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In Vitro Constitution of Esophageal Muscle Tissue with Endocyclic and Exolongitudinal Patterns

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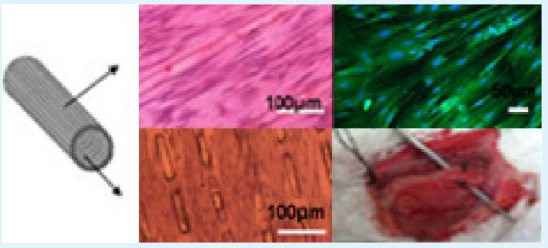
ABSTRACT: Smooth muscle tissue is the main functional structure of the esophagus and comprises of the endocircular and exolongitudinal muscle layers. To construct a tissue engineered smooth muscle by mimicking the esophageal muscle tissue, we have designed a silicon wafer where a daughter mold was prepared using soft PDMS. The daughter mold was, in turn, casted with poly(ester urethane) (PU) solution to fabricate the tissue scaffolds. The casted PU scaffolds were available in two configurations. Prototype 1 (P1) have microchannels of 100 μm width and discontinuous channel wall with gaps of 30 μm at regular intervals. Prototype 2 (P2) have microchannels of 200 μm width and continuous channel walls. The wall thickness and depth of the microchannels are 30 μm. A tubular scaffold with micropattern P1 in the lumen and micropattern P2 on the exterior was fabricated with the aim of regenerating muscle tissue with endocircular and exolongitudinal muscle architecture. After grafting with natural silk fibroin (SF), the PU micropatterned scaffold demonstrated the ability to promote smooth muscle cell (SMC) growth and differentiation; differentiation is believed to contribute to maintain the contractile function of SMCs. Results from the preliminary in vivo test revealed that the tubular scaffold patterned with microchannels is capable of supporting esophageal muscle regeneration.



KEYWORDS: *smooth muscle cells, tubular scaffold, endocircular and exolongitudinal muscle layers, esophageal tissue engineering*

1. INTRODUCTION

The esophagus is a muscular tube of ~18−26 cm, as measured from the upper esophageal sphincter to the lower sphincter. Histologically, the esophageal wall consists of four layers: luminal mucosa, submucosa, muscularis propria, and adventitia. At the upper esophagus, the muscularis propria consists predominantly of striated skeletal muscle to provide motor function. The middle section of the esophagus consists of a mix of striated and smooth muscle, while the lower esophagus consists predominantly smooth muscle. The esophageal muscularis propria consists of endocircular and exolongitudinal muscle layers found in a bilaminar arrangement. This arrangement is essential in propelling food boluses or fluid into the stomach. Sequential contraction of the circular muscle layer occludes the lumen to push the ingested bolus longitudinally along the esophagus, and sequential contraction of the longitudinal muscle to shorten the esophagus and increase luminal cross-sectional area. The contraction of longitudinal muscle layer also increases the density of the circular muscle fibers and, in turn, increases the efficiency of the circular muscle contraction.[1,2]

In our previous study, we explored the use of scaffolds with surface-immobilized proteins to tissue engineer a basement membrane to promote esophageal epithelium regeneration. These scaffolds were found to be cytocompatible for primary esophageal cells including epithelial cells, smooth muscle cells (SMCs), and fibroblasts.[3,4] This current study is designed to tissue engineer esophageal muscle tissue with the objective of combining the use of both techniques to tissue engineer a clinically functional esophagus consisting of artificial epithelium and muscle tissue in the future.

SMC is a mononucleus muscle cell with spindlelike morphology. It is the main functional cell found in endocircular and exolongitudinal bilayer muscle tissue. Previous studies have reported favorable phenotypic and biochemical responses by SMCs, which were dependent on scaffold geometry.[5] Li et al. revealed that SMC cultures in three-dimensional (3D) collagen (type I) gels exhibited decreased in proliferation rate and increased in collagen synthesis compared with SMCs cultured on two-dimensional (2D) collagen substrates, suggesting the importance of scaffold geometry on modulating SMC phenotype.[6] In another study, Shen et al. reported that SMCs cultured on scaffolds with microchannels of 100 μm width or smaller were shown to be uniformly aligned along the microchannel. The uniformly aligned SMCs cultured in the microchannels were also found to have increased expression of smooth muscle α-actin (α-SMA), suggesting that these aligned cells are shifting from synthetic phenotype to the contractile phenotype.[7]

In this work, we focus on the fabrication of a tubular scaffold with both circular and longitudinal microchannel structures using flexible polyurethane as the substrate. To evaluate its feasibility, primary esophageal SMCs were cultured on the scaffold to assess the phenotypic expression of the SMCs and

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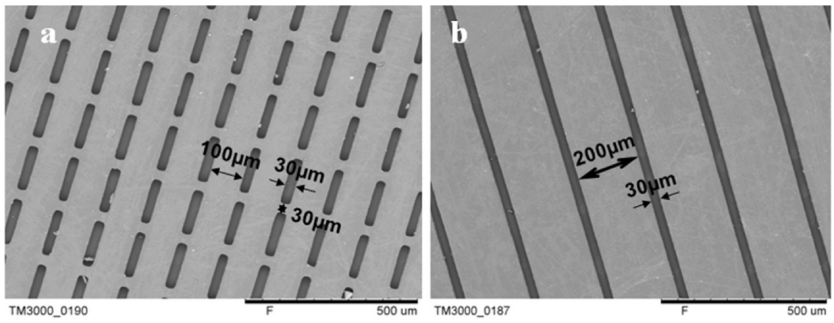

Figure 1. Scanning electron microscopy (SEM) images of PU scaffold surface with (a) P1 (microchannels of 100 μm width with discontinuous channel walls, 30 μm gap in channel walls at regular interval, wall thickness and depth of 30 μm) and (b) P2 (microchannels of 200 μm width with continuous channel walls, wall thickness and depth of 30 μm).

its effect on muscle tissue formation. Since SMCs exist ubiquitously throughout the entire body, we believe that the results presented in this study will be valuable, both experimentally and theoretically, for the tissue engineering of other tissues and/or organs.

2. EXPERIMENTAL SECTION

2.1. Materials. Poly(ester urethane) (PU) was purchased from Estane Co., China (58213 NAT 022). 1,4-Dioxane, 1,6-hexanediamine, and gelatin were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Isopropanol, glutaraldehyde (GA), paraformaldehyde, and sodium hypochlorite were purchased from Aladdin Reagent Co. (China). Penicillin and streptomycin were purchased from Beyotime Biological Technology Co. (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Bayer Biological Technology Co. (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco (Invitrogen Co., USA). Anti-α-smooth muscle actin (α-SMA), fluoresein isothiocyanate (FITC), SABC kit, DAB kit were purchased from Wuhan Boster Bioengineering Co., China. All cell culture reagents were from HyClone Co. (USA), unless otherwise specified. Water used in study was filtered through a Barnstead Nanopure system (Thermo Scientific Co., USA).

Silk fibroin (SF) was extracted from natural silkworm cocoon (Zhejiang Province, PRC), using a traditional method previously reported.[8] In brief, silkworm cocoons were boiled in an aqueous Na_2CO_3 solution (0.5 wt %) for 1 h and rinsed with water to remove sericin. The extracted silk fibers were subsequently dissolved in calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) at 80 °C to yield a homogeneous solution (5%, w/w). The dissolved silk fiber solution was dialyzed against water using a cellulose tubular membrane (MW cutoff, 12000−14000, Sigma) for 3 days at room temperature (RT); water was renewed every 4 h to completely remove residual salts. The dialyzed silk fiber solution was then freeze-dried to obtain a silk fibroin sponge.

New Zealand adult rabbits (~1 year old) used in the experiment obtained from the Experimental Animal Center of Ningbo University in compliance to the Animal Protection Act.

2.2. Fabrication and Protein Immobilization of PU Patterned Scaffold. A soft polydimethylsiloxane (PDMS) daughter mold was prepared from a silica wafer with two predetermined microchannel prototypes. Prototype 1 (P1) is configured with microchannels 100 μm wide and discontinuous channel walls with intermittent gaps of 30 μm at regular intervals. Prototype 2 (P2) has microchannels that are 200 μm wide with continuous channel walls. The wall thickness and channel depth of both prototypes are 30 μm. The scaffolds were fabricated by casting PU solution at the concentration of 0.16 g/mL in 1,4-dioxane over the PDMS mold and slowly dried at 37 °C for 24 h, followed by completely drying in a vacuum oven for 24 h to remove residual solvent. The prepared PU scaffold was then released from the mold. To confirm the presence of microstructures, the PU scaffold was observed using scanning electron microscopy (SEM) (Model S-3400N, Hitachi, Japan) (see Figures 1a and 1b).

To promote the scaffold's biocompatibility, SF was immobilized on the PU scaffold's surface, using the aminolysis and GA cross-linking method developed by our team.[9,10] In brief, the scaffold was immersed in 95% alcohol for several minutes to remove grease and surface contaminants, and then it was dried in a vacuum oven. It was then immersed in 0.06 g/mL of 1,6-hexane diamine/isopropanol solution for 10 min at 37 °C and rinsed with large quantity of water to remove free hexanediamine. This aminolyzed PU sheet was then immersed in 1 wt % GA aqueous solution for 3 h at RT, followed by rinsing with large amounts of water to remove free GA. Lastly, the sheet was incubated in SF/phosphate buffered solution (PBS) for 24 h at 2−4 °C before rinsing with water to remove any ungrafted SF to produce a SF-grafted PU scaffold (PU-SF).

2.3. Primary Esophageal Smooth Muscle Cell (SMC) Culture. Primary SMCs were obtained from the middle to lower esophagus of an adult rabbit using the tissue-explant method. Muscle tissue was obtained from esophagus by removing the mucosa layer and the adventia, and this tissue was washed thrice with PBS containing 1000 IU/mL of penicillin−streptomycin. The muscle tissue was then sterilized sequentially using 75% aqueous alcohol for 1 min, 3‰ NaClO for 3 min, followed by washing with PBS containing 1000 IU penicillin−streptomycin. The sterilized muscle tissue was then cut into cubes of ~1 mm × 1 mm × 1 mm and attached onto a culture plate. After the tissues have attached to the culture surface, DMEM containing 15% FBS and penicillin−streptomycin (100 IU) was added. After 7 days, SMCs explanted from the muscle tissue were observed under a phase contrast light microscope (Figure 2a). Immunofluorescence staining with α-SMA antibody was used to confirm the origin of the explanted cells as SMCs (Figure 2b). These primary SMCs were then collected and subcultured for the following applications.

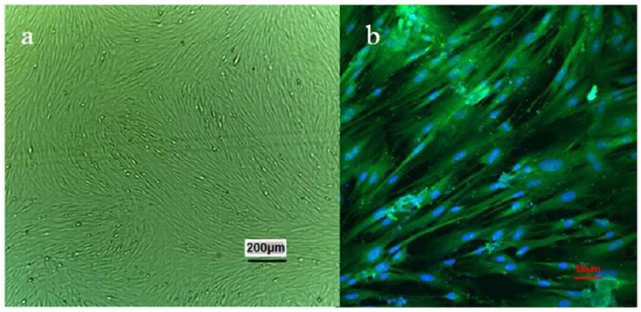

Figure 2. (a) Phase contrast micrograph of smooth muscle cells (SMCs) isolated from primary rabbit esophagus tissue (scale bar = 200 μm). (b) Immunofluorescence microscopy of SMC using α-SMA antibody (green) and cell nucleus (blue from DAPI staining) (scale bar = 50 μm). Cells were incubated at 37 °C in humidified air with 5% CO_2. (The same conditions were followed for all cell cultures.)

PU scaffolds with P1 and P2 micropatterns were punched into a disk of the size of a 96-well plate well. The scaffolds were then sterilized in 95% aqueous alcohol for 2 h, then in 75% aqueous alcohol for 4 h, before being rinsed overnight with PBS. SMC (from the third to fifth passages) were seeded on the scaffolds at the density of 5×10^4/mL.

To construct a tubular scaffold, PU scaffolds with both P1 and P2 prototypes were placed back to back and rolled up into a tube, and the edges were adhered using 1,4-dioxane solution. In this work, we fabricated a 1 cm × 2 cm (diameter × length) tubular scaffold using P1 to mimic the endocyclic pattern in the lumen and P2 to mimic the exolongitudinal pattern on the tube exterior. SMCs was first seeded on the scaffold lumen at a density of 5×10^4/mL. After 1 day, we repeated the cell seeding on the outer surface with the longitudinal microchannels. The seeded tubular scaffolds were cultured in petri dishes using DMEM containing 15% FBS and penicillin−streptomycin (100 IU). The medium was changed every 3 days.

To observe the cell morphology, the SMC-cultured tubular scaffold was rinsed three times with PBS and fixed with 4% paraformaldehyde solution for 30 min. The fixed cell-scaffold contruct was then rinsed with water and air-dried overnight in a chemical fume hood. After gold metallization with ion sputter (Model E-1010, Hitachi, Japan), the cells were observed under a SEM system (Model S-3400N, Hitachi, Japan) at an accelerating voltage of 10 kV.

The animals used in this study were treated in compliance with the requirements set by the Ethical Committee of Ningbo University and NIH's Principles of Laboratory Animal Care.

2.4. Mitochondrial Activity Assay. The mitochondrial activity of the SMCs on each scaffold was assayed using the MTT method. After MTT solution (0.5 mg/mL) was added to the cultures (in 96-well plate) and incubated at 37 °C for 4 h in darkness, 150 μL of dimethylsulfoxide (DMSO) was added to each culture well to dissolve the purple formazan crystal. The absorbance at wavelength of 490 nm was determined using an ELISA reader (MaxM5, Spectra). The absorbance of the same solvent in an unseeded well (without formazan crystal) was used as blank reference. SMCs seeded on the ungrafted PU scaffold and tissue culture polystyrene (TCPS) were used as negative and positive controls, respectively. The average of triplicates for each measured sample was used.

2.5. Hematoxylin and Eosin (HE) Staining. HE staining was conducted on cell-cultured scaffolds to observe the morphology of the SMCs. Cells were rinsed with PBS thrice before fixing in 4% paraformaldehyde for 4 h, and rinsed again with DDW to completely remove residual paraformaldehyde. Subsequently, the cultures were immersed in PBS containing 0.5% Triton-X100 for 15 min before rinsing with water. The cells were stained with hematoxylin for 30 min, followed by removal of the excess dye in 1% hydrochloric acid/alcohol solution for 30 s. Finally, the samples were rinsed with water and immersed in eosin solution for 1 min to stain the cytoplasm. Light microscopy (Model CX40, Olympus, Japan) was used to capture the HE staning images.

2.6. Immunohistochemical Staining. Immunohistochemical staining was performed on the cell-scaffold construct using anti-α-SMA as the primary antibody. A standard immunostaining protocol was used. Samples were washed with PBS for thrice for 5 min for each time and fixed in 4% paraformaldehyde for 30 min at 4 °C. After rinsing with PBS thrice, the samples were bleached in 3% H_2O_2 solution for 10 min at RT, rinsed, and blocked with 5% bovine serum albumin (BSA)/PBS for 20 min at RT. The BSA solution was then gently removed and anti-α-SMA (dilution 1:200) was added and incubated for 1 h at 37 °C. After rinsing with PBS thrice for 5 min each time, the samples was incubated in peroxidase-conjugated goat antirabbit IgG (dilution 1:1000) for 30 min followed by Strept Avidin−Biotin Complex (SABC) for 20 min at 37 °C. Finally, the samples were incubated in diaminobenzidine (DAB) solution or goat antirabbit IgG (1:1000) conjugated with FITC for 5 min at RT and rinsed four times with PBS for 5 min each time. The nuclei of immunohistostained cells were revealed by staining with hematoxylin solution for 20 min and rinsed with PBS.

2.7. In Vivo Biocompatibility Testing of Microchannelled Scaffolds. Adult rabbits (1 year old, 4−6 kg) were anesthetized with 3% pentobarbital sodium (intravenous injection, 12 mg/kg). PU-SF scaffolds with P1 and P2 micropatterns on both surfaces, shown as Figure 8e (presented later in this work), were used to evaluate its in vivo compatibility and its effect on cell alignment through contact guidance for SMC proliferation. The sheet was inserted between the muscle bilayers, with the P1 micropattern facing to endocyclic muscle and the P2 micropattern facing to exolongitudinal muscle (see Figure 9a, insert (presented later in this work)). The rabbits were kept at Animal Test Center of Ningbo University. After 60 days, the rabbits were anesthetized and the PU-SF sample was resected with a small amount of surrounding tissue (Figure 9a (presented later in this work)). The sample was fixed in 4% paraformaldehyde for 24 h at 4 °C and subsequently frozen at −80 °C. The frozen sample was sectioned at a slice thickness of 15 μm (scaffold and regenerated tissue will be separated if sliced too thinly, because of mechanical shear force) using a freezing microtome machine (Hubei Bernard Medical Technology Co., BL-800A). These sections were analyzed with HE staining, immunofluorescence staining, and SEM observation. All animals used in this study were treated in compliance to NIH Principles of Laboratory Animal Care of Ningbo University.

3. RESULTS AND DISCUSSION

3.1. Cytocompatibility of PU Scaffolds with P1 and P2 Micropatterns. As reported in previous literature, micropatterned surfaces are known to be able to control phenotypic and genotypic expression of living cells.[11] Vascular SMCs cultured on surfaces with microchannels have demonstrated parallel alignment to the channel walls, although cell proliferation was shown to be restricted in cases where the micropatterns or microchannels are too narrow.[7,11] In a previous study conducted by our group, biocompatible substrates with microchannels have been shown to help promote the alignment of esophageal cells, such as SMCs and fibroblasts, through contact guidance and induce cell differentiation. However, results from in vivo experiments showed that scaffolds with channels configured with continuous (solid) walls had significantly less periphery tissue infiltration, compared to that observed on smooth surface scaffolds. Therefore, in this work, a PDMS mold capable of producing both microchannels with continuous walls as well as discontinuous walls with intermittent gaps was developed to allow the fabrication of planar or tubular scaffolds with these predetermined microchannels (see Figures 1 and 8 (presented later in this work)). The use of micropattern P1 will reduce the compressive forces within the lumen surface, as well as the tensile force at the outer surface of the tubular scaffold.[7,11]

Poly(ester-urethane) (PU) is widely used in biomedical engineering applications, because of its favorable mechanical properties, good hemocompatibility, and biodegradability.[12,13] However, its poor wettability and intrinsically inert surface result in poor cell−material interaction. In contrast, silk fibroin (SF), which is a fibrous protein found in natural silkworm cocoon, has been used in a variety of biomedical applications. such as biomaterials for implants and scaffolding in tissue engineering, as well as for drug delivery, because of its favorable mechanical properties, remarkable biocompatibility, and controllable degradation rate.[14] Moreover, SF is also known to favor the adhesion, growth of human adult fibroblasts, as well as the performance of specific metabolic functions without eliciting proinflammatory cytokines responses.[15] Hence, grafting of SF may potentially increase the cytocompatibility of PU scaffolds for esophageal SMCs. In previous studies conducted by our group, esophageal fibroblasts and epithelial cells have

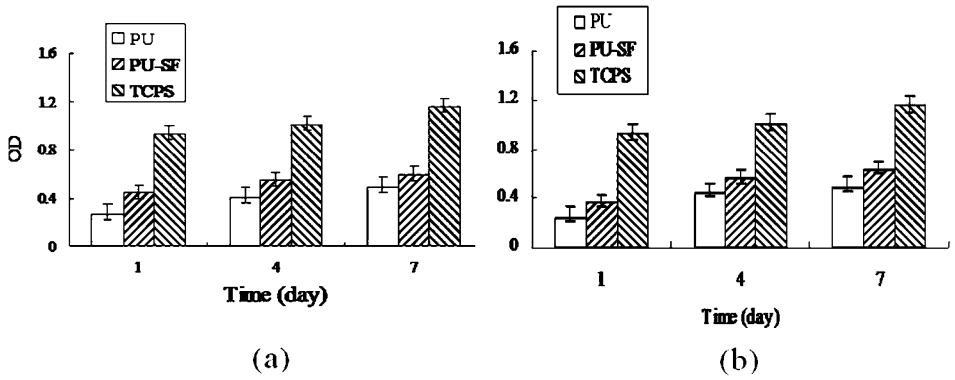

Figure 3. Results of MTT assay for SMCs seeded on micropatterned PU scaffolds: (a) P1 and (b) P2. The cell seeding density was 5×10^4/mL. TCPS was used as positive control.

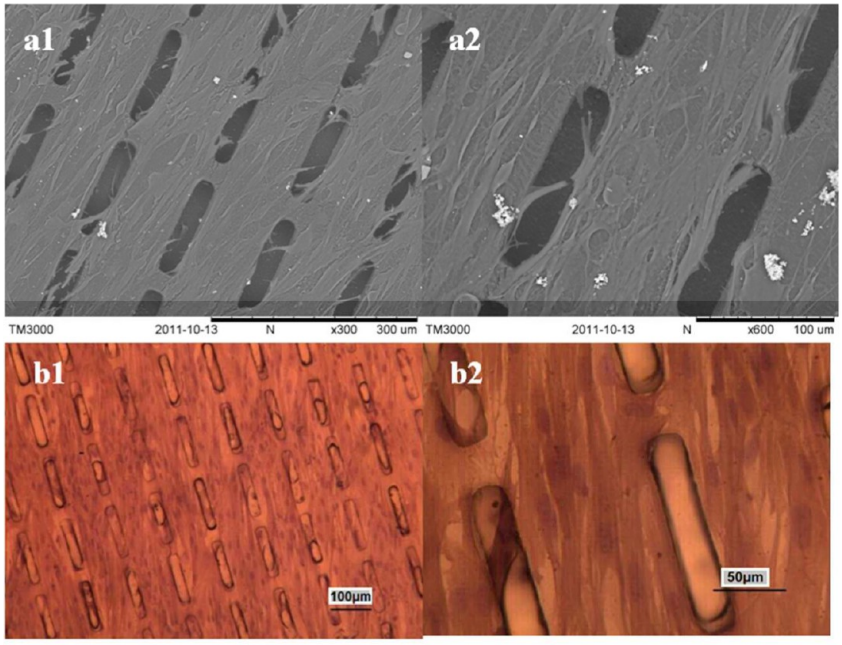

Figure 4. SMC morphology observed with (a) SEM under different magnifications, and (b) immunohistochemistry with anti-α-SMA as the primary antibody followed by peroxidase conjugated goat antirabbit IgG (1:1000) and detected with DAB. Cells were seeded on PU-SF with micropattern P1 for 7 days at the seeding density of 5×10^4/mL.

been successfully cultured on SF-grafted PU scaffolds (unpublished data). To graft SF on the surface of PU scaffolds, we used the aminolysis and GA cross-linking method previously developed by our group.[9,10] In brief, the ester-containing polymer surface (PU) was first aminolyzed with 1,6-hexanediamine, providing one amino group to react with the −COO− group to form a covalent bond, −CONH−, while the other unreacted amino group of the diamine was provided to react with one aldehyde group of the GA molecule. Finally, an amino-containing protein (SF in this case) can be grafted through reaction with the other aldehyde group from the GA molecule and the amino group from SF molecule. The density of unreacted amino groups after the completion of the aminolysis reaction was found to be $\sim 3.9 \times 10^{-7}$ mol/cm^2 as evaluated using the ninhydrin test.[9] The grafting of SF was confirmed qualitatively using Rhodamine B isothiocyanate labeling and the increase in the wettability of the PU surface (the static contact angle changed from 101.93° ± 1.59° [PU] to 76.03° ± 2.03°[PU-SF]).[16]

Before the PU scaffolds with micropatterns P1 and P2 are used to fabricate tubular scaffolds with endocyclic and exolongitudinal patterns in the lumen and exterior surfaces, respectively, they were first separately evaluated for their effects on cell attachment, cell growth, and phenotypic expression. The proliferation of SMCs over 7 days of cell culture on PU scaffolds (both SF-grafted and SF-ungrafted) with micropatterns P1 and P2 are shown in Figures 3a and 3b, respectively. PU-SF scaffolds were found to have relatively higher rates of cell attachment (day 1) and proliferation (days 4 and 7) versus that of the control PU for each micropattern investigated, but lower than that for TCPS. The difference in cell attachment rate may be due to the reduction in effective area available for cell attachment, because of the surface area occupied by the microchannel walls. The slight adsorption of PU matrix for the purple color also induced slightly lower absorbance than that on TCPS. However, between scaffolds with the same micropattern and same matrix, the increases in cell attachment and cell proliferation rates are indicative of improved cytocompatibility resulting from grafting of SF.

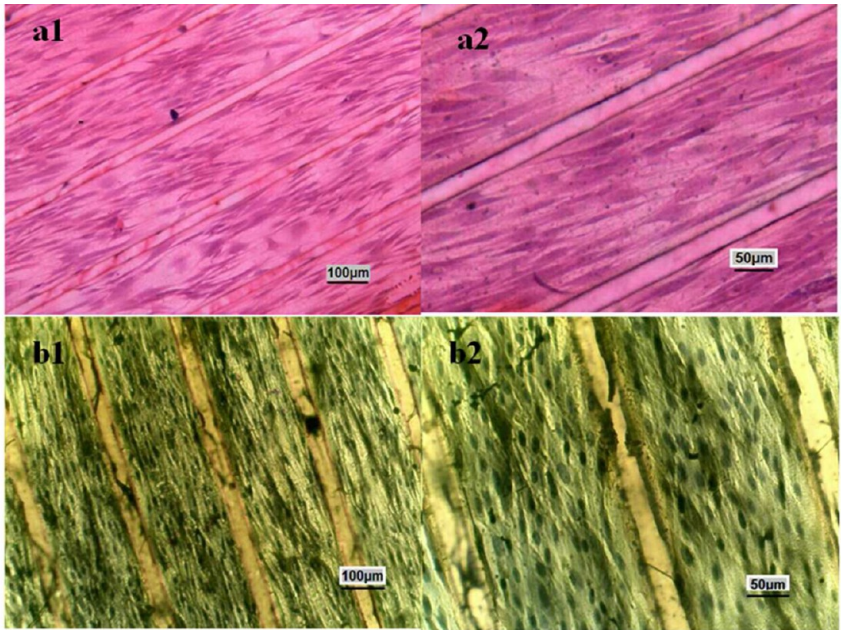

Figure 5. SMCs revealed by (a) H&E staining and (b) immunohistochemistry. Cells were seeded on PU-SF with micropattern P2 for 7 days at the seeding density of 5×10^4/mL. Anti-α-SMA was used as the primary antibody, and peroxidase conjugated goat antirabbit IgG (1:1000) as the secondary antibody. The nucleus was stained using hematoxylin solution.

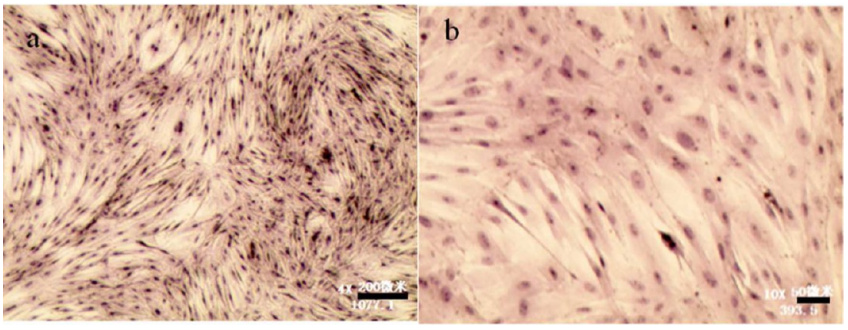

Figure 6. Micrograph of SMCs cultured on planar PU-SF scaffold (planar PU-SF) after 7 days at a seeding density of 5×10^4/mL. Pictures were taken after H&E staining.

After 7 days, SMCs cultured on both micropatterned scaffolds displayed spindlelike morphology and was predominantly aligned parallel to the channel walls, except for a few overlapping cells found at the intermittent gaps of the channel walls in scaffolds with micropattern P1 (see Figure 4a).

To investigate the phenotypic expression of the cultured SMCs, smooth muscle α-actin (α-SMA) antibody was used for immunohistochemistry analysis. α-SMA, an isoform typical of smooth muscle cells (SMC), is located at the microfilament bundles of SMCs. Immunohistochemical staining revealed that α-SMA is abundantly expressed in the cytoplasm of SMCs cultured on PU-SF scaffold, suggesting that the grafting of SF onto PU scaffolds promotes SMC differentiation into its contractile phenotype (see Figure 4b).

Similar outcomes were observed for PU-SF scaffolds on the P2 micropatterns. After 7 days, SMCs cultured on PU-SF scaffolds had achieved confluency and exhibited spindlelike morphology. In addition, the SMCs were aligned parallel to the channel walls. Figures 5a and 5b show the HE staining and α-SMA immunohistochemical staining of SMCs. In contrast, SMCs cultured on planar SF PU scaffolds (plane PU-SF) revealed random arrangement, except for minor localized alignment due to cell−cell interactions between SMCs (Figure 6, cells were stained by H&E dye in order to attain a clear figure of cells on PU-SF scaffold). Therefore, we concluded that micropatterns P1 and P2 on the PU scaffolds were able to induce cell alignment through contact guidance.

The Western blotting results of scaffolds with micropattern P2 is displayed in Figure 7. Stripes from left to right represent the α-SMA expression of SMCs on P2-patterned PU and PU-SF scaffolds, using SMCs cultured on TCPS as a reference (Figure 7a). The expression of α-SMA was found to be significantly higher in SMCs cultured on PU-SF scaffolds compared with those cultured on PU scaffolds, but lower than that for cells cultured on TCPS, as measured using an optical densitometer (Figure 7b). This result is in agreement with the findings from the MTT assay. However, the difference in α-SMA expression is much smaller between PU-SF and TCPS compared to the difference in cell proliferation results. This can be attributed to the effect of the microchannel in promoting SMC differentiation.

3.2. Tubular Scaffold with Endocircular and Exolongitudinal Architecture. After evaluating the cytocompatibility of the PU-SF scaffold with micropattern P1 or P2, a tubular

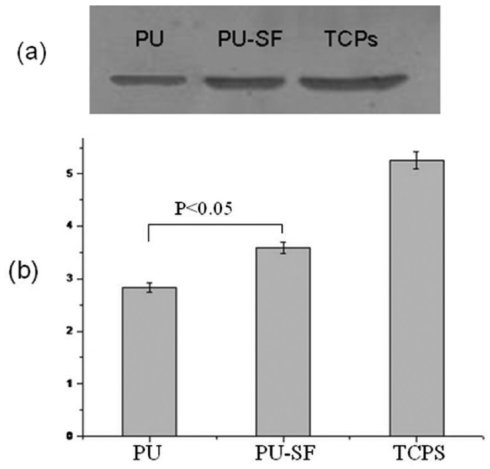

Figure 7. α-SMA expression of SMCs on scaffolds as measured by Western blotting: (a) expression of α-SMA on PU, PU-SF, and TCPS (left to right), after 7 day culture; (b) quantitative analysis of α-SMA expression using ImageJ software. The *P*-value is <0.05.

scaffold with luminal endocircular pattern and exterior exolongitudinal pattern was fabricated. The schematic diagram of the tubular scaffold is shown in Figure 8a, with an overview shown in Figure 8d. A transverse view of the tubular scaffold, as observed under SEM, is shown in Figure 8e, revealing the cross-section of the scaffold channels.

To evaluate the ability of the scaffold to support the culture of SMCs, cells were seeded on the microchannels of scaffold lumen (P1) and exterior (P2). After 7 days of culture, SMCs on both surfaces exhibited spindlelike smooth muscle morphology and were aligned parallel to the channel walls, and these appeared to be the same cell phenotype as that of SMC separately seeded on P1- and P2-micropatterened scaffolds. The alignment of the SMCs may contribute to its contractile function.

3.3. In Vivo Biocompatibility Test. To ascertain the in vivo biocompatibility of the patterned PU-SF scaffold, as well as to obtain information valuable for muscle tissue regeneration relating to this scaffold design, a scaffold without seeded cells was transplanted into the esophagus of adult rabbits. Although inflammation occurred within the first three days of the operation, the rabbits recovered from the operation wound, with fur regenerated at two weeks after the operation. After 60 days, the rabbits were sacrificed and the scaffold sample was explanted with some regenerated tissue. The explanted scaffolds were then sectioned using a freezing microtome at slice thickness of 15 μm and stained with H&E and immunofluoresence. In addition, samples were gold coated for SEM observation (Figure 9). Figure 9a revealed that the scaffold was encapsulated by the regenerated tissue after 60 days of implantation. Regenerated tissue was attached tightly to the

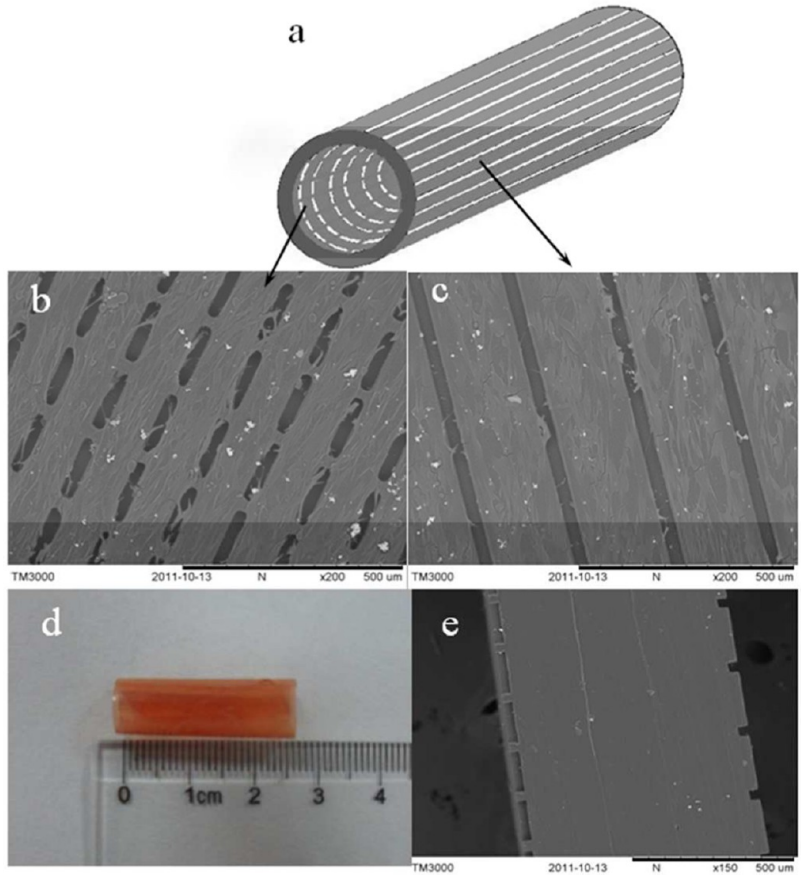

Figure 8. (a) Schematic diagram of tubular scaffold with micropatterns P1 and P2. (b) Morphology of cultured SMCs after 7 days on P1-micropatterned PU-SF in the lumen of tubular scaffold, as observed via SEM. (c) Morphology of cultured SMC after 7 days on P2-micropatterned PU-SF on the scaffold exterior, as observed via SEM. (d) Overview image of tubular scaffold captured with digital camera. (e) Transverse view of the scaffold cross-section, as observed via SEM.

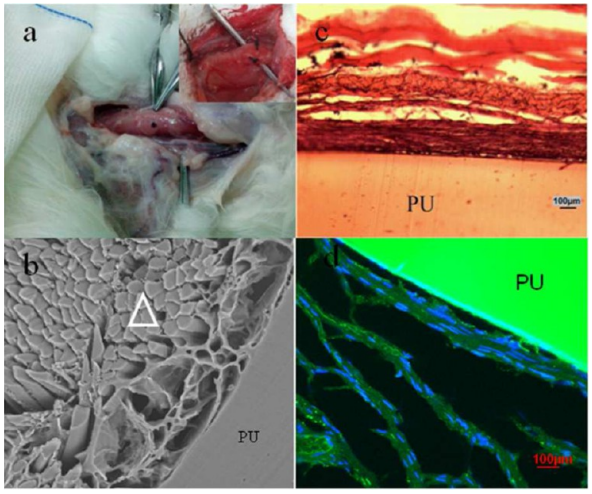

Figure 9. (a) Image of PU-SF scaffold with micropatterns P1 and P2 after 60 days of implantation (the insert shows a picture taken after operation). (b) SEM image of scaffold with regenerated tissue (the white triangle denotes the muscle fascicula along the microchannels). (c) H&E staining of the explanted scaffold after implantation for 60 days, with the channel aligned parallel to the image. (d) Immunofluorescence microscopy of regenerated smooth muscle tissue around the implanted scaffold. Green denotes α-SMA and blue denotes the cell nucleus (stained by DAPI).

scaffold surface; many SMC fascicula were packed on scaffold's channel (see the region outlined by the white triangle in Figure 9b). H&E staining revealed that many fibers were piled up along the PU-SF microchannels and were closely connecting to the scaffold surface (see Figure 9c). Immunofluorescence staining confirmed these fibers to be smooth muscle fibers (Figure 9d). However, these fibers were loosely separated from the scaffold due to the differences in mechanical properties between the regenerated tissue and PU. Thus, we deduce that fresh muscle fibril found on the scaffold was regenerated in situ from the periphery tissue and grew along the direction of the channel found on the PU-SF scaffold. These results convinced us that the combination of P1- and P2-micropatterned scaffolds are favorable candidates for the regeneration of esophageal muscle with smooth muscle phenotype. Further studies on muscle regeneration and function are necessary to advance toward the regeneration of the entire esophagus.

4. CONCLUSION

A tubular scaffold aiming to regenerate muscle tissue by mimicking the structure of normal esophageal muscle tissue was fabricated. We have designed a silica wafer, whereby scaffolds with predetermined patterns can be successfully fabricated using PU as the substrate material. P1 is a scaffold with microchannels 100 μm wide and discontinuous channel walls with regular gaps of 30 μm, while P2 is a scaffold with microchannels 200 μm wide and continuous (solid) channel walls. The thickness of the channel wall and the channel depth are both 30 μm. Silk fibroin (SF), which is a natural protein extracted from silkworm cocoon, was grafted onto the micropatterned surfaces to greatly improve the cytocompatibility of the substrate material, independent of scaffold architecture. A tubular scaffold was fabricated by PU-SF scaffolds with micropattern P1 as the lumen surface and micropattern P2 as the outer surface. The tubular scaffold was able to support esophageal SMC growth, alignment through contact guidance and differentiation. Results from the preliminary in vivo evaluation suggested that this micropatterned tubular scaffold could be a good candidate for supporting esophageal muscle regeneration.

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Notes

The authors declare no competing financial interest.

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