Multilayered Engineered Tissue Sheets for Vascularized Tissue Regeneration

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Abstract A major hurdle in engineering thick and laminated tissues such as skin is how to vascularize the tissue. This study introduces a promising strategy for generating multi-layering engineered tissue sheets consisting of fibroblasts and endothelial cells co-seeded on highly micro-fibrous, biodegradable polycaprolactone membrane. Analysis of the conditions for induction of the vessels *in vivo* showed that addition of endothelial cell sheets into the laminated structure increases the number of incorporated cells and promotes primitive endothelial vessel growth. *In vivo* analysis of 11-layered constructs showed that seeding a high number of endothelial cells resulted in better cell survival and vascularization 4 weeks after implantation. Within one week after implantation *in vivo*, red blood cells were detected in the middle section of three-layered engineered tissue sheets composed of polycaprolactone/collagen membranes. Our engineered tissue sheets have several advantages, such as easy handling for cell seeding, manipulation by stacking each layer, a flexible number of cells for next-step applications and versatile tissue regeneration, and automated thick tissue generation with proper vascularization.

Keywords Tissue engineering · Multilayered structure · Tissue sheet · Vascularization · 3D structure

1 Introduction

The generation of vascularized functional tissues is an ultimate goal of tissue engineering and regenerative medicine [1]. There have been a number of reports on the production of 3D tissue constructs based on a variety of approaches such as conventional bulk scaffold fabrication methods [2], three-dimensional (3D) bioprinting [3, 4], and layered cell sheet

technology [5–7]. The multi-layered cell sheet concept has been translated into clinical use and produced positive results for the regeneration of organs including the cornea and mucosal layer of esophagus [8, 9]. Based on the results of clinical applications, cell sheet applications should be expanded to three dimensional tissue structures for regenerating tissues thicker than several millimeters.

We hypothesized that fibroblasts and endothelial cells in the appropriate environment could be used to induce endothelial vessel networks in engineered tissue *in vivo*. First, we developed a three dimensional laminated structure, which is multi-layered with fibroblasts or with endothelial cells seeded double-sided on a biodegradable polymer membrane.

Electrospinning has been shown to be an effective scaffold fabrication method to produce thin membranes with biodegradable polymers [10]. Electrospun membranes provide a biomimetic cell microenvironment of the extracellular matrix (ECM), facilitate cell growth, and allow efficient mass transfer of nutrients and metabolites during cell survival, cell proliferation, and tissue formation [11].

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However, there are practical difficulties in handling electrospun membranes due to their highly flexible characteristic, which is a common property of nanofiber-based thin membranes. Recent studies have tried to achieve three dimensional structures by stacking or wrapping layers to form sheets or tubular tissue configurations [12, 13].

Polycaprolactone (PCL) is an FDA-approved synthetic biodegradable polymer that is widely used as a tissue engineering scaffold [14]. As a scaffold material, PCL is a well-known synthetic and biodegradable polymer [15] and has been used in blood vessel formation and ear and muscle regeneration [16, 17]. It has been shown to improve cell proliferation and cell growth rates over time [18, 19].

Collagen, the main component of the ECM, maintains the structural integrity of tissues [20–22] and plays important roles in the cell microenvironment [23]. It has been used in connective tissue-related applications and vascular regeneration [24, 25]. Electrospun collagen has also been used in vascular tissue engineering [26].

Similarly, fibroblasts and endothelial cells have been used in vascularization and generation of 3D engineered tissues. Fibroblasts are the most common cells of connective tissue and form the structural framework or stroma, which is composed of the ECM and collagen in various tissues. Endothelial cells form the inner lining of blood vessels and provide an anticoagulant barrier between the vessel wall and blood. Fibroblasts and endothelial cells have been co-cultured in vasculogenesis research [27] and used in wound healing [28, 29].

In the present study, we present a strategy for multi-layering engineered tissue sheets for the 3D generation of laminated structures of micro-sized thick membrane with each layer composed of PCL with fibroblasts or endothelial cells. This technique requires seeding various cell types onto thin membranes prepared through electrospinning of PCL or PCL/collagen mixtures. Each electrospun membrane is then prefixed with frames, cell-seeded, and stacked for 3D structure *in vitro* and implanted *in vivo*. This simple procedure generates a 3D laminated tissue structure with a defined number and density of each appropriate cell type with easily controlled dimensions and with a thickness defined by the extent of electrospinning. We demonstrate that these 3D laminated constructs may be designed to mimic tissue structures including induced vessels *in vivo*.

2 Materials and methods

2.1 Materials

The mouse fibroblast NIH/3T3 cell line and mouse pancreatic islet endothelial cell line MS1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal

bovine serum (FBS), antibiotic–antimycotic (AA), and phosphate-buffered saline (PBS, pH 7.4) were purchased from Life Technologies Korea (Gibco, Seoul, Korea). PCL (Mn = 80,000) and hexafluoroisopropanol (HFIP) were obtained from Sigma-Aldrich (St. Louis, MO). Bovine collagen was purchased from Darim Tissen (Seoul, Korea). All other chemicals were of analytical grade and were sourced from Sigma-Aldrich.

2.2 Cell culture

Fibroblasts were cultured in DMEM with 10% FBS and 1% AA. Endothelial cells were cultured in high-glucose DMEM with 5% FBS and 1% AA. Cells were sub-cultured at 70%–80% confluency. Cells were harvested through sequential treatment consisting of trypsin–EDTA digestion, PBS washes, and removal of the liquid phase after centrifugation and were used for subsequent cell expansion or seeding onto membranes.

2.3 Engineered tissue sheets

2.3.1 Electrospinning

A 10% (w/v) solution of PCL in HFIP was prepared with stirring at 70 °C for at least 8 h. Type I collagen was separately dissolved in HFIP at a concentration of 10% (w/v) with stirring at room temperature for 24 h. Ten percent PCL solution was used without further dilution for PCL membrane electrospinning. For PCL/collagen membrane electrospinning, 10% PCL and 10% (w/v) collagen solutions were mixed at a 1:1 volume ratio. The polymer solutions were loaded into a 10-mL syringe that was fitted with a 23-gauge blunt needle tip. The solution feed was driven by a syringe pump at a flow rate of 5 mL/h, and an 18-cm working distance and DC voltage of 15 kV were applied between the needle tip and aluminum foil collector. The electrospun polymer mats were collected in the form of a random mesh on top of the frames on the collector. After removal from the collector, the frame-fixed sheets were dried overnight in a fume hood. Further drying was achieved within a vacuum chamber to aid the removal of remaining solvent from the sheets. All sheets were stored in a desiccator until further use.

2.3.2 Preparation of the tissue sheet

Frames were fabricated with a 3D printing system (Projet 3510 SD; 3D Systems Korea, Seoul, Korea) with a $10 \times 10 \text{ mm}^2$ square window with 300 μm thickness (Fig. 1). These frames were attached to the rotating drum collector of the electrospinning system to permit direct bonding between the frames and electrospun sheets. For

Fig. 1 **A** Schematic diagram of engineered tissue sheet and **B** experimental groups

sterilization, the tissue sheets were exposed to UV light for 10 min in 70% ethanol and washed three times with PBS. The tissue sheets were immersed in culture medium before cell seeding.

2.3.3 Cell seeding

The cell suspensions (fibroblasts or endothelial cells) were seeded in 30 μ L at a density of 5×10^4 cells/sheet. The cell-seeded sheets were incubated for 30 min to permit cell adhesion. Additional cells were then seeded on the other side of the membranes using the same procedure as described above. Cells seeded on sheets were placed in ultra-low adherent 6-well culture plates and incubated at 37 °C and 5% CO₂.

2.4 Multi-layering of engineered tissue sheet

A frame rig (Fig. 1) was used for fast and multilayer stacking. The 3- or 11-tissue sheets were assembled for stacking, and then cell-seeded membranes were separated from 3D-printed frames with an 8-mm diameter punch. The multilayered tissue sheet structures were incubated in ultra-low adherent 6-well plates at 37 °C in 5% CO₂. Three-layered tissue sheets were composed of fibroblast–endothelial cell–fibroblast sheets with PCL (3-PCL) or

PCL/collagen (3-PCL/Collagen) membranes (Fig. 1). The 11-layered tissue sheets were constructed using electrospun PCL membranes. Layered tissue units were tested according to the ratio of endothelial cell-seeded tissue sheets to fibroblast-seeded tissue sheets and grouped as follows: control bare PCL group (no cell); fibroblasts only (E-F 0-11); a 2:1 ratio of fibroblast tissue sheets and endothelial cell sheets (E-F 3-8); and a 4:1 ratio of fibroblast tissue sheets and endothelial cell sheets (E-F 2-9).

2.5 In vitro evaluation

The viability of seeded cells was determined using the Live/Dead cell viability assay kit (Life technologies) according to the manufacturer's instructions. Fluorescence images were obtained using a confocal microscopy (CLSM, LSM710; Carl Zeiss). Cell viability was calculated by counting the number of live cells (green) and dead cells (red). Proliferation of the cells on the sheets was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Japan) assay 1, 5, and 8 days after cell seeding. A 96-well plate containing cell-seeded PCL and PCL/collagen tissue sheets was incubated at 37 °C in a humid atmosphere containing 5% CO₂ for 2 h, and the absorbance of the supernatant was measured with a

spectrophotometer at an excitation and observation wavelength of 450 nm.

2.6 *In vivo* experiments

The animal experiment protocol was reviewed and approved by the institutional animal care and use committee of Asan Medical Center (protocol number: 2014-12-003). All institutional and national guidelines for the care and use of laboratory animals were followed. Eight-week-old male nude mice were housed in an animal room in a specific pathogen-free facility with controlled temperature and relative humidity. Mice were anaesthetized with isoflurane. Two (3-layered tissue sheets) or four (11-layered tissue sheets) subcutaneous pockets were made via 1.5-cm midline incisions on the right and left back of the mice ($n = 3$ mice). The tissue sheets were implanted in the pockets. The implanted tissue sheets were harvested with the surrounding tissue 1, 2, 4, or 8 weeks after implantation and then fixed in 4% paraformaldehyde at 4 °C overnight and embedded in optimal cutting temperature, frozen, and stored at -80 °C. We subsequently obtained 12- μm sections using a cryostat, which were stained using standard hematoxylin and eosin staining methods. Sections were viewed using routine bright-field light microscopy. Cell number, area fraction, and distribution were analyzed using the ImageJ software using ITCN plugin and plot profile after converting to 8-bit images.

2.7 Immunofluorescence staining

The sections were blocked and incubated for 1 h at 37 °C with rabbit anti-VE-cadherin (1:100) and mouse anti-E-Cadherin (1:100) primary antibodies. The sections were then further incubated for 1 h at room temperature with either Alexa Fluor 488 or Alexa Fluor 568 goat anti-mouse or goat anti-rabbit secondary antibodies (1:100). The F-actin of the tissue sheets was visualized with a 1:40 dilution of Alexa Fluor 594 phalloidin and cell nuclei were counterstained using DAPI nucleic acid stain. Sections were viewed using an LSM710 confocal microscope. Cell images were analyzed with digital software (Zen2011; Carl Zeiss) and ImageJ software.

2.8 Statistical analysis

All data are reported as the mean $\pm$ standard deviation (SD). Differences between experimental groups were

analyzed with ANOVA of Origin and a two-sample t test of Excel, and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Engineered tissue sheets and multilayering

The process for generation of the engineered tissue sheets is shown in Fig. 1A. The engineered tissue sheets were prepared by fixing electrospun biodegradable membranes to the rigid frames through direct electrospinning. Once the sheets were bonded to the frame, handling of the wet electrospun sheet was simple, and little time was needed for multi-layering of the tissue sheets. Multi-layering of the tissue sheets was facilitated by the use of holes in the corners of the frame and could be easily assembled with the help of guide rods on the stacking rig (Fig. 1A, Fig. S1). Even without training, less than 2 min were required for manual stacking of 11 layers of the tissue sheets (Movie S1). After stacking, the total thicknesses of the layered sheet structures were 206.3 ± 16.9 μm for the 3-layered tissue sheets and 755.9 ± 40.6 μm for the 11-layered tissue sheets (Table 1). The thickness of each electrospun membrane was 68.7 ± 4.4 μm with less than a 3- μm filament diameter (data not shown).

3.2 *In vitro* cell viability and proliferation on the engineered tissue sheets

The cell proliferation rates of the 11-layered tissue sheets revealed that structures including endothelial cell sheets increased values of CCK-8 5 days after stacking and *in vitro* culturing (Fig. 2A). The live/dead cell viability assay revealed that over 90% of the seeded cells were alive on the 11-layered tissue sheets at 1 day after cell seeding (Fig. 2B). Also, expression of F-actin in the 11-layered tissue sheets revealed that the cells were distributed along the edge of the PCL sheets 7 days after *in vitro* seeding (Fig. 2C). However, only fibroblast-seeded constructs (E-F 0-11) showed a lack of F-actin in the middle section of the construct 7 days after seeding, while F-actin fibers were detected in the interior of other constructs (E-F 2-9 and E-F 3-8) that had endothelial cell sheets.

3.3 *In vivo* tissue formation

3.3.1 Cell distribution and numbers

Hematoxylin and eosin staining after *in vivo* implantation showed that the cells were linearly distributed along the edges of the layered sheets in the constructs (Fig. 3). Blood

Table 1 Thickness of the multi-layered tissue sheets

Number of layers	Total thickness (μm)
3	206.3 ± 16.9
11	755.9 ± 40.6

Fig. 2 *In vitro* 11-layered engineered tissue sheet. **A** Proliferation of cells on the sheets for 5 days. $*p < 0.05$. **B** Live/Dead assay 1 day after seeding. Live cells are green and dead cells are red. Scale bar, 400 μ m. **C** Immunofluorescence staining of tissue sheets 7 days after

in vitro culture. The white dashed lines indicate the location of the border line of the cultured tissue sheets. Green is E-cadherin, red is VE-cadherin, and blue is DAPI. Scale bars, 50 μ m. (Color figure online)

vessels (white dotted line) in the middle of the structure were formed 8 weeks after implantation only in the E-F 3-8 group. To assess the potential of our approach, the number of cells in the laminated constructs were analyzed using ImageJ. Quantitative analysis of the cell numbers, based on the nucleated cells within the structures, is shown in Fig. 4A, B. The differences between distributions of cells in the engineered structures showed that including endothelial cell sheets (E-F 2-9 and E-F 3-8 groups) in the structures also increased the numbers of cells in the middle of the implanted structures at 8 weeks (Fig. 4A). Also, the numbers of positive cells, representing the extent of cellular components in the implanted structures, was significantly larger in the E-F 3-8 group than in the no cell and PF groups. These results indicate that the cellular component was larger in the construct that had an endothelial cell sheet compared to the only structures with only fibroblasts. Cell numbers in the E-F 2-9 and E-F 3-8 groups increased at 4 weeks compared to the control group (no cell, 22 ± 10 ;

E-F 0-11, 31 ± 13 ; E-F 2-9, 77 ± 5 ; and E-F 3-8, 79 ± 11) (Fig. 4B). The retrieved structures showed that no surrounding tissues were observed in the no cell and E-F 0-11 groups at 1 week. However, the E-F 2-9 and E-F 3-8 groups showed red blood cells and vessels in the surrounding tissues at 1, 4, and 8 week (Fig. S2) for the construct to integrate with the host tissues.

3.3.2 Endothelial cell distribution

VE-cadherin expression in the implanted constructs at 4 weeks showed that endothelial cells were lined in the middle area of the constructs in the E-F 3-8 and E-F 2-9 groups (Fig. 5A–D). Intensity distribution of VE-cadherin showed that endothelial cell distribution in the middle section of implanted structure was higher in the E-F 2-9 and E-F 3-8 groups. Also, quantitative analysis of the areas of VE-cadherin-expressing cells within the tissue sheets at 4 weeks showed that the E-F 3-8 group had a higher number of VE-

Fig. 3 Eleven-layered tissue sheets. Hematoxylin & eosin staining of 11-layered tissue sheets based on a PCL membrane. The white dashed lines indicate the location of the border line of the generated vessel in the tissue sheets. Scale bars, 200 μ m

cadherin-positive cells than the other groups. However, there was no significant difference between the E-F 3-8 and E-F 2-9 groups in VE-cadherin expression levels (Fig. 5E).

3.4 Collagen-modified tissue sheets

To confirm vessel formation in the modified tissue sheets, collagen/PCL sheets were examined. The proliferation of seeded cells (fibroblasts) on the tissue sheets differed between 3-PCL and 3-PCL/collagen sheet materials. The 3-PCL/collagen membrane showed higher fibroblast proliferation than the 3-PCL membrane at 1 day after seeding (absorbance values: 0.15 ± 0.0021 for the control, 0.19 ± 0.0010 for 3-PCL membranes, and 0.22 ± 0.0021 for 3-PCL/collagen membranes) (Fig. S1D). As indicated by the hematoxylin and eosin staining of cross-sections of laminated structures after implantation of the 3-layered tissue sheets (Fig. 6), the cells were linearly distributed along the edge of each sheet in the constructs in the 3-PCL group, and most of the 3-PCL sheets did not degrade for 4 weeks (Fig. 6C). However, the structure of the 3-PCL/Collagen group degraded, and red blood cells were observed in the samples at 1 and 2 weeks (Fig. 6D, E). Endothelial cells were distributed throughout the layers in both the 3-PCL and 3-PCL/collagen groups, but vessels

were formed only in the 3-PCL/collagen group at 1 week (Fig. 6D, E; black arrows). To confirm EC distributions in the tissue sheets, the tissue sheets were stained with VE-cadherin EC-adherens junction. VE-cadherin staining revealed the distribution of endothelial cells in the retrieved tissue sheets.

4 Discussion

4.1 Engineered tissue sheet (ETS)

We generated an ETS based on cell-seeded biodegradable electrospun micromembranes for 3D thick engineered tissues. This novel method allows multilayered 3D structures to be generated in a short working time. Cell sheet engineering has recently been developed in order to avoid the limitations of tissue reconstruction using biodegradable scaffolds or single cell suspension injection [30–32]. However, cell sheet technology is limited by the time-consuming and complex process for multi-layered structure generation [6]. For example, using automated system for multi-layering, cell sheet transfer to gelatin block substrate requires at around 30 min [33]. Multi-layering of 10 layers will require almost 5 h (≈ 300 min). This time consuming

Fig. 4 **A** Quantification of the nucleated cell number per 100 μm thickness of the engineered tissue sheets. **B** Quantification of the total cells in the engineered tissue sheets. * $p < 0.05$; ** $p < 0.01$

Fig. 5 Immunofluorescence staining of 11-layered tissue sheets 4 weeks after implantation. We stained the sections from multilayered samples with anti-VE-cadherin to detect endothelial cells. Red is VE-cadherin and blue is DAPI. **A, E** No cell group. **B, F** E-F 0-11 group. **C, G** E-F 2-9 group. **D, H** E-F 3-8 group. Red blood cells

(white arrows) are distributed along the border of the implanted structures in all samples. **E–H** The distribution of endothelial cells in the sheet structures showed in 2.5D-plots. **(I)** Quantitative analysis of VE-cadherin, based on the areas of VE-cadherin-expressing cells in **A–D**. * $p < 0.05$; ** $p < 0.01$. (Color figure online)

multi-layer engineering step has potentially negative effect on cell survival and activity. In contrast, ETS does not need temperature change nor cell sheet detachment time. Further, ETS handling is basically gripping the frame part with forceps and multi-layering is very easy and does not take long time. Under laboratory environment, we could stack 10 layers of ETS in 2 min. This result implies that ETS based tissue engineering has much higher efficiency and safety to the cells during transplantation. The additional advantage of our technique is that, as in this study, when eleven sheets were stacked together and implanted in the body, blood vessels were generated into the stratified structure, which makes it easy to construct thick stratified structures at once. As mentioned above, our present study findings indicate that mass multi-layering is quick and could be used to stack the tissue sheets using a frame rig in

an only single process. The frame-fixed micro-sized sheets generated by this method could be easily manipulated by grasping the frame with forceps when seeding cells onto both sides of the sheet. Additionally, different cell types can be used to seed the ETS. These attributes mean that cell-specialized sheets can be prepared for immediate use in clinical applications (Fig. S3). In addition, the size of the sheets and their constituent materials could be adapted to the needs of organ-specific morphologies.

4.2 *In vitro* tissue formation approach

Tissue sheets were prepared *in vitro* through seeding two types of cells onto the polymeric membranes. Constructs containing a higher ratio of endothelial cell-sheets to

Fig. 6 Hematoxylin and eosin (H&E) and Immunofluorescence staining of 3-layered tissue sheets: fibroblast–endothelial cell–fibroblast structures based on **A, B, C** a PCL membrane and **D, E, F** a PCL/collagen membrane. H&E staining: The PCL/collagen-based tissue sheet exhibited endothelial cell-lined channels that

contained red blood cells (*black arrows*) in the implanted constructs at 1 and 2 weeks. *Scale bar*, 200 μm . Immunofluorescence staining: *Red* is VE-cadherin, and *blue* is DAPI. Red blood cells (*black arrows*) are distributed in the 3-layered tissue-like sheets based on PCL/collagen. *Scale bar*, 100 μm . (Color figure online)

fibroblast cell-sheets showed increased cell proliferation and cell distribution in the 11-layered structures *in vitro* culture (Fig. 2). These *in vitro* results are highly relevant to the vasculogenesis and cellular distribution seen *in vivo*. Also, a higher cell number was found in the middle of the layered *in vivo* constructs than in the *in vitro* context when compared at 1 week after stacking. These results may be due to differences between *in vivo* and *in vitro* culture environments.

4.3 Tissue formation *in vivo*

In our current experiments, both endothelial cells and fibroblasts were used to construct thick tissues and form blood vessels in the implanted constructs. Endothelial cells are major components of the endothelium of vessels [34]. Fibroblasts not only directly regulate endothelial cells, but also secrete various soluble factors that regulate angiogenesis [35, 36]. In addition, both fibroblasts and

endothelial cells play key roles in microvessel formation [27, 37]. Neo-vessels in constructs were observed in the E-F 3-8 group 4 weeks after *in vivo* implantation (Fig. 3). Constructs containing a higher ratio of endothelial cell-sheets to fibroblast cell-sheets showed improved angiogenesis compared to constructs containing a lower ratio.

The number of sheets in the 11-layered constructs contributed to a thickness of the engineered constructs of over 750 μm despite nanofiber-based membranes. Lamellar tissues with various tissue compositions could be generated by stacking prepared cell-specialized tissue sheets. However, no neo-vessels in the 11-layered constructs composed of only 3-PCL were seen in the tissue sheet structure at 1 and 4 weeks. Thus, further assessment is needed to rapidly establish vessels into the structure of tissue sheets composed of 3-PCL.

The cell numbers of the implanted structures that contained endothelial cell sheets increased in the E-F 2-9 and E-F 3-8 groups after 8 weeks. In contrast, the cell numbers of the E-F 0-11 group, which lacked endothelial cells, gradually decreased over 8 weeks while the No Cell group showed no significant change during the same period. This indicates that the use of an endothelial cell sheet implanted into the 11-layered structure may enhance cell survival rate and/or migration. Additionally, at 8 weeks, vessels were visible in the constructs in the E-F 3-8 group (Fig. 3), suggesting that cell numbers were influenced by the formation of vessels. In contrast, the cell numbers in the E-F 0-11 group decreased between 1 and 4 weeks. Constructs composed of fibroblasts alone could not recruit host cells, unlike structures containing endothelial cells [38], and the implanted fibroblasts underwent apoptosis by necrosis. However, the detailed cellular behavior in the implanted constructs requires further analysis.

4.4 Collagen-modified tissue sheets

Our *in vivo* studies have shown favorable vascularization via collagen-composited sheets and endothelial sheets. Three-layered tissue sheets in the 3-PCL/collagen group showed vessel formation in the middle of the structure 1 week after *in vivo* implantation. However, the 11-layered tissue sheets showed formation of vessels at 8 weeks. These differences may be due to differences in the materials used for the sheets and in the number of stacked sheet. 3-PCL alone was not a fully suitable scaffold material for the *in vivo* creation of vessel tissues. In conventional tissue engineering, electrospun 3-PCL is generally coated or mixed with other growth factors or ECM components such as collagen and gelatin when used for tissue regeneration [39, 40]. In our current experiments, collagen was used in the 3-layered tissue sheets to promote proliferation and vessel morphogenesis in concert with endothelial cells and fibroblasts. Similarly, previous reports have shown that natural polymers have been used in the

regeneration of tissues damaged by disease or trauma and, in some cases, to create new tissues and replace failing ones [41].

4.5 Potential of ETS

In the current study, we generated 11 layers of tissue sheets with PCL membranes, and these constructs were shown to maintain cell viability over a long-term period. We then further demonstrated, as a proof-of-concept, that three layers of tissue sheets based on PCL/collagen membranes can form blood vessels in *in vivo* implanted constructs. However, further assessment is needed to assess the formation of vessels in the fabricated constructs. There are some challenges regarding vascularization in our system. For example, growth factors or soluble factors for angiogenesis and vasculogenesis are released from a fabricated thick structure. A bioreactor for pre-formed *in vitro* vessels shows promise in vessel morphogenesis. These methods are applicable to various organs and tissues, including elastic tissues, stiff tissues, thin tissues, and tough tissues.

In conclusion, the generation of 3D lamellar structures is a challenging issue for tissue engineering. 3- or 11-layered constructs composed of endothelial and fibroblast sheets made of PCL and collagen can be rapidly assembled using tissue sheets *in vitro* and used to induce vascularization in mouse tissue. Tissue sheets can generate 3D tissue-specific structures using a variety of cells and materials. Our current findings suggest a potential new strategy for constructing 3D lamellar tissues containing various cell types and ECM material specific to the target tissue for tissue engineering and clinical applications.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest.

Ethical Statement The animal experiment protocol was reviewed and approved by the institutional animal care and use committee of Asan Medical Center (protocol number: 2014-12-003).

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