

Evidence for *In Vivo* Growth Potential and Vascular Remodeling of Tissue-Engineered Artery

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Nondegradable synthetic polymer vascular grafts currently used in cardiovascular surgery have no growth potential. Tissue-engineered vascular grafts (TEVGs) may solve this problem. In this study, we developed a TEVG using autologous bone marrow-derived cells (BMCs) and decellularized tissue matrices, and tested whether the TEVGs exhibit growth potential and vascular remodeling *in vivo*. Vascular smooth muscle-like cells and endothelial-like cells were differentiated from bone marrow mononuclear cells *in vitro*. TEVGs were fabricated by seeding these cells onto decellularized porcine abdominal aortas and implanted into the abdominal aortas of 4-month-old, bone marrow donor pigs ($n = 4$). Eighteen weeks after implantation, the dimensions of TEVGs were measured and compared with those of native abdominal aortas. Expression of molecules associated with vascular remodeling was examined with reverse transcription-polymerase chain reaction assay and immunohistochemistry. Eighteen weeks after implantation, all TEVGs were patent with no sign of thrombus formation, dilatation, or stenosis. Histological and immunohistochemical analyses of the retrieved TEVGs revealed regeneration of endothelium and smooth muscle and the presence of collagen and elastin. The outer diameter of three of the four TEVGs increased in proportion to increases in body weight and outer native aorta diameter. Considerable extents of expression of molecules associated with extracellular matrix (ECM) degradation (i.e., matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase) and ECM precursors (i.e., procollagen I, procollagen III, and tropoelastin) occurred in the TEVGs, indicating vascular remodeling associated with degradation of exogenous ECMs (implanted decellularized matrices) and synthesis of autologous ECMs. This study demonstrates that the TEVGs with autologous BMCs and decellularized tissue matrices exhibit growth potential and vascular remodeling *in vivo* of tissue-engineered artery.

Introduction

VASCULAR GRAFTS fabricated from nondegradable synthetic polymers such as expanded polytetrafluoroethylene and polyethylene terephthalate have been clinically used to replace diseased blood vessels.¹⁻³ However, these synthetic grafts have shortcomings, including thrombus formation, calcium deposition, and infection.⁴ In addition, because these vascular grafts do not degrade *in vivo*, they are not capable of growing *in vivo*. In surgery for pediatric patients with congenital cardiovascular diseases, an autologous living vascular graft that has growth capability is required.

Tissue-engineered vascular grafts (TEVGs) could overcome the shortcomings of nondegradable synthetic polymer vascular grafts. TEVGs are fabricated by seeding autologous vascular cells⁴⁻⁶ or stem cells⁷⁻¹⁰ onto biocompatible and biodegradable polymer scaffold that allows the cells to regenerate autologous vascular tissues including endothelium *in vivo*. The polymer scaffolds degrade completely *in vivo*, resulting in autologous tissue formation without foreign materials. These TEVGs are antithrombogenic, biocompatible, and capable of growing and self-repair. These grafts might be appropriate for child patients with congenital cardiovascular defects.

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In the present study, we investigated whether TEVGs, which are fabricated with autologous bone marrow–derived cells (BMCs) and decellularized tissue matrices, exhibit growth potential *in vivo*. BMCs were induced to differentiate into endothelial-like cells and smooth muscle (SM)–like cells prior to cell seeding. The vascular cells were seeded onto porcine decellularized aortas and implanted in the abdominal aortas of young bone marrow donor pigs. Eighteen weeks after implantation, vascular tissue regeneration in the TEVGs was examined by histological, immunohistochemical, and electron microscopic analyses. The growth of the implanted TEVGs was investigated by comparing the dimensions (outer diameter and length) of the grafts with those of native abdominal aortas. Vascular remodeling in the implanted TEVGs was assessed by a reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemical analyses for various extracellular matrices (ECMs) and ECM degradation–related molecules.

Materials and Methods

Fabrication of decellularized aorta matrices

Decellularized vascular matrices (7–8 mm in internal diameter, 24–32 mm in length) were prepared as previously described.¹⁰ In brief, freshly harvested, young (4 months old) porcine abdominal aortas were washed with distilled water for 1 h to remove all blood elements. The aortas were then immersed in a decellularization solution [0.5% (v/v) Triton X-100 (Sigma, St. Louis, MO) and 0.05% (v/v) ammonium hydroxide (Sigma) in distilled water] with shaking at 4°C for 5 days. To remove the residual detergent, the decellularized vessels were washed in distilled water with shaking at 4°C for 3 days. The resultant matrices were lyophilized for 3 days and sterilized with ethylene oxide gas at room temperature.

BMC isolation and culture

Bone marrow (50 mL for each pig) was aspirated from the humeri of healthy young pigs (3 months old, $n = 4$) with a syringe containing heparin (100 unit heparin/mL bone marrow). The aspirated bone marrow was mixed with an equal volume of phosphate buffered saline (PBS; Sigma), and centrifuged on a Ficoll-Paque density gradient (Amersham Biosciences, Arlington Heights, IL) for 20 min at 1500 rpm. Bone marrow mononuclear cells (BMMNCs) were isolated from the layer between the Ficoll-Paque reagent and blood plasma, and washed three times in PBS solution. For differentiation into SM-like cells, BMMNCs were cultured for 3 weeks in Medium 199 (Gibco BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 unit/mL penicillin (Gibco BRL), and 0.1 mg/mL streptomycin (Gibco BRL) in humidified air with 5% CO₂ at 37°C.⁹ For differentiation into endothelial-like cells, BMMNCs were cultured for 3 weeks on culture dishes coated with 1 µg/cm² human fibronectin (Sigma) in EGM-2 medium (Cambrex, Walkersville, MD) supplemented with human vascular endothelial growth factor (VEGF, 10 ng/mL; PeproTech, Rocky Hill, NJ), human basic fibroblast growth factor (bFGF, 2 ng/mL; PeproTech), human epidermal growth factor (10 ng/mL; PeproTech), human insulin-like growth factor-1 (5 ng/mL; PeproTech), and ascorbic acid.⁹ Vascular cells differentiated from BMMNCs were cultured *in vitro* for 3 weeks before cell seeding.

BMC characterization

Endothelial-like cells and SM-like cells differentiated from BMMNCs were characterized by immunochemical staining. Endothelial-like cell and SM-like cell staining was performed using primary antibodies against von Willebrand Factor (vWF; Santa Cruz Biotechnology, Santa Cruz, CA) and SM α -actin (DAKO, Carpinteria, CA), respectively. The staining signal was visualized with an avidin–biotin complex immunoperoxidase kit (Vectastain[®] Elite ABC kit; Vector Laboratories, Burlingame, CA) and a 3,3'-diaminobenzidine (DAB) substrate solution kit (Vector Laboratories).

Vascular cells differentiated from BMMNCs were also characterized by RT-PCR. Total RNA was extracted from endothelial-like cells and SM-like cells. These cells were lysed in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted with 200 µL of chloroform and precipitated with 500 µL of 80% (v/v) isopropanol. After removing the supernatant, the RNA pellet was washed with 75% (v/v) ethanol, air-dried, and dissolved in 0.1% (v/v) diethyl pyrocarbonate-treated water. The RNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer. A reverse transcription reaction was performed with 5 µg pure total RNA using SuperScript[™] II reverse transcriptase (Invitrogen). The synthesized cDNA was amplified by PCR using the following primers: (1) vWF¹¹: sense 5'-TGG TGA GAA GGG TGA GAA-3', antisense 5'-AGA TCT TGG TAA AGC GAA TG-3'; (2) SM α -actin¹²: sense 5'-TGC AGC TTC CTT CTC ACC TTG A-3', antisense 5'-TCC TGG CCC AGT ATG AAG GAA ATC-3'; (3) β -actin¹³: sense 5'-GCA CTC TTC CAG CCT TCC TTC C-3', antisense 5'-TCA CCT TCA CCG TTC CAG TTT TT-3'. PCR was carried out for 30–35 cycles of denaturing (94°C, 30 s), annealing (55–60°C, 30 s), and extension (72°C, 45 s), with a final extension at 72°C for 7 min. The sizes of the RT-PCR products for vWF, SM α -actin, and β -actin were 298, 965, and 282 bp, respectively. The PCR products were visualized by electrophoresis on 2% (w/v) agarose gels containing 0.5 µg/mL ethidium bromide.

Cell seeding and in vitro maintenance of cell-seeded matrices

SM-like cells (4.0×10^7 cells/mL) suspended in 1 mL of culture medium were uniformly seeded onto decellularized tissue matrices. Two hours later, endothelial-like cells (1.0×10^7 cells/mL) suspended in 1 mL of culture medium were seeded onto the luminal sides of the matrices. Prior to implantation, the BMC-seeded vascular grafts were cultured in Medium 199 supplemented with 20% (v/v) FBS, human VEGF (10 ng/mL), and human bFGF (2 ng/mL) for 1 week in humidified air with 5% CO₂ at 37°C.⁹

TEVG implantation

Four-month-old, bone marrow donor pigs (30–40 kg, $n = 4$) were anesthetized with injections of intramuscular ketamine (30 mg/kg) and intravenous pentobarbital (30 mg/kg), and ventilated with a mixture of O₂, N₂, and isoflurane during the operation. Through a midline abdominal incision, abdominal aortas were exposed. After heparin (100 units/kg) had been administered intravenously, the proximal and distal portions of the abdominal aortas were clamped. Segments (20 mm) of the abdominal aortas were resected and replaced by the

TEVGs by an end-to-end anastomosis using a 6–0 polypropylene suture (Ethicon, Somerville, NJ). No anticoagulants or antiplatelets were administered postoperatively. All care and handling of the animals were provided according to the Guide for the Care and Use of Laboratory Animals of Yonsei University.

Computed tomography (CT)

CT was performed as previously described.¹⁴ The implanted TEVGs were examined with a 16-slice CT scanner (SOMATOM Sensation 16; Siemens, Erlangen, Germany) using the following scan parameters: collimation, 16×0.75 mm; slice thickness, 2 mm; rotation time, 500 ms; tube voltage, 120 kV; effective tube current, 200 mAs_{eff}; pitch factor, 1.05. For image reconstruction, axial images were reconstructed with a smooth reconstruction kernel value (Siemens, B31f), a slice thickness of 1 mm, and an increment of 0.7 mm.

Measurement of the TEVG and native aorta dimensions

The initial dimensions (length and outer diameter) of the TEVGs were measured 1 week after cell seeding. Native abdominal aorta segments dissected upon TEVG implantation were used to measure the initial outer diameter of native aortas. Immediately after retrieval of TEVGs and native aortas at 18 weeks after implantation, the outer diameters of the TEVGs and native aortas were measured and compared with their initial outer diameters. The TEVG length was also measured at 18 weeks and compared with the initial TEVG length.

Histological and immunohistochemical analyses

Mid-portion segments of the TEVGs retrieved at 18 weeks after implantation were fixed in 10% (v/v) buffered formaldehyde solution, dehydrated with a graded ethanol series, and embedded in paraffin. The specimens were cut into 4-μm-

thick sections and stained with hematoxylin and eosin (H&E). Collagen in the retrieved TEVGs was stained using Masson's trichrome method. The specimens were also stained immunohistochemically for platelet/endothelial cell adhesion molecules (PECAM; Santa Cruz Biotechnology) and SM (α-actin (DAKO)). For staining of ECM precursors, the specimens were immunohistochemically stained with primary antibodies against procollagen type I (Chemicon, Temecula, CA), procollagen type III (Chemicon), and elastin (Sigma; this antibody is specific for tropoelastin¹⁵). The staining signal was visualized with avidin–biotin complex immunoperoxidase (Vectastain[®] ABC kit) and a DAB substrate solution kit (Vector Laboratories).

Semi-quantitative RT-PCR

Total RNA was extracted from the TEVGs retrieved at 18 weeks after implantation. A reverse transcription reaction was performed with 5 μg pure total RNA using SuperScript[™] II reverse transcriptase (Invitrogen). The synthesized cDNA was amplified by PCR using the primers listed in Table 1. PCR was carried out for 30–35 cycles of denaturing (94°C, 30 s), annealing (55–60°C, 30 s), and extension (72°C, 45 s), with a final extension at 72°C for 7 min. The PCR products were visualized by electrophoresis on 2% (w/v) agarose gels, and photographed. The images were scanned and saved as tagged image files using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA). Densitometric analysis of scanned images was carried out using the Scion Image program (NIH Image; Scion Corporation, Frederick, MD). The band intensities of each cDNA molecule were normalized as ratios to β-actin cDNA in the same sample.

Transmission electron microscopy

The specimens of the native abdominal aortas and the TEVGs retrieved at 18 weeks after implantation were fixed in 2.5% (v/v) buffered glutaraldehyde for 2 h and embedded in

TABLE 1. PRIMER SEQUENCES AND PCR PRODUCT SIZES FOR GENES RELATING TO VASCULAR REMODELING

Gene	Primer sequence (sense and antisense)	Product size	Reference
MMP-1	5'-GAA GAT GTG GAC CGT GCC AT-3' 5'-TGC CAT CAA TGT CAT CTT GG-3'	384 bp	16
MMP-2	5'-CAA GGA CCG GTT CAT TTG GCG-3' 5'-ATG GCA TTC CAG GCA TCT GCG-3'	375 bp	13
MMP-9	5'-GTA TTT GTT CAA GGA TGG GAA GTA C-3' 5'-TGC CGG ATG CAG GCG TAG TGT TT-3'	515 bp	13
TIMP-1	5'-CTG TTG TTG CTG TGG CTG ATA G-3' 5'-CTT TTC AGA GCC TTG GAG GAC-3'	507 bp	13
TIMP-2	5'-TCT GGA AAC GAC ATT TAT GG-3' 5'-GTT GGA GGC CTG CTT ATG GG-3'	508 bp	13
TIMP-3	5'-CTT CTG CAA CTC CGA CAT CGT G-3' 5'-TGC CGG ATG CAG GCG TAG TGT TT-3'	459 bp	17
Procollagen type I	5'-GGC TCC TGC TCC TCT TAG CG-3' 5'-CAT GGT ACC TGA GGC CGT TC-3'	132 bp	18
Procollagen type III	5'-CCA GTA CAA GTG ACC AAC TA-3' 5'-TAG CAC CAT TGA GAC ATT-3'	182 bp	18
Tropoelastin	5'-TGG AGC CCT GGG ATA TCA AG-3' 5'-GAA GCA CCA ACA TGT AGC AC-3'	369 bp	19
β-actin	5'-CAG GCA CCA GGG CGT-3' 5'-ATG GCT GGG GTG TTG AAG-3'	282 bp	13

epoxy resin. Sections (70 nm in thickness) were double stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-1200EX-II; Nihon Denshi, Tokyo, Japan).

Statistical analysis

Quantitative data are expressed as mean \pm standard deviation. Statistical analysis was performed by the unpaired Student's *t*-test using InStat software (InStat 3.0; GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ defined statistical significance.

Results

Characterization of decellularized vascular matrices

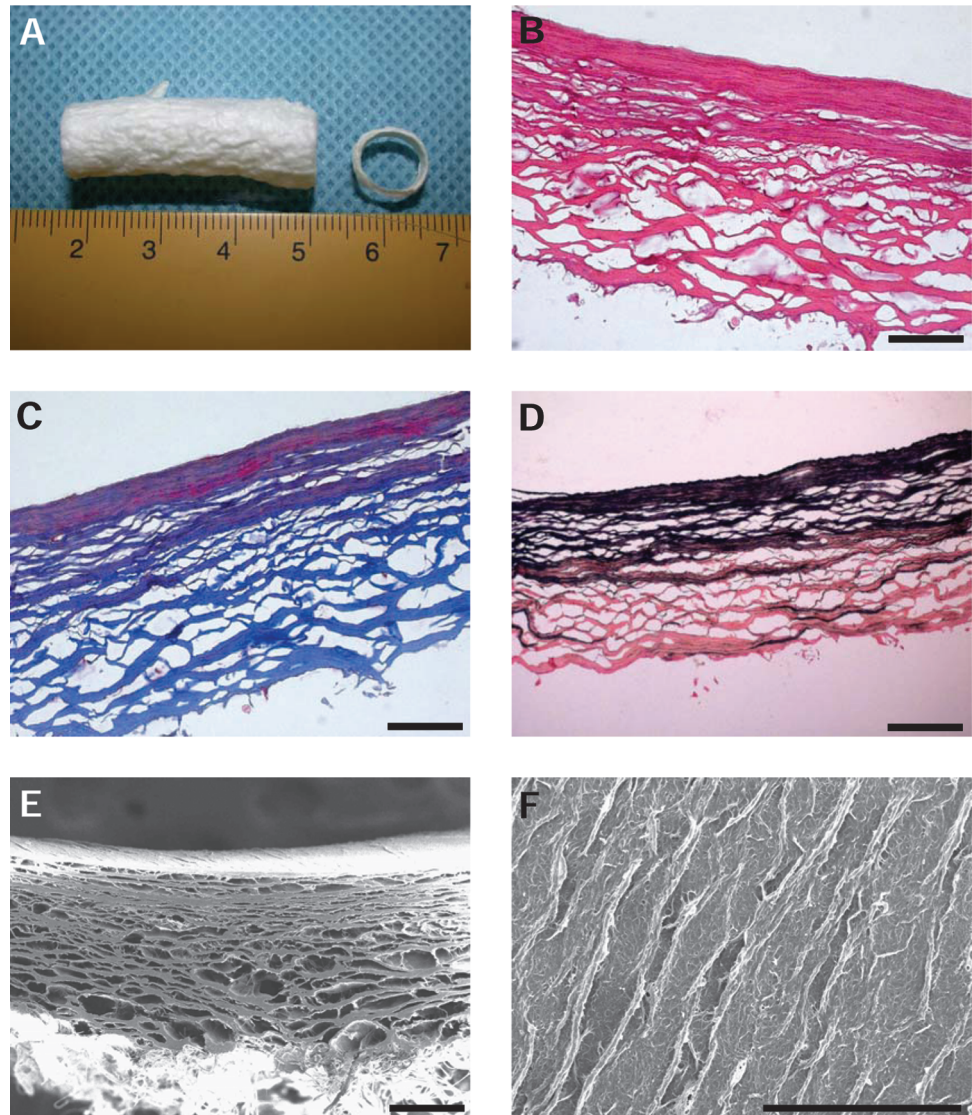
Vascular graft matrices (Fig. 1A) were fabricated by decellularization of porcine abdominal aortas. Through a decellularization process using nonionic detergent (Triton X-100), all cellular components were removed from the aortas

(Fig. 1B), leaving native ECMs such as collagen (Fig. 1C) and elastin (Fig. 1D). Scanning electron microscopic examination of decellularized tissue matrices indicated porous and multi-layer structures in the cross section of the matrices (Fig. 1E), which provide surfaces for cell adhesion upon cell seeding and three-dimensional space for tissue formation upon implantation. Endothelium was not observed on the luminal surfaces of the decellularized matrices (Fig. 1F).

Characterization of BMCs

BMMNCs were differentiated into endothelial-like cells and SM-like cells *in vitro*. The endothelial-like cells showed cobblestone morphology, which is a typical endothelial cell (EC) morphology (Fig. 2A), and expressed vWF (Fig. 2B), which is a mature EC phenotypic marker. The SM-like cells showed morphology similar to that of mature smooth muscle cells (SMCs) (Fig. 2C) and expressed SM α -actin (Fig. 2D), which is an SMC phenotypic marker. The mRNA expression of vWF was expressed only in the BMCs cultured in the EC culture condition (Fig. 2E). The mRNA expression of SM

FIG. 1. Vascular matrix prepared by decellularization of porcine abdominal aorta. (A) A gross view of the decellularized tissue matrix (8 mm in internal diameter, 32 mm in length). The scale is in centimeters. (B) H&E staining showed removal of cellular components from canine abdominal aorta ($\times 100$). (C) Masson's trichrome staining ($\times 100$) and (D) van Gieson's staining ($\times 100$) of the collagen and elastin in the decellularized tissue matrices. Scanning electron micrographs of (E) the cross section ($\times 100$) and (F) the luminal surface ($\times 300$) of the decellularized tissue matrix. The scale bars indicate 200 μm . All images were taken after lyophilization using a freezing dryer. Color images available online at www.liebertonline.com/ten.



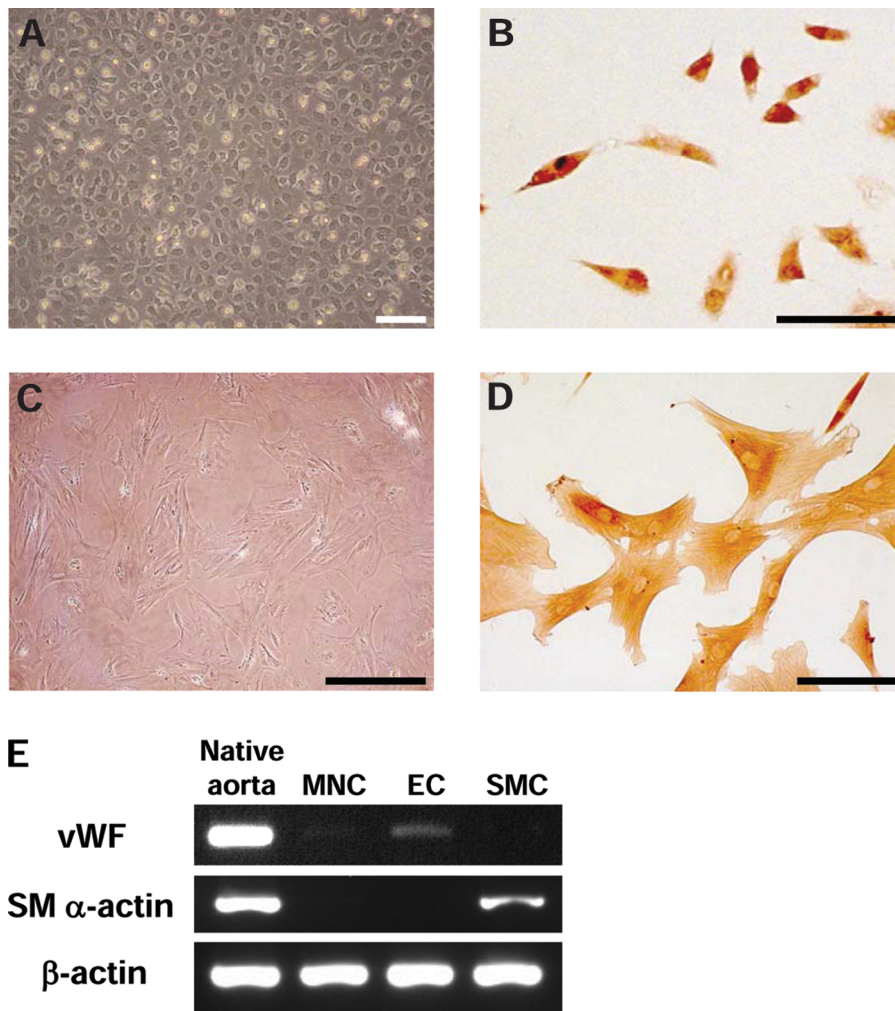


FIG. 2. Characterization of differentiated bone marrow-derived cells (BMCs). (A) Endothelial-like cells derived from bone marrow mononuclear cells (BMMNCs) showing a cobblestone morphology ($\times 100$) and stained positively for (B) vWF ($\times 400$). Scale bars in (A) and (B) indicate 50 and 30 μm , respectively. (C) SM-like cells derived from BMMNCs exhibiting the morphology of mature SMCs ($\times 200$) and stained positively for (D) SM α -actin ($\times 200$). Scale bars in (C) and (D) indicate 50 μm . (E) RT-PCR for detection of the mRNA expression of vWF and SM α -actin in BMCs cultured in EC and SMC culture conditions. MNC, BMMNCs; EC, endothelial-like cells derived from BMMNCs; SMC, SM-like cells derived from BMMNCs. Color images available online at www.liebertonline.com/ten.

α -actin was expressed only in BMCs cultured in the SMC culture condition (Fig. 2E).

Pre- and postimplantation examination of TEVGs

Vascular graft matrices were seeded with endothelial-like cells and SM-like cells differentiated from BMMNCs and maintained *in vitro* under static culture conditions for 1 week to allow for cell adherence. The seeded BMCs adhered well onto the vascular graft matrices. H&E staining of the cross section of the BMC-seeded vascular grafts showed that a high density of cells were present on the luminal sides of the matrices (Fig. 3A). Scanning electron microscopic examination of the luminal surfaces of the grafts revealed a confluent layer of BMCs (Fig. 3B). The BMC-seeded vascular grafts (Fig. 3C) replaced segments of the porcine abdominal aortas without matrix rupture or deformation (Fig. 3D). The implanted TEVGs were all patent at 18 weeks. No sign of thrombus formation, dilatation, or stenosis were observed in the explanted grafts (Fig. 3E, F).

Vascular tissue regeneration in TEVGs

Histological and immunohistochemical analyses of the TEVGs retrieved at 18 weeks of implantation revealed formation of the three vascular components (endothelium,

media, and adventitia) that are similar to those of native aorta (Fig. 4). H&E-stained sections of the retrieved TEVGs showed regeneration of vascular tissues similar to that of native abdominal aortas (Fig. 4A, E). Masson's trichrome staining revealed the presence of a significant amount of collagen throughout the vascular grafts (Fig. 4B). Immunohistochemical analysis showed that cells on the luminal sides of the TEVGs stained positively for PECAM (Fig. 4C), indicating endothelium regeneration. Cells in the medial parts of the TEVGs stained positively for SM α -actin (Fig. 4D), indicating SM regeneration. Transmission electron microscopic examination of the TEVGs revealed the presence of elastin (arrows) and collagen (arrowheads) fibers in the TEVGs (Fig. 5A). These ECM structures of the TEVGs (Fig. 5A) were similar to those of native aortas (Fig. 5B).

In vivo growth of TEVGs

The outer diameter and length of the TEVGs were measured to investigate whether the TEVGs have growth potential *in vivo*. CT scan images of the TEVGs immediately, 9 weeks, and 18 weeks after implantation showed patent TEVGs interposed into the abdominal aortas (Fig. 6). The body weight of the piglets and outer diameter of the native abdominal aortas increased during 18 weeks after implantation (Fig. 7A, B), and the outer diameter of three of the four

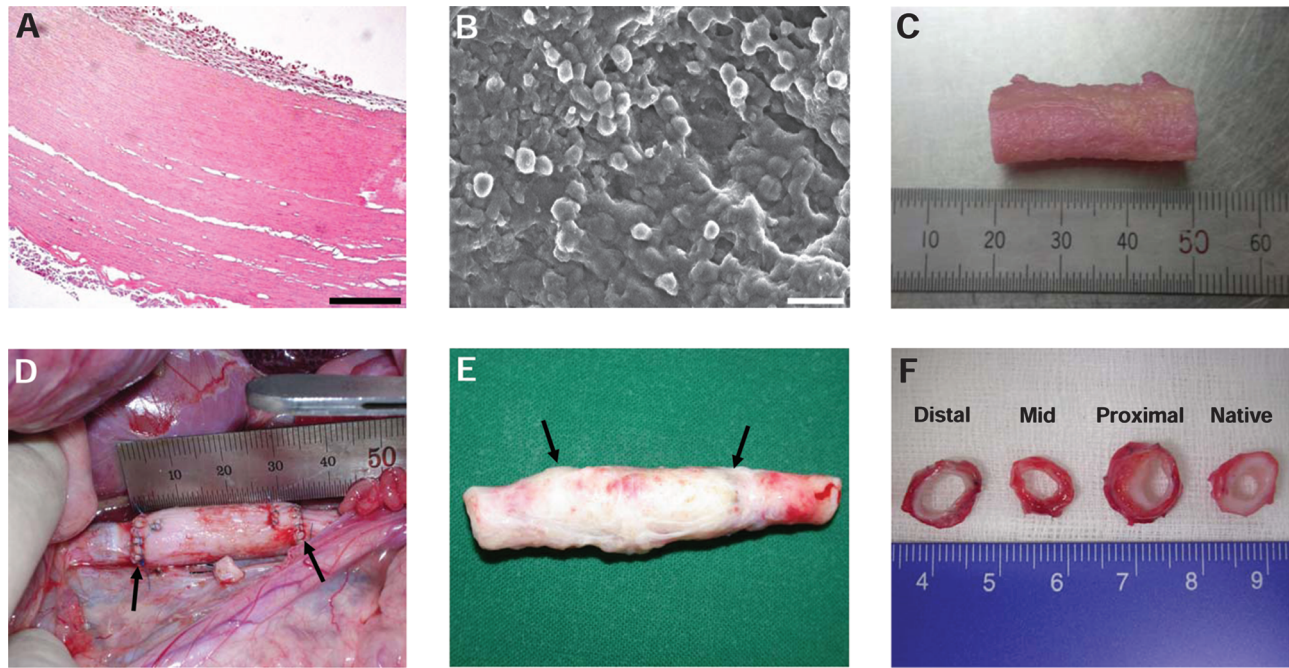


FIG. 3. Examination of TEVGs at pre- and postimplantation. (A) H&E-stained section of the TEVG cultured *in vitro* for 1 week after cell seeding ($\times 200$). The scale bar indicates 200 μm . (B) Scanning electron micrograph of the luminal surface of the vascular graft 1 week after cell seeding ($\times 300$). The scale bar indicates 30 μm . (C) A gross view of TEVG maintained *in vitro* for 1 week after cell seeding. The scale is in centimeters. (D) Surgical implantation of the TEVG. TEVGs were interposed into the abdominal aortas of bone marrow donor pigs by end-to-end anastomosis. The arrows indicate the TEVGs implanted to abdominal aortas. The scale is in centimeters. (E) Gross view of TEVG retrieved at 18 weeks after implantation. The arrows indicate the TEVGs interposed to abdominal aortas. (F) Gross view of segments of the TEVG (proximal, mid, and distal portions) retrieved at 18 weeks after implantation and a segment of native abdominal aorta. The scale is in centimeters. Color images available online at www.liebertonline.com/ten.

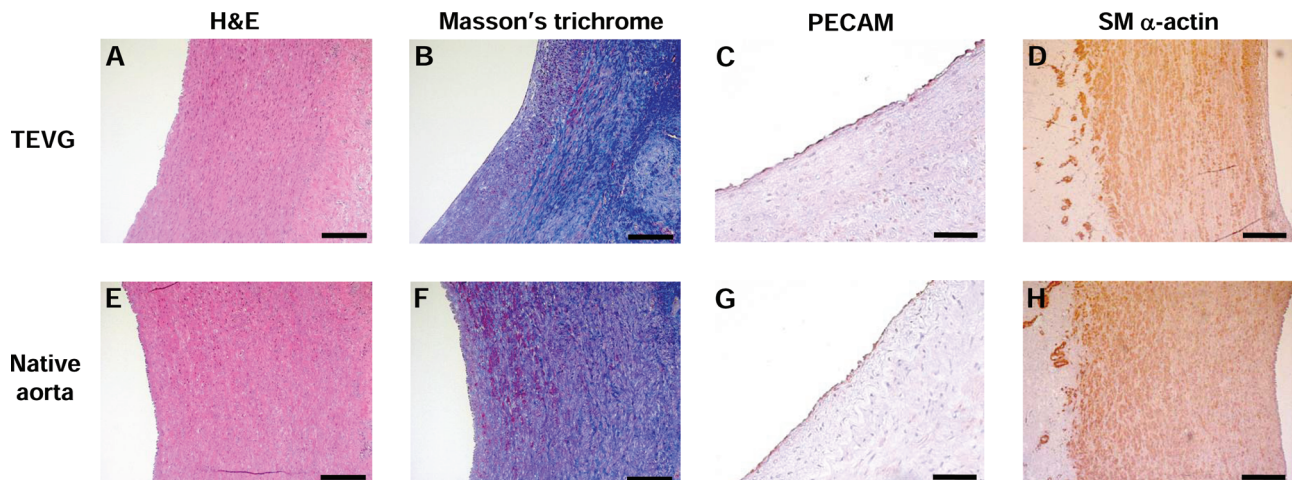


FIG. 4. Histological and immunohistochemical analyses of TEVGs and native aortas retrieved at 18 weeks after implantation. (A) H&E staining of retrieved TEVGs showing regeneration of vascular tissues ($\times 100$). (B) Masson's trichrome staining indicates collagen regeneration in the TEVGs ($\times 100$). (C) Cells on the luminal sides of the retrieved TEVGs stained positively for PECAM, indicating endothelium regeneration ($\times 400$). (D) Immunohistochemical staining for SM α -actin ($\times 100$) showing regeneration of SM tissue in the medial parts of the TEVGs. (E) H&E ($\times 100$) and (F) Masson's trichrome ($\times 100$) staining of native abdominal aortas. Immunohistochemical staining for (G) PECAM ($\times 400$) and (H) SM α -actin ($\times 100$) of native abdominal aortas. The scale bars in (A), (B), (D), (E), (F), and (H) indicate 200 μm . The scale bars in (C) and (G) indicate 50 μm . Color images available online at www.liebertonline.com/ten.

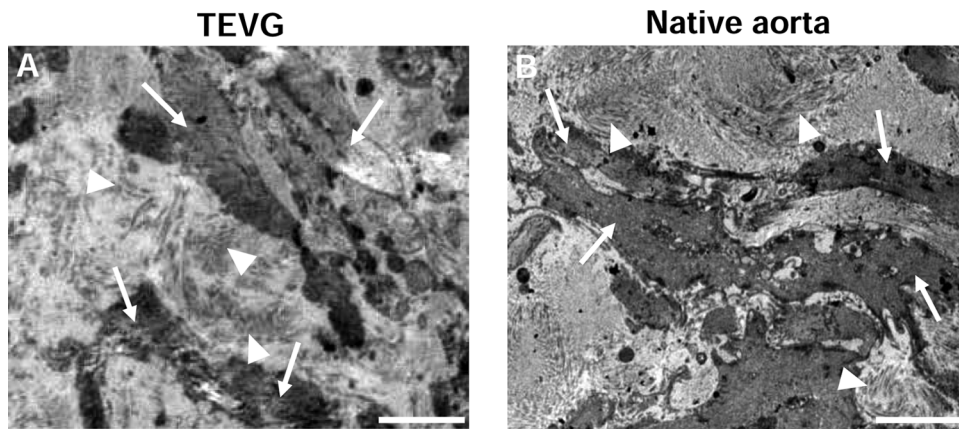


FIG. 5. Transmission electron microscopic examination of the TEVGs retrieved at 18 weeks after implantation. (A) Vascular ECM structures (collagen and elastin fibers) can be observed in the vascular wall of the retrieved TEVGs ($\times 10,000$). Arrows and arrowheads indicate elastin and collagen fibers, respectively. (B) ECM structures in the vascular walls of native abdominal aortas ($\times 10,000$). The scale bars indicate 2 μm .

TEVGs increased during the same period (Fig. 7C). However, the length of three of the four TEVGs did not increase (Fig. 7D). The inner diameter of three TEVGs except for one graft did not increase at 18 weeks after implantation (pig #1, 7.2 mm \rightarrow 7.1 mm; pig #2, 8.9 mm \rightarrow 6.5 mm; pig #3, 7.0 mm \rightarrow 5.8 mm; and pig #4, 6.5 mm \rightarrow 8.0 mm).

Vascular remodeling in the implanted TEVGs

RT-PCR assays for molecules associated with ECM degradation such as matrix metalloproteinase (MMP) and tissue inhibitor of matrix metalloproteinase (TIMP) were performed to examine the expression pattern of the genes during vascular remodeling. Vascular remodeling in the TEVGs is regulated by the reciprocal effects of MMP and TIMP series. The mRNA expression of MMPs was slightly enhanced in the TEVGs at 18 weeks after implantation compared with native abdominal aortas, but not significantly ($p > 0.05$) (Fig. 8A, B). TIMP-3 mRNA expression in the TEVGs was significantly higher ($p < 0.05$) than that in native aortas (Fig. 8C), but there

was no difference in the mRNA expression between TIMP-1 and TIMP-2 (Fig. 8A, C).

ECM synthesis in the implanted TEVGs

Immunohistochemical staining of the TEVGs retrieved at 18 weeks for various ECM precursors (i.e., procollagen type I, procollagen type III, and tropoelastin, which are precursors of collagen type I, collagen type III, and elastin, respectively) showed that those ECM precursors were produced actively in the TEVGs (Fig. 9A). RT-PCR analysis for ECM precursors also revealed that the mRNA of the ECM precursors was expressed at a higher (procollagen I) or similar (procollagen III and tropoelastin) extent in the TEVGs compared with the native abdominal aortas (Fig. 9B, C), indicating synthesis of autologous ECMs in the implanted TEVGs.

Discussion

TEVGs could address the shortcomings of the currently available nondegradable synthetic polymer vascular grafts that

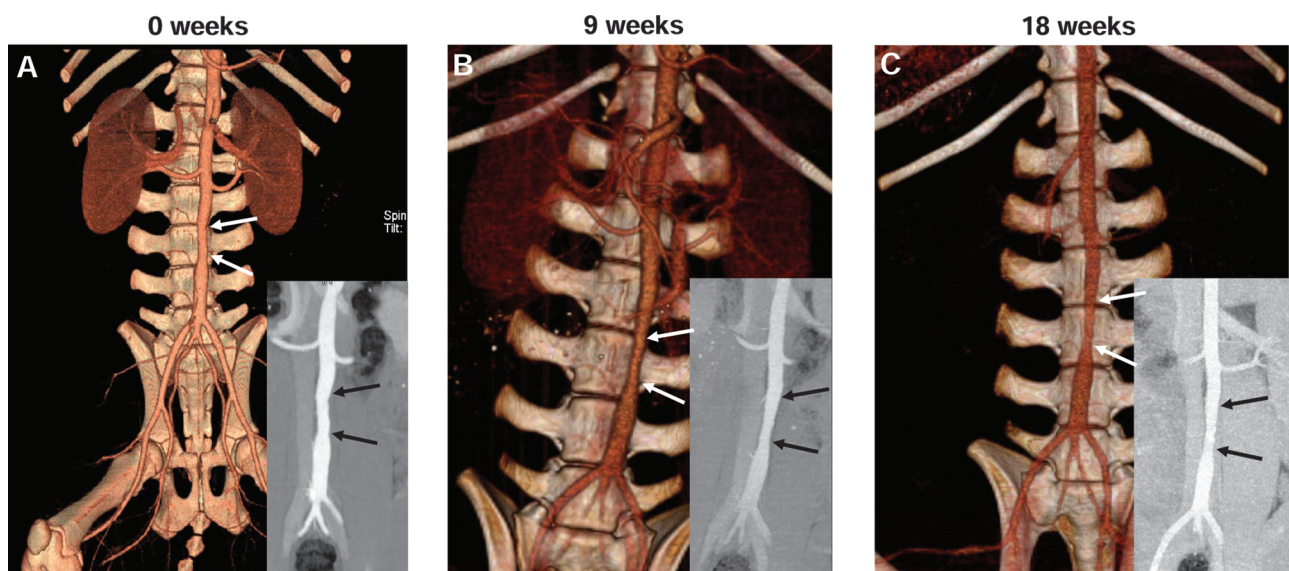


FIG. 6. CT scanning images of the TEVGs after implantation at (A) 0 weeks, (B) 9 weeks, and (C) 18 weeks after implantation. CT images showed patent TEVGs in abdominal aorta interposition. Arrows indicate the implanted TEVGs. Color images available online at www.liebertonline.com/ten.

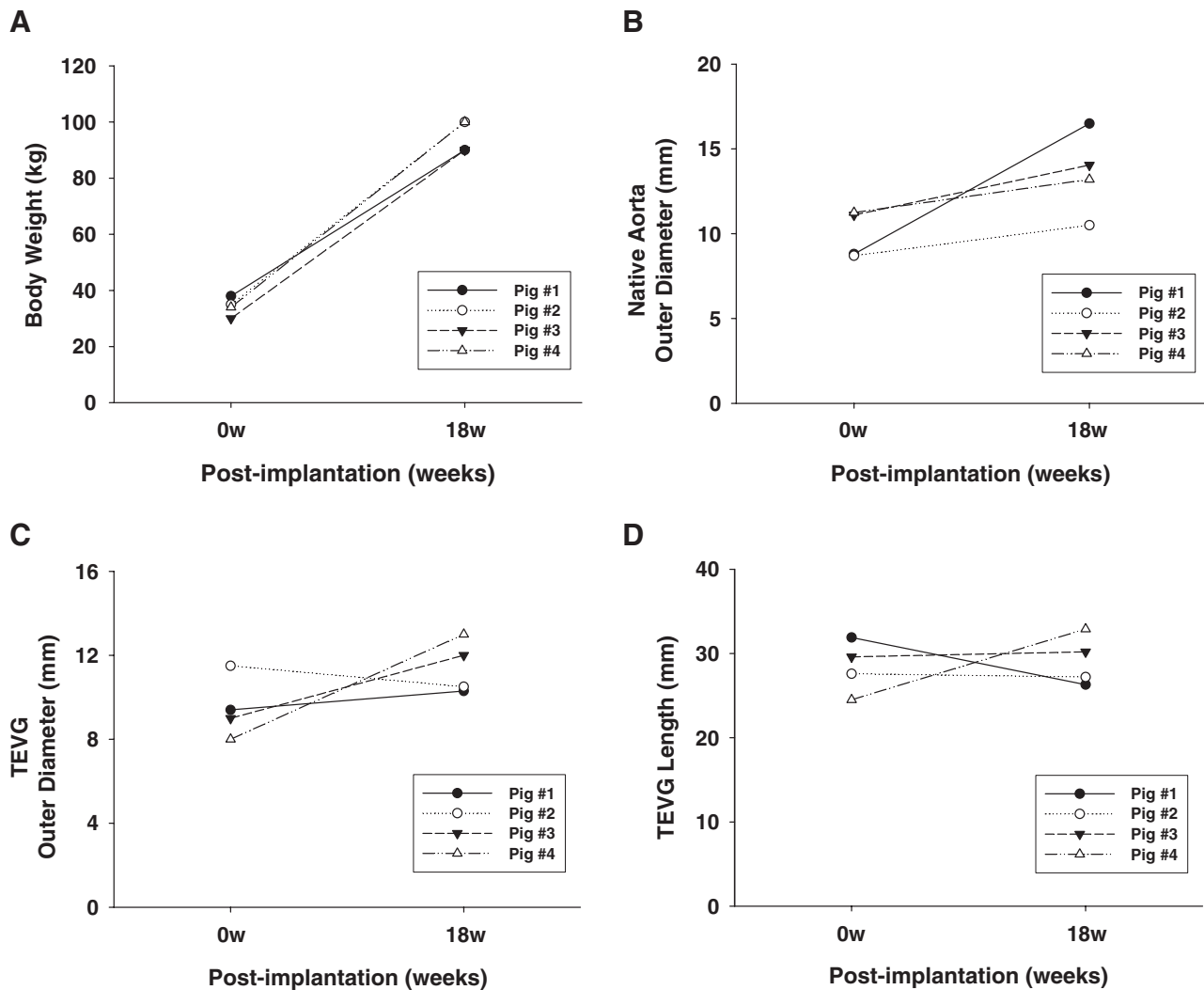


FIG. 7. Dimension change in the TEVGs and native abdominal aortas over an 18-week implantation period. (A) Body weight of the pigs receiving TEVG implantation. (B) Outer diameter of the native abdominal aortas. (C) Outer diameter of the implanted TEVGs. (D) Length of the implanted TEVGs.

have no growth potential. In this study, we developed a TEVG using autologous BMCs and decellularized tissue matrices, and examined the *in vivo* growth potential of the TEVGs in a porcine abdominal aorta model. Upon implantation into rapid-growing animals for 18 weeks, the TEVGs with autologous cells exhibited their growth potential and remodeling *in vivo*, which are critical requirements of vascular grafts for pediatric patients undergoing cardiovascular surgery.

Decellularized tissue matrices provide potential for growth *in vivo*. The ECMs in native blood vessels are degraded *in vivo* by proteolytic enzymes such as MMPs.^{20–22} Upon implantation, exogenous ECMs in decellularized tissue matrices degrade and are gradually replaced with autologous ECMs synthesized by implanted autologous cells and ingrowing host cells (e.g., SMCs or fibroblasts).^{20,23} Because the resultant autologous vascular tissues after exogenous ECM degradation and vascular remodeling are living tissues integrated in host tissues without foreign materials, these tissues could have growth and repair potential. In allogenic matrix transplantation, decellularized matrix biodegradation occurs at a

slower rate than synthesis of autologous ECMs,^{21,23} thus TEVGs using decellularized tissue matrices may not show aneurysmal degeneration or dilation.

BMCs could be an alternative cell source for tissue engineering of vascular grafts. It has been reported that BMCs can differentiate into ECs and vascular SMCs *in vivo*^{24–27} and *in vitro*.^{28–31} In the present study, TEVGs with autologous BMCs showed regeneration of vascular tissues, including endothelium, collagen, elastin, and SM (Figs. 4 and 5). Our previous studies using canine models have shown *in vivo* vascular tissue formation in vascular tubular grafts^{9,10} and vascular patches⁸ engineered with BMCs and biodegradable scaffolds, indicating the feasibility of TEVGs using BMCs in large animal models. These results are consistent with results reported by Shin'oka and co-workers in which autologous TEVGs using BMMNCs and biodegradable polymer scaffolds showed vascular tissue regeneration in canine models.⁷ They have also reported that biodegradable polymer vascular grafts engineered with autologous BMMNCs had successful post-operative results in humans.^{32,33} The use of BMCs as the cell

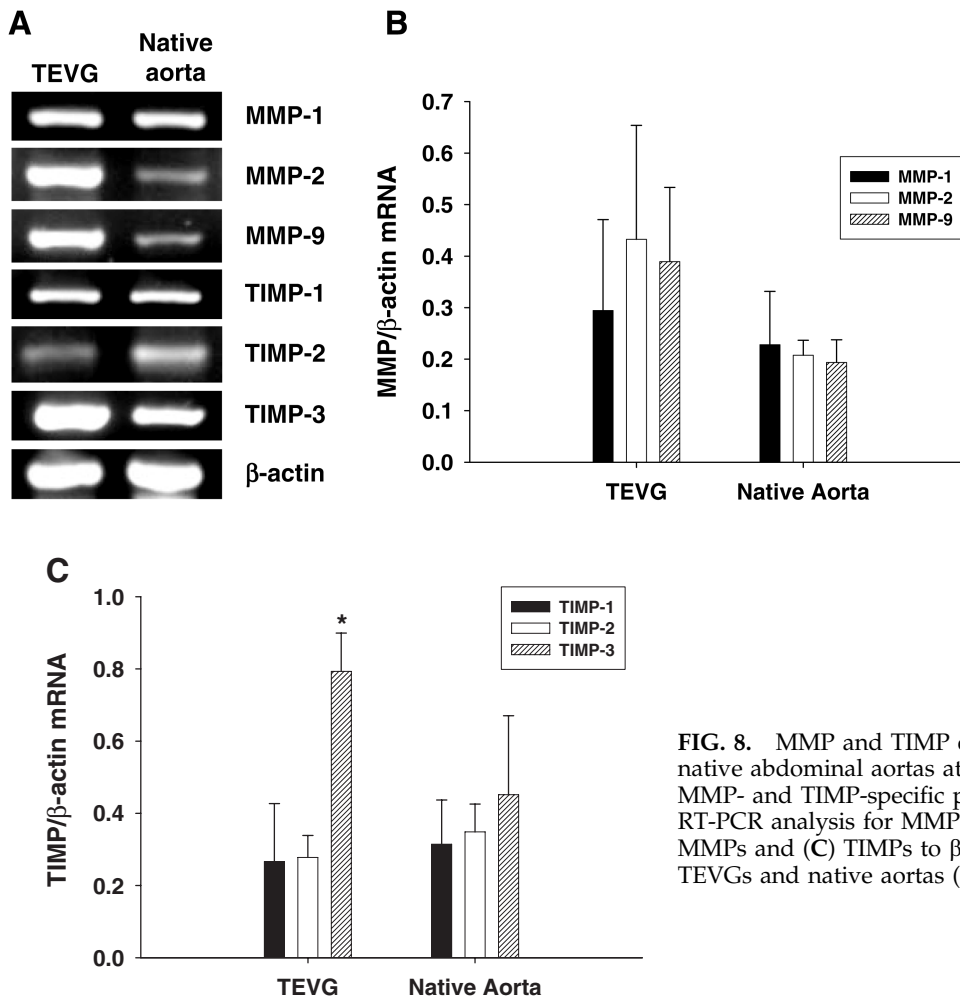


FIG. 8. MMP and TIMP expression in the TEVGs and native abdominal aortas at 18 weeks. (A) RT-PCR using MMP- and TIMP-specific primers. (B, C) Semi-quantitative RT-PCR analysis for MMPs and TIMPs. The ratios of (B) MMPs and (C) TIMPs to β -actin mRNA expression in the TEVGs and native aortas ($*p < 0.05$ versus native aortas).

source is generally considered as less invasive than harvesting of vascular cells from autologous blood vessels. In addition, BMCs could be utilized as a cell source when patients do not have blood vessels suitable for harvesting due to preexisting vascular disease or vessel use in previous procedures.

The *in vivo* recellularization by host cell migration is another contribution to vascular tissue reconstruction in the implanted TEVGs. Host vascular cells migrating from surrounding vessels and vascular progenitor cells mobilized from circulating blood contribute to vascular tissue regeneration in the implanted TEVGs as well as BMCs seeded onto the matrices before implantation. Decellularized tissue matrices without cell seeding have been shown to be successfully remodeled into cellularized vessels after implantation into large animal models.^{34–36} This might indicate that spontaneous healing mechanism such as *in vivo* recellularization by host cells regenerates vascular tissues in the decellularized matrices following implantation. However, these results may not be relevant to humans, because cell outgrowth toward the vascular grafts from adjacent vessels is typically slow in human, and especially endothelium development on the grafts in humans is much slower than in animals.³⁷ Therefore, *in vitro* recellularization strategy through cell seeding would be required to maintain the patency and facilitate tissue regeneration during initial time frame without

host cell migration after graft implantation in clinical application. Long-term cell labeling method could be used to reveal which process of *in vitro* recellularization (by preseeded cells) and *in vivo* recellularization (by host cells) is more critical for vascular tissue regeneration by showing the *in vivo* distribution of seeded cells. Reporter gene transfection (e.g., green fluorescent protein gene transfection using viral vectors) could be considered for *in vivo* cell tracing. TEVGs should be also analyzed to confirm the presence of seeded cells at short or middle time point.

The expression pattern of MMP, TIMP, and ECM precursor genes demonstrates ongoing vascular remodeling in the implanted TEVGs. Our study has shown that the activity of MMPs was slightly higher than in the native aortas and the activity of TIMP-3 was significantly higher in the TEVGs than in the native aortas (Fig. 8). These results suggest that degradation of exogenous ECMs (i.e., implanted decellularized matrices) occurs in the TEVGs and that the TEVGs may not have aneurysmal dilatation.³⁸ The significant expression of procollagen and tropoelastin in the retrieved TEVGs (Fig. 9) suggests ongoing autologous ECM production by implanted autologous cells and ingrowing host cells in vascular walls following exogenous ECM degradation. Therefore, the ECM structures originally existing in the decellularized matrices of implanted TEVGs may be replaced by newly formed

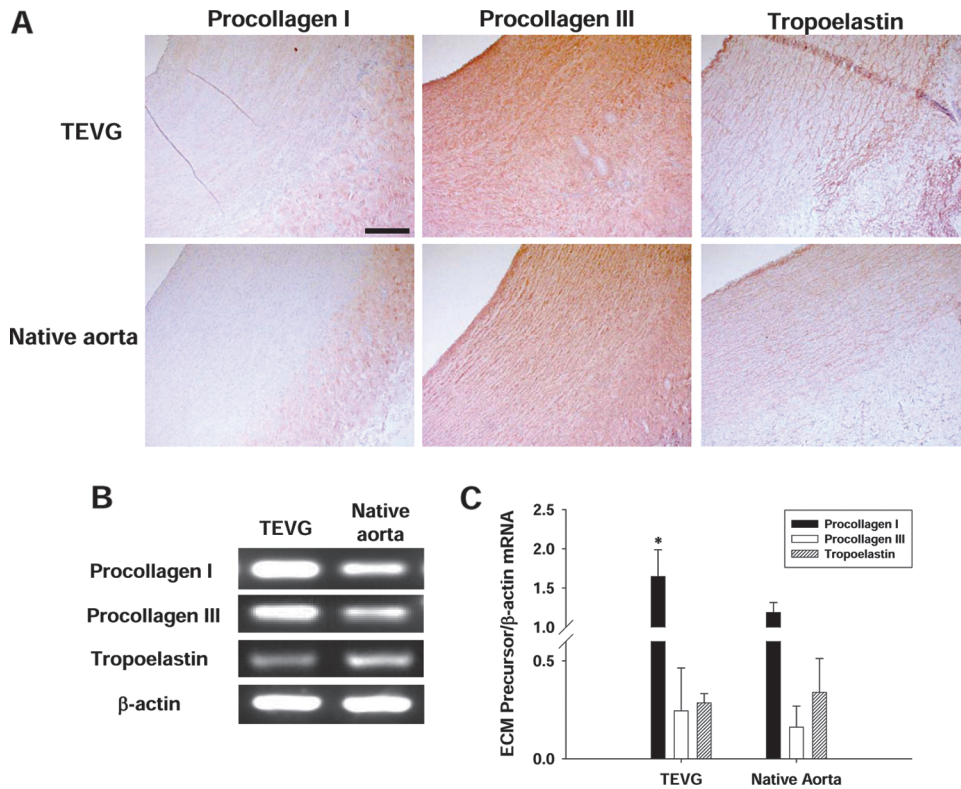


FIG. 9. ECM precursor expression in the TEVGs and native abdominal aortas at 18 weeks. **(A)** Immunohistochemical staining for ECM precursors (procollagen I, procollagen III, and tropoelastin). The image in the left top of the photomicrograph is the endothelium. The scale bar indicates 200 μ m. All the photomicrographs were taken at the same magnification. **(B)** RT-PCR using ECM precursor-specific primers. **(C)** Semi-quantitative RT-PCR analysis for expression of ECM precursor genes. The ratios of ECM precursors to β -actin mRNA expression in the TEVGs and native aortas ($*p < 0.05$ versus native aortas). Color images available online at www.liebertonline.com/ten.

autologous ECM structures, which could explain a possible mechanism for *in vivo* TEVG growth. In the present study, the accumulation of procollagen type I in adventitia, a common phenomenon of vascular remodeling after birth¹⁵ or vascular injury,³⁹ was observed in the TEVGs at 18 weeks after implantation (Fig. 9A).

TEVG diameter could increase pathologically through aneurysmal dilation besides normal matrix remodeling. In general, expression of MMPs in aneurysmal dilated vessel is significantly upregulated, compared with that in normal vessel.³⁸ The expression ratio of MMPs to TIMPs increases in vessel with aneurysmal dilation. Unlike these typical patterns of MMP/TIMP expression in aneurysm, the levels of MMP and TIMP expression in the TEVGs were not significantly different from those in native blood vessels (Fig. 8). Although the diameters were not even along whole length of the TEVGs (Fig. 3F), significant deformation of the matrices was not observed in the TEVGs at 18 weeks. Thus, the diameter increase was likely due to vascular remodeling process by seeded BMCs and migrating host cells. However, because implantation for 18 weeks may be not enough to prove any progress in dilation, additional studies with long-term implantation would be required to reveal the occurrence of aneurysmal dilation.

A recent study demonstrated the growth potential of TEVGs with vascular cells isolated from autologous vessels and biodegradable synthetic polymer scaffolds in a sheep model.⁴⁰ The diameter and length of the TEVGs were approximately 30% and 40% greater than their initial values. In contrast, only the diameters of the TEVGs in our study increased after implantation (Fig. 7C). This discrepancy may be due to the difference between degradation rates of scaffolds. Scaffolds fabricated from decellularized vessels might have a slower degradation rate compared with poly(glycolic acid)/

poly-4-hydroxybutyrate scaffolds used in the previous study. To evaluate more precisely the *in vivo* growth of grafts, digital imaging or morphometry techniques capable of measuring exactly the *in vivo* dimensions of the TEVGs should be applied in future studies. To the best of our knowledge, the present study is the first to demonstrate the growth potential of the TEVGs with autologous stem cells. Furthermore, our study describes the vascular remodeling process with regard to MMPs, TIMPs, and ECM precursors, which could explain a potential mechanism associated with *in vivo* growth of vascular grafts tissue-engineered with adult stem cells.

The present study provides preliminary evidence of *in vivo* growth and vascular remodeling of vascular grafts tissue engineered with autologous adult stem cells in a rapid-growing large animal model. The TEVGs could be applied to treat pediatric patients with congenital cardiovascular diseases. However, additional studies are needed to evaluate the clinical potential of these TEVGs. Because this study is a preliminary work with a small number ($n = 4$) of animals, the TEVGs should be further tested in a large number of animals for longer periods of time. Negative control groups such as nonseeded graft or decellularized venous graft should be contained in future studies to evaluate *in vivo* recellularization and compare with decellularized arterial graft. Vascular graft characteristics such as patency, thrombosis, calcification, vasoreactivity, and aneurysmal dilatation should also be addressed in long-term animal studies.

Acknowledgments

This study was supported by grants (A050082, 02-PJ10-PG8-EC01-0016) from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea.

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Received: March 24, 2008

Accepted: June 17, 2008

Online Publication Date: September 8, 2008