Proteomic Profiling of Tissue-Engineered Blood Vessel Walls Constructed by Adipose-Derived Stem Cells

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Adipose-derived stem cells (ASCs) can differentiate into smooth muscle cells and have been engineered into elastic small diameter blood vessel walls *in vitro*. However, the mechanisms involved in the development of three-dimensional (3D) vascular tissue remain poorly understood. The present study analyzed protein expression profiles of engineered blood vessel walls constructed by human ASCs using methods of two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). These results were compared to normal arterial walls. A total of 1701 ± 15 and 1265 ± 26 protein spots from normal and engineered blood vessel wall extractions were detected by 2DE, respectively. A total of 20 spots with at least 2.0-fold changes in expression were identified, and 38 differently expressed proteins were identified by 2D electrophoresis and ion trap MS. These proteins were classified into seven functional categories: cellular organization, energy, signaling pathway, enzyme, anchored protein, cell apoptosis/defense, and others. These results demonstrated that 2DE, followed by ion trap MS, could be successfully utilized to characterize the proteome of vascular tissue, including tissue-engineered vessels. The method could also be employed to achieve a better understanding of differentiated smooth muscle protein expression *in vitro*. These results provide a basis for comparative studies of protein expression in vascular smooth muscles of different origin and could provide a better understanding of the mechanisms of action needed for constructing blood vessels that exhibit properties consistent with normal blood vessels.

Introduction

WEINBERG AND BELL FIRST REPORTED in 1986 the *in vitro* construction of a complete biological blood vessel by the tissue engineering approach.¹ Since then, many attempts have been made to engineer functional arteries using various seed cells under different culture circumstances, including physical loading, hemodynamic forces, and chemical stimuli.^{2–5} These conditions for engineering vascular grafts are complicated and difficult to control. Thus, engineering of a vascular graft that acquires structural similarities to native vessels, such as the matrix component, spatial distribution of cells and matrix, and the mechanical strength, remains a major challenge. A better understanding of the molecular events within the process of neo-tissue formation is critical for blood vessel tissue engineering. To this end, selection of an ideal technique for investigating protein expression profile, post-translational modification, and their functions is prerequisite for identifying the influencing factors that correspond with development of constructed vascular grafts.

A proteomic analysis could provide such a method for quantitative and qualitative mapping of the entire proteome, as well as help to elucidate the molecular mechanisms in-

involved in biological processes.^{6,7} For instance, the proteome of human adipose-derived stem cells (ASCs) was characterized using two-dimensional gel electrophoresis (2DE) and tandem mass spectrometry (MS/MS),⁸ revealing that the identity of >200 proteins in both the undifferentiated and adipose differentiated cells. Also, the human ASC proteome shares features in common to that reported for fibroblasts, bone marrow mesenchymal stem cells, and other lineages.^{9–11} Meanwhile, a number of studies have analyzed the proteomic profiles of tissues from physiological or pathological human vascular walls.^{12,13} These findings have furthered our understanding of the processes involved in vasculature development and progression of cardiovascular diseases. However, to date, proteomic approaches have not been utilized for total protein analysis of tissue-engineered vascular walls.

In the present study, we utilized 2DE and ion trap mass spectrometry to identify profiles of protein from engineered vessel walls and normal arterial walls, respectively. A total of 38 proteins were identified to be differentially expressed in vessel walls engineered from human ASCs *in vitro*. The significance of some engineered vascular wall-specific proteome fingerprint was discussed, which would provide a basis for further study of the mechanisms responsible for the *in vitro*

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generation of engineered vessel walls, and thereby to develop improved methods for blood vessel tissue engineering.

Materials and Methods

Human adipose-derived stem cell isolation and expansion in vitro

Fresh human lipoaspirates were obtained from five healthy patients (average age of 30 years), who received abdominal liposuction in the Department of Plastic and Reconstructive Surgery of Shanghai 9th People's Hospital. All protocols involving human tissue handling were approved by the Research Ethics Committee of the Hospital. Processed lipoaspirate cell isolation and cultures were performed as previously described. Briefly, fresh lipoaspirates were extensively washed with phosphate-buffered saline (PBS), and then digested with 0.075% collagenase type I (Sigma-Aldrich) at 37°C for 1 h. After inactivation of the collagenase, the samples were centrifuged at 1200 g for 10 min to obtain a high-density stromal vascular fraction, which was further filtered through 50- μ m mesh filters, and then centrifuged again at 600 g for 10 min. The supernatant was discarded and the pellet was resuspended in a low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin (Sigma-Aldrich), and 100 mg/mL streptomycin (Sigma-Aldrich) (defined as a growth medium), followed by culture in 5% CO₂ at 37°C. The cultured cells were referred to as P0 cells. When cells were almost confluent, the cells were washed with PBS and harvested by using 0.25% trypsin/1 mM EDTA. The cells were then subcultured at 70%–80% confluency, and cells from passage 4–7 were used for further studies.

Smooth muscle differentiation from human adipose-derived stem cells

Human adipose-derived stem cells (hASCs) were induced to differentiate into smooth muscle cells as previously described.¹⁴ Briefly, hASCs were cultured in an inducing medium composed of LG-DMEM, 1% FBS, 5 ng/mL recombinant human transforming growth factor- β 1 (TGF- β 1; R&D Systems), and 2.5 ng/mL recombinant human bone morphogenetic protein 4 (BMP4; R&D Systems) (defined as differentiated hASCs). Cells cultured in the growth medium served as the negative control (defined as undifferentiated hASCs). The culture medium was replaced every 2 days for a period of up to 7 days. Human umbilical artery smooth muscle cells (hUASMCs) were isolated as previously described¹⁵ and served as the positive control.

Contractility of undifferentiated and differentiated hASCs

The contractile response of cells was analyzed according to previously described methods.¹⁶ Cells, including undifferentiated hASCs, differentiated hASCs, and hUASMCs, were treated under different culture conditions for 7 days, and then washed with PBS, followed by stimulation with the growth medium or the inducing medium containing 1 mM carbachol (C4382; Sigma-Aldrich) for 1 min. Cell contractility was observed with a Nikon microscope (Nikon Y-FL), and images from identical fields before and after carbachol treatment were collected.

Vessel structure construction in a vascular bioreactor

Nonwoven meshes of polyglycolic acid (PGA) fibers (Shanghai Ju Rui Biomaterials) with a 15- μ m diameter were used as templates for seeding differentiated hASCs. The scaffold was sterilized by ethylene oxide and subsequently trimmed into a sheet measuring 60 $\times$ 45 $\times$ 0.4 mm. The differentiated hASCs-SMCs were collected and seeded onto PGA sheets. Thereafter, cell/PGA constructs were incubated for 4 h to allow for cell adhesion to the PGA fibers. After 7 days incubation in a CO₂ incubator, the PGA sheet associated with differentiated hASCs-SMCs was wrapped onto a silicone tube in the culture chamber of the vascular bioreactor, and incubated in the LG-DMEM medium containing 10% FBS, penicillin–streptomycin, 40 μ g/mL ascorbic acid, and 5 ng/mL TGF- β 1. The cells were incubated in the pulsatile bioreactor culture for 8 weeks ($n=4$). Pulsatile flow rates and pressure were regulated to gradually increase from a mean flow rate of 70–80 mL/min and mean pressure of 0.01 MPa to eventually 100–120 mL/min and 0.02 MPa. The medium was exchanged once every 7 days.

Immunofluorescent staining

For immunofluorescent staining, differentiated hASCs (cultured in the inducing medium for 7 days) were fixed and incubated with mouse monoclonal anti- α -smooth muscle actin (SMA) (1:100, C6198; Sigma-Aldrich), rabbit monoclonal anti-calponin (1:100; Abcam), and mouse monoclonal anti-myosin (1:50, M7786; Sigma-Aldrich) primary antibodies. The cells were then washed with PBS and subsequently incubated in FITC-conjugated goat anti-rabbit secondary antibodies for calponin (1:100; Millipore), and goat anti-mouse for α -SMA, myosin (1:100; Millipore). Cell nuclei were counterstained with propidium iodide.

Scanning electron microscopic examination and hASCs labeling

The cell-PGA scaffold samples were harvested after 7 days in culture and fixed with 2.5% glutaraldehyde solution (pH 7.2) for 2 h, followed by two PBS washes. The samples were then postfixed in 1% osmic acid for 2 h at 4°C. Following fixation, the samples were dehydrated by dipping the samples for 10 min in 50%, 70%, 80%, and 90% aqueous ethanol solution, respectively, followed by three times in absolute ethanol. The dehydrated samples were mounted on aluminum supports and sputter-coated with gold, and the sections were then examined with a scanning electron microscope (SEM, Quanta 200; FEI).

The differentiated hASCs-SMCs were prelabeled before seeding with fluorescent 3,30-diiododecylcarbocyanine perchlorate (DiO) dye (Molecular Probes) at 37°C for 20 min following the manufacturer's instructions. The labeled cells were then seeded onto a PGA scaffold as described above. Cell distribution on the scaffold was then observed using a laser-scanning confocal microscope (CLSM; Leica Microsystems) at 7 days after seeding.

Histological and immunohistochemical examinations

Following culture in the bioreactor for 8 weeks, the engineered vessel walls ($n=4$) were harvested and fixed in 10% formalin, embedded in paraffin, and sectioned. Using standard

histological techniques, the specimens were stained with hematoxylin and eosin (H&E), Masson trichrome, and Gomori staining, respectively. For immunohistochemical staining, frozen cross sections of samples were treated with 3% H₂O₂ for 15 min at room temperature to block endogenous peroxidase. The sections were then blocked in 0.1% trypsin and 1:10 normal goat serum (Sigma-Aldrich) for 30 min at 37°C, respectively, followed by incubation with mouse monoclonal anti- α -SMA (1:100, C6198; Sigma-Aldrich) or mouse monoclonal anti-myosin (1:50, M7786; Sigma-Aldrich) primary antibodies for 1 h at 37°C. After washing with PBS, the sections were incubated with goat anti-mouse IgG horseradish peroxidase (Santa Cruz) for 30 min, and subsequently color-developed with Liquid DAB+Substrate Chromogen System (Dako K3467; Dako) before counterstaining with hematoxylin.

Biomechanical properties

Mechanical properties of tissue-engineered blood vessels were examined using a tensile tester (Instron-4456) according to a previous report.¹⁷ Briefly, tissue-engineered vessels, as well as the native human saphenous vein (HSV) ($n=4$), were sectioned in a circumferential direction and the ring was opened. The circumferential tissue strips (3 mm in width and 8 mm in length) were mounted onto a customized holder in a relaxed state and tested in a humid condition at a tissue-extension rate of 10 mm/min. Ultimate strength and elastic modulus were analyzed according to parameters of specimen strain–stress behavior. For suture retention strength testing, the samples were measured using 5-0 polypropylene sutures (Ethilon; Ethicon) that were placed in four quadrants of the vessel wall ($n=4$) 1 mm from the vessel edge. Constant elongation (3 mm/min) was applied along the longitudinal axis of the vessel until complete rupture. For burst pressure testing, the vessel ($n=4$) was pressurized with PBS, and hydrostatic pressure was increased by 0.02 MPa/s until vessel failure. Burst pressure was recorded in MPa.¹⁸ For tissue collagen quantification, the samples were digested in a pepsin solution (pepsin dissolved in 0.5 M acetic acid, 10 mg/mL) at a 1:10 ratio (pepsin:tissue, wet weight) with vigorous stirring at room temperature overnight. The samples were then treated with a Sircol Soluble Collagen Assay kit (Biocolor) according to the manufacturer's instructions and the fluorescence was measured by a spectrophotometer at 540 nm. The collagen content of the samples was interpolated from the standard curve and expressed in microgram units per gram of wet tissue weight ($\mu\text{g/g}$ wet weight). Normal HSV (4-mm diameter) served as the control.

Human radial artery samples

The radial artery ($n=3$) was harvested from patients undergoing extremity amputation due to trauma, and the arteries were extensively washed in cold saline to remove blood. The arteries were mixed with M199 media (Life Technologies) containing penicillin (50 U/mL) and streptomycin (50 $\mu\text{g/mL}$) (Sigma-Aldrich). This study was approved by the Research Ethics Committee at Shanghai 9th People's Hospital.

Sample preparation for 2DE

Connective tissue was removed from radial artery sections (about 2 cm), and the lumen was longitudinally opened. The

endothelium was then removed by scraping the open lumen with a scalpel and washing with PBS. The media layer was peeled from the adventitia using forceps, and the dissected normal arterial tissue and explanted constructed vessel wall ($n=3$) were immediately stored at -70°C before preparation for 2DE. Protein extraction was performed according to previously described methods.¹⁹ Briefly, frozen tissue samples were pulverized in liquid N₂ into a fine powder using a pestle and mortar. The resulting powder was collected and dissolved in the lysis buffer containing 8 M urea, 2 M thio-urea, 4% CHAPS, 40 mM Tris, 65 mM dithiothreitol (DTT), and protease inhibitor cocktails. The sample then was sonicated on ice for 5 min and centrifuged at 12,000 g and 4°C for 1 h. The supernatant was then transferred and stored. Protein concentrations were determined using the Bradford assay.

2D gel electrophoresis

A total of 150 μg protein was loaded onto the gels. First-dimension isoelectric focusing (IEF) was performed on non-linear gradient IPG-strips (18 cm, pH 3–10) at 30 V for 12 h, 100 V for 1 h, 1000 V for 1 h, and then 8000 V for 4 h with a limiting current of 50 μA per strip. Once IEF was complete, the strips were equilibrated in the equilibration buffer I (50 mM Tris-HCl, pH 6.8, 15 mM DTT, 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue) for 15 min, followed by the equilibration buffer II (same as buffer I, but DTT was replaced by 2.5% iodoacetamide) for 15 min. Subsequently, second-dimension electrophoresis was performed on 12.5% SDS-PAGE gels at 30 mA until the bromophenol blue front migrated off the lower end of the gels.

Protein staining and gel analysis

Proteins were separated by analytic 2DE and were visualized following silver diamine staining as previously described.²⁰ Data analysis was performed as previously described.²¹ Briefly, silver-stained 2DE gels were scanned using a GS710 imaging densitometer (Bio-Rad). Image Master software (GE Health Care) was used for spot detection, quantification, and matching. Spot intensities from tissue-engineered vessel walls and normal arterial walls were compared on each gel. To account for experimental variation, three gels for each group were analyzed (constructed vessel walls or normal arterial walls), and only spots with >2.0-fold expressional changes were selected.

Sample preparation for MS analysis

Spots that differed in intensity between tissue-engineered vessel walls and normal arterial walls, were excised from the preparative gel, destained in 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1 v/v), washed in Milli-Q water, and dehydrated by lyophilization. The dried samples were then digested in 12.5 ng/mL trypsin in 0.1 M NH₄HCO₃ at 37°C overnight to generate sufficient peptides.

Mass spectrometry analyses of protein gel bands

The protein gel bands were analyzed using an Agilent 1200 Series nano-LC system coupled through an orthogonal nanospray ion source Chip Cube to an Agilent 1200 Series LC/MSD Trap XCT Ultra ion trap mass spectrometer, as previously described.²² Briefly, sample enrichment, desalting,

and peptide separation by RP chromatography (75 $\mu\text{m} \times 150$ mm) were completed within the Chip Cube. The peptides were eluted at a flow rate of 300 nL/min in a gradient beginning with 5% solvent B (0.1% formic acid in acetonitrile) and 95% solvent A (0.1% formic acid in water), followed by a gradient from 5%–15% B over 2 min, 15%–30% B over 13 min, 30%–90% B over 10 min, 90%–90% for 2 min, and down to 5% B over 3 min. The LC/MSD Trap XCT Ultra was operated in the standard-enhanced scan mode. The ionization mode was positive. Drying gas flowed at 4 L/min at a temperature of 325°C. The capillary was set to 1800 V with the skimmer at 40 V, and the capillary exit was set to 158.5 V. The trap drive was set to 82.9 V. The ion charge control was at a maximum accumulation time of 200 ms, the smart target was 500,000, and the MS scan range was 400–1800, with an average of 2. Automatic MS/MS was performed in an ultrascan mode, with the number of precursor ions set at three, fragmentation amplitude at 1.00 V, active exclusion on (after two spectra for 0.5 min), exclusion of singly charged ions on, a MS/MS scan range of 100–2200, and ultrascan on.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation, and SPSS 10.0 software was used for statistical analysis. A paired *t*-test (Student's *t*-test) was performed, and a *p*-value < 0.05 was considered statistically significant.

Results

Smooth muscle differentiation of hASCs

Optimal smooth muscle differentiation was observed when hASCs were cultured in the inducing medium (containing 5 ng/mL TGF- β 1, 2.5 ng/mL BMP4, and 1% FBS). After 1 week, hASCs cultured in the growth medium exhibited a flat morphology (Fig. 1A-a). Cells cultured in the presence of the inducing medium acquired a spindle-like morphology and grew in a "hill and valley" pattern (Fig. 1A-b) similar to primary isolated hUASMCs (Fig. 1A-c). To determine if TGF- β 1 and BMP4 induced hASC differentiation to SMCs, the effect of TGF- β 1 and BMP4 on SMC-specific marker expression was analyzed by immunofluorescence staining. Figure 1B shows that TGF- β 1 and BMP4 stimulation increased expression of α -SMA, calponin, and SM-MHC, which was significantly higher than that in cells cultured in the growth medium (undifferentiated hASCs). These results suggested that α -SMA, calponin, and SM-MHC expression was dramatically upregulated (at a level similar to hUASMCs) during hASC differentiation to smooth muscle-like cells. Furthermore, to determine whether induced hASCs acquired contractile function, undifferentiated and differentiated hASCs were subjected, respectively, to 1 mM carbachol stimulation for 1 min. As shown in Figure 1C, undifferentiated hASCs did not exhibit changes in the cell shape. However, a large proportion of hASCs induced with TGF- β 1 and BMP4 (differentiated hASCs) responded to carbachol treatment by exhibiting a shortened or rounded morphology that is similar to hUASMCs.

Elastic blood vessels generated in the pulsatile bioreactor culture

Differentiated hASCs were collected and seeded onto the prepared PGA mesh (Fig. 2A). At 7 days after culture, a

smooth muscle-like sheet was observed to be formed. SEM and microscopic observation showed that a considerable number of cells adhered to and spread along PGA fibers with secreted extracellular matrix (ECM) filling the space between the fibers (Fig. 2B–D). Within 8 weeks culture in the bioreactor, the engineered vessels received a gradually increased stimulation of pulsatile pressure (ID = 4 mm) and acquired a distinctly similar appearance to native vessels, showing as the homogeneous tubular tissue with significant elasticity. In addition, when a compression force was applied to the engineered wall and allowed to be released, the vessel wall repeatedly rebound to its original luminal shape (Fig. 2E, F).

Engineered blood vessels acquired similar morphology and strength to native vessels

After 8 weeks in the bioreactor culture, H&E staining revealed that the engineered vessel wall consisted of a dense muscle-like structure in the middle and outer layer. While, in the inner region, only relatively loose muscle fibers could be observed (Fig. 3A-a). Spindle-shaped cells were found to distribute randomly within the neo-generated tissue. PGA fibers could no longer be detected (Fig. 3A-b). Furthermore, Masson Trichrome staining revealed that the collagen matrix deposited in a relatively organized way in the outer and middle region of the engineered wall. Whereas, no elastin was detected by Gomori staining (Fig. 3A-c, d, e, f). It was shown by immunohistochemical staining that, the engineered vessel walls were well populated with α -SMA and myosin staining positive cells, respectively. However, distribution of these cells was in a disorganized manner as compared with that in native vessels (Fig. 3B).

For biomechanical test, the engineered tissue samples were obtained in a circumferential direction and the parameter was compared with that of the HSV. As shown in Table 1, ultimate strength, ultimate strain, and elastic modulus were determined from the stress–strain curves of engineered vessels and HSV, respectively. These results demonstrated that the biomechanical properties of the engineered vessels reached 55%–65% of that of native HSV. In addition, collagen deposition in the engineered vessel walls reached 60% of that of freshly collected HSV as determined by a quantitative test.

Comparative analysis of proteins by 2DE

Proteins in the supernatants of extraction from engineered and normal arterial were separated, respectively, via IEF as the first dimension and SDS-PAGE as the second dimension, followed by fluorescence staining. Scanned gels were analyzed using Image Master 2D Platinum 5.0 software. Gel spots were numbered and automatically matched, and the intensity of each gel spot was determined. To ensure reproducibility of samples in the 2D gels, the experiments were performed in triplicate, leading to highly reproducible results. Figure 4 shows analyses by 2D gels (pH 3–10) of protein profiles from two different samples. A total of 1701 ± 15 and 1265 ± 26 protein spots were detected in gels from normal arterial walls and engineered walls samples, respectively. These differentially expressed proteins were considered for protein identification by PMF. As previously described, proteins with an altered abundance ratio > 2.0-fold were chosen.¹² For 2DE analysis, a total of 20 spots, expression of which showed a 2.0-fold

FIG. 1. Human adipose-derived stem cells (hASCs) characterization and functional evaluation were performed at 7 days *in vitro*. **(A)** Phase-contrast images of undifferentiated hASCs as a negative control **(a)**, differentiated hASCs induced by DMEM supplemented with 5 ng/mL transforming growth factor- β 1 and 2.5 ng/mL bone morphogenetic protein 4 for 7 days **(b)**; primary human umbilical artery smooth muscle cells (hUASMCs) as a positive control **(c)**. The differentiated hASCs exhibit a typical “hills and valleys” growth pattern similar to hUASMCs. Scale bars: 100 μ m. **(B)** Cells were subjected to immunofluorescent staining with antibodies specific to α -smooth muscle actin (SMA), calponin, and myosin (green), as well as a nuclear counterstaining with propidium iodide (PI) (red), respectively. Representative example from more than 5 experiments. Scale bars: 50 μ m. **(C)** Cargill-induced contraction of undifferentiated hASCs and differentiated hASCs. hUASMCs served as the positive control. Images were collected before **(a–c)** and after **(d–f)** 1 mM Cargill addition for 1 min. Arrowheads show representative contracted cells in the differentiated hASC group and the hUASMC group. Scale bars: 50 μ m. Color images available online at www.liebertpub.com/tea

change at minimum, were selected for protein identification. Of these analyzed spots, 38 proteins were found to be downregulated in engineered vessel walls relative to normal arteries by MS analysis and database searching. Protein expression patterns are shown in Table 2, in which a list of identified proteins with their respective spot ID, protein name, number of matched peptides, percentage of sequence cover-

age, MS/MS search score, spectra, and functional classification are included.

Functional classification of regulated proteins

Classification of the 38 identified differential proteins was based on annotations from the UniProt Knowledgebase

FIG. 2. Polyglycolic acid (PGA) smooth muscle-like cell constructs *in vitro*. **(A)** Gross and morphological view of PGA scaffold. **(B)** Scanning electron micrographs of PGA seeded with cells and cultured for 7 days. The inset image shows that cells fill the void between fibers with extracellular matrix (ECM) production at day 7. Arrow indicates secreted ECM. **(C)** Fluorescent images of DiO-labeled differentiated hASCs on PGA at 7 days postseeding. Scale bars: 200 μ m. **(D)** At 7 days, PGA/cell scaffolds were observed by laser-scanning confocal microscope. The inset image shows a cross section of the same PGA/cell construct by confocal microscopy. Scale bars: 50 μ m (inset: 100 μ m). **(E, F)** The process of cell-PGA scaffolds in the bioreactor. With degradation of PGA fibers, a tubular smooth muscle-like tissue gradually forms, and eventually an elastic vessel wall is formed after 8 weeks of dynamic engineering (scale bars: 1 mm). Representatives from four independent experiments are shown. Color images available online at www.liebertpub.com/tea

(www.ebi.uniprot.org/index.shtml; Fig. 5 and Table 2). Among these, the largest group that comprises 19 proteins (50%) belongs to cytoskeleton and cytoskeleton-related proteins, such as HSP, actins, transgelin (SM-22 α), and alpha-centractin. Analysis also revealed that a group of proteins involved in glycolytic metabolism was expressed differentially, such as alpha-enolase, beta-enolase, gamma-enolase, and pyruvate kinase isozymes M1/M2.

In addition, differentially expressed proteins also refer to that a panel of signal transduction molecules, such as phosphatidylethanolamine-binding protein 1, that a group of anchored proteins, such as Filamin-A, and that proteins related to oxidative stress and protein binding, such as alpha-crystallin B chain, protein DJ-1, and selenium-binding protein 1, which play an important role in protecting cells against oxidative stress and cell death. Proteins associated with complement system activation, such as complement C3 precursor, which has been reported to be able to induce smooth muscle contraction and increase vascular permeability, were also identified.

Some proteins with unknown functions were recognized, such as serotransferrin precursor, Ig kappa chain C region, S-formylglutathione hydrolase, and Ig lambda chain C regions.

Discussion

With increased morbidity of cardiovascular diseases in modern society, tissue-engineered small blood vessels could

provide great potential for treating such vascular diseases in future. To date, this approach has been successfully demonstrated in constructing engineered vascular grafts for blood vessel replacement in animal models.^{2,4,17,23} In the present study, an elastic small diameter vessel wall (4 mm in diameter) was generated with SMC-differentiated hASCs under the stimulation of physiological pulsatile pressure in a bioreactor system. However, results showed that mechanical properties of the vessel walls constructed under the dynamic culture reached only 55%–65% of that of native vessel (human HSV) (Table 1). In addition, elastic fibers were not observed in tissue-engineered vessel walls. Therefore, a better understanding of the molecular mechanisms involved in regulating SMC differentiation, orientation, and ECM synthesis, is critical for facilitating the development of blood vessel tissue engineering. Proteomic profiling analysis provides an efficient and systematic method to identify important protein candidates that may be involved in SMC differentiation and 3D vessel tissue development *in vitro*. Using proteomic approaches based on 2D electrophoresis and ion trap mass spectrometry, 38 differentially expressed proteins were identified between engineered vessels and normal arterials.

In the present study, the largest group identified was cytoskeletal and cytoskeleton-related protein, such as actin (α -SMA), myosin, and actin-related proteins, including actin, lamin, centractin, and heat shock protein beta-1 (HSP β -1). These results suggested that an altered cytoskeleton

FIG. 3. Histological analyses and immunohistochemical staining of engineered blood vessel walls cultured under pulsatile stimulation ($n=4$) for 8 weeks. **(A)** Histological characterization of engineered blood vessel walls (**a, c, e**). Human umbilical arteries (normal) (**b, d, and f**) served as positive controls. Section were stained with hematoxylin and eosin (**a, b**), Masson trichrome staining (**c, d**), and Gomori staining (**e, f**). Arrow indicates elastic fibers. Scale bars: 100 μm . **(B)** Immunohistochemical staining of engineered blood vessel walls (**a, c**) and human normal umbilical arteries (**b, d**) for α -SMA (**a, b**) and myosin (**c, d**). Arrows show representative-positive cells that express α -SMA and myosin. Scale bars: 50 μm . Color images available online at www.liebertpub.com/tea

integrity in induced ASCs may represent one of the major influential factors for blood vessel engineering. In response to contractile and mechanical stimulation, actin filaments polymerized and assembled, which subsequently participated in contractile force development in the arterial smooth muscle.^{24–27} Moreover, actin assembly increased the number of contractile units and length of actin filaments. In addition, newly polymerized filaments involved in the reorganization processes, which allowed for the rapid adjustment of stiffness and tension in tissues containing smooth muscle cells.^{28–31} As an actin-related protein, β -contractin was found to cap the growing filament and play an important role in the formation and stabilization of the immunological synapse.³² Constitutive expression of HSP beta-1 (also called HSP27) in muscle is a notable difference between muscle and non-muscle cells. Phosphorylation of HSP beta-1 is necessary for

formation of F-actin after growth factor stimulation of non-muscle cells.³³ McGregor *et al.* found that HSP27 induction might be part of the adaptation of the actin cytoskeleton of venous smooth muscle cells transplanted into an arterial bed during coronary bypass surgery.¹³ Taken together, these studies indicated that differentiation of ASCs to SMCs and their participant in formation of vessel walls was concurrently associated with development and reorganization of the actin cytoskeleton under dynamic biomechanical stimulations. In the present study, one concerned issue is that induced ASCs within engineered vessel walls exhibited a less organized distribution compared with that in the native walls, which presents a densely orientated structure with well-populated SMCs. Thus, it is of importance to further explore whether this less organized structure of engineered vessel is a result of decreased expression of cytoskeleton and

TABLE 1. SUMMARY OF RESULTS OF ULTIMATE TENSILE TESTING PERFORMED ON ENGINEERED BLOOD VESSEL WALLS COMPARED TO HUMAN SAPHENOUS VEINS

Vessel type	Collagen ($\mu\text{g/g}$ wet weight)	Suture retention (N)	Ultimate strength (MPa)	Ultimate strain	Elastic modulus (MPa)	Burst pressure (MPa)	Wall thickness (mm)
Pulsatile	48.35 (± 6.65)	0.93 (± 0.04)	0.63 (± 0.09)	0.75 (± 0.15)	10.89 (± 1.23)	0.12 (± 0.01)	0.67 (± 0.06)
HSV	78.15 (± 5.57)	1.62 (± 0.07)	0.98 (± 0.08)	0.52 (± 0.11)	14.78 (± 1.87)	0.22 (± 0.02)	0.46 (± 0.04)

Tissue-engineered vessel walls ($n=4$), as well as native HSV, were measured in a circumferential direction. HSV samples served as controls. HSV, human saphenous vein.

FIG. 4. Typical protein profiles of two-dimensional (2D) PAGE for tissue-engineered blood vessel walls constructed by hASCs and normal human arterial walls using pH 3–10 IPG strips. Three 2D electrophoresis gels after fluorescence staining are shown here as representatives. **(A)** Engineered blood vessel walls; **(B)** normal arterial walls served as positive control. Identified proteins are listed in Table 2. Horizontal axes designate the pH value, and vertical axes designate molecular mass.

thereafter, whether to modulate expression of cytoskeleton proteins by adjusting stimulation parameters would facilitate generating well-organized vessel walls *in vitro*.

In addition to their role in regulation of cytoskeleton structure, HSPs have also been shown recently that, as molecular chaperon with many other proteins, they are important modulators in controlling cellular apoptosis. In a previous study, Salinthon *et al.* demonstrated that overexpression of HSP27 confers airway SMCs resistance to hydrogen peroxide cytotoxicity.³⁴ Moreover, as a member of the small HSP family, α B-crystallin has been reported to facilitate refolding of non-native proteins.³⁵ By suppression of caspase3 activation, α B-crystallin was found to function as a novel negative regulator of myogenic apoptosis that occurred at early stage of myogenic differentiation.³⁶ In line with these notions, HSPs expression in engineered walls indicates that ASCs stimulated with pulsatile pressure in a bioreactor acquired, in some degree, self-protective functions against apoptosis.

As an essential component in vessel wall responsible for maintenance of blood pressure, SMCs cyclically undergo contraction and relaxation in response to mechanical strains as a result of pulsatile pressure. During this process, sufficient energy generated from ATP synthesis via activation of glycolytic pathway is vital for SMCs performing their contraction functions. The present results showed that some proteins linked to glycolysis and pyruvate metabolism, such as enolase (α , γ , and β -enolase), and pyruvate kinase isozymes M1/M2, are less expressed in engineered vessel walls as compared with that in native ones. Pyruvate kinase is a rate-controlling enzyme of the glycolytic cascade, which catalyses the formation of pyruvate and ATP from phosphoenol pyruvate and ADP.³⁷ In addition, enolase, which is composed of three distinct subunits (α , β , and γ), is an abundant glycolytic enzyme present in vascular tissues.³⁸ Based on these studies, we speculate that upregulation of these proteins would accelerate glucose metabolism and the energy production, resulting in an increased number of metabolic active SMCs. Consequently, further work should be done to investigate whether increased expression of

enolase and pyruvate kinase isozymes M1/M2 could serve as reliable biomarkers indicating an active metabolic status of engineered vascular tissues constructed in the bioreactor.

Furthermore, we have identified some other interesting proteins, such as peroxiredoxin-2, protein DJ-1, prolargin precursor, which have functions involved in cell apoptosis/defense, protein modification, and destination of molecular anchoring. Peroxiredoxins family proteins are antioxidant enzymes within cells, and are essential for cell survival in injured brain and other organs by modulating cellular redox status and transcription factor activity.³⁹ Protein DJ-1, also known as PARK 7 for its gene name, is a multifunctional oxidative stress response protein, which protects cells against reactive oxygen species and mitochondrial damage.⁴⁰ During oxidative stress, DJ-1 has been demonstrated to modulate the expression of genes, such as glutamate cysteine ligase, extracellular superoxide dismutase (SOD3), MnSOD, and to exert cyto-protection against Bax-induced, caspase-dependent cell death.^{41,42} Thus, these results, including ours, indicate that *in vitro* generated smooth muscle tissues acquired self-protective functions against oxidative stress. Meanwhile, as a molecular anchor to basement membranes in connective tissue, prolargin has been documented to be distributed in intima of arteries, and play a role in regulating smooth muscle cells adhering to endothelial cells (or basement membranes).⁴³ Identification of prolargin is, therefore, of significance in that engineered vessels obtained an important molecule for endothelial cell-SMCs or endothelial cell-ECM cross talks, which is a prerequisite in structure for normal vessel functions.

It is noteworthy that, among the 20 differentially expressed spots of normal arterial walls, some proteins, including serotransferrin precursor, Ig kappa chain C region, S-formylglutathione hydrolase, and Ig lambda chain C regions, have not been previously detected in the proteome of vascular tissue. Identification of these proteins and their functions in vascular tissue could help to broaden our knowledge of vascular physiology in future.

The present study still has some limitations. First, differentiated hASCs acquiring contractile phenotype would undergo dedifferentiation to a synthetic phenotype in such a long duration of culture *in vitro*. Thus, some markers identified in this study may be a result of dedifferentiation. In future studies, more specific functional identification need to be confirmed by cytochemistry or Western blot and, be explored with detection of concomitant changes in their mRNA expression level. Second, according to previous studies, several of the detected proteins, including HSPs, enolase, are not vascular smooth muscle cell specific and are present in other cell types, such as erythroblasts cells, myocardial cells, and skeletal muscle cells.^{44,45} It remains to be determined whether grouping two or more of these detected novel markers could synergistically increase the sensitivity for identification. This is due to that some functional classes of protein identified in the present study were coregulated during SMC differentiation. Therefore, Gene Ontology terms mapping may help to understand cross talks between these different classes of protein coregulated.⁴⁶ Finally, the proteome of engineered vessel walls constructed under static culture was required to make comparisons with dynamic cultures and normal vessel walls. With help of these future studies, we could find specific proteins as potential biomarkers for predicting full differentiation of ASCs into contractile SMCs and,

TABLE 2. DIFFERENTIAL PROTEIN IDENTIFICATION FROM CONSTRUCTED BLOOD VESSEL WALLS AND NORMAL ARTERIAL WALLS

<i>Uniprot</i>	<i>Dot NO.</i>	<i>Protein name</i>	<i>Distinct peptides</i>	<i>Distinct summed MS/MS search score</i>	<i>% AA coverage</i>	<i>Spectra</i>	<i>Functional classification</i>
P04792	1	Heat shock protein beta-1	2	26.12	13	2	Actin organization
Q01995	3	Transgelin	2	32.27	9	2	Actin-binding protein
P02511	5	Alpha-crystallin B chain	2	37.67	13	2	Cell defense/ stress
P30086		Phosphatidylethanolamine-binding protein 1	2	36.73	18	2	Signal transduction
P30086	6	Phosphatidylethanolamine-binding protein 1	2	28.17	17	2	Signal transduction
Q99497	7	Protein DJ-1	3	39.51	30	3	Oxidative stress response protein
P04792	8	Heat shock protein beta-1	2	30.95	23	2	Actin organization
P61163	16	Alpha-centractin	6	96.35	24	7	Actin-related protein
P42025		Beta-centractin	3	47.66	9	3	Actin-related protein
P06733	18	Alpha-enolase	9	136.82	28	12	Glycometabolism (energy production)
P09104		Gamma-enolase	3	43.2	10	4	Glycometabolism (energy production)
P13929		Beta-enolase	3	43.2	10	4	Glycometabolism (energy production)
P01024	22	Complement C3 precursor	7	110.86	7	7	Complement system
Q5TCI9		Lamin-A/C	2	26.79	3	2	Intermediate filament family
Q5TCI9	23	Lamin-A/C	5	89.18	13	6	Intermediate filament family
D3JIA6		Serotransferrin precursor	4	68.14	10	6	Other
P21333	24	Filamin-A	26	406	14	39	Anchor protein
P32119	28	Peroxiredoxin-2	2	27.8	8	2	Peroxidase (anti-oxidant enzyme)
P68133		Actin, alpha skeletal muscle	2	28.23	6	2	Cytoskeleton proteins
P68032		Actin, alpha cardiac muscle 1	2	28.23	6	2	Cytoskeleton proteins
P62736	29	Actin, aortic smooth muscle	2	28.23	6	2	Cytoskeleton proteins
P63267		Actin, gamma-enteric smooth muscle	2	28.23	6	2	Cytoskeleton proteins
P01834		Ig kappa chain C region	2	25.95	34	2	Other
P01834	30	Ig kappa chain C region	2	24.02	34	2	Other
P68133		Actin, alpha skeletal muscle	3	42.48	10	3	Cytoskeleton proteins
P68032		Actin, alpha cardiac muscle 1	3	42.48	10	3	Cytoskeleton proteins
P62736		Actin, aortic smooth muscle	3	42.48	10	3	Cytoskeleton proteins
P63267	31	Actin, gamma-enteric smooth muscle	3	42.48	10	3	Cytoskeleton proteins
P63261		Actin, cytoplasmic 2	3	42.48	10	3	Cytoskeleton proteins
P60709		Actin, cytoplasmic 1	3	42.48	10	3	Cytoskeleton proteins
P0CG04		Ig lambda chain C regions	2	31.44	28	2	Other
P35232		Prohibitin	2	27.03	8	2	Cell cycle
P10768	33	S-formylglutathione hydrolase	3	47.18	20	4	Other
P10768	34	S-formylglutathione hydrolase	5	80.34	27	6	Other
Q13228	36	Selenium-binding protein 1	2	28.13	6	2	Protein trafficking and secretion
Q5TCI9	39	Lamin-A/C	4	66.61	8	4	Intermediate filament family
P51888		Prolargin precursor	2	26.97	6	2	Molecular anchor protein
P14618	40	Pyruvate kinase isozymes M1/M2	5	84.26	17	6	Glycolysis metabolism

MS/MS, tandem mass spectrometry.

for improving construction of more elastic blood vessels under physiology comparable stimulations.

Conclusion

In the present study, using human ASCs as seed cells, we have engineered an elastic small diameter vessel wall with certain functional and mechanical properties in a bioreactor

system that mimics physiologically pulsatile pressure stimulation. In addition, protein expression profiles of engineered vessel walls and normal arterial walls were analyzed, respectively. A total of 38 proteins were identified, which were downregulated in engineered vessel walls compared to that in native ones, as determined by MS detection and bioinformatic analysis. By comparing these differentially expressed proteins, this approach improved our understanding

FIG. 5. Distribution and functional classification of proteins identified by 2D gel electrophoresis and mass spectrometry in engineered blood vessel walls and normal arterial walls. Distribution of all identified proteins according to their annotation in the Swiss-Prot Knowledgebase. The smaller figure shows subclasses of cellular organization. Color images available online at www.liebertpub.com/tea

of protein profile changes in large scale when the 3D arterial tissues are engineered. Future works should focus on identification of more specific proteins that would be used to determine the molecular mechanisms underlying *in vitro* generation of vessel walls with comparable biomechanical properties.

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Disclosure Statement

No competing financial interests exist.

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