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Engineering of perfusable double-layered vascular structures using contraction of spheroid-embedded hydrogel and electrochemical cell detachment

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Perfusable vasculatures are essential for engineering three-dimensional thick tissues and organs in the fields of tissue engineering and regenerative medicine. Here, we describe an approach for the fabrication of double-layered vascular-like structures (DVSs) composed of a monolayer of human vascular endothelial cells (HUVECs) covered with a dense human smooth muscle cell (SMC) layer. HUVECs were attached to a gold needle via the oligopeptide self-assembled monolayer and grown to form a HUVEC monolayer that was subsequently embedded in a photo-crosslinkable gelatin hydrogel containing SMC spheroids in a culture chamber. During four days of culture, the hydrogel significantly contracted and formed a dense SMC layer around the needle. The binding between the HUVEC layer and the gold needle was cleaved by applying a negative potential to desorb the oligopeptide and the needle was extracted from the chamber, resulting in a perfusable DVS composed of HUVEC and SMC layers. The DVS was cultured under perfusion, and the cells in the DVS showed greater expressions of SMC-specific genes compared to those of spheroids. The DVS possessed a dynamic contraction ability in response to acetylcholine as observed in the *in vivo* SMC layer. This study proposes a promising approach for the fabrication of perfusable vasculatures for the engineering of fully vascularized tissues and organs.
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[**Key words:** Oligopeptide; Double-layered vascular structure; Electrochemical cell detachment; Human vascular endothelial cell; Smooth muscle cell]

Organs such as the liver, pancreas, and kidney contain vascular networks for mass transport, constantly delivering oxygen and nutrients throughout solid tissues and organs. Many approaches have been reported to replicate vascularized tissues *in vitro*. A typical approach is to encapsulate vascular endothelial cells in a hydrogel that can spontaneously form vascular networks. Successful engraftment and curative effects have been demonstrated using prevascularized tissue constructs prepared by encapsulating human vascular endothelial cells (HUVECs) with iPS-derived hepatocytes in a hydrogel (1). The vasculatures in the constructs were connected to *in vivo* vascular beds for blood to flow into the tissue constructs. However, because the diameter of these spontaneously formed vasculatures is typically less than 50 μm, it is difficult to connect an external pump to perfuse culture medium, limiting further the expansion of this approach to larger dimensions and higher cell densities.

Approaches for perfusable vasculature fabrication have been proposed, including microchannel preparation with sacrificial templates or needles in a hydrogel and subsequent seeding of vascular endothelial cells inside microchannels (2–4). We also reported an approach for perfusable vascular fabrication where a unique electrochemical reaction was used to detach cells from a needle (5,6). Vascular endothelial cells were first attached to a gold-coated needle via an electro-active oligopeptide layer which could then be cleaved from the needle by the application of a negative potential and transferred to a hydrogel, resulting in the fabrication of a microchannel enveloped by vascular endothelial cells (7,8). The advantage of this electrochemical approach is that the cells and cell layers could be rapidly detached and transferred to a hydrogel within 2–10 min (9–11). This rapid fabrication is significant when organ cells such as hepatocytes and pancreatic cells are encapsulated at a high density in a hydrogel to replicate these tissues, because calculations regarding the dissolved oxygen concentration and oxygen consumption of hepatocytes suggest that oxygen in the medium is depleted within an hour even when cells are encapsulated at one-tenth the density of the liver (7). In the conventional subsequent cell seeding approach, perfusion of culture medium should be stopped for at least a few hours to let endothelial cells attach onto the microchannels. In the proposed approach, the transferred vascular endothelial cells migrated and sprouted into the surrounding hydrogels in following perfusion culture to form branched and perfusable vascular networks (12,13). However, the drawback of this approach is that the vasculatures consist only of an endothelial cell layer, and a rigid culture chamber is necessary to mechanically support it against hydraulic pressure generated by perfusion of culture medium. This may be problematic when a fabricated tissue is transplanted into the body.

In relatively large vasculatures *in vivo*, vasculatures contain an SMC layer that provides mechanical tolerance to blood pressure and regulates the diameter of vasculatures by modulating its contraction

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and relaxation. The SMC layer is also closely involved in various vascular diseases, such as arteriosclerosis. Thus, the ability to replicate such double-layered vascular-like structures (DVSs) *in vitro* is beneficial for engineering fully-vascularized three-dimensional thick tissues for transplantation and for understanding the mechanisms of vascular diseases. Although there are several reports for fabricating DVS using 3D printing (14), aggregation of microgels and spheroids (15), and polymer scaffolds (16), a vascular endothelial cell layer was typically added after the SMC layers were fabricated. Considering that vasculatures are inserted into high-cell dense constructs for engineering vascularized thick tissues, this endothelialization approach may cause a shortage of oxygen because the perfusion of culture medium should be stopped during seeding of vascular endothelial cells onto an SMC layer for at least few hours.

In the present study, we took an approach of direct fabrication of DVS constructs by first culturing a tubular HUVEC layer followed by encapsulation with SMC spheroids. The rapid detachment and formation of the HUVEC tubular layer allowed maintaining oxygen supply during the process and the use of SMC spheroids provided high cell seeding density (Fig. 1). We believe that this could be a practical approach for the fabrication of perfusable and robust vasculatures with thick SMC layers for engineering three-dimensional tissues with vasculature networks.

MATERIALS AND METHODS

Cell culture GFP-labeled human umbilical vein endothelial cells (GFP-HUVECs; CC-2517, Cambrex Bio Science, Walkersville, MD, USA) were grown in EGM-2 culture medium (Lonza, Basel, Switzerland), exchanged every 2–3 days. The cells were passaged using 0.25% trypsin–EDTA (Thermo Fisher Scientific Inc., Tokyo, Japan) before 80% confluence. GFP-HUVECs at passages 6 to 9 were used for experiments. Normal human aorta smooth muscle cells (SMCs; CRL-1999, ATCC, Manassas, VA, USA) were grown in Ham's F-12 culture medium (Sigma–Aldrich, Tokyo, Japan) supplemented with endothelial cell growth supplement (ECGS, Sigma–Aldrich), 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and 1% penicillin-streptomycin (Life Technologies). Culture medium was replaced every 2–3 days. Cells were passaged using 0.25% trypsin–EDTA before cells reached 90% confluence. SMCs from passages 2 to 5 were used for the experiment. EGM-2 culture medium was used for co-culturing two types of cells.

Fabrication of microwell array culture plate and SMC spheroid formation A microwell array plate (Fig. 1B) was fabricated using a two-step molding process as previously described (17). Briefly, hemispherical concave microwells (2000 wells in 20 × 20 mm region) were drilled on an olefin plate (ZEONOR 1430 R, Zeon Co., Tokyo, Japan) with a ball-end mill (φ500 μm) using a milling machine (MDX-540S, Roland DG Co., Hamamatsu, Japan). The microwell configurations were then transferred to an epoxy resin by pouring its solution (epoxy:curing agent, 2:1; Shinetu, Niigata, Japan) and curing at room temperature for 48 h. Using this protruding mold, a microwell array culture plate was fabricated by casting a polydimethylsiloxane (PDMS; Shinetu) prepolymer solution (silicon elastomer:curing agent, 10:1) and baking at 80 °C for 20 min. The PDMS microwell array plate was then immersed in pure water and autoclaved (120 °C for 1 h) to remove potentially harmful chemicals including uncured PDMS and catalyst, to sterilize for subsequent cell culture. To make the surface of microwells cell-repulsive, a sterilized Pluronic F-127 solution (5 ml of 4% aqueous solution; Sigma–Aldrich) was poured in the microwells and incubated for 8 h at room temperature. Prior to cell seeding, the plate was washed three times with PBS (Thermo Fisher Scientific Inc.) to remove excess Pluronic F-127. SMCs were seeded on the plate at a density of 4×10^6 cells/2000 wells/4 ml (2000 cells/well). After 1, 2, and 3 days of culture, SMC spheroids were collected from the microwells by gentle pipetting.

Preparation of gelatin methacrylate Photo-crosslinkable gelatin methacrylate (GelMA) was prepared as described previously (18,19). A solution containing

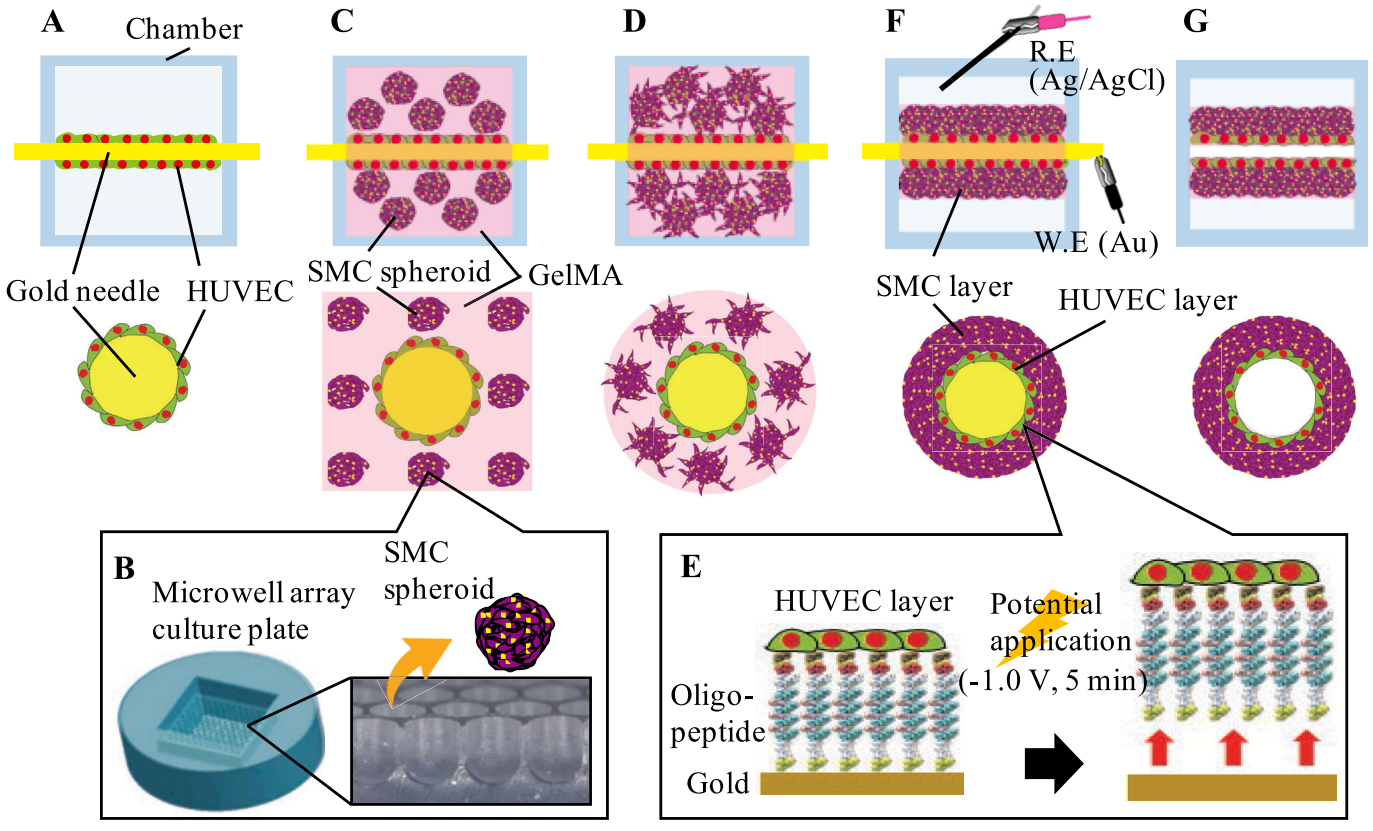

FIG. 1. Steps for fabrication of double-layered vascular-like structures (DVS). (A) Seed HUVECs on a gold needle coated with an oligopeptide self-assembled monolayer. (B) Prepare SMC spheroids with a PDMS microwell array plate. (C) Encapsulate SMC spheroids around the HUVEC-covered gold needle in GelMA in a culture chamber. (D) Incubate for four days. During this culture period, SMCs migrate into GelMA, which subsequently contract the hydrogel toward the needle via the attraction forces of SMCs. (E, F) Detach HUVECs from the gold needle along with electrochemical desorption of the oligopeptide layer. (G) Extract the needle and start perfusion of culture medium using a syringe pump.

either 5% or 7% GelMA and 0.1% photoinitiator (Irgacure 2959, Fuji Film Wako Pure Chemical, Co., Osaka, Japan) was sterilized using a 0.22 μm-pore filter (TPP, Trasadingen, Switzerland).

Characterization of GelMA by encapsulating SMC spheroids To characterize GelMA in terms of migration of SMCs, SMC spheroids prepared by culturing for 1 and 3 days in the microwell plate were encapsulated at a density of 50 spheroids/200 μl in 5% GelMA solutions. The hydrogels were then crosslinked by UV exposure at 3 different intensities (5.0 mW/cm^2−50 s, LOW; 7.0 mW/cm^2−40 s, MID; and 7.0 mW/cm^2−50 s, HIGH) with a UV lamp (Ushio Inc., Tokyo, Japan). The spheroids were cultured for 3 days and the number and length of migrated cells from the surface of spheroids were quantified by taking phase-contrast microscopic images and analyzed using IMARIS software.

Oligopeptide design and gold needle preparation The oligopeptide sequence CGGGKEKEKEKGRGDSP (Scrum Inc., Tokyo, Japan) was designed for electrochemical cell detachment (Fig. 1E) (20). The oligopeptide is composed of three functional domains, C, KEKEKEK, and GRGDSP (21). The cysteine (C) domain contains a thiol group, through which the oligopeptide is anchored to a gold surface with a gold−thiolate (Au−S) bond. The alternating lysine (K) and glutamic acid (E) domains induce a close packing of the oligopeptide layer on a gold surface by electrostatic intermolecular interactions, leading to a high-density self-assembled oligopeptide layer (22). The GRGDSP domain promotes integrin-mediated cell adhesion. The stainless needles were sputter-coated with a gold layer and modified with the oligopeptide by immersing in a 50 μM oligopeptide aqueous solution at 4 °C for 12 h. After sterilizing with 70% ethanol solution for 5 min and washed three times with PBS, the needles were fixed in a culture chamber (see below). HUVECs were seeded on the needle with EGM-2 culture medium and cultured until cells covered the whole surface of the needle.

Preparation of culture chamber A culture chamber was designed for the fabrication of a single DVS and subsequent perfusion culture (Fig. 1A). The chamber was made of PDMS and the volume was 80 μl (2 mm in height, 20 mm in width, 2 mm in depth). Two glass pipes (650 μm in inner diameter) were embedded into two walls of the chamber to guide the insertion and extraction of the needles and to connect silicone tubes (1.0 mm in inner diameter) from a syringe pump to a waste bottle. The PDMS chamber was fabricated using an acrylic plate mold cut using a computer-aided laser (Laser PRO C180; GCC Inc., New Taipei, Taiwan) and bonded with an acrylic binder. After baking to cure PDMS in an 80 °C oven for 30 min, the acrylic mold was removed and the glass pipes (diameter, 500 μm; length, 8 mm each; Hirschmann Laborgeräte, Eberstadt, Germany) were inserted into two holes in the opposite chamber walls and sterilized by autoclaving. Subsequently, the entire inner surface of the chamber was treated with 4% Pluronic F-127 solution for 8 h to prevent cell adhesion. The chamber was rinsed three times with PBS to remove the extra Pluronic F-127 solution.

Fabrication of DVS SMC spheroids collected at day 1 (S1) or day 3 (S3) of the culture were suspended in 80 μl of 5% GelMA solution at a density of 50,000 spheroids/ml. Further, the gold needle with HUVECs was set into the PDMS chamber and the GelMA solution with SMC spheroids was poured into the chamber. The GelMA was crosslinked with UV irradiation at 7.0 mW/m^2 for 50 s. The shrinkage of the hydrogel was assessed for 4 days of culture. The Ag/AgCl reference electrode was inserted into the chamber and a potential of −1.0 V was applied for 5 min to detach and transfer HUVECs from the needle to the surrounding spheroid-embedded hydrogel. The gold needle was then extracted through the glass guide pipe carefully to prevent damage of the inner HUVEC layer. For the perfusion culture, the glass pipe was connected to the micro-syringe pump and the EGM-2 culture medium was perfused at 20 μl/min. The structure was observed with a phase-contrast, fluorescent microscope (IX-71, Olympus, Tokyo, Japan), and a confocal laser scanning microscope (LSM700, Carl Zeiss Microscopy, Tokyo, Japan).

Contraction of SMC spheroids and DVS in response to drug stimulation Fabricated spheroids and DLS were exposed to 500 nM acetylcholine chloride (A6625; Sigma−Aldrich) for 30 min and the changes in the diameters were recorded every 30 s by taking phase-contrast microscopic images. The images were analyzed with Image J.

Immunofluorescence staining Cultures were fixed with 4% paraformaldehyde (Sigma−Aldrich) in PBS for 12 h, treated with 0.2% Triton X100 for 6 h, and then stained with rhodamine phalloidin (Cytoskeleton, Inc., Denver, CO, USA) and 4′,6-diamidino-2-phenylindole (DAPI) for 12 h at 4 °C. After washing three times with PBS, fluorescent images were taken with a fluorescence microscope and a confocal laser scanning microscope. For the SMC layers, the cultures were fixed with 4% paraformaldehyde and then incubated with mouse anti-α-actin antibody (1:100) (Thermo Fisher Scientific Inc.) for 24 h at 4 °C. After washing three times with PBS, cells were incubated with a secondary antibody, Alexa Fluor 488 anti-mouse IgG antibody (Thermo Fisher Scientific Inc.) for 2 h. During the last 30 min, DAPI was added to the culture.

Elastin staining Elastin staining was performed with an elastin stain kit (Modified Verhoff's; ScyTek Laboratories Inc., Logan, UT, USA) by following the manufacturer's instructions. Frozen sections were hydrated with distilled water, to which working solution was added. These were then incubated for 15 min. After washing with tap water, 10−15 drops of 2% ferric chloride differentiating solution were continuously added and then washed with tap water followed by 2 dips in distilled water. 4−5 drops of 5% sodium thiosulfate solution were applied and incubated for 1 min. After washing with tap water and following 2 dips in distilled water, 4−5 drops of Van Gieson's solution were added and incubated for 5 min. The section was rinsed with 95% ethanol and observed under a light microscope.

Gene expression analysis Reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the relative changes in the expression of SMC-specific genes, including α-smooth muscle actin (*αSMA*), calponin, and smooth muscle myosin heavy chain (*SM-MYH*). These genes encode the smooth muscle isoforms of contractile apparatus proteins. Total RNA was extracted from samples using an RNeasy mini kit (Qiagen K.K., Tokyo, Japan). In the extraction of mRNA from SMC spheroids, a disposable homogenizer (Nippi, Tokyo, Japan) was used to physically grind cells. cDNA was synthesized via reverse-transcription using a ReverTraAce RT-qPCR kit (Toyobo, Osaka, Japan). Real-time RT-PCR was performed with Step One (Applied Biosystem, Singapore) and detected using SYBR Green (Qiagen) according to the manufacturer's instructions. The sequences of forward and reverse primers were 5′-agccacatcgctcagacac-3′ and 5′-gcccaatacgaccaaatcc-3′ for glyceraldehyde-3-phos-phate dehydrogenase (*GAPDH*), 5′-ctgttccagccatccttcat-3′ and 5′-tcatgatgctgttgtaggtggt-3′ for *αSMA*, 5′-ccaaccatacacaggtgcag-3′ and 5′-tcaccttgtttccttttcgtctt-3′ for calponin, 5′-aactcgtgtccaacctggaa-3′ and 5′-ttctcctcggctaacaactga-3′ for *SM-MYH*. All primers were purchased from Takara Bio Inc. (Shiga, Japan). The results were analyzed by using Step One Software v.2.2 (Applied Biosystem). PCR measurements were repeated in triplicates from 3 experiments.

RESULTS AND DISCUSSION

Characteristics of SMC spheroids Schematics for engineering DVS are shown in Fig. 1. Following this scheme, we initially prepared SMC spheroids with the PDMS microwell array plate to later encapsulate them at a high density in GelMA. In the PDMS plate, the cells aggregated in each microwell after 1 day of culturing and formed condensed spheroids after 3 days (Fig. 2A). A large number of spheroids (300 spheroids/culture plate) with a relatively uniform diameter were prepared with this PDMS plate, because almost the same number of cells enters the microwells simultaneously. PDMS has typically been used in the fields of lab-on-a-chip and BioMEMS mainly owing to its cytocompatibility, transparency, micro workability, and oxygen permeability (23). Because muscle cells generally have a high demand of oxygen and oxygen shortage can be readily caused in the core of spheroids when cells are aggregated, the PDMS microwell array plate was designed to supply oxygen not only from the top of the culture medium but also from the bottom through the PDMS (24). The diameter of spheroids decreased from ∼150 μm to ∼92 μm during the 3 days of culture (Fig. 2B), which is equivalent to a 67% compaction in volume. This significant change could affect the SMC phenotype and SMC-induced hydrogel shrinkage.

Changes in gene expression of smooth muscle markers were analyzed using RT-PCR. The analysis revealed that the expression of *calponin* and *SM-MYH* reached its highest peaks on day 1, while the expression of *αSMA* decreased over time (Fig. 2C). These marker genes were expressed as a differentiated contractile phenotype (25,26) and their drastic decrease is correlated to the undifferentiation to a proliferative phenotype (27). In a typical SMC culture, SMCs alter the phenotype from differentiated to undifferentiated phenotypes when they form aggregates such as spheroids (28). This is consistent with the results obtained in this study, except that we found peaks of *calponin* and *SM-MYH* expressions on day 1 of the culture. The spheroids were then prepared in the plate for 1 and 3 days and examined on the level of contraction in response to a muscle concentration inducer, acetylcholine. Immediately after the stimulation, the spheroid diameter decreased up to 12% in 10 min (Fig. 2D). The spheroids after 3 days of culture (S3) contracted only 6% 15 min after stimulation (data not shown). These results suggest that SMC alter their characteristics after 3 days of spheroid culture.

Migration of SMC spheroids in GelMA The culture period for SMC spheroid preparation and UV exposure for crosslinking GelMA

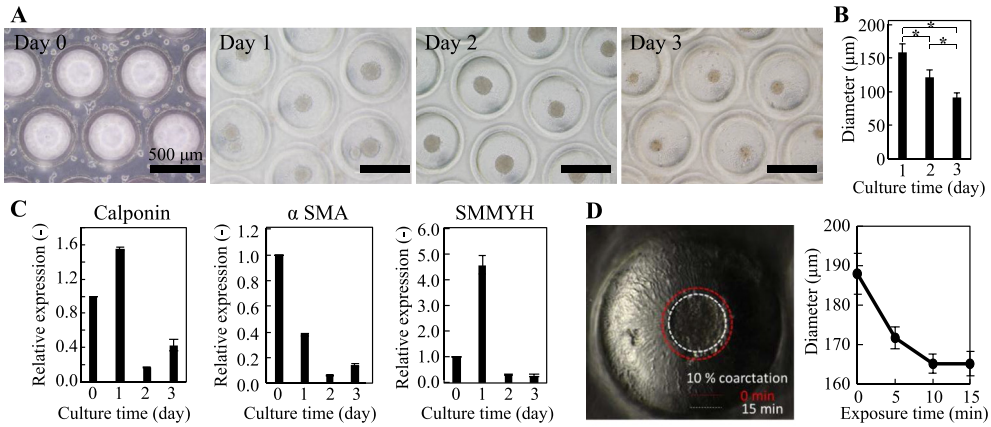

FIG. 2. Preparation of SMC spheroids. (A) Cell aggregation and subsequent compaction in the spheroid culture plate. (B) Quantification of the spheroid diameter in panel A. Error bars represent the standard deviations calculated from total 10 spheroids in three independent experiments. Numerical variables were statistically evaluated by the Steel–Dwass test. $p < 0.05$ was considered significant. (C) Changes in gene expressions of SMCs. Error bars represent the standard deviations calculated from three independent experiments. (D) Contraction response against stimulation with acetylcholine chloride. The red and white circles in the image indicate the diameter of SMC spheroid before and 15 min after the stimulation, respectively. The graph indicates changes in the diameter. Error bars represent the standard deviations calculated from total 5 spheroids in three independent experiments.

might play a role in the SMC-driven shrinkage of the hydrogel and thus in the successful fabrication of a high cell dense SMC layer around the needle. We examined these influences using the spheroids cultured for 1 and 3 days (S1 and S3) then encapsulated in GelMA under 3 different crosslinking conditions (5.0 mW/cm^2 for 50 s, LOW; 7.0 mW/cm^2 for 40 s, MID; and 7.0 mW/cm^2 for 50 s, HIGH). During 3 days of culture after the encapsulation, SMCs in S1 were found to migrate at least several hundred micrometers in GelMA in all three crosslinking conditions. Interestingly, the results of comparisons among three crosslinking conditions were in opposition to our expectations. We had predicted that a softer gel would facilitate more cell

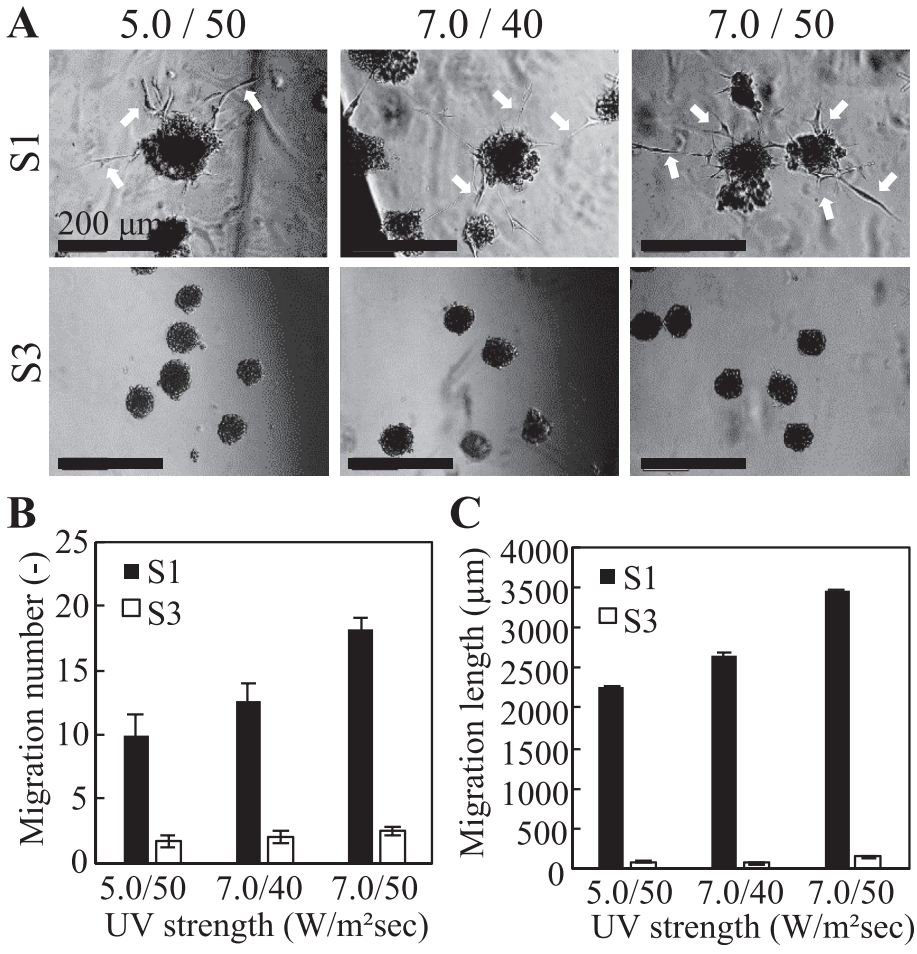

FIG. 3. Migration of SMCs in GelMA. (A) Comparisons of migration of SMCs from spheroids after 5 days of culture in GelMA. S1 and S3 indicate that spheroids were previously cultured in the microwell array plate for 1 and 3 days, respectively. The values on the top indicate UV irradiation intensity (mW/cm^2)/exposed time (sec). (B) The number and length of migrated SMCs from the surface of spheroids. Error bars represent the standard deviations calculated from 10 spheroids in three independent experiments.

extension and migration, but the migration number and length, in fact, increased by increasing the UV exposure intensity and time (Fig. 3B and C). Further extension of UV exposure time (70, 80, 90 s, 7.0 mW/cm^2) was found to hinder cell extension and migration completely. SMCs in S3 did not migrate into the GelMA and the morphology of these spheroids remained in the original shape, independent of the crosslinking conditions. Based on these results, we decided to use S1 and 7.0 mW/cm^2 for 50 s in the following experiments.

DVS fabrication and characterization As shown in Fig. 1A, HUVECs were seeded on the needle coated with the oligopeptide self-assembled monolayer and grown to cover the surface. Subsequently, a GelMA solution containing SMC spheroids (S1) was poured into the chamber containing the needle. The thickness of the hydrogel was 1.9 mm after the crosslinking (Fig. 4A). We carried out the same experiments with S3. The shrinkage of the hydrogel containing S1 was rapid and significant compared to that of S3 (Fig. 4B). This was expected, as shown in Fig. 3, because the SMCs in S1, but not in S3, readily migrated into the hydrogel. The hydrogel with S1 shrunk by almost 50% during the 4 days of culture and the cell density became 6.0 $\times$ 10^8 cells/ml. This cell density is over 50 times greater than that of previous studies where single SMCs were encapsulated in a tubular hydrogel (29).

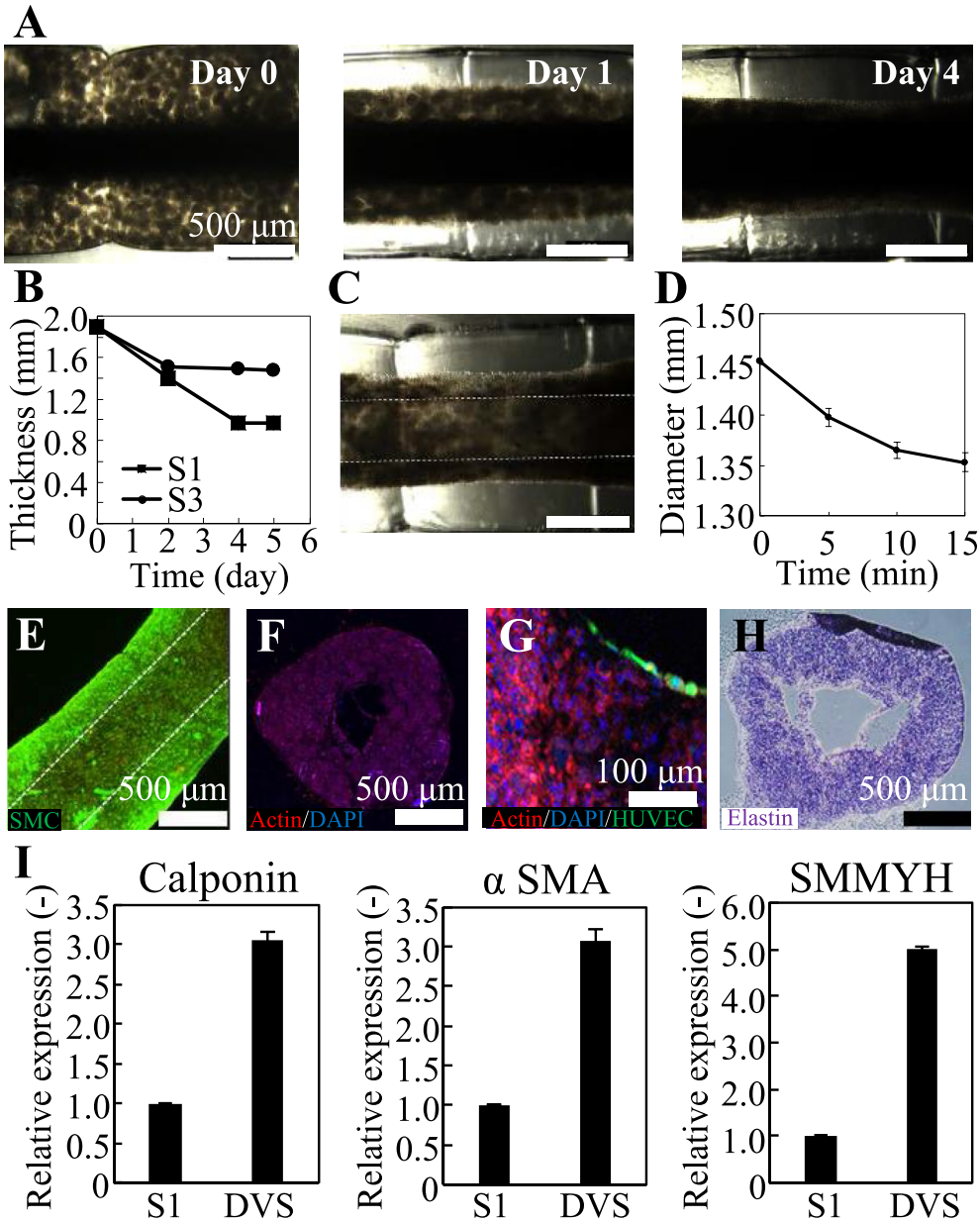

FIG. 4. DVS. (A) Shrinkage of SMC spheroid-embedded GelMA layer around a needle during 4 days of culture. (B) Quantitative analysis of changes in the thickness of the hydrogel. Error bars represent the standard deviations calculated from three independent experiments. (C) DVS after extracting a gold needle. (D) Coarctation of the DVS in response to stimulation of 500 μM acetylcholine chloride. Error bars represent the standard deviations calculated from three independent experiments. (E–G) Confocal microscopic images of the structure. Three-dimensional configuration of smooth muscle layer stained with Alexa F 488 conjugated anti α-actin antibody (E). Cross-section stained with DAPI and rhodamine phalloidin (F) and its magnified view additionally merged with an image of GFP-HUVEC layer in the same region (G). (H) Cross-section stained for elastin. (I) Gene expressions of SMCs at 1 day of culture in the microwell array plate (S1) and subsequent 5 days of perfusion culture as a DVS. Error bars represent the standard deviations calculated from three independent experiments.

After 4 days of culturing in the chamber, an electrical potential of −1.0 V vs. Ag/AgCl was applied to the gold-coated needle for 5 min. The needle was then carefully extracted from the chamber (Fig. 4C), resulting in a DVS composed of a dense SMC layer enveloped in a monolayer of HUVECs (Fig. 4E–G). The length and inner and outer diameters were 18.78 ± 0.29 mm, 972.2 ± 26.7 μm, and 488.0 ± 10.2 μm, respectively. The standard deviations were calculated from the results of three independent experiments. We have previously fabricated a DVS by dip-coating and crosslinking GelMA on a needle covered with HUVEC, with the subsequent electrochemical detachment of the HUVEC layer (19). However, this approach did not result in a thick SMC layer. We repeated the GelMA coating process to increase the thickness of layer, but this only resulted in a low cell density layer. There are several studies that report on fabricating an SMC tube with a cell-dense thick layer. In one approach, multiple ring-like SMC aggregates were previously prepared on a microfabricated plate and then assembled with a support needle (30). In another approach, a SMC cell sheet was prepared and wrapped on a support needle (31). Both approaches were able to fabricate SMC tubes with a thick layer. However, when seeding vascular endothelial cells onto an internal surface of the fabricated SMC tubes, the flow of culture medium should be stopped for a couple of hours so that cells can settle down and attach to the surface. However, this may cause a shortage of the oxygen supply and serious ischemia-reperfusion injuries once perfusion is restarted. The advantages of the approach used in the present study are: (i) SMCs are encapsulated at a relatively high density but there are still pathways to diffuse oxygen and nutrients between the spheroids; (ii) immediately after the transfer of an endothelial layer, perfusion of culture medium can be started; (iii) in the perfusion culture, mechanical loads are applicable on the constructed DVS for maturation with an external pump. We have previously demonstrated that the electrochemical transfer of vascular endothelial cell layers is applicable not only to a single needle but also to spatially aligned multiple needles (12). We prepared a multi-needle consisting of 9 stainless steel needles (diameter, 500 μm; pitch, 500 μm; length, 10 mm, 3 × 3 arrangement), with which 9 endothelialized microchannels were simultaneously fabricated in a square hydrogel block of dimensions 3 mm × 3 mm × 10 mm. We believe that the same approach can be used to fabricate multiple DVSs by placing a spacer with lattice architecture between needles to compartmentalize each needle.

After 3 days of perfusion culture, the contractility of the DVS was examined by the addition of 500 μM acetylcholine in the culture medium. Changes in the appearance of DVS were recorded for the following 15 min. A dynamic contraction up to 7% was observed after 10 min (Fig. 4D), suggesting that the fabricated DVS possesses an adequate contractility similar to that of *in vivo* blood vessels (32). The elastin staining of the DVS cross-sections indicates that there are accumulated elastic fibers secreted by SMCs (Fig. 4H). The gene expression analysis of DVS after 3 days of perfusion culture revealed that *αSMA*, calponin, and *SM-MYH* were upregulated up to 3.1, 3.1, and 5.0 times compared to those of S1 before the encapsulation, respectively (Fig. 4I). Further studies may be needed to clarify the mechanisms responsible for the upregulation of these gene expressions. There could be complex interactions through various cues, such as mechanical loading generated by perfusion, and signaling between HUVECs. The interactions between endothelial cells and smooth muscle cells or pericyte *in vivo* are well studied (33,34). Signaling through soluble factors and direct contacts such as angiopoietin and notch signaling is essential for morphogenesis during development and maintenance of vascular functions in mature vessels.

In conclusion, we have demonstrated a novel approach that is scalable for the three-dimensional alignments of DVS by assembling multiple needles as previously reported (12). Our next task is to examine whether such multiple DVSs can be used to induce perfusable and complex vascular networks *in vitro* and supply oxygen and nutrients to parenchymal cells encapsulated between the needles in a hydrogel. We believe this perfusable DVS fabrication approach could open up a new avenue for engineering vascularized tissues *in vitro* for regenerative medicine applications, such as liver and kidney tissues.

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