaffold (Fig. 10B). In addition, a secondary harvested cell sheet can be subsequently layered onto the first cell sheet such that multiple cell sheets can continued to be wrapped around the graft. To obtain a more mature tissue, the pulsatile flow was applied in the previous study. The pressure to the cell sheet-vascular scaffold leads to tissue maturity that can withstand the level of blood flow from a native artery. The combination of cell sheet and scaffold-based engineering also allows engineering of a 3D bladder patch for bladder regeneration [138]. Multiple adipose-derived stem cell (ASC) sheets were layered and attached onto a polyglycolic acid (PGA) scaffold (Fig. 10C). Whereas various kinds of engineered tissue constructs have been developed for urinary bladder reconstruction, conventional approaches are still required to be improved to achieve transplantation of therapeutic cells to target sites. Since the ASC sheet transplantation provides a large number of ASCs with the porous scaffold and then promotes significant regeneration of the urothelium in a rat model, this tissue-engineered bladder patch has great potential for future bladder regeneration.

10. Cell behaviors in 3D environment made of multilayer cell sheets

10.1. Vascular-like cellular network formation

In order to prevent necrosis within large-scale engineered tissues, many research groups have reported methods to engineer vascularized tissue constructs [78–81]. In the cell sheet-based approach, tissue constructs greater than four-layered cell sheets (>100 μm in thickness) have been limited in thickness due to hypoxia; however, advanced bioreactor systems can be used to create thicker cell sheet constructs [83,84]. In multilayered cardiomyocyte cell sheets, co-cultured ECs form vascular-like branching networks. This cellular network can mature in the bioreactor system, and the newly formed vasculatures supply oxygen and nutrients into the vascularized tissue construct. Therefore, ECs incorporated within tissue constructs play an important role to accelerate vascularization in tissue constructs. As an alternative approach,

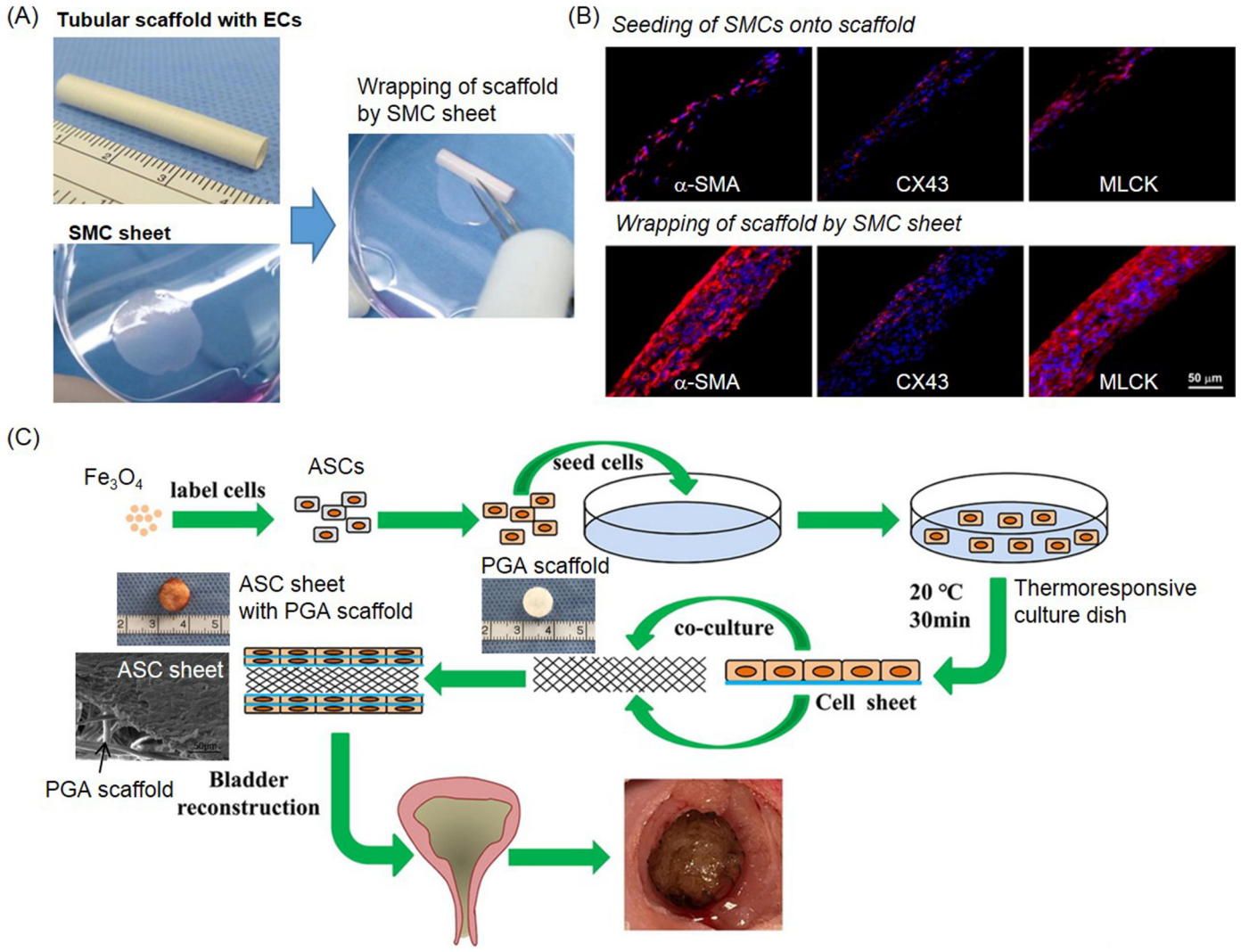

Fig. 10. (A) Fabrication of smooth muscle cell (SMC) layers on a vascular scaffold based on cell sheet engineering. By wrapping a tubular scaffold composed of PCL/collagen with SMC sheets, a mature smooth muscle layer can be efficiently produced, compared to one simply fabricated by seeding of SMCs onto a scaffold. (B) Fluorescence images of SMC-specific proteins including α-smooth muscle actin (α-SMA), connexin 43 (CX43), and myosin light chain kinase (MLCK) generated at a higher level by cell sheet wrapping when compared with that by single cell seeding. (C) Schematic illustration of fabrication of a bioengineered bladder patch composed of a porous PGA scaffold and multilayered adipose-derived stem cell (ASC) sheets. Adapted with permission from [137,138].

ECs can be sandwiched between multiple cell sheets to incorporate them within the construct [50,82,139]. For example, when human umbilical vein endothelial cells (HUVECs) were cultured between two myoblast sheets via the cell sheet layering process, they formed branching networks within the cell sheet construct. Interestingly, by seeding HUVECs onto a single cell sheet, they simply adhere as a single cells onto a cell sheet without the cell sheet layering (Fig. 11A). On the other hand, the cell sheet layering triggers the formation of a branching structure (Fig. 11B). This indicates that the 3D environment provided by cell sheets is important for the induction of the network formation and can only be obtained by the layer-by-layer construction technique. Since this scaffold-free cell-dense architecture allows communication between myoblasts and ECs, the cells incorporated within the construct can recognize their 3D cell environment. Moreover, cells also recognize the anisotropy of their tissue environment. When HUVECs are incorporated in multilayer cell sheets composed of aligned myoblasts, they form anisotropic branching structures according to the myoblast orientation (Fig. 11C). It is important that the recognition of the tissue anisotropy by ECs and the subsequent self-organization is due to the cell-dense tissue architecture made up of only cells and ECM proteins.

10.2. Arrangement of 3D orientation using cell sheet layering techniques

In some native tissues, 3D anisotropy is essential for specific mechanical and biological functions. Native myocardial tissue consists of multiple layers of aligned cardiomyocytes that are oriented in various directions throughout the whole tissue [116,117,140]. This 3D structure is well-designed to generate the electrical propagation in the native myocardium. Cell sheets composed of aligned cells can be layered while maintaining the designed orientation [45,122]. For example, gelatin casting method allows cell sheets to be stacked without the 3D scaffold. Since a cell sheet attaching to a gelatin gel can be transferred with maintaining the aligned orientation, 3D perpendicularly orientated tissue constructs can be created without the use of microfabricated materials (Fig. 12A and B) [122]. A gelatin gel coated plunger is also used to layer multiple cell sheets in different directions [125]. Whereas 3D microfabricated scaffolds are usually used to produce 3D oriented tissues, these cell sheet techniques allow the creation of a variety of tissue architectures that does not require any scaffolds. As described above, the cell-dense tissue structure is important for cells to communicate with each other within the layered cell sheet construct. In addition, this unique environment enables the introduction of a unique behavior

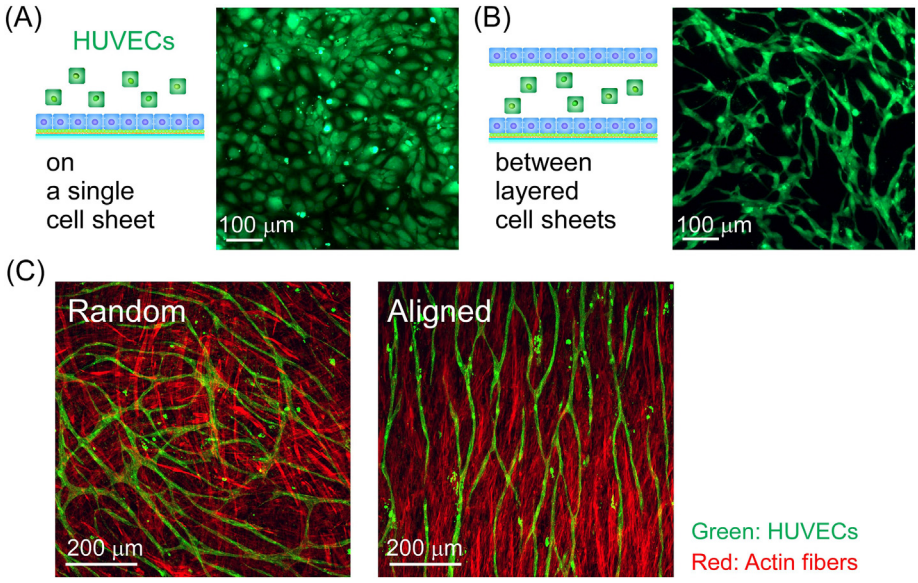

Fig. 11. (A) HUVECs adhering on a single myoblast sheet at Day 1 after cell seeding. (B) Formation of vascular-like branching networks within two-layered cell sheet construct at Day 1 after cell layering. HUVECs were stained with CellTracker Green. (C) Branching network formations of HUVECs incorporated between cell sheets composed of randomly oriented myoblasts (Random) or aligned myoblasts (Aligned). Images were taken at Day 5 after layering. Adapted with permission from [50].

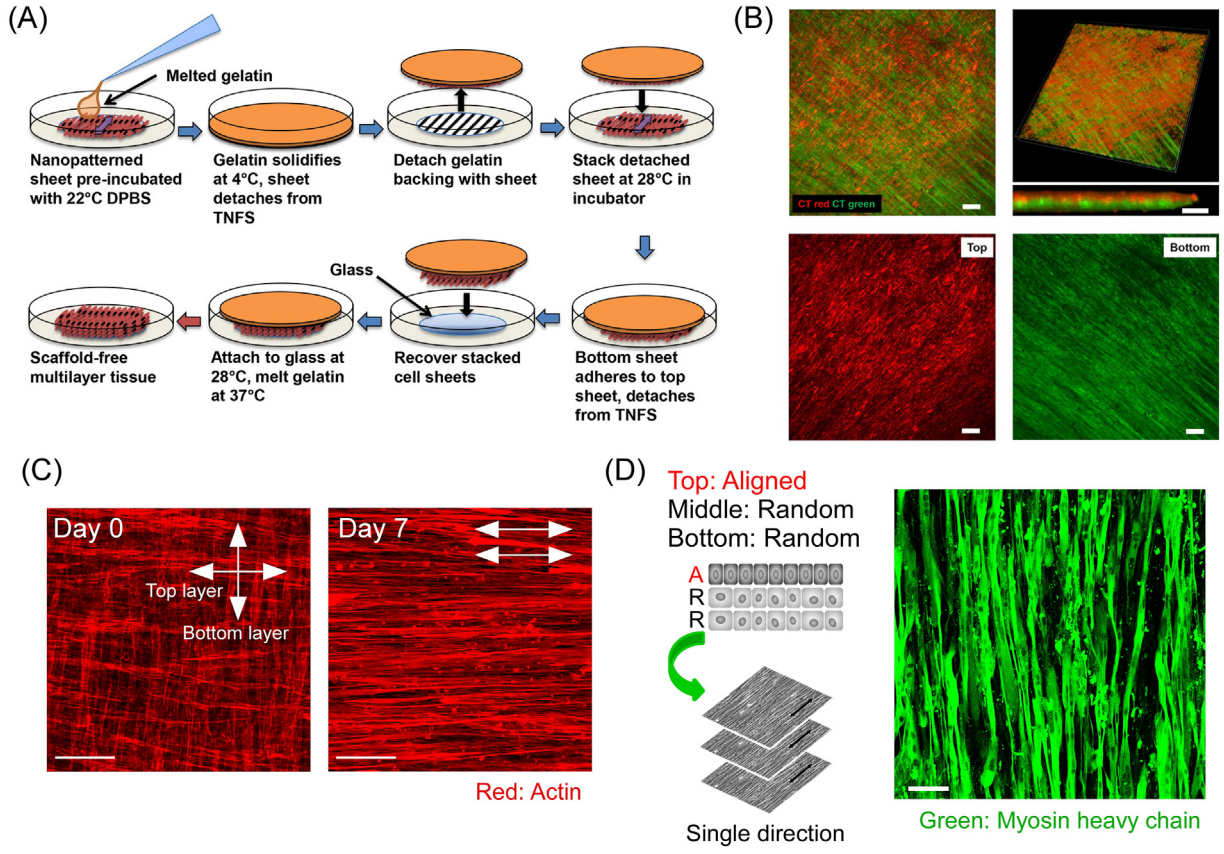

Fig. 12. (A) Schematic illustration of cell sheet layering method using a gelatin gel to transfer cell sheet anisotropy. (B) Fluorescence image of perpendicularly layered cell sheets produced by cell sheet layering. Top and bottom cell sheets were stained with red and green, respectively. (C) Fluorescence images of perpendicularly layered myoblast sheets produced by cell sheet layering. Actin fibers of cell sheets were stained just after (Day 0) and after 7 days of the cell sheet layering (Day 7). Only the single orientation can be observed within multilayered myoblast sheets at Day 7. (D) Myotube construct produced through differentiation of triple-layered myoblast sheets. Since random cells rearranged their orientation by themselves, all myotubes formed aligned orientation within the multilayered cell sheet construct. Scale bar: 100 μm. Adapted with permission from [49,122].

of myoblasts previously unknown [49]. When perpendicularly oriented myoblast sheets were produced, only a single direction was observed within the oriented cell sheets at Day 7 after cell sheet layering (Fig. 12C). This was because that the myoblasts recognized the aligned environment and then self-organized their orientation. Interestingly, in all cases the bottom myoblasts changed their orientation to align with the direction of the top myoblasts. Therefore, when random myoblasts are layered with a cell sheet composed of aligned myoblasts, all cells align by themselves in the same direction as the aligned cells in the top sheet (Fig. 12D). This self-organization process shows an important mechanism in regulation of cell/ECM orientation in native muscle tissues. Furthermore, this cell behavior gives us simpler method to produce 3D myotube constructs having a single orientation. Myoblasts in the multilayered cell sheet construct are able to self-adjust their 3D orientation, and then the 3D tissue construct finally forms an aligned structure of myotubes (Fig. 12D). This unique behavior of myoblasts was found in a scaffold-free cell-dense environment, and the unique tissue structure allows the cells to recognize tissue anisotropy and flexibly self-organize their orientation.

11. Conclusions

Cell sheet-based tissue engineering has been able to expand its unique features and advantages for use in regenerative medicine and tissue modeling. Nano-scale PIPAAm grafting provides culture substrates with a thermoresponsive property which allows it to thermally regulate cell adhesion and detachment. PIPAAm hydrogels conjugated with cell-adhesive biomolecules can also be used to produce a single cell sheet. Importantly, these cell sheets can be fabricated with intact cell-cell junctions and the associated ECM. Based on this technology, cell sheet therapy can deliver various therapeutic cells to damaged sites and have already been applied in a number of human clinical studies. Moreover, the cell sheet transplantation approach will be applied to other types of tissue regeneration including myocardium, liver, and endometrium in the near future. In addition, cell sheet layering process can be used to produce 3D tissue constructs without the use of 3D scaffolds. To successfully scale-up these cell-dense tissues, in-vitro vascularization techniques using a vascular bed have been developed. In fact, twelve-layer thick cardiac cell sheet constructs can be produced on a vascular bed, and the same procedure will be applicable for other types of cell sheet constructs. On the other hand, scaling-up cell sheet constructs requests significant number of cell sheets. Compared to scaffold-based strategies, the preparation and layering of them are very time consuming when thick tissue constructs are produced. Therefore, it remains problem in the current cell sheet strategies. On the other hand, cell sheet-based tissue engineering is also effective for tissue modeling researches. Well-organized tissue models will be used to better understand the mechanism of specific diseases and develop therapeutic agents to treat a wide variety of diseases. Recently, based on iPS cell technology, human cell-based tissue models can be fabricated. Tissue engineering researchers believe that tissue models using disease-specific human iPS cells will become a powerful tool for producing a personalized tissue model. As an example, development of the culture method for iPS cell expansion and cardiac differentiation has enabled the production of human cell-based cardiac cell sheet constructs. With the vascularization method, thick human heart tissues are expected to be fabricated and will clearly be useful in future tissue modeling. In addition, techniques to construct more complex tissues with specific microstructures are required to produce tissue models that more closely mimic native tissues. The use of micropatterned thermoresponsive substrates provides anisotropy within the engineered skeletal muscle tissue, which will provide well-organized tissue constructs having mechanically and biologically biomimetic useful features. In addition to human clinical studies, the progress in cell sheet technology is expected to broaden the number of target diseases in regenerative medicine and enable us to produce human cell-based tissue models for treatment of refractory diseases. Whereas various kinds of cell types are expected to be appropriate cell sources to engineer tissue constructs, some kinds of cell types are still difficult to be used to produce a well-defined single cell sheet. Therefore, advanced techniques are continuously required for cell sheet preparation.

Competing interests

Teruo Okano, Ph.D. is a founder and Director of the Scientific Advisory Board of CellSeed Inc., which has licenses for certain cell sheet-related technologies and patents from Tokyo Women's Medical University.

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