Published in final edited form as:
FASEB J. 2008 June ; 22(6): 1635–1648. doi:10.1096/fj.07-087924.

Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs)

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Abstract

Using biodegradable scaffold and a biomimetic perfusion system, our lab has successfully engineered small-diameter vessel grafts using endothelial cells (ECs) and smooth muscle cells (SMCs) obtained from vessels in various species. However, translating this technique into humans has presented tremendous obstacles due to species and age differences. SMCs from elderly persons have limited proliferative capacity and a reduction in collagen production, which impair the mechanical strength of engineered vessels. As an alternative cell source, adult human bone marrow-derived mesenchymal stem cells (hMSCs) were studied for their ability to differentiate into SMCs in culture plates as well as in a bioreactor system. In the former setting, immunofluorescence staining showed that MSCs, after induction for 14 days, expressed smooth muscle α-actin (SMA) and calponin, early and mid-SMC phenotypic markers, respectively. In the latter setting, vessel walls were constructed with MSC-derived SMCs. Various factors (*i.e.*, matrix proteins, soluble factors, and cyclic strain) in the engineering system were further investigated for their effects on hMSC cell proliferation and differentiation into SMCs. Based on a screening of multiple factors, the engineering system was optimized by dividing the vessel culture into proliferation and differentiation phases. The vessel walls engineered under the optimized conditions were examined histologically and molecularly, and found to be substantially similar to native vessels. In conclusion, bone marrow-derived hMSCs can serve as a new cell source of SMCs in vessel engineering. Optimization of the culture conditions to drive SMC differentiation and matrix production significantly improved the quality of the hMSC-derived engineered vessel wall.—Gong, Z., Niklason, L. E. Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs).

Keywords

vessel engineering; smooth muscle cell; bioreactor; cyclic strain; extracellular matrix; soluble factors

In cardiac or peripheral bypass surgery, diseased arteries are replaced with autologous veins or, less frequently, arteries. However, some patients in need of such operations do not have suitable veins as replacement due to the systematic pathological changes in their vascular system, or previous vein harvest (1). Although synthetic vascular prostheses, such as Dacron and expanded polytetrafluoroethylene (ePTFE), have their own merits as conduit in high-flow low-resistance conditions (such as large peripheral arteries), their performance as small diameter vessel graft is far from satisfactory (2–5). Biological vascular grafts may be a viable solution, being composed of cellular components and able to respond physiologically to various hemodynamic forces and chemical stimuli. Tremendous progress has been made in vascular engineering field since Weinberg and Bell constructed one of the first blood vessels 20 years ago using endothelial cells (ECs) and smooth muscle cells (SMCs) in gelatin (6). Since that

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time, various approaches to culture vascular grafts have been developed, including gelatin-based, biodegradable scaffold-based, and sheet-based grafts.

Using polyglycolic acid (PGA) as a scaffold and a biomimetic system, our lab has successfully engineered vessels with excellent mechanical strength in various species, including bovine, porcine, and canine (7–9). However, translating vascular engineering techniques from animal to human vascular cells has met with some difficulties. We recently reported the impact of telomerase (hTERT) gene therapy on culturing human vessels (10,11) Although hTERT expression enabled the culture of engineered human blood vessels, extension of cellular life span did not appear to alter the intrinsic aging-associated cellular changes of vascular SMC, such as decline in collagen synthesis and consequent decreased burst strength.

Based in part on these observations, it seems reasonable to seek alternative cell sources that may be suitable for vessel engineering. Human stem and progenitor cells have been isolated from a wide range of sources. Their autologous origin, high proliferative capacity, and potential to differentiate into vascular phenotypes have generated significant attention. Mesenchymal stem cells (MSCs) are variously defined as multipotent adult stem cells that are present in the bone marrow (12,13) and other tissues (14–16) and have the ability to differentiate into multiple cell lineages including osteoblasts, adipocytes, chondrocytes, myoblasts, and early progenitors of neural cells (13–15,17,18). The ability of MSCs to differentiate into myocytes has been shown previously. For instance, mesenchymal precursor 10T1/2 cells differentiated into skeletal myoblasts after treatment with 5-azacytidine (19). Galmiche *et al.* documented that stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate along a vascular smooth muscle differentiation pathway (20). MSCs from the adult rat have been shown to have a potential to differentiate into SMCs when exposed to TGFβ1 (21). Human MSCs also exhibited enhanced differentiation with increased contractility in response to TGFβ1 (22). In addition to growth factors, cell-cell contact also plays an important role in the creation of an environment conductive to SMC differentiation. Hirschi and colleagues showed that heterotypic cell-cell interactions mediated EC-induced recruitment of 10T1/2 cells and their differentiation to smooth muscle cells (23). In another MSC-EC coculture experiment, direct contact with ECs augmented SMA expression (24).

Beyond these studies of differentiation potential of MSCs, the application of bone marrow-derived stem cells *in vitro* and *in vivo* for vascular engineering is just emerging (25–32). Populations of marrow-derived cells have been used to regenerate vessels in canine and bovine models, but in some cases these populations are not fully characterized and the differentiation impact of various factors (such as substrate and mechanical stimulation) are not well understood (29,31). In several studies involved in the engineering of heart valves, the isolation, expansion, and phenotype confirmation hMSCs were well documented before scaffold seeding (26–28).

We speculated that various factors that are associated with the intact or regenerating vascular wall may be involved in directing the differentiation of hMSCs toward an SMC phenotype. Specifically, growth factors that are elaborated by platelets and vascular cells after vessel injury (PDGF, TGF-β1, and bFGF), extracellular proteins found in native vessel wall, and cyclic mechanical strain were all examined for their impact on SMC differentiation from hMSCs. Intriguingly, many of these factors were found to influence extent of differentiation toward an SMC fate, implying that local cues within injured vessels *in vivo* may direct hMSCs toward a vessel reparative function.

To test whether human bone marrow-derived MSCs can directly differentiate into SMCs that are functional for arterial engineering, vessel walls were engineered using human bone marrow-derived MSCs in a biomimetic culture system *in vitro*. A vessel engineering protocol,

having proliferation and differentiation phases, was designed to promote hMSC proliferation and SMC differentiation, respectively. The cultured cells and the engineered vessel wall were examined histologically for extracellular matrix (ECM) components, as well as molecularly for the expression of SMC phenotypic markers. When induced to expand and then differentiate into SMCs in the bioreactor, hMSCs proved to be an excellent starting material for arterial engineering.

Materials and Methods

Isolation and cell culture of hMSCs

hMSC cell culture was established as described previously (33) from six different donors (ages 22–45 y, three for each gender). Briefly, fresh unprocessed human bone marrow (Lonza, Basel, Switzerland) was slowly loaded onto Ficoll-Paque Plus density media (StemCell Technologies, Vancouver, BC, Canada) and fractionated at room temperature for 30 min at 1200 *g* with no brake. The mononuclear cell layer at the interface was removed and washed once with Dulbecco's phosphate buffered saline (PBS; Life Technologies, Inc., Gaithersburg, MD, USA) before being plated in 75 cm (2) flasks in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Inc.) containing 10% selected lot of fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) and 1% penicillin-streptamycin-glutamate (Life Technologies, Inc.). The screening of the FBS lot was based on the support of cell proliferation. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed 2×/wk. Cultures were passaged to 75 cm^2 flasks for protein analyses or in chamber slides for histochemical analysis.

Fluorescence-activated cell sorting (FACS) analysis

Cells were tested for purity by flow cytometry after isolation. FACS analysis was performed on cultured hMSCs from three different donors. Fluorescein isothiocyanate (FITC) -conjugated mouse anti-human IgGs (CD14, CD45, and CD34 from Abcam Inc., Cambridge, MA, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA; and Miltenyi Biotec Inc., Auburn, CA, USA, respectively) were utilized. Proper isotype IgGs were served as controls. For SH2 and SH3 staining, 100 μl supernatants from SH2 and SH3 hybridoma cell (American Type Culture Collection, Manassas, VA, USA) culture were utilized. After staining, the cells were fixed in 4% paraformaldehyde, and quantitative FACS was performed on a FACStar flow cytometer (BD, Franklin Lakes, NJ, USA).

Confirmation of hMSCs phenotype

MSCs are commonly defined by their ability to differentiate into osteogenic and adipogenic lineages.

Osteogenic induction—hMSCs (3×10^4) were seeded in 2 ml mesenchymal stem cell medium (MSCGM, Lonza) per well of 6-well plates and allowed to adhere to the culture surface for 24 h before replacing the MSCGM with the osteogenesis induction medium (Lonza). The induced hMSCs were fed every 3–4 days for 3 wk by completely replacing the medium with fresh osteogenesis induction medium. The noninduced control hMSCs were fed with MSCGM on the same schedule.

Alkaline phosphatase (ALP) and von Kossa staining (for mineralization nodule-formation) was performed to examine osteogenesis in hMSC culture. Mineralized bone nodules were identified by double labeling for von Kossa stain and ALP as described (34).

Adipogenesis induction—hMSCs (2×10^5) were seeded in 2 ml MSCGM medium per well of a 6-well plate and fed every 3–4 days until the culture reached confluence. At full confluence,

three cycles of induction/maintenance treatment were performed to stimulate optimal adipogenic differentiation. Each cycle consisted of feeding the hMSCs with adipogenesis induction medium (Lonza) for 3 days followed by 3 days of culture in adipogenic maintenance medium (Lonza). Noninduced control hMSCs were fed with adipogenic maintenance medium only on the same schedule. After three complete cycles of induction/ maintenance, the extent of adipogenesis was examined under the microscope by fixation with 10% NBF and stained with 1 μg/ml Nile red for 5 min for lipid formation.

Induction of hMSCs into SMCs

hMSCs were grown in basal medium containing low-glucose DMEM (Life Technologies, Inc.) supplemented with 10% selected lot of premium fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin-glutamine (Life Technologies, Inc.) on glass chamber slides (Nunc, Roskilde, Denmark). SMC induction was performed by adding 1 ng/ml TGFβ1 to the basal culture medium. The control culture was grown in parallel in basal medium. Immnofluorescence staining was performed (as described below) for smooth muscle α-actin (SMA) and calponin on day 14. Coronary artery smooth muscle cells (CASMCs) were cultured as positive controls in smooth muscle growth medium (SmBM) supplemented with SmGM-2 SingleQuots (Lonza) and stained with hMSCs.

Immunofluorescence studies

Immnofluorescence staining for SMC markers was performed as described previously (24). The paraformaldehyde-fixed chamber slides were incubated with monoclonal primary antibodies (1:100 dilution for both SMA and calponin, and 1:50 for SM-MHC, smooth muscle myocin heavy chain; Dako, Copenhagen, Denmark) for 1 h at room temperature. After three washes with PBS, the secondary antibody, a goat anti-mouse IgG conjugated with FITC (1:2000, Santa Cruz Biotechnology) was added and incubated for 30 min at room temperature. Nuclei were stained with 4′,6-diamidine-2-phenylindole (DAPI), which was contained in the Vectashield Mounting Medium for Fluorescence with DAPI kit (Vector Laboratories, Inc., Burlingame, CA, USA).

Effect of various matrix proteins on hMSCs

In these 2-D culture studies, hMSCs were seeded at 2×10^4/ml in DMEM plus 10% FBS medium on 6-well plates untreated or coated with collagen type I, IV, elastin, fibronectin, and laminin (Flexcell International, Hillsborough, NC, USA). After 7 days, the cells were detached and enumerated with 3% acetic acid with methylene blue (StemCell Technologies) on a hemacytometer. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer with Triton X-100 (Boston BioProducts, Boston, MA, USA) with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Protein lysates were quantified by Bradford assay, using the Quick Start Bradford Dye Reagents (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol, and stored at −80°C for Western blot for SMA and calponin.

Effect of various soluble factors on hMSCs

hMSCs were seeded in 6-well plates at $5.6 \times 10^3/cm^2$ in DMEM plus 5% FBS medium with one of the following supplements: 0 (control), 0.01, 0.1, 1, or 10 ng/ml transforming growth factor β1 (TGFβ1, R&D Systems, Inc., Minneapolis, MN, USA); 10 ng/ml PDGF-BB, PDGF-CC, and bFGF (R&D Systems, Inc.); or 50 μg/ml ascorbic acid. Media were changed on day 4. After 7 days of treatment, cells were harvested for cell proliferation and differentiation analysis.

Effect of cyclic strain on hMSCs

hMSCs seeded (4×10^4 cells/ml for cell proliferation and 2×10^4 cells/ml for cell differentiation) in DMEM plus 10% FBS were subjected to cyclic strain using a Flexcell 4000T unit (Flexcell International) in the presence or absence of 10 ng/ml PDGF-BB. The stain unit is a computer-regulated device that applies cyclic tensile strain to the cell culture through regulated vacuum pressure on the bottom culture plates with a flexible membrane that is untreated or pre-treated with fibronectin and type I collagen (Flexcell International). The strain causes the flexible plate to stretch across a cylindrical loading post to provide equibiaxial strain to the cells. hMSCs were subject to equibiaxial cyclic strain for 5 days at a frequency of 0.5 Hz, resulting in ~8–12% substrate elongation. Unstrained controls were hMSCs prepared in an identical manner and cultured on unstrained untreated or collagen I or fibronectin-coated flexible plates for 5 days. At the end of the experiment, cell enumeration, protein isolation, and Western blot were performed.

Blood vessel wall culture

Basic engineering protocol—As an initial control study to determine the behavior of hMSCs in bioreactor conditions, human blood vessels (n=6) were engineered from hMSCs using techniques similar to those previously described (7,8). To prepare for blood vessel culture, hMSCs were preconditioned in enhanced DMEM medium (8) supplemented with 10 ng/ml PDGF-BB and bFGF for 48 h before they were seeded on scaffolds in the bioreactor. The enhanced DMEM, supplemented with 20% serum, 10 ng/mL bFGF, and PDGF-BB has been characterized previously as stimulating SMC growth and collagen matrix production during engineered vessel growth (8).

Blood vessel bioreactors and PGA mesh scaffolds (Concordia Manufacturing LLC, Coventry, RI, USA) were prepared as described previously (7,8) and seeded with hMSCs (5×10^6 cells for each vessel). Bioreactors were filled with enhanced DMEM medium and fed with fresh enhanced DMEM for half of the bioreactor volume 1×/wk. The pulsatile perfusion system applied ~5% cyclic strain (7) from wk 2–8 of culture. Ascorbic acid was added to the bioreactor 3×/wk. Vessel culture continued for 8 wk, at which time the vessels were harvested from the bioreactors.

Optimized culture protocol—Based on the results from matrix protein and soluble factor screen and cyclic strain experiments (summarized in Fig. 6A), the engineering system was optimized to induce SMC differentiation, as shown in Fig. 6B. Seven vessels were engineered based on the optimized protocol and compared with the ones generated with the original protocol.

Endothelialization of engineered vessel wall

To show feasibility of EC adhesion to the engineered vessel lumen, endothelial seeding was performed on one engineered vessel. human umbilical cord-derived endothelial cells (HUVECs; 2.6×10^6) resuspended in DMEM/10% FBS were seeded into the lumen of the vessel with a syringe. The ends of the vessel were clamped, and the bioreactor was manually rotated every 15 min for 40 min at different positions to allow even distribution of the HUVEC cells. Then the bioreactor was returned to the incubator. After 8 h, flow was restarted to allow the perfusion of endothelialized vessel lumens with enhanced DMEM medium at a flow rate that gradually increased from ~0.02 to 0.1 ml/s (shear stress 0.25–1 dyne/cm^2) over 18 h of duration.

Scanning electron microscopy (SEM)

Vascular grafts (endothelized and nonendothelialized) were harvested, fixed, and prepared as described previously (8). The dried samples were coated with gold using a Cressington C108

autocoater (Cressington Scientific Instruments, Ltd., Watford, UK) and examined by SEM (XL30ESEM-FEG; FEI Company, Hillsborough, OR, USA).

Analysis of the engineered vessel walls

Each engineered vessel was cut into three segments, whose weight was measured and recorded. One segment of each vessel was fixed immediately in 10% neutral buffered formalin (NBF) for 1 h followed by dehydration and embedding in paraffin. Sections (4 μm thick) were cut, deparaffinized, and stained with hematoxyline and eosin (H&E), Masson Trichrome for collagen, Verhoeff-Van Gieson (VVG), and Movat stains for elastin production and immunohistochemical staining. The tissue sections from the engineered human vessel walls were also stained immunohistochemically for proliferating cell nuclear antigen (PCNA) using the PCNA Staining Kit (Zymed Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions.

Total protein lysates were isolated from segments of each engineered vessel with RIPA buffer containing protease inhibitor cocktail as described above.

Collagen analysis

Collagen analysis was performed according to a protocol previously published (10,35). A 1:10 w/w ratio of hydroxyproline and collagen was used to calculate the collagen content of the vessels. The collagen content was calculated as the percentage of the dry weight. Human umbilical cord artery was included as control.

Western blot analysis

Lysates containing 25 μg of protein from each vessel or cell culture were separated on 10% SDS-PAGE precast gel (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). The membranes were incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in TBST buffer) and then incubated with mouse anti-human primary antibodies, 1:200, 1:200, 1:50, and 1:30 dilution in 1% nonfat dry milk (Bio-Rad) in TBST buffer for SMA, calponin, SM-MHC, and smoothelin (HyCult Biotechnology, Uden, The Netherlands), respectively. Then the blots were probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2000; Santa Cruz Biotechnology) for 30 min at room temperature. After washing, the blots were developed using the SuperSignal West Pico Chemiluminescent detection system (Pierce, Rockford, IL, USA) and exposed to X-ray film to detect the protein bands. After visualization, membranes were stripped in Restore™ Western blot Stripping Buffer (Pierce) at room temperature for 5–15 min and reprobed for β-actin (1:5000 dilution, Sigma), which was used as equal loading control. Quantification of Western blots was performed using Image J (National Institutes of Health, Bethesda, MD, USA). The results were presented as relative density after correction with β-actin.

Statistical analysis

All quantitative results were obtained from five samples for cell proliferation analyses and from triplicate samples for Western blot analyses. Data were expressed as the mean ± SD. Statistical analysis was performed using Student's *t* test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Phenotype of isolated hMSC

The surface marker expression of the isolated hMSCs from the bone marrow was analyzed by FACS (Fig. 1A). hMSCs were negative for CD14, CD34, and CD45, known markers for monocyte/macrophage, hematopoietic progenitor cells, and differentiated nonerythrocyte hematopoietic cells, respectively. However, they were positive for SH2 and SH3, antibodies that have been shown to recognize CD105 (endoglin) and CD73 on human MSCs, respectively, but are nonreactive to osteoblasts and osteocytes (36).

hMSC phenotype was further confirmed by osteogenic and adipogenic induction. Osteogenic induced hMSCs exhibited changes in cell morphology from spindle shape to cuboidal shape as they differentiated and mineralized. Osteogenesis was further examined after 3 wk induction by ALP and von Kossa staining, where ALP-positive areas appeared red, while the mineralized areas were brown to black in color in osteogenic culture, exhibiting high ALP enzyme activity and mineralization (Fig. 1C). In contrast, no ALP activity or mineralized bone nodules were found in the control culture (Fig. 1B). Confluent hMSC cultures were also treated with adipogenic induction media. After 8 days of adipogenic induction/maintenance treatment, lipid vacuoles started to appear in the confluent hMSC culture (data not shown). By day 18, after three cycles of adipogenic induction and maintenance, lipid-containing adipocytes became very prominent, which stained positive for Nile red, a lipophilic fluorescence dye (Fig. 1E). In contrast, no Nile-red positive cells were found in the hMSC control culture (Fig. 1D). These studies, in combination with the FACS data, confirm the mesenchymal stem cell identity of the starting cellular material.

Induction of hMSCs into SMCs

hMSCs expressed basal levels of SMA and calponin when cultured in control conditions (DMEM plus 10% FBS) (Fig. 2a, d). hMSCs were partially induced into an SMC phenotype by exposure to 1 ng/ml TGFβ1. The partial phenotypic switch from MSC to SMC was demonstrated by immunofluorescence staining for smooth muscle α-actin (SMA) and calponin, which are early and mid-SMC markers, respectively. After 14 days of exposure to TGFβ1, hMSCs were stained positively for SMA (Fig. 2b) and calponin (Fig. 2e) but not for SM-MHC (a late marker of SMC lineage, not shown), indicating a committed yet immature stage of SMC differentiation from MSCs after 14 days of exposure. The distribution of SMA and calponin expression in MSCs was similar to that of CASMCs (Fig. 2c, f), which served as positive control.

Effect of various matrix proteins on hMSCs

To determine the impact of various ECM proteins that are present in the vascular wall on hMSC differentiation, we cultured hMSCs on Flexcell 6-well culture plates that had been precoated with ECM molecules (and not subjected to cyclic strain). None of the examined matrix proteins (collagen type I, collagen type IV, laminin, elastin, and fibronectin) had a significant effect on hMSC proliferation as compared to untreated surfaces. The presence of 10 ng/ml PDGF-BB, which is an important mitogen and chemoattractant for mesenchymal cells (37–38), increased hMSC proliferation similarly across all the different matrix proteins studied (Fig. 3A).

As assessed by immunoblotting, no difference was found in SMA expression between each matrix protein and the untreated surface, with or without PDGF-BB (Fig. 3B, n=5). ECMs including collagen I, IV, elastin, and fibronectin significantly increased calponin expression compared to untreated surfaces. This is an interesting finding, given that these four matrix proteins are present in native vasculature at different locations. In contrast, laminin exerted the opposite effect on calponin compared to control (Fig. 3C, D). The inhibitory effect of PDGF-

BB on SMA was confirmed in hMSCs cultured on uncoated or elastin and fibronectin-coated plates (Fig. 3B), while PDGF-BB decreased calponin on almost all coating matrix proteins except elastin (Fig. 3C).

Effect of various soluble factors on hMSCs

The enhanced culture medium that is used to grow engineered arteries (7,8) contains soluble factors such as PDGF-BB, bFGF, vitamin C, and a high percentage of serum. Serum, in turn, contains a variety of growth factors, including those liberated from platelets, that affect mesenchymal cell behavior (*e.g.*, TGF-β1 and PDGF). Seven days of exposure to various soluble factor revealed that TGFβ1 induced a dose-dependant inhibition of hMSC cell proliferation at concentrations between 0.1 and 10 ng/ml. In contrast, other factors, including PDGF-BB, PDGF-CC, bFGF, and vitamin C, significantly increased hMSC cell number over a 7-day period (Fig. 4A).

TGFβ1, although not affecting SMA protein expression (Fig. 4B), significantly increased calponin expression in hMSCs, in a dose-dependent fashion, from 0.1 to 10 ng/ml (Fig. 4C, D). Of note, expression levels of calponin in hMSCs exposed to 10 ng/ml of TGF-β1 were similar to levels expressed by CASMCs with similar protein loading. The differentiation effect of TGFβ1 is consistent with results reported by other groups. However, PDGF-BB, PDGF-CC, and bFGF all significantly reduced both SMA (Fig. 4B, D, E) and calponin (Fig. 4C, D) levels. Since both PDGF-BB and bFGF are included in the enhanced DMEM medium that is used for basic vessel culture, it might be anticipated that these factors would inhibit hMSC differentiation into SMC.

Effect of cyclic strain on hMSCs

Native vessels, and engineered vessels during culture, are exposed to cyclic mechanical strain. Cyclic strain inhibited cell proliferation after 5 days application when hMSC were cultured on three different kinds of matrices—untreated, pronectin (fibronectin), and collagen type I (Fig. 5A). In the absence of matrix coating, cyclic strain alone significantly decreased SMA expression. In the presence of PDGF-BB, cyclic strain displayed opposite effect on SMA expression: an increase on collagen I matrix and a decrease on fibronectin (Fig. 5B). In the absence of PDGF-BB, calponin level was significantly decreased on collagen I but increased on fibronectin by cyclic strain (Fig. 6C). The inhibition of PDGF-BB on SMC protein expression was further confirmed regardless of cyclic strain and different matrix coating, suggesting that PDGF-BB should be avoided during SMC differentiation despite its potent mitogenic effect on hMSCs. The observation that fibronectin increased calponin expression in the absence of cyclic strain (Fig. 5C comparison of calponin in S− B− in nontreated *vs.* fibronectin-coated, $P<0.05$) is concordant with the results from Fig. 3C.

In summary, while cyclic strain inhibited hMSC proliferation independent of ECM (untreated, collagen type I, or fibronectin), its influence on hMSC differentiation was significantly tied to the presence of ECM as well as PDGF-BB.

Optimization of the engineering system

Based on the above results, we developed an “optimized” protocol for engineering vessels from hMSCs that was divided into two phases. Under the optimized conditions, instead of culturing the vessel walls in the same enhanced DMEM medium for 8 wk, the culture period was divided into 4 wk of proliferation phase and another 4 wk of differentiation phase. Before cell seeding, PGA scaffold was precoated with 10 $\mu g/cm^2$ fibronectin (BD Biosciencies, San Jose, CA, USA), which in combination with cyclic strain stimulated hMSC differentiation into SMC (Fig. 5C). The basic components of the enhanced DMEM medium were essentially the same as the original protocol, except bFGF was removed from the culture medium due to its potent

inhibition on SMC differentiation. Another potent hMSC mitogen, PDGF-BB, was retained during the first 4 wk of culture. After 4 wk, PDGF-BB was substituted with 1 ng/ml TGFβ1, which had been seen to significantly enhance SMC differentiation from hMSCs (Fig. 4C). The pulsatile cyclic strain in the bioreactor was not initiated until wk 4, when the differentiation phase started, due to its observed inhibition of hMSC cell proliferation.

Histological comparison of vessels walls engineered before and after optimization

Engineered vessels were cultured using previously described protocols and using the "optimized" protocol described above. Thirteen SMC vessel walls were engineered in total from six different donors: half according to the original protocol (n=6), and the rest using the optimized protocol (n=7). After 8 wk of bioreactor culture, the histological appearance (H&E stain) of the vessel walls cultured according to the original protocol had some similarity to that of native vessels (Fig. 7a, c). Masson's trichrome stain of the engineered vessel revealed some production of collagen (blue stain, Fig. 7b, d). Remnants of the PGA polymer near the lumen of the vessel wall were stained dark blue, indicating incomplete degradation of PGA after 8 wk of culture, which has been observed previously (7). Movat and VVG stains did not reveal the deposition of elastin (data not shown).

With optimization, both cellularity and collagen production were substantially improved in the engineered vessel wall based on H&E (Fig. 7e, g) and Masson's Trichrome stains (Fig. 7f, h), respectively. PCNA stain showed that more cells were proliferating after optimization, which distributed evenly through the vessel wall, in contrast to few proliferative cells only in proximity to the lumen of the vessel wall before optimization (Fig. 7i, j). The result that more cells are proliferating at the end of culture using the "optimized" conditions is somewhat surprising, given that conditions were chosen to favor differentiation during the latter part of culture. Local paracrine factors secreted by differentiating SMC may have contributed to the observed increase in replication and cellularity under "optimized" conditions.

Collagen production was quantified by hydroxyproline assay. Without optimization, the average collagen content of engineered vessels was 5.1 ± 2.5% of total dry weight (n=6). After optimization, collagen significantly increased to 22.1 ± 7.8% of dry weight (P<0.05, Fig. 8A, n=4), which is roughly half of the collagen content of native vessels (7). But based on our mechanical testing of burst pressure on two vessels engineered from each protocol, the vessel walls engineered with the optimized protocol had higher burst pressures (7–8 psi) compared to those engineered with the original protocol (~3–4 psi). Both the burst pressure and handling of the vessel walls engineered with the two-phase protocol were superior to the ones with original protocol.

All of the engineered vessels expressed SMA and calponin proteins by immunoblotting (Fig. 8B). While SMA may be expressed by myofibroblasts and pericytes, calponin expression generally connotes true smooth muscle cell differentiation. After optimization, the expression levels of SMA and calponin were substantially increased to levels comparable with the CASMC control. SM-MHC, late SMC marker, and smoothelin, a cytoskeletal protein that is only found in contractile smooth muscle cells (39), were not detected in any of the engineered vessel walls (data not shown), indicating an incomplete differentiation into an SMC phenotype. Though collagen production was robust in engineered vessels, Western blots for tropoelastin did not reveal this elastin monomer (data not shown), and mature elastin was not detected by staining. This result is in contrast to results from differentiated human SMC used for vascular engineering, wherein tropoelastin production is detectable throughout culture, despite lack of mature, insoluble elastin (10).

Scanning electron microscopy

Without endothelialization, sheets of tightly packed collagenous ECM produced by hMSCs were evident on the inner lumen of the engineered vessel (Fig. 9A). In contrast, the lumen of the endothelialized vessel wall appeared very smooth, with flattened ECs covering the whole lumen and little evidence of the underlying ECM and hMSCs (Fig. 9B). This result shows the feasibility of producing a complete engineered vessel from hMSCs and luminal ECs.

Discussion

A long-term goal of this study was to explore the feasibility of using hMSCs from adult marrow as a new cell source to generate a vascular smooth muscle cell wall in engineered arteries for adult humans. Consistent with previous reports, we showed that factors such as TGFβ1 induced a partial conversion of hMSC to an SMC phenotype. We also analyzed the impact of other growth factors, substrate molecules and mechanical stimulation on the phenotypic differentiation of hMSC. Using information gleaned from the study of individual factors, we devised an “optimized” protocol whereby hMSC proliferation and differentiation could be controlled in the vessel engineering bioreactor. Under the optimized protocol, we observed significantly more cellularity and collagen deposition than those obtained under standard conditions. Importantly, many of the factors that drove both hMSC proliferation and differentiation into an SMC phenotype are factors that are found in the native or regenerating vessel wall.

Interestingly, evidence of partial induction of hMSCs into SMC was obtained after 2 wks' culture in enhanced DMEM medium that has been used to grow vessels in multiple species. By immunofluorescence and Western blotting, we noticed some basal level expression of SMA and calponin in hMSC cultured in control condition (DMEM plus 10% FBS). This finding is in agreement with previous report that bone marrow stromal cells have a phenotypic similarity to a subset of vascular smooth muscle cells (20). Because of the high TGFβ1 level in our selected serum lot (~2 ng/ml in 10% FBS medium), which significantly increased SMA and calponin expression, we believe TGFβ1 might be the critical factor that led to the basal expression of SMC markers. Since it is inevitable to have TGFβ1 in any lot of FBS, it is not surprising that SMA and calponin are expressed in other MSC cultures (40,41).

The fact that we did not observe SM-MHC, late SMC marker, after 2 wks' exposure to TGFβ1 suggests that the MSC-derived SMC were still at an early differentiation stage. Furthermore, PGA scaffold hydrolysis has been found to induce dedifferentiation of SMC in the proximity of polymer remnants in engineered vessels, which may have contributed to the incomplete differentiation observed in engineered vessels under both original and optimized conditions (8).

In investigating factors that may drive hMSC differentiation toward a vascular SMC phenotype, we focused on those soluble, matrix and physical factors that may be associated with the intact or regenerating vessel wall. Specifically, growth factors elaborated by platelets and vascular cells after vessel injury (PDGF, TGF-β1, and bFGF), extracellular proteins found in native vessel wall, and cyclic mechanical strain were all examined. Intriguingly, many of these factors were found to influence the extent of differentiation toward an SMC fate, implying that local cues within injured vessels *in vivo* may direct hMSC toward a vessel reparative function.

Inadequate collagen synthesis by the partly differentiated SMC (5% by dry weight) under the original protocol contrasted with the average vessel collagen content under the optimized protocol (~20% by dry weight). This increase in collagen synthesis is consistent with a more complete conversion to SMC phenotype under the optimized protocol. Although all the

engineered vessel walls expressed SMA and calponin, these appeared to be more highly expressed in vessels cultured under optimized conditions, as compared to original conditions. Indeed, though myosin heavy chain and smoothelin were not detectable in hMSC-derived vessels cultured for 8 wks, levels of alpha actin and calponin were comparable to those of CASMC.

Whereas one principle function of SMC is contraction, this cell also has an important synthetic function. It is the major source of the ECM components of the blood vessel wall (42–44), which contributes importantly to the mechanical strength of the vessel. The contractile and synthetic functions of SMCs appear to be inversely correlated and may be described by the term phenotypic modulation (45). The extent of SMC phenotypic modulation and plasticity appears to be dependent on many factors, among which are soluble factors, ECMs, and mechanical forces (46). The ECM that surrounds cells is a highly organized and dynamic structure that contributes to the control of cellular function and is involved in the maintenance of SMCs' state of proliferation and differentiation (47). Among the five ECMs we've examined, although none of them affected hMSC proliferation and SMA expression, all of the ECMs (including fibronectin, collagen I and IV, and elastin) significantly increased calponin expression except laminin, which, in contrast, significantly decreased calponin level.

SMCs on various ECMs, such as collagen, Matrigel (a basement membrane-rich matrix material) and fibronectin, evoked changes in SMC morphology consistent with a change in the differentiated phenotype (48–50). ECM proteins such as fibronectin and collagen I were found to promote proliferation of human airway SMCs and suppress contractile protein expression. In contrast, basement membrane elements such as laminin inhibited proliferation and supported a more contractile phenotype (51). The discrepancy between other results and our findings may reflect the difference in cell source (differentiated SMCs *vs.* bone marrow-derived hMSCs) as well as the limitations of the *in vitro* culture system and the specific experimental conditions and matrices examined. Further exploration and confirmation is clearly needed in this complex area.

Components of our enhanced DMEM medium, PDGF-BB, bFGF, and vitamin C, were examined for their effect on hMSC proliferation and differentiation. In addition, PDGF-CC, a newly discovered member of the VEGF/PDGF superfamily, which has been shown to induce the differentiation of bone marrow cells into smooth muscle cells and stimulate their growth during vessel sprouting (52), was examined. TGFβ1, a potent multifunctional cytokine that coordinately up-regulates a variety of SMC differentiation marker genes in cultured SMCs and is released from platelets after vascular injury (53,54), was also included in the study. Surprisingly, among all the factors examined, vitamin C, a cofactor for collagen synthesis, stimulated hMSC proliferation the most. PDGF-BB is a key mediator of SMC phenotypic switching. It has been shown to potently suppress expression of SMC marker genes as well as to increase the rate of proliferation and migration in cultured SMCs (55–58). Whereas both PDGF-BB and bFGF elicited a potent proliferative response in hMSCs, they significantly decreased SMA and calponin protein expression. bFGF reduced SMA and calponin levels to nearly undetectable levels by immunoblotting. TGFβ1 inhibited proliferation of hMSCs in a dose-dependent manner (59). However, calponin expression was significantly elevated by TGFβ1 at concentration between 0.1 and 10 ng/ml, consistent with previous reports on multipotent adult potential cells (MAPCs) cultured in serum-free condition (60). PDGF-CC exerted similar effect on hMSC proliferation and differentiation as PDGF-BB.

Cyclic strain has been shown to enhance extracellular matrix remodeling and synthesis, as well as cellular proliferation of differentiated SMCs (61–66). For example, Birukov *et al.* (61) demonstrated potentiation of SMC proliferation in serum-activated cultures. In contrast, Chapman *et al.* (67) reported that physiological cyclic stretch causes cell cycle arrest in cultured

vascular SMCs, which was verified by others (68,69). In the present study, while cyclic strain inhibited hMSC proliferation independent of ECM (untreated, collagen type I or fibronectin), its influence on hMSC differentiation was significantly tied to the presence of ECM as well as PDGF-BB. For example, in the absence of matrix coating, cyclic strain alone significantly decreased SMA expression. In the presence of PDGF-BB, cyclic strain displayed the opposite effect on SMA expression: an increase on collagen I matrix and a decrease on fibronectin (Fig. 5B).

Between the two major SMC markers, SMA and calponin, SMA is less specific due to its transient expression in early stages of cardiac and skeletal myocytes (44,70), as well as in myofibroblasts in healing wounds (71) and tumors (72). In addition, treatment of ECs and myofibroblasts with TGFβ can also induce SMA expression (73,74). Thus, SMA alone does not provide definitive evidence for SMC lineage. In contract, in adult organisms, the expression of calponin appears to be restricted almost exclusively to vascular smooth muscle (45). The different specificity of these two SMC markers might explain the different changes of the two proteins in response to each factor.

In summary, human vessel walls have been successfully constructed with hMSCs, providing a new cell source to generate small-diameter vessel grafts. This is the first report of utilizing hMSCs for engineered vessel culture in a bioreactor setting. These vessel walls exhibit some similarities in terms of morphology, histology, and protein synthesis to native counterparts, which are mainly composed of SMCs and their ECM. Further investigation is necessary to improve the mechanical strength of vessels derived from MSCs, and this will likely come from improvements in SMC differentiation and matrix synthesis. Based on our initial success on the endothelialization of these hMSC-derived vessel walls, endothelialization with EPC-derived ECs, and *in vivo* implantation may be the next steps in achieving the goal of a marrow-derived vascular replacement.

Acknowledgements

The authors are grateful for Dr. Clay Quint and Mr. Thomas Petersen for their help with the FACS analysis, Dr. Clay Quint for the endothelialization and SEM imaging, and Dr. Thomas Hitchcock for his assistance with the histological images. This work was funded by U.S. National Institutes of Health grants RO1HL083895 and HL063766 (both to L.E.N.).

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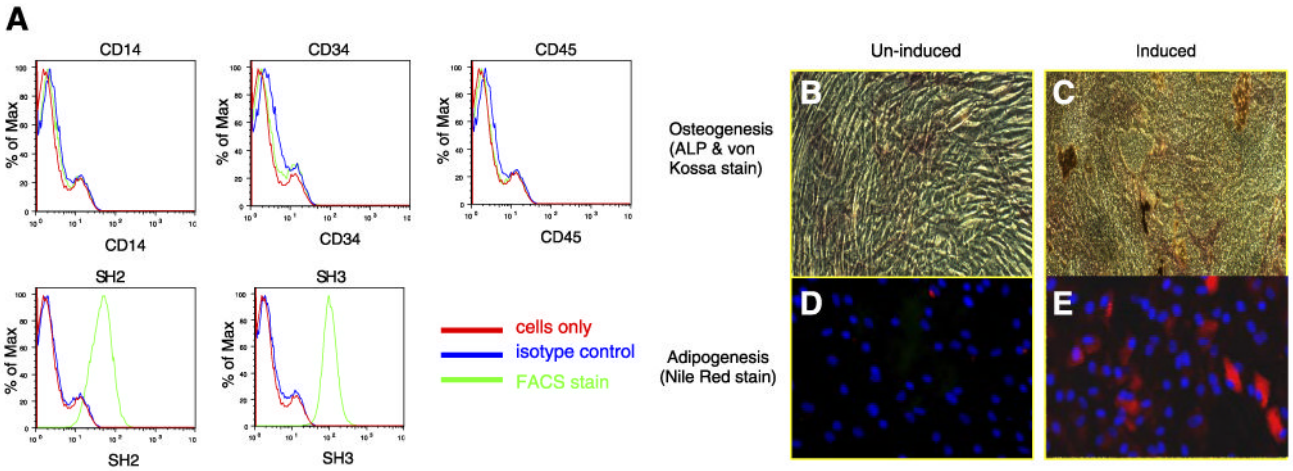

Figure 1.

Confirmation of hMSC phenotype. *A*) Representative flow cytometric analysis showing reactivity of SH2 and SH3 antibody with hMSCs isolated from human bone marrow (green curve). hMSCs were negative with CD14, CD34, and CD45 antibody (green curve). Staining with the isotype-matched control antibody is shown in blue; cells-only controls are shown in red. *B*–*E*) Osteogenic and adipogenic induction of hMSC culture was performed. Osteogenesis was examined 3 wk after treatment in control (*B*) or induction (*C*) cultures by ALP and von Kossa staining. After three complete cycles of induction and maintenance, the extent of adipogenesis was examined by Nile red staining of control (*D*) and induction (*E*) cultures.

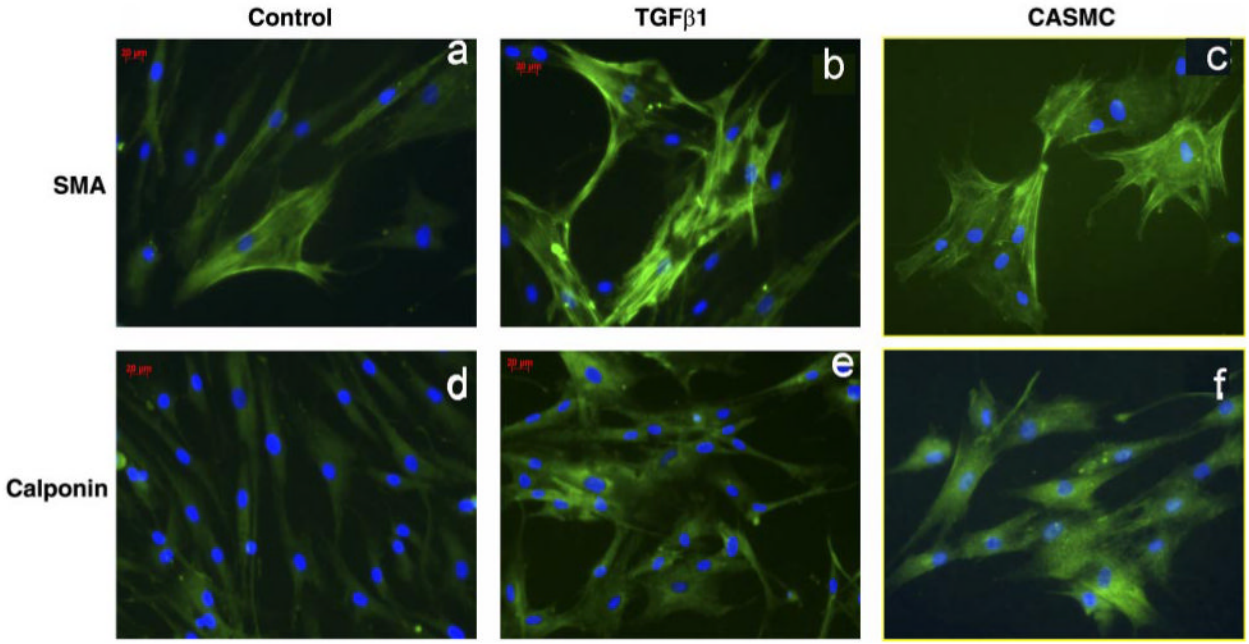

Figure 2.
SMC induction in hMSC culture. hMSCs were grown in basal DMEM medium on glass chamber slides without (*a*, *d*) or with (*b*, *e*) the addition of 1 ng/ml TGFβ1. Immnofluorescence staining (green) was performed for SMA (*a*–*c*) and calponin (*e*, *f*) on day 14. Nuclei were stained with DAPI (blue). CASMCs were stained for SMA (*c*) and calponin (*f*) as positive controls.

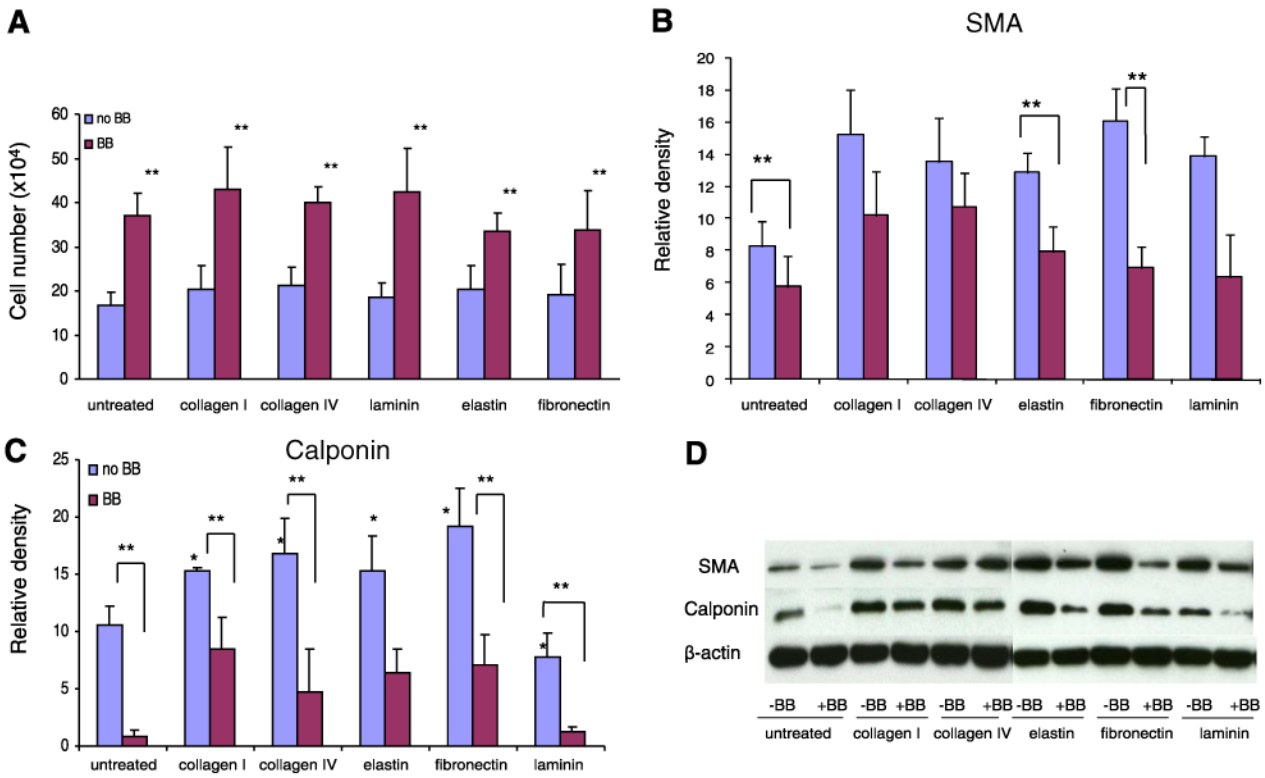

Figure 3.

Effect of various matrices on hMSC cell proliferation and differentiation. hMSCs were seeded at 2 × 10^4/ml in DMEM plus 10% FBS medium on 6-well plates untreated or coated with collagen type I, IV, elastin, fibronectin, and laminin in the presence or absence of 10 ng/ml PDGF-BB. *A*) After 7 days, cell numbers were counted with methylene blue on a hemacytometer for cell proliferation (**P<0.05, PDGF-BB treatment *vs.* no BB; no difference between each matrix and untreated with or without PDGF-BB; n=5). *B, C*) Western blots were performed on the protein lysates from each treatment group on SMA (*B*) and calponin (*C*, *P<0.05 *vs.* untreated; **P<0.05, PDGF-BB *vs.* no BB; n=3). *D*) Representative Western blot on SMA, calponin, and β-actin.

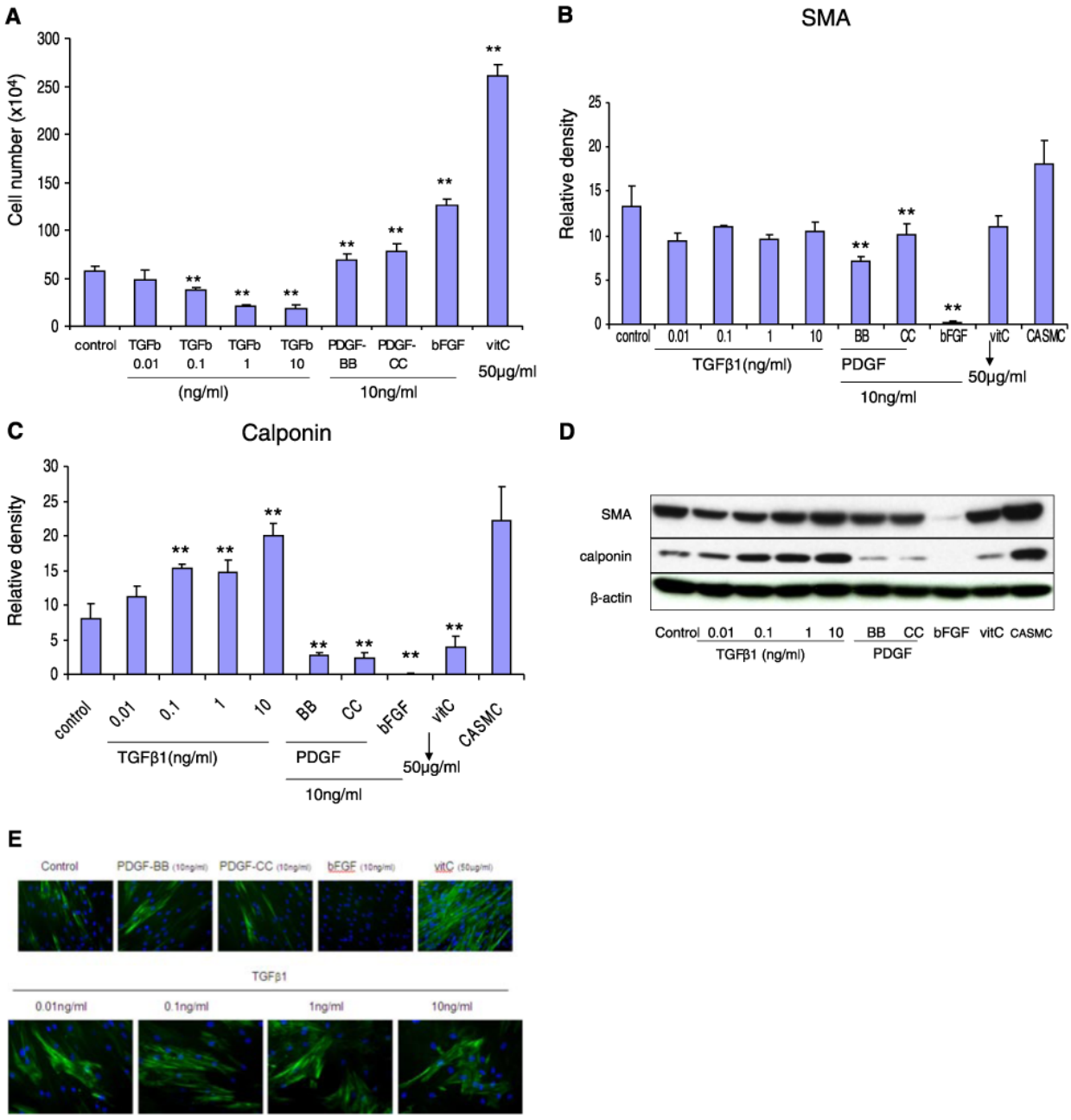

Figure 4.

Effect of various factors on hMSC cell proliferation and differentiation. hMSCs were seeded at $5.6 \times 10^3/cm^2$ in DMEM plus 5% FBS medium with one of the following supplements: 0 (control), 0.01, 0.1, 1, or 10 ng/ml TGFβ1; 10 ng/ml PDGF-BB, PDGF-CC, bFGF; or 50 μg/ml ascorbic acid. *A*) After 7 days, the cells were enumerated with 3% acetic acid with methylene blue on a hemacytometer (***P*<0.05 *vs.* control; *n*=5). *B*, *C*) Western blots were performed on the protein lysates from each treatment group on SMA (*B*) and calponin (*C*; ***P*<0.05 compared to control, *n*=3). *D*) Representative Western blot on SMA, calponin, and β-actin. *E*) Immunofluorescence staining for SMA in hMSC culture after treatment with TGFβ1,

PDGF-BB, PDGF-CC, bFGF, and vitamin C in addition to control for 7 days. Concentration of each factor is labeled in the figure.

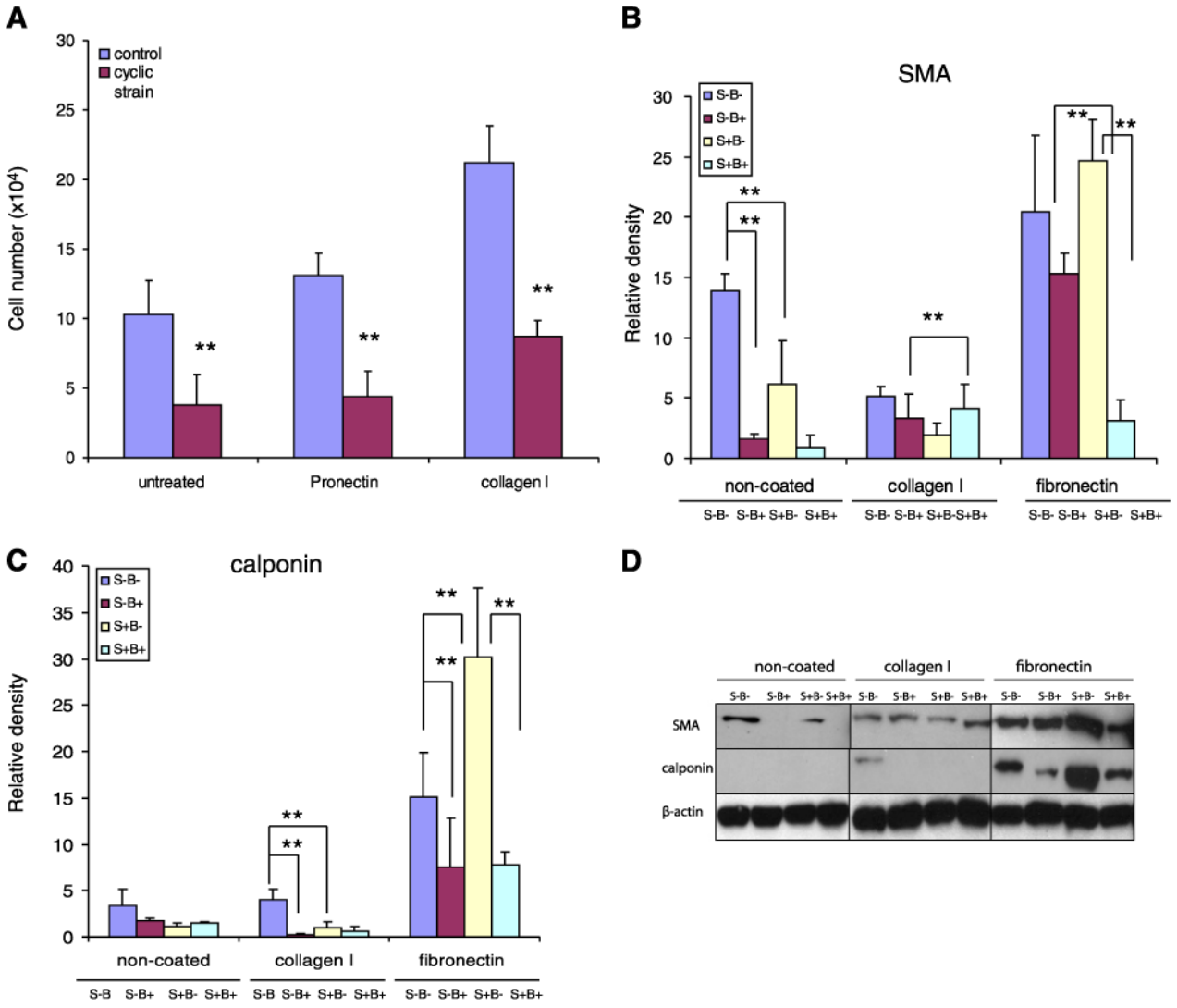

Figure 5.

Effect of cyclic strain on hMSC cell proliferation and differentiation. hMSCs were cultured in 6-well plates noncoated or coated with collagen I or fibronectin. In each surface treatment group, there were four culture conditions: control (S− B−), PDGF-BB only (10 ng/ml, S− B +), cyclic strain only (S+ B−), and PDGF plus cyclic strain (S+ B+). *A–C*) Cells were subject to the detailed condition for 5 days and harvested for cell number (*A*) and Western blot on SMA (*B*) and calponin (*C*). *D*) Representative Western blot film (*n*=3).

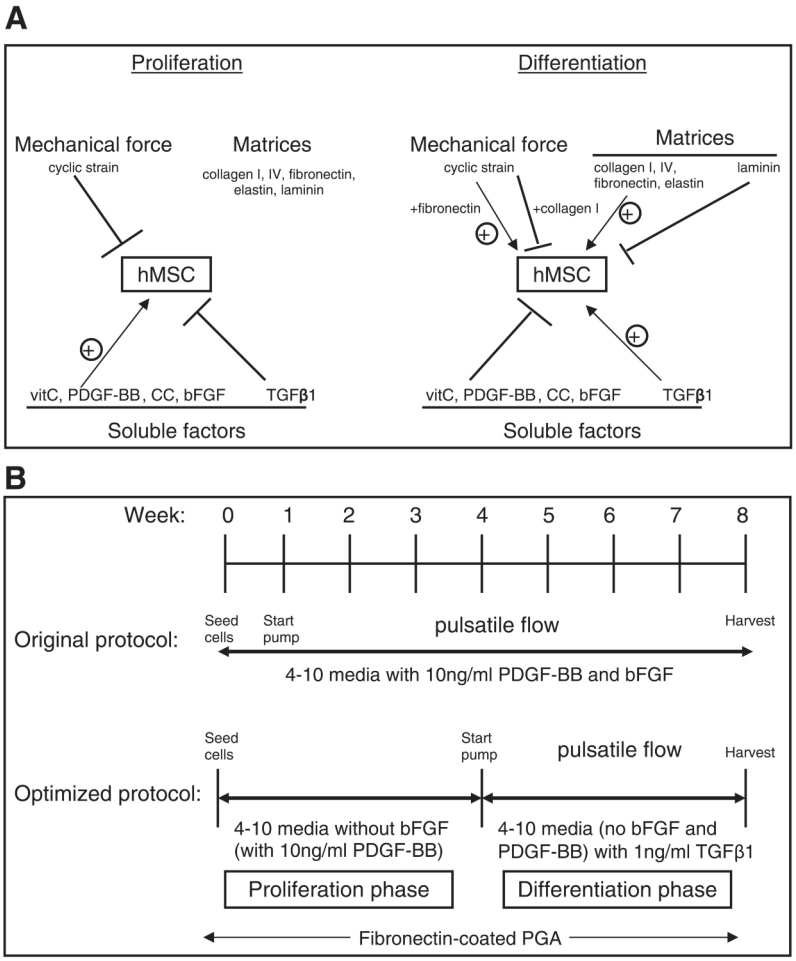

Figure 6.

A) Effects of various soluble factors, matrix proteins, and cyclic strain on hMSC cell proliferation and differentiation. *B*) Optimization of the engineering system to grow a vessel wall using hMSCs.

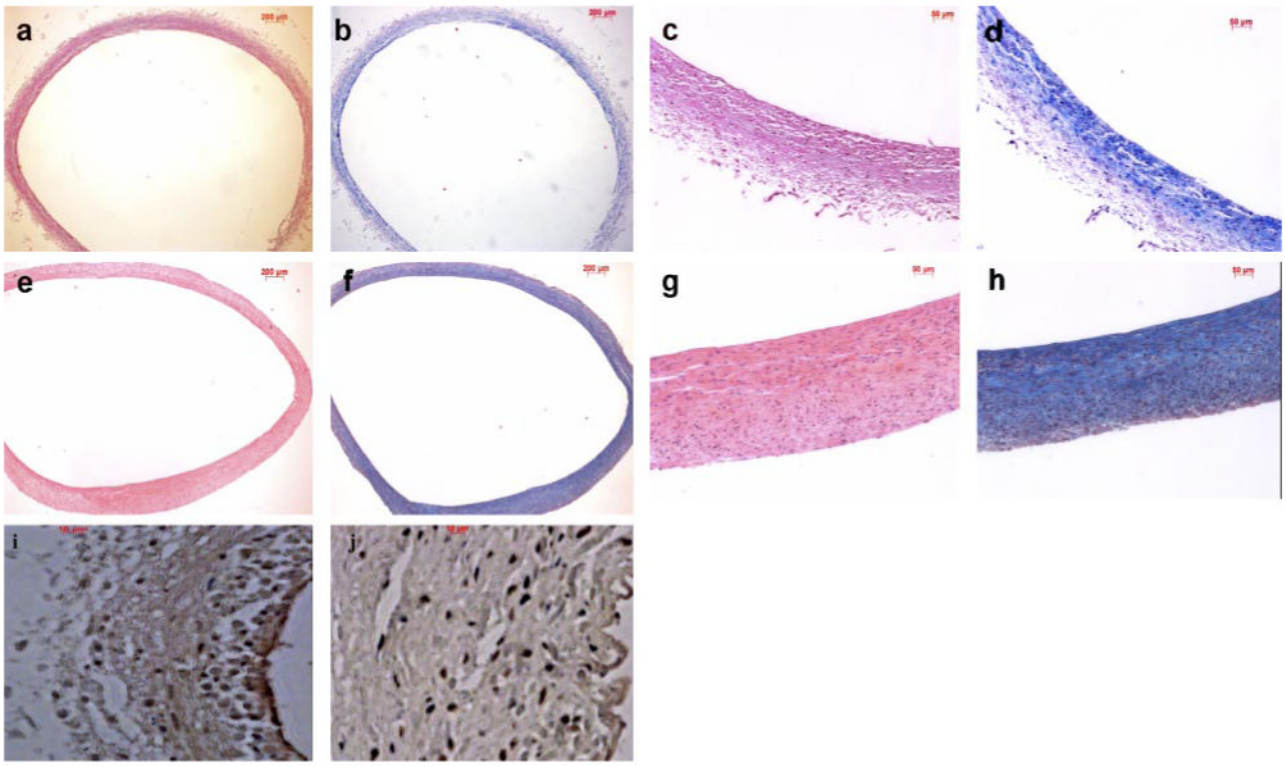

Figure 7.
Representative histological stainings on engineered vessel walls before (*a–d*, *i*) and after (*e–h*, *j*) the optimization of engineering conditions: H&E (*a*, *c*, *e*, *g*), Masson's Trichrome (*b*, *d*, *f*, *h*), PCNA (*i*, *j*). Scale bar is shown in each picture.

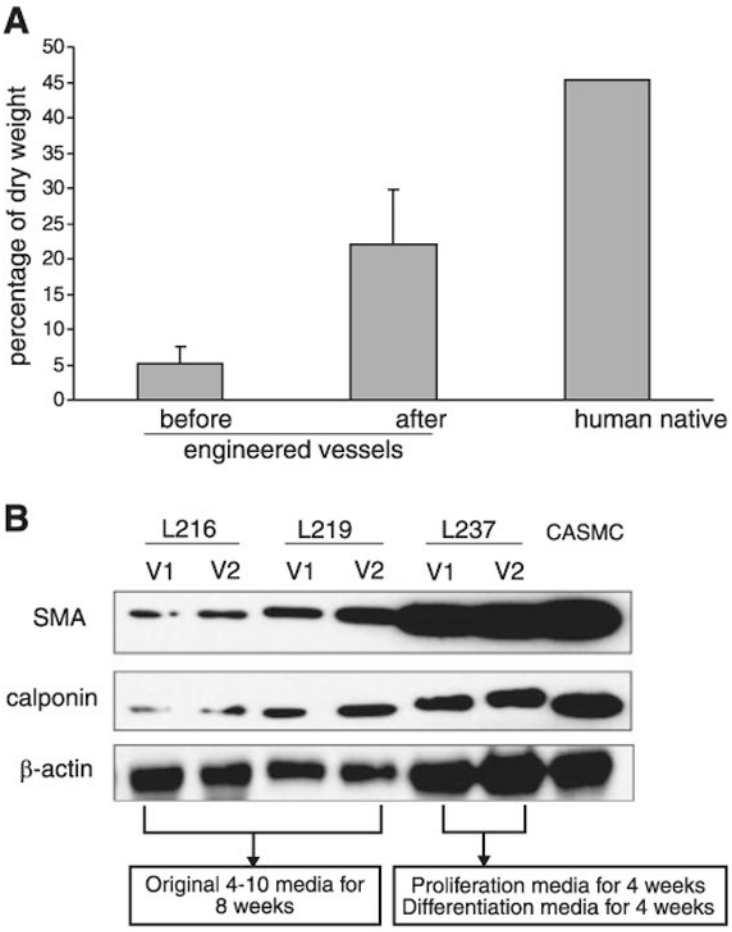

Figure 8.

Collagen and Western blot analysis on engineered vessels. *A*) Collagen assay on the engineered vessel walls before (n=6) and after optimization (n=4). Human umbilical artery served as positive control. *B*) Western blot on protein lysate from six engineered human vessels for SMA and calponin. L216 v1 and v2, L219v1 and v2, and L237 v1 and v2 were the pairs of vessels grown in the same bioreactor from the same donor-derived MSCs. Lysates from CASMCs were used as positive control. β-Actin served as equal loading control.

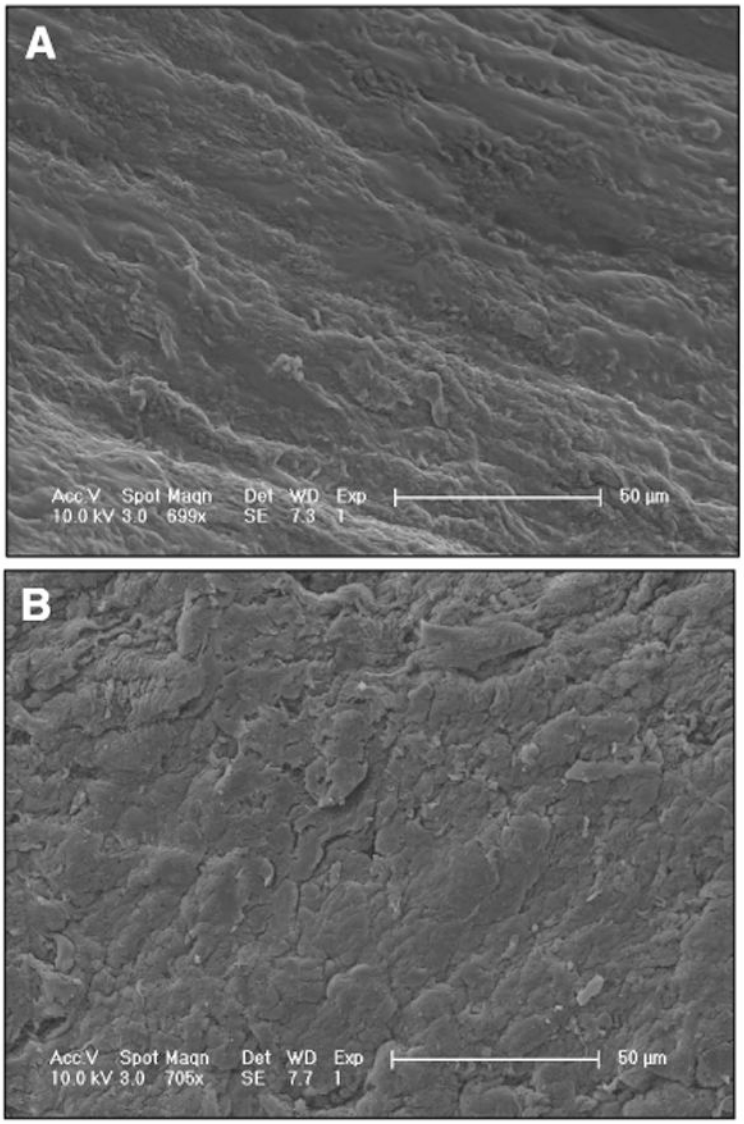

Figure 9.
SEM images of the luminal surface of nonendothelialized (*A*) and endothelialized (*B*) vessels engineered from hMSCs using the optimized protocol. Scale bars = 50 µm.