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Construction of three-dimensional vascularized cardiac tissue with cell sheet engineering

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ABSTRACT

Construction of three-dimensional (3D) tissues with pre-isolated cells is a promising achievement for novel medicine and drug-discovery research. Our laboratory constructs 3D tissues with an innovative and unique method for layering multiple cell sheets. Cell sheets maintain a high-efficiently regenerating function, because of the higher cell density and higher transplantation efficiency, compared to other cell-delivery methods. Cell sheets have already been applied in clinical applications for regenerative medicine in treating patients with various diseases. Therefore, in our search to develop a more efficient treatment with cell sheets, we are constructing 3D tissues by layering cell sheets. Native animal tissues and organs have an abundance of capillaries to supply oxygen and nutrients, and to remove waste molecules. In our investigation of vascularized cardiac cell sheets, we have found that endothelial cells within cell sheets spontaneously form blood vessel networks as *in vivo* capillaries. To construct even thicker 3D tissues by layering multiple cell sheets, it is critical to have a medium or blood flow within the vascular networks of the cell sheets. Therefore, to perfuse medium or blood in the cell sheet vascular network to maintain the viability of all cells, we developed two types of vascular beds; (1) a femoral muscle-based vascular bed, and (2) a synthetic collagen gel-based vascular bed. Both vascular beds successfully provide the critical flow of culture medium, which allows 12-layer cell sheets to survive. Such bioreactor systems, when combined with cell sheet engineering techniques, have produced functional vascularized 3D tissues. Here we explain and discuss the various processes to obtain vascular networks by properly connecting cell sheets and the engineering of 3D tissues.

1. Introduction

Rapid progress has been made recently in tissue engineering toward new medical treatments and drug screening procedures since the discovery of embryo stem cells and induced pluripotent stem cells. Various tissue engineering technologies are currently being used, such as "top down" approaches where cells are seeded to biodegradable scaffolds [1,2], and "bottom up" approaches where cells are shaped into fiber and spheroid forms to construct organs [3–5]. In our laboratory, cells are shaped into a sheet, which has a highly efficient regenerating function because of its higher cell density and transplantation efficiency, compared with other cell-delivery methods [6–8]. Consequently, cell sheet engineering has attracted the attention of researchers around the world as a novel bottom-up approach.

Cell sheet engineering has only become possible since the development of new culture dishes. The surface of these new culture dishes alters the surface characteristics from hydrophobic to hydrophilic by simply changing the temperature, which allows cells to attach or detach from the surface. The surface of temperature-responsive culture dishes is grafted with a nanoscale-thick polymer layer (~20 nm) of poly (*N*-isopropylyacrylamide) by electron beam irradiation. At 37 °C, the dish surface becomes hydrophobic and allows cells to attach; in contrast, under 32 °C, the surface becomes hydrophilic and allows cells to detach from the surface while preserving the cell membrane connections and cell–cell communication [9].

Cell sheet engineering has already been successfully used in clinical applications of regenerative medicine for the cornea, heart, esophagus, periodontal membrane, and cartilage [10–15]. Future progress of cell-sheet treatments for serious injury and tissue deficiency, such as organ transplantation therapy, now depends on the development of 3D tissues. Therefore, we are now constructing not only single-layer cell sheets, but layering many cell sheets to fabricate highly functional three-dimensional and multi-layered cell-sheet tissues. However, the thickness of a multi-layered cell sheet has limitations, because of the possible necrosis inside the cell sheet due to the lack of capillaries to supply oxygen and nutrients [16,17]. Our laboratory found that endothelial cells spontaneously form blood vessel networks as capillaries in a single-layer cell sheet, as well as between sandwiched cell sheets. Furthermore, methods to optimize endothelial network formation are also

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being investigated by sandwiching oriented cell sheets. In this article, we explain and discuss the processes we use for constructing 3D tissues using cell sheet engineering and vascular bed bioreactors.

2. Vascular network formation in cell sheets

Since native *in vivo* capillaries have multiple functions such as supplying oxygen and nutrients for maintaining tissue viability, engineered 3D tissues also need capillaries to maintain their functions and viability. We have developed a functional cardiomyocyte cell sheet with the ability to contract, because the cell membrane functions were preserved [18]. The challenge to create thicker cardiac tissues, with high-functionality and stronger contractile ability, is entirely dependent on the number of capillaries created in the tissues. The human adult heart has ~10% of the entire body's capillaries and has an extremely high density of capillaries where separation distances are in the order of ~15 μm [19]. In a conventional culture dish, thin cardiac cell sheets with a thickness of approximately 20 μm, which is comparable to that of a single cell, can survive by simple diffusion of oxygen and nutrients in a culture medium, without capillaries. An innovative technology is required to break through this diffusion limit in 3D tissue engineering. Endothelial cells co-cultured with cardiac cells spontaneously formed a network structure (Fig. 1A) [20]. Cell sheets co-cultured with endothelial cells released more cytokines, such as vascular endothelial growth factor (VEGF), than cell sheets without endothelial cells (Fig. 1B) [21]. When a vascularized cardiac cell sheet was transplanted to the back of a nude rat, the cell sheet attached to the host tissue after 1–2 days, and the newly-created vascular network in the sheet has been confirmed to connect to the host capillaries. In contrast, transplanted cell sheets without endothelial cells did not produce a vascular network and subsequently showed a marked decrease in survival [21]. Furthermore, after transplantation to the infarcted myocardial tissue of an adult rat, a vascularized cell sheet was found to improve function of the infarcted heart. Fig. 1C demonstrates that vascular networks in a cell sheet connected to the host capillaries [22]. The green fluorescence color indicates the vascular network of the cell sheet and the red blood cells were from the host (the lower right photograph of Fig. 1C). Moreover, co-cultures of fibroblasts and endothelial cells, and myoblast and endothelial cells have also been found to offer sufficient vascularization and were highly effective for transplantation [23]. These results demonstrated how critical vascularization was to successful transplantation of highly functional 3D tissue.

3. Controlled orientation of vascular cells in sandwiched cell sheets

The spontaneous network formation of endothelial cells in a co-culture with cardiomyocytes, fibroblasts, and myoblasts has been reported. However, the cause of *in vitro* vascular formations is still poorly understood, and the formation in cell sheets is known to be uncontrollable. Therefore, we have investigated the possibility of controlling the shape of endothelial cell networks by using various types of patterned cell sheets having a single orientation. A culture dish grafted with a

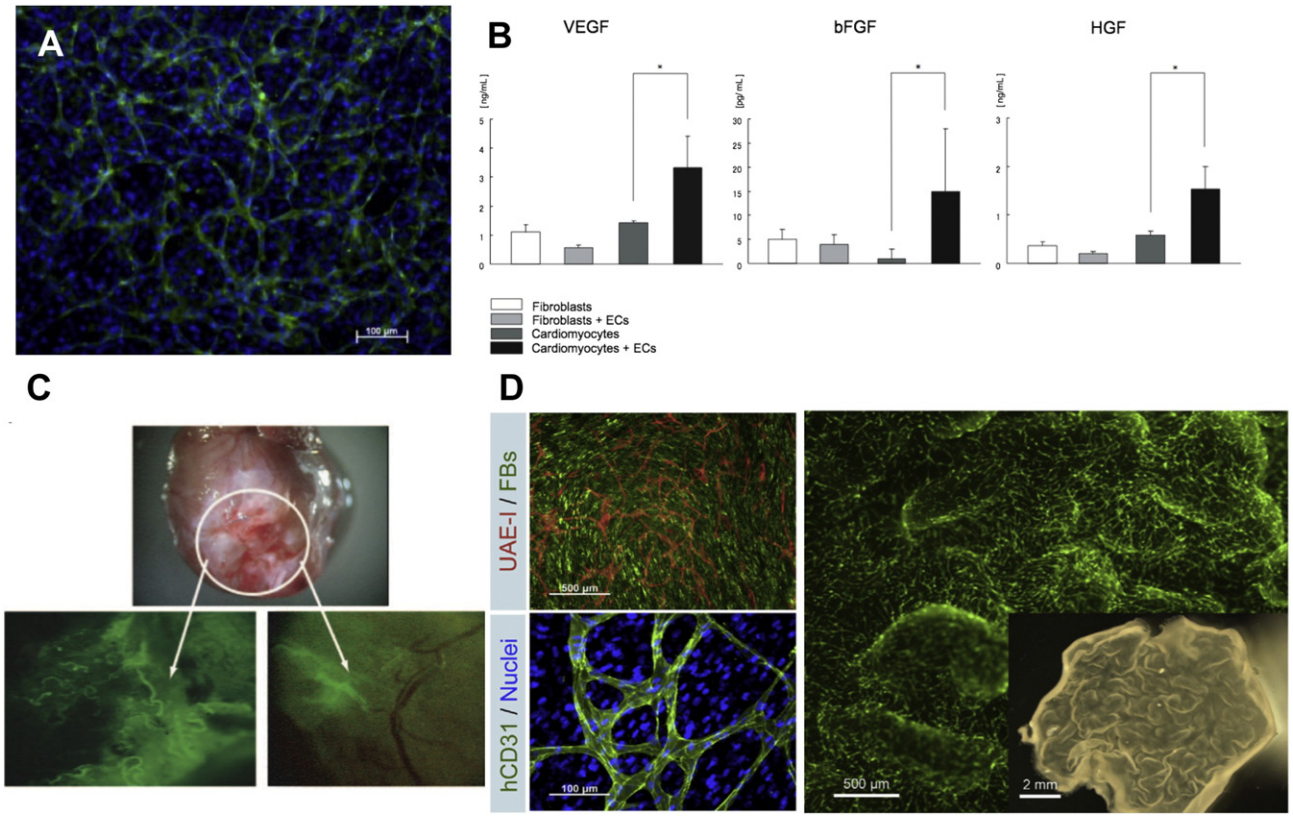

Fig. 1. Vascular networks in cell sheets. (A) Vascular network of endothelial cells in cardiac cell sheets treated with 10 mol/L rat recombinant vascular endothelial growth factor after 4 days. (B) Secretions of vascular growth factors from cardiac cell sheets. Amounts of cytokines secreted from cardiomyocyte and fibroblast sheets co-cultured with endothelial cells are measured by enzyme-linked immunosorbent assay. Protein expressions for vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor are demonstrated. Cardiac cell sheets containing endothelial cells secrete a significantly larger amount of all three angiogenesis growth factors than the sheet without endothelial cells ($n = 12$). Error bars indicate SD (*, $P = 0.05$). (C) Contribution of cell sheet with a vascular network to the neovascularization of ischemic hearts. Macroscopic view of capillary formation is shown. Green fluorescent protein-expressing vessels migrate into the host myocardium and connect to the capillaries of the host heart. White circle indicates the position of the transplanted cardiac cell sheets. (D) Fabrication of double-layered fibroblast sheet with vascular human aortic endothelial cells (HAECs). HAECs and fibroblast sheets strained with Ulex Europaeus Agglutinin 1 and anti-prolyl-4-hydroxylase antibody, respectively (upper left photograph). The networked cells are stained with anti-human CD31 antibody, and the nuclei are counterstained with Hoechst 33342 (lower left photograph). The photograph on the right shows harvested cell sheets consisting of a double-layered fibroblast sheet with HAECs.

parallel line pattern using different amounts of polymer, produced a fibroblast or myoblast cell sheet having a well aligned orientation, which was maintained for 7 days. Moreover, when an aligned cell sheet was put on a randomly aligned cell sheet, the bottom sheet aligned itself to the upper single-oriented cell sheet (Fig. 2A) [24]. Accordingly, the orientation of endothelial cells was also affected by sandwiching between two well-aligned cell sheets. This result showed that stripe-patterned endothelial cells were maintained along the orientation of the aligned fibroblast sheets. In contrast, stripe-patterned endothelial cells spread out when placed on a randomly orientated cell sheet and became a randomly oriented cell sheet (Fig. 2B) [25]. These results indicate (1) the possibility to control the endothelial cell network formation by sandwiching two aligned cell sheets, and (2) the possibility to design optimal vascular networks within engineered tissues.

4. *In vivo* construction of 3D tissue

Endothelial cells spontaneously form vascular networks in co-culture with cardiac cells, fibroblasts, and myoblasts, and the formed vascular networks effectively connect to the host capillaries after transplantation. However, because vascularized cell sheets also have a thickness limitation, cell sheets having more than three layers develop necrosis. Therefore, after angiogenesis from the host to the neovascular vessels of the transplanted triple-layered cell sheet was confirmed, another triple-layered cell sheet was transplanted onto the previously transplanted cell sheet, and then repeated. This method produced cardiac tissue approximately 1-mm thick, consisting of 30 layers of cell sheets with firm vascularization and synchronized pulsatility (Fig. 3A) [26]. Engineered vascularized cardiac tissues prepared by the method described above have been retransplanted to a different location in the host and confirmed to function properly (Fig. 3B) [26]. In another application, a tube-shaped 3D cardiac tissue has been created for assisting cases of heart failure and for drug screening. Specifically, a cardiac tube was prepared by wrapping cardiac cell sheets around an abdominal aorta, which was taken from an adult rat, and the cardiac tube was transplanted back to the original position of the aorta (Fig. 3C) [27]. Consequently, as the cardiac-tube beat it could be monitored electrically as well as inspected visually, and amazingly produced

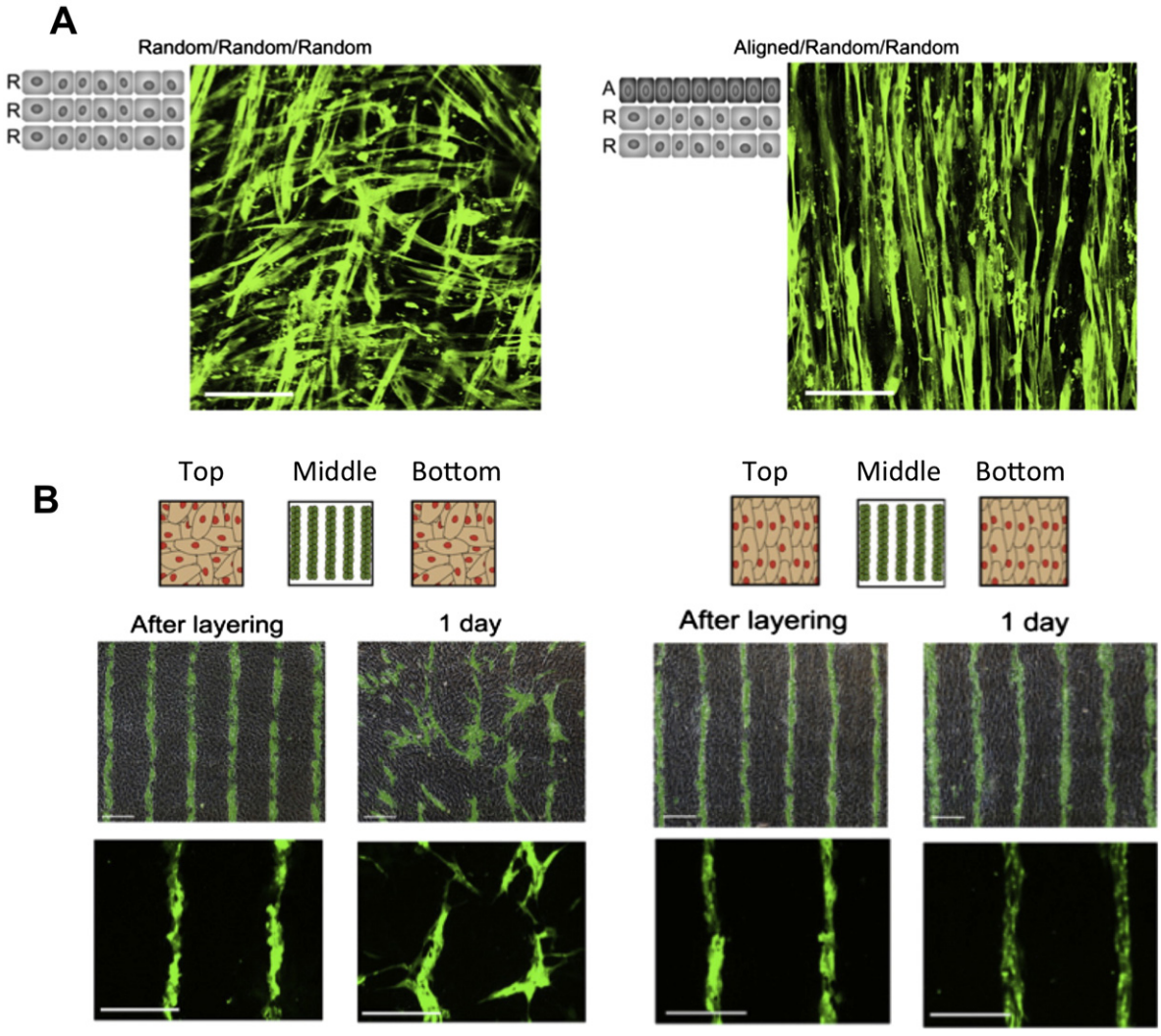

Fig. 2. Effect of cellular orientation. (A) Formation of aligned myoblast cell sheets though a self-organization process. An aligned sheet is layered onto two randomly oriented sheets, and then incubated in growth media for 2 days (Aligned/Random/Random). As a control, three randomly aligned sheets are layered together (Random/Random/Random). Both of these triple-layered myoblast sheets are cultured in differentiation media for 5 days, and then the myosin heavy chain was stained fluorescent green. Scale bar: 200 mm. (B) Movement of the patterned green fluorescent protein positive-human umbilical vein endothelial cells (GFP-HUVECs) in the multi-layered cell sheet with normal human dermal fibroblast (NHDF) sheets. Illustration at the top shows the cell arrangement in multi-layered tissues. The photographs on the upper row are merged photographs of the tissue by phase and fluorescent microscopy, and photographs on the bottom row show high magnification fluorescent photographs of GFP-HUVECs. GFP-HUVECs sandwiched between non-aligned NHDF sheets keep their original pattern just after being sandwiched, but they lose the original pattern at 1 day after layering. However, GFP-HUVECs sandwiched with aligned NHDF keep their original pattern just after being sandwiched, even at 1 day.

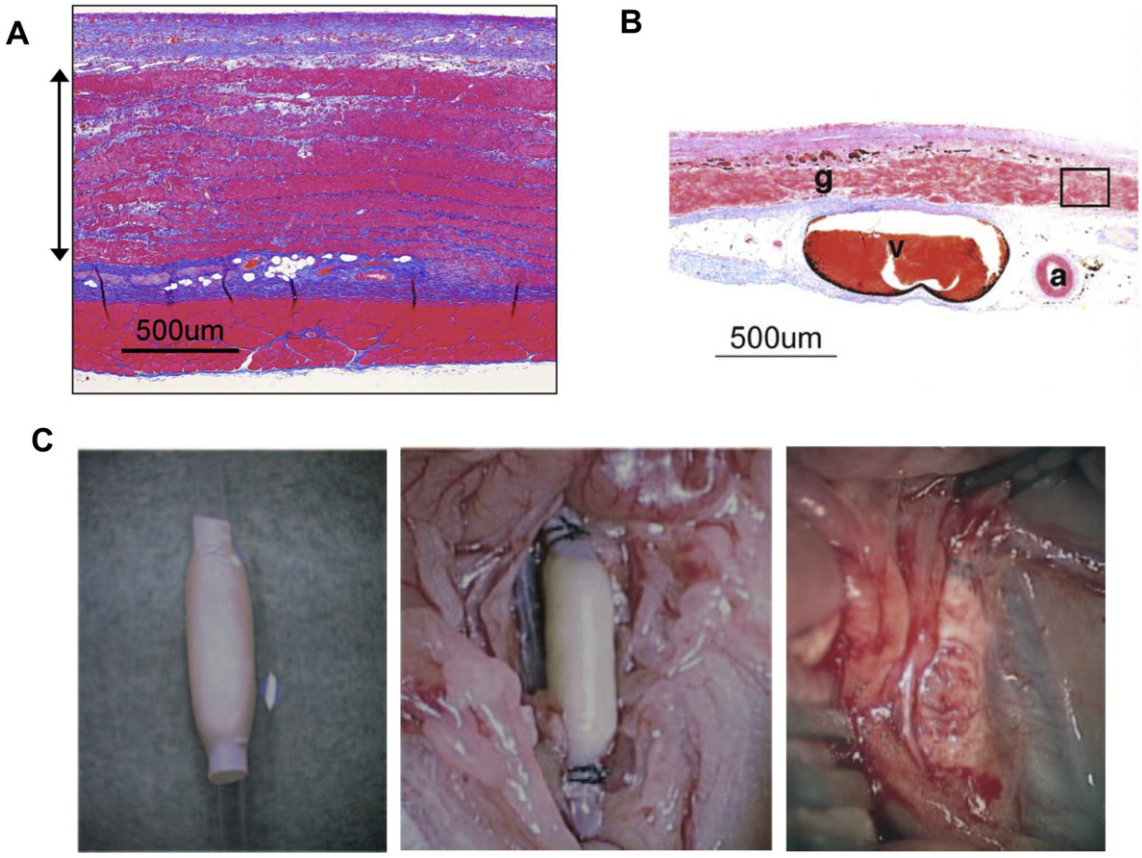

Fig. 3. *In vivo* construction of three-dimensional tissue. Thick cell-dense tissues made by poly-surgery repeated 10 times. Azan staining shows that after repeated poly-surgery with a 1-day interval, multi-layered cell-dense myocardium with well-organized microvessels was created. Bidirectional arrow indicates viable myocardial cell sheet layers. (B) Myocardial tissue graft created over top of the surgically accessible blood vessels. Azan staining shows a multi-layered myocardial tissue graft (g) over the caudal epigastric artery (a) and vein (v), which was fixed at 2 weeks after the poly-surgery procedure. (C) Fabrication and transplantation of myocardial tubes. After the noninvasive low-temperature harvest, individual cardiomyocyte sheets were sequentially wrapped around a resected thoracic aorta to produce a sextuple-layered myocardial tube *in vitro* (left photograph). The myocardial tube is anastomosed to the host vasculature (middle photograph). At 4 weeks after aortic replacement, the myocardial tube is integrated with the host tissues (right photograph).

a pressure of approximately 6 mm Hg. This work has shown that thick 3D tissues consisting of cell sheets could be successfully recreated in an *in vivo* situation. However, the repeated layering of cardiac cell sheet is not useful for clinical applications and drug screening, because the poly-surgery and quantification are too difficult on the patient. Therefore, we have been investigating the production of engineered cardiac tissues in an *in vitro* situation.

5. *In vitro* construction of 3D tissue

Three-dimensional tissues made by layering cell sheets *in vitro* are beneficial not only for the treatments of damaged tissues and failed organs, but also for drug screening. In recent years, many huge grants for research projects have been provided for drug efficiency tests with artificial 3D tissues, because drug-screening methods with 3D tissue models are recognized to be more useful due to the short processing time and low cost. Therefore, many large-scale and venture companies are entering the drug screening market with 3D tissue models [28]. However, the creation of such 3D tissues having more complex biomimetic structures is much more difficult in conventional culture methods using simple static culture dishes. Recently, bioreactor technologies have attracted the attention of the public as well as tissue engineering researchers. Numerous types of bioreactors have been developed which can provide various conditions such as high hydro-pressure, perfusion, and mechanical stretching to mimic an *in vivo* condition, to be used in the fabrication of functional 3D tissues. We also developed two different types of bioreactors, having (1) a femoral muscle based vascular bed, and (2) a collagen-gel based vascular bed, to provide oxygen and nutrients to 3D tissues through the newly formed blood vessels in the tissue. In the first type of bioreactor, femoral muscle was resected from ~8 week-old rats, and a triple-layered cardiac cell sheet put on the femoral muscle with an artery–vein loop for re-transplant. After 3–5 days of perfusion-cultivation through an artery–vein loop, the newly created vascular networks in the cell sheet reached and connected to the capillaries of the vascular bed, and then, flesh culture medium flowed into the vascular network in the cell sheet. After the connection between the vascular network in the cell sheet and the capillaries in the femoral muscle was confirmed, another cell sheet was layered on the cell sheet. As a result, this repeated procedure produced thicker multi-layered cell sheets than those by a single-step procedure. Triple-layered cell sheets were layered four times, resulting in a 12-layered cell sheet [29]. In the second type of bioreactor, collagen-based microchannels (~300 μm inner diameter) were used for perfusing the vascular networks in the cell sheets. After a triple-layered cell sheet was cultured on the collagen gel-based vascular bed with microchannels, endothelial cells migrated and formed a vascular network as capillaries throughout the cell sheet and the collagen gel. Finally, the endothelial cell network reached and integrated with the microchannels in a manner similar to that of the femoral muscle bed. To confirm the flow in the newly created endothelial cell network, blood, fluorescence beads, and epoxy resin (before curing reaction) were perfused into the microchannels. The flow of blood in the newly created vascular network was observed, and the total integration between the network and microchannels was confirmed. Similar to the femoral muscle, the procedure was repeatedly performed. Triple-layered cell sheets were layered on previously layered cell sheets,

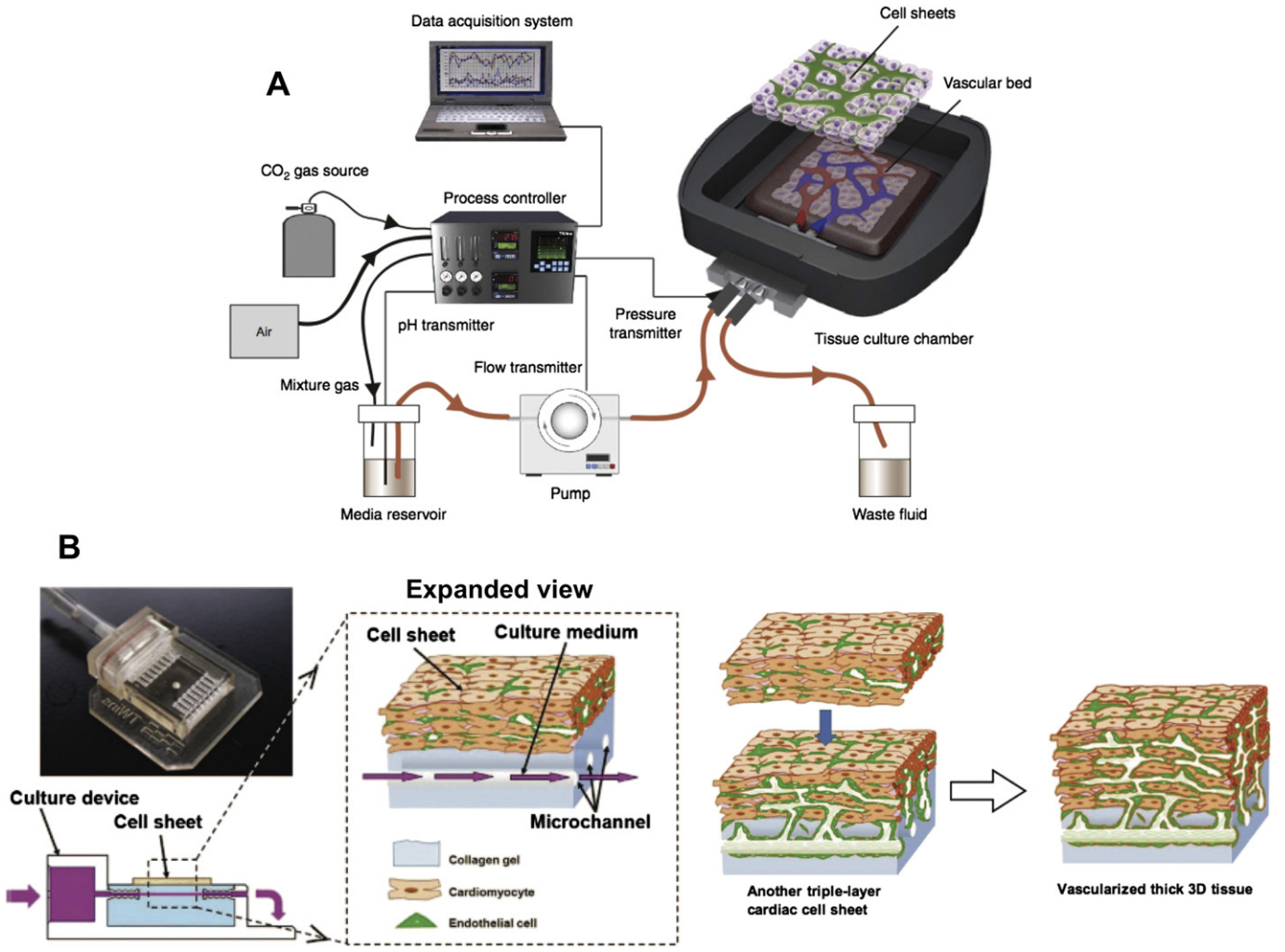

Fig. 4. Development of bioreactors to perfuse cell sheets. (A) A multi-layered cell sheet is being perfused in a femoral muscle-based vascular bed bioreactor. The bioreactor is a one-pass system consisting of a delivery pump, custom-made tissue culture chamber, pH transmitter, flow transmitter, pressure transmitter, CO_2 gas source, process controller, and data acquisition system. (B) This collagen gel vascular bed with microchannels imitates the subcutaneous conditions. In the expanded view, the blue area shows collagen gel that can imitate a subcutaneous extracellular matrix; the pink arrows indicate the direction of culture medium flow. The photograph on the left shows the culture device. After newly formed microvessels connect with the collagen gel microchannels, another triple-layered cardiac cell sheet is placed on the existing cell sheet. By repeating the sheet layering process, the subsequently added cell sheets are perfused with fresh medium through the newly-created vessels (right illustration).

resulting in a 12-layered cell sheet, without any sign of necrosis (Fig. 4B) [30]. As various technologies for constructing 3D tissues are investigated and developed, the optimization of the technologies for preparing 3D tissues will be able to fabricate ideal artificial-organs that can exhibit the same functions as native organs.

6. Conclusion

In summary, the authors can make vascularized cell sheets containing endothelial cells and successfully construct 3D vascularized cardiac tissues by repeating the procedures with the new perfusion bioreactors *in vitro*. However, the next generation of 3D tissue engineering still needs new technologies (1) allowing the use of a much larger number of cells, (2) providing perfect culture media to supply oxygen and nutrients to 3D tissues with a feedback system, and (3) controlling a shape of vascular formation for optimizing perfusing cultivation. In regard to control vascular formation, Fig. 2 indicates the maintenance of a patterned shape of endothelial cells using cell sheet with oriented pattern. Therefore, a novel technology with the combination of vascular bed technology and the patterned cell sheet technology would have a possibility to control a vascular formation in a 3D tissue. Moreover, the number and type of endothelial cell could control the vascular formation. These new technologies will provide new opportunities to develop even more effective organ engineering, which will be able to create high metabolic-rate transplantable tissue such as the heart, liver, and kidney.

Disclaimer on conflict of interest

Dr. Teruo Okano is a founder and director of the board of CellSheed Inc. Dr. Teruo Okano and Dr. Tatsuya Shimizu are shareholders of CellSeed Inc. Tokyo Women's Medical University is receiving research funds from CellSeed, Inc.

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