Functional tissue-engineered blood vessels from bone marrow progenitor cells

Jin Yu Liu [a], Daniel D. Swartz [b], Hao Fan Peng [a], Sylvia F. Gugino [c], James A. Russell [c], Stelios T. Andreadis [a,*]

[a] *Bioengineering Laboratory, 908 Furnas Hall, Department of Chemical and Biological Engineering, University at Buffalo, State University of New York, Amherst, NY 14260, United States*
[b] *Women and Children's Hospital of Buffalo, University at Buffalo, The State University of New York, Buffalo, NY, United States*
[c] *Department of Physiology and Biophysics, University at Buffalo, The State University of New York, Buffalo, NY, United States*

Abstract

Objective: Stem cells have significant potential for development of cell-based therapeutics for cardiovascular tissue regeneration.
Methods: We developed a novel method for isolating smooth muscle cells (SMC) from ovine bone marrow using a tissue-specific promoter and fluorescence-activated cell sorting.
Results: As compared to vascular SMC, bone marrow-derived smooth muscle progenitor cells (BM-SMPC) exhibited similar morphology, showed higher proliferation potential and expressed several SMC markers including α-actin, calponin, myosin heavy chain, smoothelin, caldesmon and SM22. When embedded in fibrin hydrogels, BM-SMPC contracted the matrix and displayed receptor- and non-receptor-mediated contractility, indicating that BM-SMPC can generate force in response to vasoreactive agonists. We also prepared tissue-engineered blood vessels from BM-SMPC and BM-derived endothelial cells and implanted them into the jugular veins of lambs. As early as five weeks post-implantation, grafted tissues displayed a confluent endothelial layer overlaying the medial layer in which BM-SMPC were aligned circumferentially and synthesized significant amounts of collagen. In contrast to previous results with vascular SMC, BM-SMPC synthesized high amounts of elastin that was organized in a fibrillar network very similar to that of native vessels.
Conclusions: Our results suggest that BM-SMPC may be useful in studying SMC differentiation and have high potential for development of cell therapies for the treatment of cardiovascular disease.
© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Stem cells; Bone marrow; Mesenchymal stem cells; Alpha actin promoter; Vascular tissue engineering; Ovine; Blood vessels; Transcriptional activation; Ovine animal model

1. Introduction

Cardiovascular disease is the leading cause of mortality in western countries and around the world increasing the demand for small diameter blood vessels as replacement grafts. Although venous grafts are currently the golden standard, they suffer several major disadvantages: (i) availability may be limited, especially for repeat grafting procedures; (ii) there is pain and discomfort associated with the donor site; (iii) the replicative capacity of cells from older donors is limited [1,2]; and (iv) the ten-year failure rate is high [3]. Tissue engineering can provide an alternative to existing technologies by providing autologous tissue engineered vessels (TEV) for vascular repair and regeneration.

Three major approaches have been proposed for tissue engineering of vascular grafts: (i) decellularized blood vessels;

---

* Corresponding author. Tel.: +1 716 645 2911x2204; fax: +1 716 645 3822.
E-mail address: sandread@eng.buffalo.edu (S.T. Andreadis).

(ii) cell sheet engineering; and (iii) biodegradable scaffolds from natural or synthetic polymers. Scaffolds derived from decellularized blood vessels have been implanted directly or after addition of endothelial and smooth muscle cells to improve patency and vascular reactivity [4–6]. Cell sheet engineering does not employ a scaffold but relies on the ability of the cells to form highly interconnected sheets when grown to high densities. When these sheets were wrapped around a mandrel and cultured for several weeks, they yielded multi-layered cylindrical tissues with high mechanical strength and vascular reactivity [7,8]. Finally, synthetic and natural polymers have been used as scaffolds to support cell growth and provide mechanical support necessary for implantation. Polyglycolic acid (PGA) and co-polymers of PGA with poly-L-lactic acid, polycaprolactone or poly-4-hydroxybutyrate have been used with various degrees of success [9–16]. Natural biomaterials such as collagen and fibrin have also been employed because they can polymerize in the presence of cells and contain inherent biological signals that influence cellular activity [17–19]. Using fibrin hydrogels, we recently demonstrated that fibrin-based small-diameter TEV can be implanted in an ovine animal model [20]. After only two weeks in culture TEV exhibited significant reactivity in response to several vasodilators and vasoconstrictors and developed considerable mechanical strength to withstand interpositional implantation in the jugular veins of lambs, where they remained patent for 15 weeks and displayed significant matrix remodeling.

Despite significant progress toward development of bio-materials and methods to cultivate 3D vascular constructs, cell sourcing remains a major problem. Isolation of smooth muscle and endothelial cells from autologous vessels injures the donor site and may also be limited by the health and age of the patient. Indeed, adult somatic cells were shown to exhibit limited replicative capacity especially when they originated from older donors, the population more likely to suffer from cardiovascular disease [1,2]. Therefore, an autologous source of progenitor vascular cells with high proliferative capacity is necessary to enable isolation and expansion of cells to large numbers necessary for preparation of TEV.

Stem cells have tremendous potential as autologous, non-immunogenic cell source for tissue regeneration. Specifically, adult stem cells from bone marrow provide a promising alternative as they can be isolated from the same patient avoiding immune rejection and long-term immunosuppression. Bone marrow derived stem cells have high proliferation potential, can home into sites of vascular injury where they differentiate into vascular cells [21–25] and can even be allografted to histocompatible receivers [26]. Finally, adult stem cells are not compounded by the ethical considerations of embryonic stem cells and they are readily available for research.

In this communication, we obtained a highly purified population of ovine bone-marrow derived smooth muscle progenitor cells (BM-SMPC) using fluorescence-activated cell sorting (FACS) to separate bone marrow mononuclear cells (BM-MNC) that expressed EGFP under the control of smooth muscle α-actin (SMαA) promoter. These cells exhibited high proliferation potential and expressed early, intermediate and late markers of vascular smooth muscle cells. BM-SMPC were embedded in fibrin hydrogels, which were polymerized around 4-mm diameter mandrels to engineer cylindrical TEV, denoted as BM-TEV. These engineered blood vessels exhibited vascular reactivity in response to KCl and norepinephrine (NE) and mechanical properties that were comparable to those of TEV from vascular smooth muscle cells. Endothelial cells were also isolated from ovine BM-MNC and seeded in the lumen of BM-TEV that were subsequently implanted as interpositional grafts into the jugular veins of lambs. At 5 weeks post-implantation, explanted BM-TEV displayed a confluent endothelial monolayer, circumferential alignment of smooth cells in close proximity to the lumen and remarkable matrix remodeling. Specifically, BM-TEV showed high levels of collagen and fibrillar elastin very similar to native veins. Our results demonstrate that BM-derived progenitor cells can be used to engineer vasoreactive and implantable TEV thus providing a source of highly proliferative, autologous cells for cardiovascular tissue engineering.

2. Materials and methods

2.1. Cloning of SMαA promoter

Rat smooth muscle alpha actin (SMαA) promoter DNA was amplified from rat genomic DNA (Clontech, Mountain View, CA) using high fidelity PCR with forward primer: ACGGTCCTTAAGCATGATAT; and reverse primer: CTTACCCTGATGGCGACTGGCTGG [27]. The PCR re-action was carried out with denaturation for 30 s at 94 °C; annealing for 30 s at 55 °C and extension for 90 s at 72 °C. The PCR product was cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and was subsequently excised with Xho1 and BamH1 and subcloned into the same sites of the promoterless EGFP reporter vector (pEGFP-1, Clontech).

2.2. Isolation of smooth muscle progenitor cells from ovine bone marrow

Bone marrow mononuclear cells (BM-MNC) from a newborn lamb were separated from bone marrow aspirate using histopaque (1.077 g/ml) density-gradient centrifugation (Sigma, St. Louis, MO). BM-MNC cells were cultured in DMEM (Gibco, Grand Island, NY) containing 10% FBS (Gibco) for 3–4 days until they reached 70% confluence. At that time, BM-MNC were transfected with SMαA-EGFP plasmid DNA using lipofectamine (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. Briefly, BM-MNC were washed three times with serum-free, antibiotic-free DMEM. Lipofectamine:DNA (8 μl:2 μg) complex was prepared in 0.2 ml serum-free, antibiotic-free DMEM and incubated at room temperature for 30–45 min. For transfection, 0.8 ml of serum-free DMEM was added into the lipid:DNA solution and overlaid on BM-MNC for 5 h at 37 °C. After incubation, the

transfection mixture was removed and replaced with DMEM containing 10% FBS (Invitrogen Corporation). Then the next day, the culture medium was replenished again and at 72 h post-transfection EGFP-expressing cells were observed by fluorescence microscopy and sorted by FACS. These cells were termed bone marrow derived smooth muscle progenitor cells (BM-SMPC).

Ovine vascular smooth muscle cells (V-SMC) were isolated from umbilical veins of near-term fetal lambs as described previously [20] and served as positive controls. Unsorted BM-MNC served as negative controls.

2.3. Isolation of endothelial cells from ovine bone marrow

BM-MNC were separated from a bone marrow aspirate as described above, seeded onto a 100 mm tissue culture dish coated with 20 ng/ml of human fibronectin (Calbiochem, La Jolla, CA) and cultured in DMEM containing 20% of FBS at 10% CO_2, 37 °C. The next day, non-adherent cells were transferred onto a new fibronectin-coated plate and cultured in the same medium for 24 h before the non-adherent fraction was transferred again to a third fibronectin-coated plate. The adherent cells were cultured for another 10–14 days. At that time, individual colonies containing cells that displayed cobblestone morphology were isolated using trypsin-soaked cloning disks (Scienceware, Santa Ana, CA), and each one was transferred into one well of a 6-well plate each in the same medium. The next day the medium was replaced with serum-free, basal endothelial growth medium (EGM) supplemented with 10 μg/ml of human plasma fibronectin (Calbiochem), 10 ng/ml of epidermal growth factor (EGF; BD Biosciences, Bedford, MA) and 20 ng/ml of human basic fibroblast growth factor (bFGF; BD, Biosciences). The cells stained positive for DiI-Ac-LDL, CD31, CD144 and vWF (see Histology and immunohistochemistry section below). These cells were termed BM-EC and used for experiments between 4 and 6 passages.

2.4. Reverse transcription polymerase chain reaction

RT-PCR was conducted with RNA from BM-SMPC and V-SMC using primers and conditions as described previously [28]. For smoothelin the primers were taken from [29]. The PCR products were subjected to electrophoresis on 2% agarose gels. To ensure that there was no DNA contamination of mRNA, PCR was performed using mRNA as template (no reverse transcription) and no product was detected with any of the primer sets. Each PCR reaction included a sample in which H_2O was used as template (negative control) and a sample with cDNA from V-SMC (positive control). Each PCR reaction was conducted at least twice with RNA from two independent experiments.

2.5. Histology and immunohistochemistry

Histology and immunostaining were performed as described previously [30–32] using the following antibodies

in PBS containing 1% BSA and 0.01% triton X-100: mouse monoclonal anti-human smooth muscle actin (1:100 dilution; SeroTec, Oxford, UK); anti-human smooth muscle calponin (1:100 dilution; DakoCytomation, Carpinteria, CA); mouse anti-ovine FITC-conjugated CD31 (1:10 dilution; Serotec), rabbit anti-human FITC-conjugated
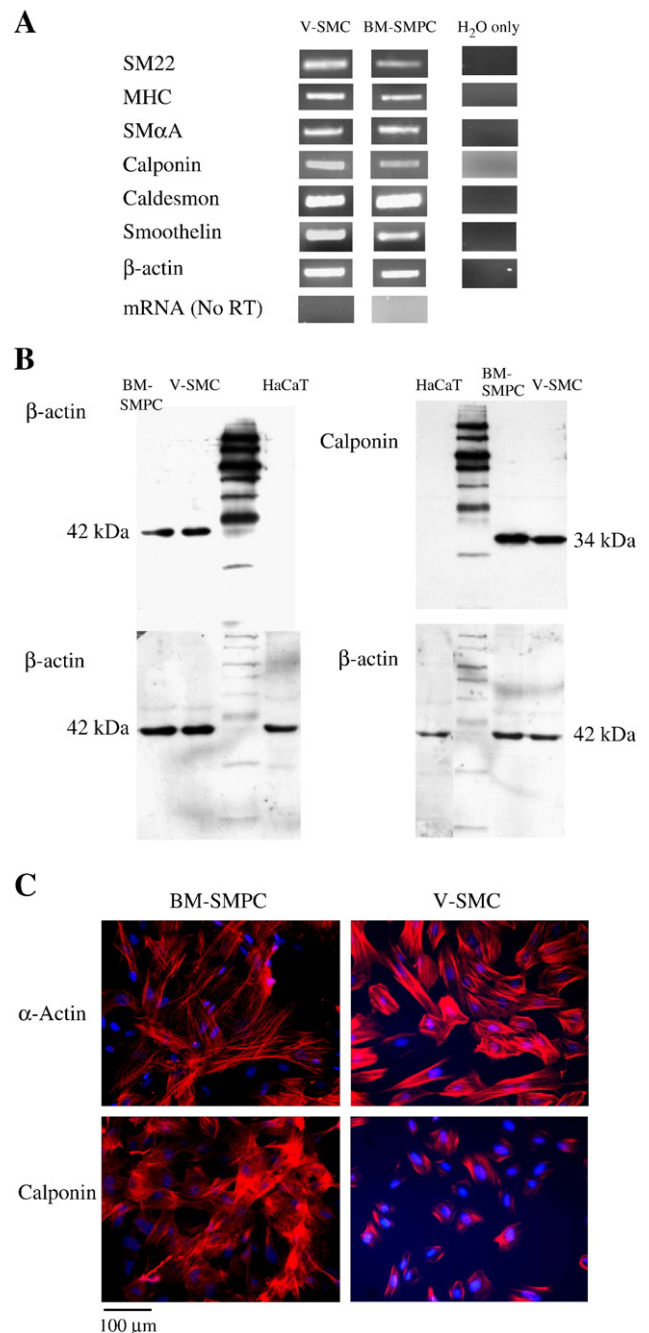

Fig. 1. BM-SMPC express biochemical markers of smooth muscle cells. (A) RT-PCR showed that BM-SMPC expressed smooth muscle cell markers as indicated. (B) Western blot for smooth muscle α-actin and calponin; beta-actin served as loading control. (C) Immunocytochemistry for smooth muscle α-actin and calponin. V-SMC from umbilical veins of near-term lambs were used as positive control. Representative results from three independent experiments are shown.
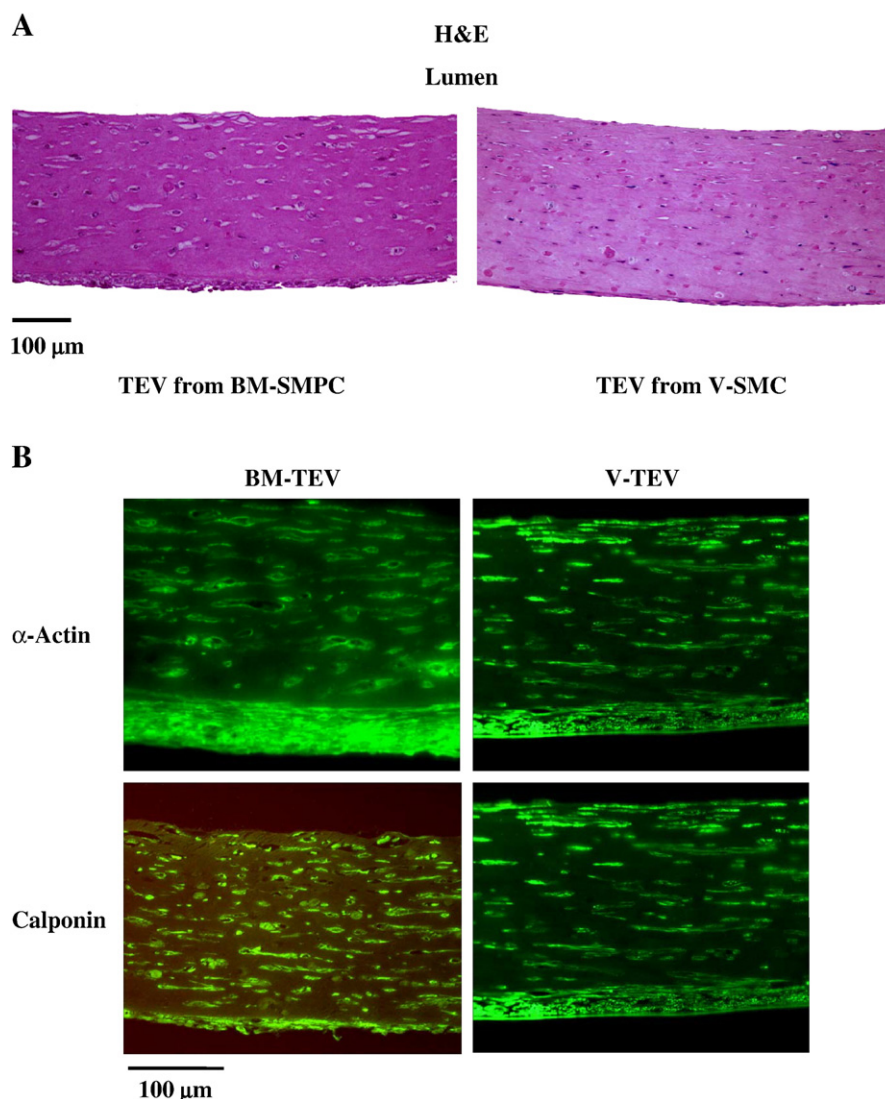
Fig. 2. BM-TEV displayed similar morphologic and biochemical characteristics as TEVs from V-SMC. BM-SMPC or V-SMC were embedded in fibrin hydrogels and cultured around 4-mm mandrel for 2 weeks to form cylindrical tubes. (A) Hematoxylin and eosin (H&E) staining showed that BM-SMPC were distributed uniformly in fibrin hydrogels (bar=100 μm). (B) Immunostaining of BM-TEV and TEV from V-SMC for smooth muscle α-actin and calponin (bar=100 μm).

CD144 (1:10 dilution; Serotec), polyclonal rabbit anti-human von Willebrand Factor (1:10 dilution; DakoCytomation). Secondary antibodies goat anti-mouse IgG (1:100 dilution; Molecular Probes) were conjugated with Alexa Fluor488 or Alexa Fluor594. No fluorescence was observed when cells or tissues were stained with secondary antibody only (no primary antibody; negative control). Finally, ovine vascular smooth muscle or endothelial cells from umbilical veins of near-term fetal lambs were isolated as described previously [20] and served as positive controls for BM-SMPC and BM-EC, respectively.

2.6. Western blots

Western blots were performed as described previously [33,34] using the following antibodies: mouse anti human smooth muscle α-actin (1:100 dilution; SeroTec) and mouse anti-human calponin (1:100 dilution; DakoCytomation) in TBS-Tween. After incubation with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000 dilution; Cell Signaling Technology, Danvers, MA) the protein bands were detected using chemiluminescence (LumiGLO; KPL, Gaithersburg, Maryland) as per manufacturer's instructions. Western blots using BM-TEV lysates were performed using the same protocol.

2.7. Tissue engineering of small diameter blood vessels

TEV from BM-SMPC were prepared as described previously [20,31]. Briefly, BM-SMPC were suspended in thrombin in the presence of calcium. The thrombin
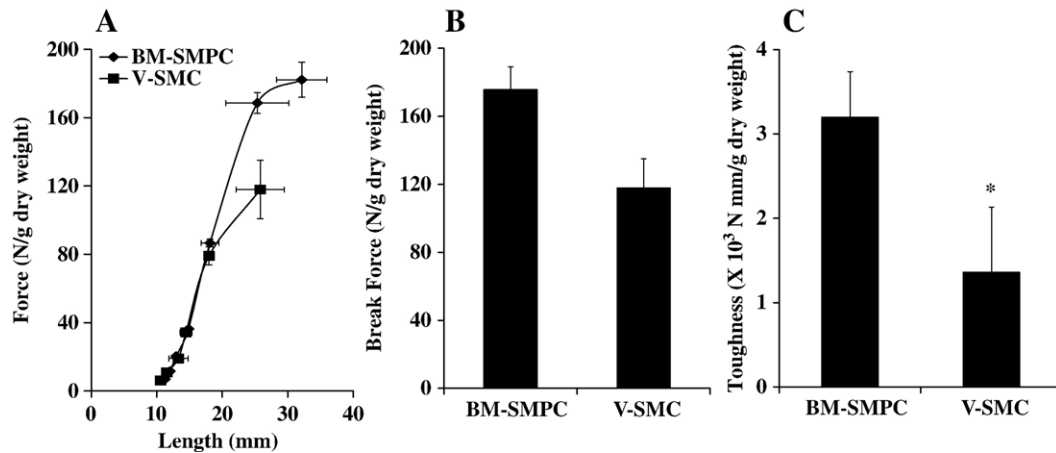
Fig. 3. BM-TEVs displayed similar mechanical properties as TEV from V-SMC. TEV from BM-SMPC or V-SMC were cultured around 4-mm mandrels for 2 weeks. Mechanical strength was measured using an isolated tissue bath system. (A) Force-length curve; (B) Break force; (C) Toughness. All values are the mean±SD of triplicate samples in a representative experiment ($n=3$). The symbol (*) indicates statistical significance ($p<0.05$).

containing BM-SMPC was mixed with fibrinogen at a ratio of 1:4, poured into a plastic mold (1 cm in diameter; 6 cm in length) surrounding a silastic tube with diameter of 4.0 mm and polymerized within 5–10 s. The final concentration of each component was: 2.5 mg/ml fibrinogen, 2.5 mM calcium and 2.5 U/ml of thrombin. The final cell density of BM-SMPC was 1×10^6 cells/ml gel. After 1 h incubation in a CO_2 incubator, BM-TEV were detached from the walls, removed from the plastic tube and transferred into a 50 ml conical tube, where they were incubated in 40 ml of DMEM medium containing 25 mM Hepes, 20% FBS and 300 μM ascorbic acid phosphate. The next day the medium was supplemented with 2 μg/ml insulin, 5 ng/ml TGF-β1 and 20 μg/ml aprotinin. Thereafter, cell culture medium was replenished every three days. After two weeks in culture the cylindrical tissues were removed from the mandrel and BM-EC were seeded in the lumen at 6×10^5 cells/cm² of luminal area as described previously [20]. The cells were allowed to adhere for 4 h under continuous rotation to ensure uniform seeding. Subsequently, the tissues were cultured in M199 containing 20% FBS for another 10 days before implantation.

2.8. Vasoreactivity and mechanical strength of BM-TEV

After two weeks in culture BM-TEV were released from the mandrel and cut in 3–4 mm segments, mounted on two stainless hooks in an isolated tissue bath and incubated in Krebs–Ringer solution. The tissues were continuously bubbled with 94% O_2, 6% CO_2 to obtain pH of 7.4, P_{CO_2} of 38 mm Hg, and $P_{O_2}>500$ mm Hg at 37 °C. Each construct was mounted on stainless steel hooks through the lumen, one was fixed, and the other one was connected to a force transducer. Tissues were equilibrated at a basal tension of 1.0 g and constant length for 30–60 min. After equilibration, potassium chloride (KCl, 118 mM) or norepinephrine (NE, 10^{-6} M) was added to the tissue bath and isometric contraction was recorded by a PowerLab data acquisition unit

and analyzed by Chart5 software (ADInstruments, Colorado Springs, CO).

Tissue segments were mounted on the force transducer and stretched incrementally until they broke, yielding the break tension and break length of the tissue. The initial tissue length corresponds to the length under a passive tension of 1.0 g. Broken constructs were dehydrated with series of ethanol washes, air dried and weighted. The force was normalized by the dry weight of each construct and expressed in units of Newton per gram of dry tissue weight (N/g dry weight). Linear modulus was calculated as the slope of the linear part of the length–tension curve. Toughness was calculated by numerically integrating the area under the length–tension curve after fitting the curve by the method of least squares, using Maple 9.0 software (Waterloo Maple, Waterloo, ON, Canada).
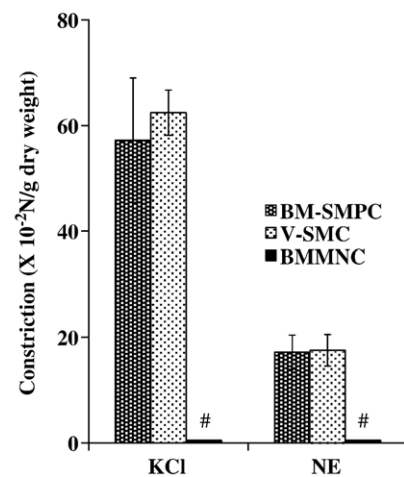

Fig. 4. BM-SMPC displayed significant contractility. TEV from BM-SMPC, BM-MNC or V-SMC were cultured around 4-mm mandrels for 2 weeks. Vascular reactivity was measured in response to KCl (118 mM) or NE (10^{-6} M). All values are the mean±SD of triplicate samples in a representative experiment ($n=3$). The symbol (#) indicates very small value close to zero for BM-MNC.
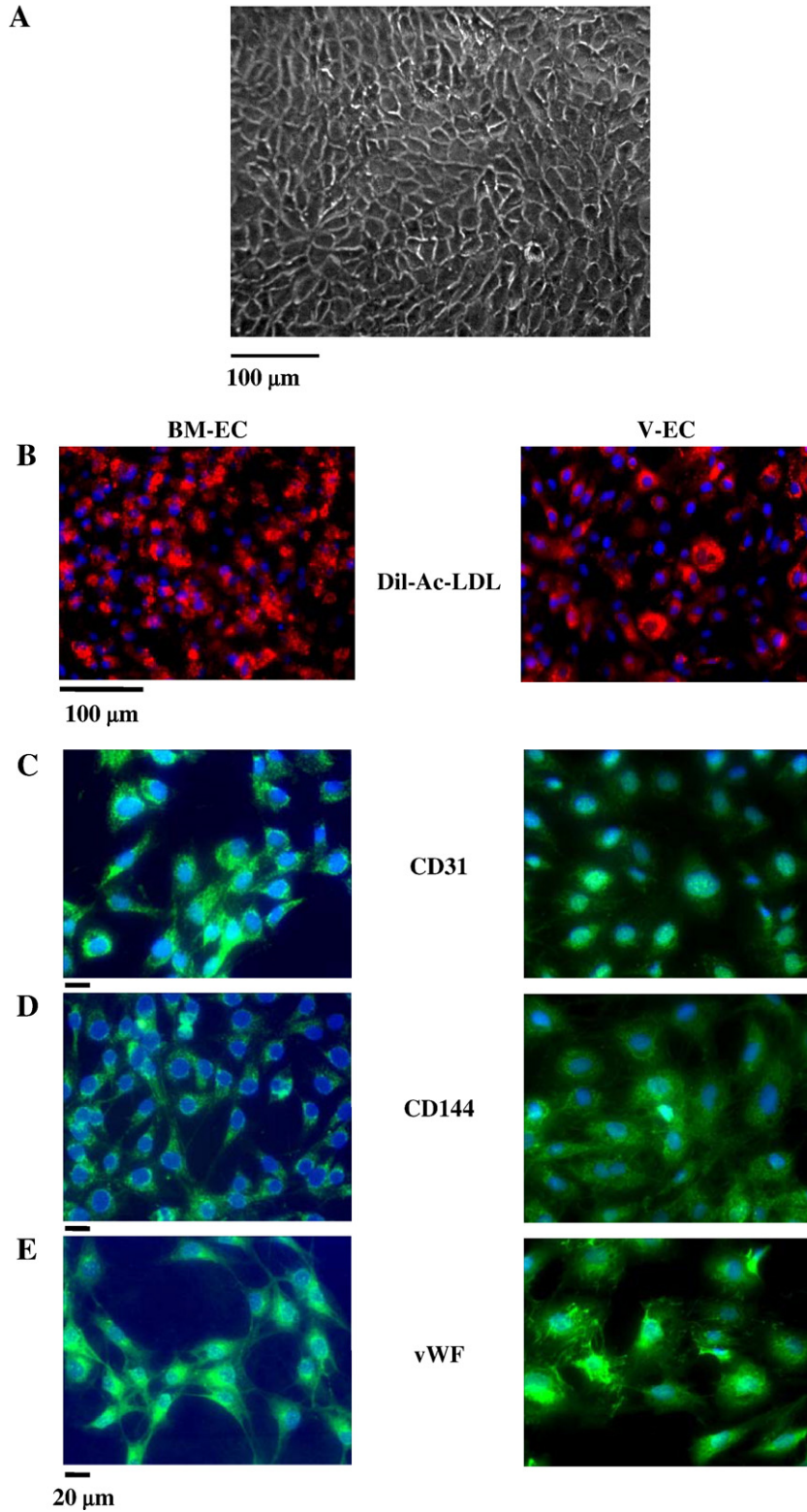
Fig. 5. BM-EC displayed morphologic and biochemical characteristics of V-EC. BM-EC were isolated from bone marrow and cultured in endothelial growth medium (EGM) supplemented with EGF, bFGF and fibronectin. (A) BM-EC displayed cobblestone morphology and formed well-organized confluent monolayers (bar=100 μm). (B) BM-EC stained positive for Dil-Ac-LDL (bar=100 μm). Immunocytochemistry showed strong staining of BM-EC for (C) CD31; (D) CD144; and (E) vWF. V-EC from umbilical veins of near-term lambs were also stained and served as positive control (bar=20 μm).
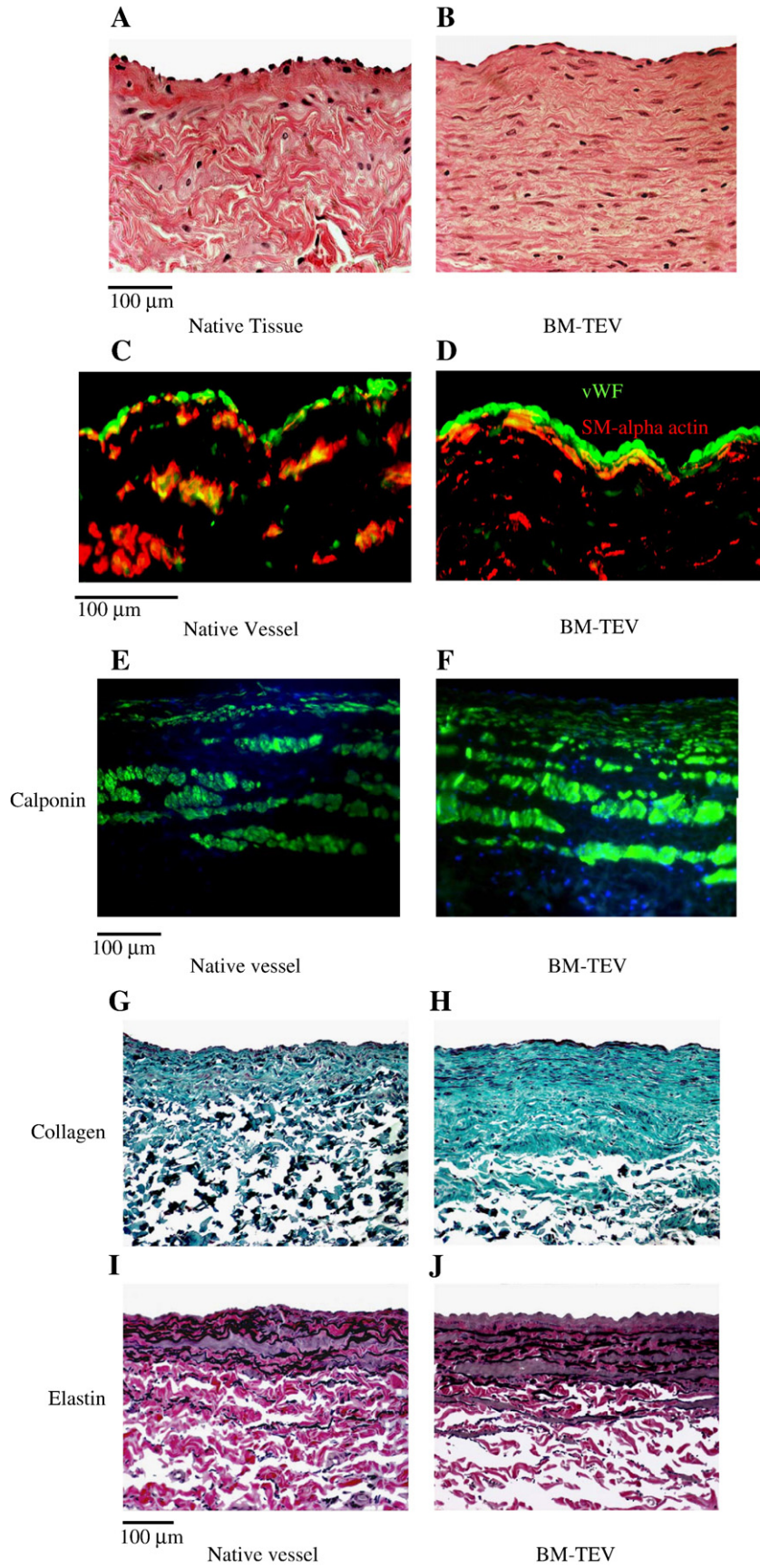
Toughness was expressed in units of (Newton × millimeters per gram tissue weight) (N mm/g dry weight).

2.9. BM-TEV implantation

BM-TEV were implanted into the jugular vein of 8-week-old lambs as we described previously [20]. Briefly, 8-week-old dorset cross castrate males (∼25 kg) were fasted 24 h prior to surgery. Anesthesia was induced with sodium pentathol (50 mg/animal) and maintained with 1.5–2.0% isoflurane through a 6.0 mm endotracheal tube using a positive pressure ventilator and 100% oxygen. The left external jugular vein was exposed through a longitudinal 8 cm incision. After tying small collateral vessels, 3000 units of heparin sulfate were administered and the proximal and distal ends of the implantation site were clamped.

The external jugular vein was transected and a 1.5–2.0 cm segment of the TEV was sutured into place using continuous running 8–0 proline cardiovascular double armed monofilament suture (Ethicon, Johnson and Johnson, Somerville, NJ). The vascular clamp was slowly removed and flow was resumed through the TEV graft. A radiopaque tie was loosely secured at the caudal end of the TEV to mark the location of the graft. The incision was closed using 2–0 vicryl in layers (facia and skin). The animal was recovered and monitored daily for adverse affects. At 5 weeks post-grafting the animals were euthanized using 10 ml concentrated sodium barbiturate (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI). TEV grafts were removed along with intact caudal and cephalic native vessel. Tissue segments were processed for histology and immunohistochemistry. All procedures and protocols in this study were approved by the Laboratory Animal Care Committee of the State University of New York at Buffalo. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.10. Statistical analysis

Data were expressed as mean±standard deviation and statistical significance (defined as $p < 0.05$) was determined using Student's *t*-test.

3. Results

3.1. Isolation of functional smooth muscle cells from bone marrow

To ensure isolation of smooth muscle cells from bone marrow mononuclear cells with no contamination from any other cell type, we propose a novel method that is based on expression of green fluorescence protein (EGFP) from the smooth muscle α-actin (SMαA) promoter. The rat SMαA promoter (Accession Number S76011) was PCR-amplified from rat genomic DNA and cloned into a promoterless vector encoding for EGFP. Bone marrow was harvested from newborn lambs and mononuclear cells were isolated by density gradient centrifugation using histopaque and grown in DMEM medium with 10% FBS. Non-adherent cells were discarded, adherent mononuclear cells were transfected with the SMαA-EGFP plasmid and EGFP + cells (<1% of total cells) were subsequently sorted using fluorescence activated cell sorting.

The sorted cells, termed bone marrow derived smooth muscle progenitor cells (BM-SMPC) were examined for expression of smooth muscle cell markers using RT-PCR. We found that BM-SMPC expressed alpha-actin, calponin, myosin heavy chain, smoothelin, SM22 and caldesmon to a similar extent as V-SMC (Fig. 1A). Western blots (Fig. 1B) and immunostaining (Fig. 1C) confirmed strong expression of smooth muscle α-actin and calponin by BM-SMPC and V-SMC. As expected, a human epidermal cell line (HaCaT) expressed neither protein (Fig. 1B).

Notably, BM-SMPC could be sub-cultured repeatedly with no apparent loss of proliferative potential even after 12 passages. In contrast, mature V-SMC terminally differentiated and stopped proliferating after 5–6 passages, suggesting that BM-SMPC have a higher proliferation potential and may be a better cell source for cardiovascular tissue engineering.

3.2. BM-SMPC generated force and compacted fibrin hydrogels

Next we examined the ability of BM-SMPC to generate force and compact three dimensional fibrin matrix. To this end, BM-SMPC (10^6 cells/ml) were embedded in fibrin hydrogels that were polymerized around 4-mm mandrels to prepare cylindrical tissue equivalents termed bone marrow based tissue engineered blood vessels (BM-TEV). BM-TEV were prepared with 2.5 mg/ml fibrinogen (FBG) and 2.5 U/ml thrombin and were cultured in the presence of insulin, TGF-β1 and aprotinin, a combination that was previously shown to promote mechanical strength and vascular reactivity of TEV generated from V-SMC [31,35].

Similar to V-SMC, BM-SMPC compacted fibrin hydrogels to approximately 5% of their original volume within 3 days in culture, indicating that these cells had developed the ability to generate force. After 2 weeks in culture, compacted tissues with wall thickness of approximately 0.5 mm were removed from the mandrel and processed for histology and

Fig. 6. Explanted TEVs exhibited similar morphology and deposition of ECM as native jugular vein. TEVs from BM-SMPC were implanted in the jugular vein of 8-week old lambs (*n*=3). (A, B) H&E of native vein and explanted BM-TEV show that BM-TEV contained multiple layers of smooth muscle cells that were overlaid by continuous monolayer of endothelial cells. (C, D) Immunostaining for vWF (green) and smooth muscle α-actin (red). (E, F) Immunostaining for calponin (green) and counterstaining of cell nuclei with Hoechst (blue). (G, H) Mason's trichrome stain showed abundant collagen (blue/green) in both native jugular vein and explanted BM-TEV. (I, J) Verhoff's elastin stain showed abundant expression and fibrillar organization of elastin (black lines) in both native tissue and explanted BM-TEV. Luminal surface is at the top of each panel and blood flows in the direction that crosses the plane of the page (bar=100 μm).

immunohistochemistry. Similar to V-SMC, BM-SMPC distributed uniformly in the fibrin hydrogel (Fig. 2A) and stained positive for smooth muscle α-actin and calponin (Fig. 2B).

We also measured the mechanical strength of the resulting TEV using an isolated tissue bath system. After 2 weeks in culture BM-TEV exhibited higher break force and toughness (integral under the force-length curve representing the energy required to break the tissue segment) as compared to TEV from V-SMC (Fig. 3A–C).

3.3. BM-SMPC displayed significant contractility

The defining property of mature SMC is their ability to contract and generate force in response to vasoactive agonists. To examine whether BM-SMPC exhibited functional properties of mature V-SMC, we used an isolated tissue bath to measure the isometric tension generated by segments of cylindrical BM-TEV that were cultured for two weeks. Both tissues showed active pathways of receptor and non-receptor mediated vascular reactivity. Specifically, BM-TEV exhibited vasoconstriction in response to KCl (118 mM) or NE (10^{-6} M) to the same extent as TEV from V-SMC (Fig. 4). In contrast, TEV generated from unsorted BM-MNC showed no reactivity in response to KCl or NE (#), suggesting that only a small fraction of the starting BM-MNC population – those with active SMαA promoter – exhibited functional properties of mature SMC.

3.4. BM-TEV exhibited high potential for tissue remodeling in vivo

Next we hypothesized that BM-TEV may show improved ability for remodeling after implantation in vivo. To address this hypothesis, we prepared BM-TEV containing BM-SMPC in the cylindrical wall and bone marrow-derived endothelial cells (BM-EC) in the lumen. To this end, endothelial cells were isolated from bone marrow based on differential adhesion of BM-MNC on fibronectin as published previously [36–38]. When grown to confluence in EGM medium supplemented with fibronectin, EGF and bFGF BM-EC exhibited cobblestone morphology (Fig. 5A) and stained positively for DiI-Ac-LDL (100%), CD31 (88%), CD144 (85%) and vWF (85%) (Fig. 5B–E). As expected, BM-SMPC did not stain for any of these proteins.

After two weeks in culture the BM-SMPC containing tissues were removed from the mandrels, BM-EC were seeded in the lumen as we described previously [20] and cultured for 10 more days before interpositional implantation into the jugular veins of 8-week-old lambs (*n*=3). At 5 weeks post-implantation, BM-TEV were removed from the animals and processed for histology. Hematoxylin-eosin stain showed that, similar to native jugular vein, explanted BM-TEV contained multiple layers of SMC which were overlaid by a continuous monolayer of endothelial cells (Fig. 6A, B). Immunostaining showed that the intimal cells were stained for

vWF (Fig. 6C, D), while cells in the medial layer stained for smooth muscle α-actin (Fig. 6C, D) and calponin (Fig. 6E, F). Since transfection results in transient transgene expression, BM-SMPC were not EGFP+ at the time of implantation, and therefore, there was no interference of EGFP fluorescence with immunostaining. BM-SMPC in close proximity to the lumen assumed circumferential orientation and produced high amount of collagen matrix throughout the tissue (Fig. 6G, H). Most important, implanted tissues contained significant amount of elastin that displayed fibrillar organization similar to the adjacent native tissue (Fig. 6I, J). In contrast, TEV from mature V-SMC exhibited very low amount of elastin and no fiber organization, even after 15 weeks in vivo [20], indicating that BM-SMPC may have higher elastogenic potential than mature V-SMC.

4. Discussion

Lack of availability of autologous vascular grafts and the pain and discomfort associated with the donor site necessitate the development of tissue engineered blood vessels for tissue regeneration. Here we demonstrated a novel method for isolation of BM-SMPC using a tissue specific promoter, SMαA, driving expression of EGFP. BM-SMPC showed high proliferative potential and displayed morphological and phenotypic properties of V-SMC as shown by expression of smooth muscle markers such as α-actin, calponin, MHC, caldesmon, SM22 and smoothelin. Notably, BM-SMPC displayed contractile properties suggesting that these cells had developed a functional mature SMC phenotype. Most important, when TEV engineered from BM-SMPC and BM-EC were implanted into the jugular veins of lambs they demonstrated remarkable ability for matrix remodeling as evidenced by production of collagen and elastin fibers.

Several animal studies suggested that bone marrow progenitor cells can infiltrate the atherosclerotic intima and differentiate to smooth muscle and endothelial cells within the atherosclerotic plaque [23–25,39]. Further support came from studies which showed that smooth muscle cells from sex-mismatched [40] or β-galactosidase-expressing [22] bone marrow transplants were recruited to a much larger extent to diseased as compared to healthy blood vessels. Collectively, these studies suggested that there are smooth muscle progenitor cells in the bone marrow and peripheral blood that could provide an autologous cell source for cardiovascular tissue engineering.

Based on these in vivo studies several investigators attempted to culture smooth muscle cells from bone marrow mononuclear cells by stimulation with cytokines and growth factors such as PDGF-BB or TGF-β1 [41–43]. Although soluble factors in the medium can direct differentiation of a fraction of cells toward the SMC lineage, these approaches have not demonstrated isolation of a pure population of functional, contractile SMC. One study used a SM22 promoter to select for SMC from bone marrow mononuclear cells [44]. Interestingly, cells with an active SM22 promoter

expressed neither immature nor mature SMC markers. Only after G418 selection for 25 days, clones of cells that expressed SMC markers were identified, suggesting that merely a fraction of cells with active SM22 promoter expressed SMC markers. In addition, functional properties of these cells such as force generation or contractility were not investigated and therefore, it was not clear whether these cells had developed functional characteristics of mature SMC. In contrast, our results show that using a SMαA promoter and fluorescence activated cell sorting we selected a highly proliferative cell population that displayed morphologic and biochemical characteristics of SMC. Most important, BM-SMPC displayed contractility, as demonstrated by compaction of fibrin hydrogels and measurements of vascular reactivity.

Interestingly, TEV from BM-SMPC showed superior mechanical properties as compared to TEV from V-SMC. Recent studies showed that vessels cultured from infant cells exhibited higher levels of proliferation, extracellular matrix deposition and enhanced physical properties as compared to vessels cultured from adult cells [45]. In addition, TEV engineered from cells originating from older donors exhibited inferior mechanical properties that prohibited implantation. Interestingly, both cell proliferation and mechanical properties of TEV were improved by expression of telomerase, an enzyme that extents cellular lifespan [1,46]. In combination with these studies, our results may suggest that the improved mechanical properties of BM-TEV may be due to functional differences between smooth muscle progenitor cells from bone marrow and mature V-SMC. As with development of other engineered tissues [33,47], high throughput methods of global gene expression profiling may prove useful in identifying the molecular basis of these functional differences.

Contractility is the defining property of mature SMC and one of the most important properties of blood vessels. When BM-SMPC were embedded in fibrin hydrogels they compacted the gels to approximately 5% of their original volume within 3 days in culture. Most important, BM-TEV displayed vascular reactivity in response to vasoconstrictors such as KCl and NE. Since KCl causes contraction by opening the L-type, slow calcium potential-dependent channels, while NE acts through α1 and α2 receptors, our results demonstrate that BM-SMPC had developed both receptor and non-receptor mediated pathways of vascular reactivity. In contrast, TEV prepared from unsorted BM-MNC displayed no contractility, indicating that bone marrow contains only a small fraction of functional SMC, which retain their biochemical and contractile properties after purification and expansion in vitro.

Functional BM-SMPC and BM-EC were used to engineer small diameter blood vessels that were implanted into the jugular veins of lambs. Histology showed that the morphology and cellular organization of the explanted BM-TEV was very similar to that of native tissues. The SMC close to the lumen were circumferentially aligned and displayed highly organized fibers of α-actin. The endothelial monolayer in the lumen appeared to be confluent and expressed vWF. Cells in the medial layer also expressed calponin, a smooth muscle specific marker that is not expressed by fibroblasts [48], suggesting that these cells were BM-SMPC that were embedded in the fibrin hydrogel and not infiltrating fibroblasts from the host. In addition, BM-SMPC remodeled fibrin and expressed high amounts of collagen throughout the medial layer. Most notably, BM-SMPC expressed significant amount of highly organized elastin fibers, very similar to the native tissue. In contrast, TEV from V-SMC expressed significantly smaller quantities of elastin even at 15 weeks post-implantation [20], suggesting that BM-SMPC may be better able to remodel the implanted tissues and contribute to their long-term function and mechanical stability. BM-SMPC may also serve as a model system to study SMC differentiation and understand the molecular attributes that may be responsible for their enhanced growth and elastogenic potential.

Acknowledgement

References

[1] Poh M, Boyer M, Solan A, Dahl SL, Pedrotty D, Banik SS, et al. Blood vessels engineered from human cells. Lancet 2005;365:2122–4.

[2] McKee JA, Banik SS, Boyer MJ, Hamad NM, Lawson JH, Niklason LE, et al. Human arteries engineered in vitro. EMBO Rep 2003;4:633–8.

[3] Gaudino M, Cellini C, Pragliola C, Trani C, Burzotta F, Schiavoni G, et al. Arterial versus venous bypass grafts in patients with in-stent restenosis. Circulation 2005;112:I265–9.

[4] Huynh T, Abraham G, Murray J, Brockbank K, Hagen PO, Sullivan S. Remodeling of an acellular collagen graft into a physiologically responsive neovessel. Nat Biotechnol 1999;17:1083–6.

[5] Bader A, Steinhoff G, Strobl K, Schilling T, Brandes G, Mertsching H, et al. Engineering of human vascular aortic tissue based on a xenogeneic starter matrix. Transplantation 2000;70:7–14.

[6] Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. [comment]. Nat Med 2001;7:1035–40.

[7] L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. [comment]. FASEB J 1998;12:47–56.

[8] L'Heureux N, Stoclet JC, Auger FA, Lagaud GJ, Germain L, Andriantsitohaina R. A human tissue-engineered vascular media: a new model for pharmacological studies of contractile responses. FASEB J 2001;15:515–24.

[9] Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, et al. Functional arteries grown in vitro. Science 1999;284:489–93.

[10] Niklason LE, Abbott W, Gao J, Klagges B, Hirschi KK, Ulubayram K, et al. Morphologic and mechanical characteristics of engineered bovine arteries. J Vasc Surg 2001;33:628–38.

[11] Kim BS, Nikolovski J, Bonadio J, Smiley E, Mooney DJ. Engineered smooth muscle tissues: regulating cell phenotype with the scaffold. Exp Cell Res 1999;251:318–28.

[12] Shin'oka T, Imai Y, Ikada Y. Transplantation of a tissue-engineered pulmonary artery. N Engl J Med 2001;344:532–3.

[13] Watanabe M, Shin'oka T, Tohyama S, Hibino N, Konuma T, Matsumura G, et al. Tissue-engineered vascular autograft: inferior vena cava replacement in a dog model. Tissue Eng 2001;7:429–39.

[14] Lee SH, Kim BS, Kim SH, Choi SW, Jeong SI, Kwon IK, et al. Elastic biodegradable poly(glycolide-co-caprolactone) scaffold for tissue engineering. J Biomed Mater Res A 2003;66:29–37.

[15] Hoerstrup SP, Kadner A, Breymann C, Maurus CF, Guenter CI, Sodian R, et al. Living, autologous pulmonary artery conduits tissue engineered from human umbilical cord cells. Ann Thorac Surg 2002;74:46–52 [discussion].

[16] Wake MC, Gupta PK, Mikos AG. Fabrication of pliable biodegradable polymer foams to engineer soft tissues. Cell Transplant 1996;5: 465–73.

[17] Barocas VH, Girton TS, Tranquillo RT. Engineered alignment in media equivalents: magnetic prealignment and mandrel compaction. J Biomech Eng 1998;120:660–6.

[18] Seliktar D, Nerem RM, Galis ZS. Mechanical Strain-Stimulated Remodeling of Tissue-Engineered Blood Vessel Constructs. Tissue Eng 2003;9:657–66.

[19] Stegemann JP, Nerem RM. Altered response of vascular smooth muscle cells to exogenous biochemical stimulation in two- and three-dimensional culture. Exp Cell Res 2003;283:146–55.

[20] Swartz DD, Russell JA, Andreadis ST. Engineering of fibrin-based functional and implantable small-diameter blood vessels. Am J Physiol Heart Circ Physiol 2005;288:H1451–60 [Electronic publication 2004 Oct 14].

[21] Galmiche MC, Koteliansky VE, Briere J, Herve P, Charbord P. Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. Blood 1993;82:66–76.

[22] Shimizu K, Sugiyama S, Aikawa M, Fukumoto Y, Rabkin E, Libby P, et al. Host bone-marrow cells are a source of donor intimal smooth-muscle-like cells in murine aortic transplant arteriopathy. Nat Med 2001;7:738–41.

[23] Hillebrands JL, Klatter FA, van den Hurk BM, Popa ER, Nieuwenhuis P, Rozing J. Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. J Clin Invest 2001;107:1411–22.

[24] Han CI, Campbell GR, Campbell JH. Circulating bone marrow cells can contribute to neointimal formation. J Vasc Res 2001;38:113–9.

[25] Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 2002;8:403–9.

[26] Liechty KW, MacKenzie TC, Shaaban AF, Radu A, Moseley AM, Deans R, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med 2000;6:1282–6.

[27] Hu B, Wu Z, Phan SH. Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. Am J Respir Cell Mol Biol 2003;29:397–404.

[28] Rodriguez LV, Alfonso Z, Zhang R, Leung J, Wu B, Ignarro LJ. Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. Proc Natl Acad Sci U S A 2006;103:12167–72.

[29] Niu Y, Xu Y, Zhang J, Bai J, Yang H, Ma T. Proliferation and differentiation of prostatic stromal cells. BJU Int 2001;87:386–93.

[30] Geer DJ, Swartz DD, Andreadis ST. In vivo model of wound healing based on transplanted tissue-engineered skin. Tissue Eng 2004;10: 1006–17.

[31] Yao L, Swartz DD, Gugino SF, Russell JA, Andreadis ST. Fibrin-based tissue-engineered blood vessels: differential effects of biomaterial and culture parameters on mechanical strength and vascular reactivity. Tissue Eng 2005;11:991–1003.

[32] Geer DJ, Swartz DD, Andreadis ST. Biomimetic delivery of keratinocyte growth factor upon cellular demand for accelerated wound healing in vitro and in vivo. Am J Pathol 2005;167:1575–86.

[33] Koria P, Andreadis ST. Epidermal morphogenesis: the transcriptional program of human keratinocytes during stratification. J Invest Dermatol 2006;126:1834–41.

[34] Singh R, Andreadis ST. EGFR activation decreases retroviral gene transfer through protein kinase C-delta. Mol Ther 2006;15:15(2):369–77.

[35] Neidert MR, Lee ES, Oegema TR, Tranquillo RT. Enhanced fibrin remodeling in vitro with TGF-beta1, insulin and plasmin for improved tissue-equivalents. Biomaterials 2002;23:3717–31.

[36] Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92: 362–7.

[37] Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952–8.

[38] Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001;7:1035–40.

[39] Saiura A, Sata M, Hirata Y, Nagai R, Makuuchi M. Circulating smooth muscle progenitor cells contribute to atherosclerosis. Nat Med 2001;7: 382–3.

[40] Caplice NM, Bunch TJ, Stalboerger PG, Wang S, Simper D, Miller DV, et al. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci U S A 2003;100:4754–9 [Electronic publication 2003 Mar 28].

[41] Simper D, Stalboerger PG, Panetta CJ, Wang S, Caplice NM. Smooth muscle progenitor cells in human blood. Circulation 2002;106: 1199–11204.

[42] Le Ricousse-Roussanne S, Barateau V, Contreres JO, Boval B, Kraus-Berthier L, Tobelem G. Ex vivo differentiated endothelial and smooth muscle cells from human cord blood progenitors home to the angiogenic tumor vasculature. Cardiovasc Res 2004;62:176–84.

[43] Cho SW, Lim SH, Kim IK, Hong YS, Kim SS, Yoo KJ, et al. Small-diameter blood vessels engineered with bone marrow-derived cells. Ann Surg 2005;241:506–15.

[44] Kashiwakura Y, Katoh Y, Tamayose K, Konishi H, Takaya N, Yuhara S, et al. Isolation of bone marrow stromal cell-derived smooth muscle cells by a human SM22alpha promoter: in vitro differentiation of putative smooth muscle progenitor cells of bone marrow. Circulation 2003;107:2078–81.

[45] Solan A, Niklason L. Age effects on vascular smooth muscle: an engineered tissue approach. Cell Transplant 2005;14:481–8.

[46] Gong Z, Niklason LE. Blood vessels engineered from human cells. Trends Cardiovasc Med 2006;16:153–6.

[47] Andreadis ST. Experimental models and high-throughput diagnostics for tissue regeneration. Expert Opin Biol Ther 2006;6:1071–86.

[48] Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004;84:767–801.