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Retention of stemness and vasculogenic potential of human umbilical cord blood stem cells after repeated expansions on PES-nanofiber matrices

Matthew Joseph^{†,1}, Manjusri Das^{†,1}, Suman Kanji¹, Jingwei Lu¹, Reeve Aggarwal¹, Debanjan Chakraborty², Chandrani Sarkar², Hongmei Yu², Hai-Quan Mao³, Sujit Basu², Vincent J. Pompili¹, and Hiranmoy Das^{1,*}

¹Stem Cell Research Laboratory, Davis Heart and Lung Research Institute, Wexner Medical Center at The Ohio State University, Columbus, OH.

²Department of Pathology, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, The Ohio State University Comprehensive Cancer Center. Columbus, OH.

³Department of Materials Science and Engineering & Whitaker Biomedical Engineering Institute, Johns Hopkins University, Baltimore, MD.

Abstract

Despite recent advances in cardiovascular medicine, ischemic diseases remain a major cause of morbidity and mortality. Although stem cell-based therapies for the treatment of ischemic diseases show great promise, limited availability of biologically functional stem cells mired the application of stem cell-based therapies. Previously, we reported a PES-nanofiber based *ex vivo* stem cell expansion technology, which supports expansion of human umbilical cord blood (UCB)-derived CD133⁺/CD34⁺ progenitor cells ~225 fold. Herein, we show that using similar technology and subsequent re-expansion methods, we can achieve ~5 million-fold yields within 24 days of the initial seeding. Interestingly, stem cell phenotype was preserved during the course of the multiple expansions. The high level of the stem cell homing receptor, CXCR4 was expressed in the primary expansion cells, and was maintained throughout the course of re-expansions. In addition, re-expanded cells preserved their multi-potential differential capabilities *in vitro* such as, endothelial and smooth muscle lineages. Moreover, biological functionality of the re-expanded cells was preserved and was confirmed by a murine hind limb ischemia model for revascularization. These

*Address correspondence to: **Hiranmoy Das, PhD**, Associate Professor of Internal Medicine, Director, Cardiovascular Stem Cell Laboratories, The Dorothy M. Davis Heart and Lung Research Institute, Wexner Medical Center at The Ohio State University, 460 W. 12th Avenue, BRT 394, Columbus, Ohio 43210, Phone: (614) 688-8711, Fax: (614) 293-5614, hiranmoy.das@osumc.edu.

[†]Equally contributed to this work

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Disclosure

Authors have no competing financial interests.

Author's contribution

Conceived and designed the experiments: MJ, MD, HD. Performed the experiments: MJ, MD, SK, JL, RA, DC, CS HY. Analyzed the data: MJ, MD, SK, JL, RA, DC, CS, HY, SB, VP, HD. Contributed reagents/materials/analysis tools: HM, VP. Wrote the paper: HD. All authors read and approved the final manuscript.

cells could also be genetically modified for enhanced vasculogenesis. Immunohistochemical evidences support enhanced expression of angiogenic factors responsible for this enhanced neovascularization. These data further confirms that nanofiber-based *ex-vivo* expansion technology can generate sufficient numbers of biologically functional stem cells for potential clinical applications.

Keywords

PES-nanofiber; Re-expansion; CD34⁺ stem cells; proangiogenic growth factors; NOD/SCID mice; limb ischemia

1. Introduction

Hematopoietic stem cells (HSC) are multipotent in nature [1]. During the past several decades, HSC transplantation has been used as standard treatment for various hematological disorders [2, 3]. Even though HSC transplantation has been applied in the clinics, regulation of HSC self-renewal and differentiation remain a major challenge that hampers *ex-vivo* expansion of HSCs, and limits generation of sufficient number of biologically functional HSCs for routine clinical use from a single unit of human umbilical cord blood (UCB). Furthermore, the maintenance, proliferation and differentiation of HSCs are mainly regulated by the microenvironment in bone marrow or stem cell niche that contains niche cells and extracellular matrix (ECM). While ECM provides the basic physical and chemical support for stem cell function, niche cells control stem cell quiescence, proliferation and differentiation [4–6]. Many of the current *ex vivo* expansion technologies are being developed mimicking bone marrow microenvironment to acquire optimum condition for survival and proliferation of HSCs with limited differentiation [5].

ECM plays very important role in stem cell regulation, survival and differentiation by supporting mechanical ultra-structure of the microenvironment present in the bone marrow. ECM interacts with stem cells through adhesion molecules, control cell geometry, mechanical property and nanotopography [7]. As for example, adhesive segments of an ECM protein fibronectin were able to enhance growth and proliferation of HSCs [8]. Mechanical signals developed within the microenvironment also alter the cytoskeletal tensions of ECM and regulate the fate of HSCs, enabling them to proliferate, differentiate, migrate or undergo apoptosis [9]. Osteoblasts residing within the bone marrow niche are the most important cells that support maintenance of HSCs by secreting various cytokines and growth factors [10]. Osteoblasts also secrete chemo-attractant, stromal cell-derived factor (SDF)-1, which binds to CXC chemokine receptor 4 (CXCR4) expressed on HSCs [11]. SDF-1 also stimulates the growth and survival of CD34⁺ progenitor cells [12, 13].

The *ex vivo* expansion of human stem cells has been studied extensively using biological or biomaterial approaches. In a biological approach, stromal layers were used for expansion of stem cells, however, secretory products from these methods are not clearly defined and additionally, anti-proliferative signals are also generated from these methods that limits proliferation of HSCs [14]. To mimic *in vivo* ECM structure, numerous synthetic polymeric biomaterial substrates such as polyethylene terephthalate (PET), tissue culture polystyrene

(TCPS), maleic anhydride, and polyether sulfone (PES) fibers are being extensively studied for *ex vivo* expansion of HSCs [15]. These materials have advantages because of their well-defined composition, reproducibility of surface chemistry topography, toxicity profile, and degradation rates. Therefore, several biomaterials have been used without modifications for the *ex vivo* expansion of HSCs with limited success [16, 17]. Thus, modifications of base materials with ECM molecules or chemical moieties and topographical patterns were applied for effective HSC expansion. Studies support that the surface chemistry and topography affect the rate of HSC proliferation and expansion [18–21]. Human UCB-derived CD34+ cells were *ex vivo* expanded on chemically modified PES substrate. PES that conjugated with amine group has shown to have different patterns of focal adhesion and supports highest expansion of HSCs compared to other chemically modified PES or unmodified PES [19].

One of the major causes of human mortality and morbidity in the world are ischemic diseases [22]. Ischemia is generally caused by occlusion of artery due to cholesterol deposition into the arterial lumen resulting in reduction of oxygen supply and nutrition leading to cellular death. Although advancement in traditional therapy in the last decade, improved life expectancy, however, a significant number of patients are not suitable for the common therapeutic approaches [23]. Thus new strategies for revascularization would be beneficial to increase blood flow via an alternative stem cell therapeutic approach for these patients. Herein, we explore the concept of therapeutic angiogenesis in which neovascularization is induced in ischemic tissues to improve blood flow and subsequently, reduce symptoms of these suboptimal patients [24]. In this study, we assess the biological functionality of re-expanded cells in a hind limb ischemic model.

2. Materials and Methods

2.1. CD133+ cell isolation

Fresh human umbilical cord bloods (70–100 ml) were obtained from The Wexner Medical Center at The Ohio State University after IRB approval and written consent from donors. Blood samples were processed following a similar protocol earlier published [20, 25–28]. In brief, the citrate phosphate dextrose-adenine 1 (CPDA-1) anti-coagulated blood was diluted with PBS and 10 ml of Ficoll-Paque plus (GE Healthcare, Piscataway, NJ) was carefully under layered. After 30 min centrifugation in a swinging bucket rotor at 14000 rpm, the upper layer was aspirated and the mononuclear cell layer was collected. Furthermore, following labeling with magnetic bead conjugated anti-CD133 (CD133) monoclonal antibody (Miltenyi Biotec Inc, Bergisch Gladbach, Germany), two cell separation cycles (with different columns) were performed using the AutoMACS cell sorter (Miltenyi Biotec) according to the manufacturer's protocol and reagents. After separation, periodic purity of the cell product was determined by flow cytometry.

2.2. Electrospinning of PES nanofiber mesh

Electrospinning, PAAc grafting and amination of PES nanofibers were performed following earlier described protocol [19]. All reagents were purchased from Sigma-Aldrich (USA) except PES granules (MW: 55,000), which were purchased from Goodfellow Cambridge

Limited. In brief, PES granules were dissolved in DMSO at 20% w/v concentration to pass through a plastic syringe with 27G needle. A pump (KD Scientific, USA) with fixed speed (0.3 ml/h) was used to feed the polymer solution into the syringe. Electrospinning was performed at 13 kV with a high voltage power supply (Gamma High Voltage Research, USA). Nanofibers were collected directly onto grounded 15 mm diameter glass coverslips (Paul Marienfeld, Germany) located at a fixed distance of 160 mm from the needle tip, over a collection time of 25 min. PES films were fabricated by dip-coating glass in 10% PES in DMSO. The deposited nanofiber and film samples were washed thoroughly in distilled water and ethanol to remove any residue of DMSO, and subsequently dried and stored in a desiccator.

2.3. Surface grafting of PES nanofiber mesh with poly acrylic acid (PAAc)

Acrylic acid (AAc) (Merck, Germany) was distilled and stored at -20°C prior to use. PAAc was grafted onto the PES nanofiber mesh surface by photo-polymerization [18]. Briefly, samples were immersed in aqueous solution containing 3% AAc solution and 0.5 mM NaIO_4 in a flat-bottom glass container. The temperature of the solution was maintained at 8°C . The samples were then exposed to UV from a 400W mercury lamp (5000-EC, Dymax, Germany) for 2 min at a fixed distance of 25 cm. The PAAc-grafted meshes were then thoroughly washed with deionised water at 37°C for over 36 h to remove any ungrafted PAAc from the surface of the scaffold and dried in a storage desiccator.

2.4. Amination of PAAc-grafted PES nanofiber mesh

Following cross-linking method the PAAc-grafted PES nanofiber mesh was conjugated with ethylene diamine (EtDA). Briefly, each scaffold was first gently shaken in 2 ml acetonitrile containing 50 mM N-hydroxysuccinimide (NHS) and 50 mM dicyclohexylcarbodiimide (DCC). After 6 h, the reaction solution was carefully aspirated and each scaffold was immediately immersed into 2 ml acetonitrile containing 0.03 mmol EtDA. After 12 h, the reaction solution was carefully aspirated and each scaffold was thoroughly washed in absolute ethanol to remove any dicyclohexyl urea (DCU), a byproduct of the reaction. All substrates were subsequently sterilized in 70% ethanol, then loaded into 24-well tissue culture plates (Nunc) and stored in sterile PBS until use. Surface amine density was quantified according to the method described earlier [29].

2.5. CD133+ hematopoietic stem cell expansion and re-expansion cultures

The StemSpan SFEM medium was purchased from StemCell Technologies (Vancouver, BC, Canada). Purified recombinant human stem cell factor (SCF), Flt-3 ligand (Flt3), TPO and IL-3 were purchased from Peprotech Inc. (Rocky Hill, NJ, USA), and low-density lipoprotein was purchased from Athens Research and Technology Inc., Athens, GA, USA. Nanofiber meshes were securely glued to the bottoms of wells of a 24-well tissue culture plate. Eight hundred CD133+ cells were seeded onto each scaffold in 0.6 ml StemSpanTM serum-free expansion medium, which consists of 1% BSA, 0.01 mg/ml recombinant human insulin, 0.2 mg/ml human transferrin, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine in Iscove's MDM, supplemented with 0.04 mg/ml low-density lipoprotein, 100 ng/ml SCF, 100 ng/ml Flt3, 50 ng/ml TPO and 20 ng/ml IL-3. Cells were cultured at 37°C in an atmosphere containing 5% CO_2 for 10 days without medium change. Cells were harvested after 10 days

of expansion. All substrates were washed once with non-trypsin cell dissociation solution and twice with 2% FBS Hanks' buffer at 5–10 min intervals between each wash. The cell suspensions collected were then concentrated through centrifugation at $500 \times g$ for 10 min. Aliquots of the concentrated cells were then used for cell counting by a hemocytometer, flowcytometric analysis, as well as for further studies. Primary expanded cells were re-expanded at least for four more times by following the same expansion methods and the same 24-well nanofiber plates.

2.6. Flowcytometry

Flow cytometric analysis for cell surface markers were performed by blocking expanded cells first with FCR Blocking Reagent (1:5; Miltenyi Biotec Inc) and followed by incubating for 20 min at 4 °C with the following antibodies: anti-CD34-PE, and anti-CD133/2 FITC (all from Miltenyi Biotec Inc), CXCR4, CD31, CD14, CD161, MHC Class-I, MHC Class-II, LFA-1, VCAM-1, CD45, CD69, CD18, CD36, CD3, CXCR2, CD71, CD45R and Isotype controls (IgG1 and IgG2a) were purchased from BD Biosciences, (USA). After incubation, cells were washed with MACS sorting buffer and analyzed using a FACS Calibur flowcytometer (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded via propidium iodide staining. At least 20,000 events were acquired. Data analysis was performed with BD Cell Quest software.

2.7. Scanning electron microscopy (SEM)

Part of the re-expanded cell cultures sample wells were gently rinsed with PBS, fixed with 3% glutaraldehyde for 30 min at 20 °C, and post-fixed with 1% osmium tetroxide for another 15 min at 20 °C. Samples were then dehydrated using a graded series of ethanol (25%, 50%, 70%, 90%, 95% and 100%) followed by HMDS drying. The samples were mounted onto aluminum stubs and gold sputter-coated before viewing under a scanning electron microscope (FEI Nova NanoSEM 400, Hillsboro, OR, USA).

2.8. Induced differentiation of re-expanded cells and immunofluorescence microscopy

To determine whether re-expanded cells retain their multipotential ability, re-expanded cells were subjected to endothelial, and smooth muscle differentiation *in vitro* using specific inducing medium such as EGM2 medium and SMGM-2 medium (Cambrex Inc / Lonza Inc, NJ) for at least 14 days on chamber slides (Labtek, Nunc International Inc) replacing fresh media in every 3rd day. All cultures were performed in quadruplicate, incubated at 37 °C in 5% CO₂ and 95% humidity, and scored after 14 days of culture by light microscopy. Cells were fixed with 4% paraformaldehyde, and blocked non-specific binding with 10% goat serum, and followed by specific staining. Immunofluorescence staining for endothelial differentiation was confirmed by CD31, and von-Willebrand factor (vWF) expression; and for smooth muscle differentiation, by expression of smooth muscle α -actin and smooth muscle myosin heavy chain (SM-MHC) (Incubated with monoclonal antibody, followed by FITC-conjugated secondary goat anti-mouse Ab, and nuclear stain with mounting solution containing DAPI). Samples were viewed under fluorescence microscope (Nikon E800 with MetaMorph version 4.5 software, Universal Imaging Corp.). Cultured human umbilical vein

endothelial cells (HUVEC) and NIH3T3 cells served as positive and negative controls, respectively.

2.9. Genetic modification of stem cells

Five times nanofiber-expanded stem cells were transfected with either GFP containing vector (pmaxGFP) or VIP vectors (VEGF IRES and PDGF in pAMFG vector) using a human CD34 cell Nucleofector kit (Amaxa Inc.) following manufacturer's protocol. In brief, $1-3 \times 10^6$ cells were transfected with 2–4 μg of plasmid DNA in 100 μl of CD34 cell Nucleofector solution and using Amaxa Electroporator programs: U-008 or U-001 (Amaxa Inc.). After transfection cells were cultured with serum-free medium or as stated for further studies.

2.10. Revascularization studies in a NOD/SCID mouse hind limb vascular injury model

All animal experiments were performed in accordance with the guidelines published in the "Guide for the Care and Use of Laboratory Animals" (NRC publication), and under the protocols approved by the Institutional Animal Care and Use Committee at Wexner Medical Center at The Ohio State University. Female NOD/SCID mice (7 weeks old) were purchased from Jackson laboratory (Bar Harbor, ME). Mice were anesthetized with an intra-peritoneal injection of a cocktail of ketamine, xylazine, and acepromazine, the proximal right femoral artery was ligated at 2 points 3 mm apart, and the artery between the ligatures was transected. Studies were performed with three groups of mice. Each group (7–9 mice) was injected with either media alone, 24-day PES-nanofiber re-expanded cells (5×10^5 cells/mouse), or VEGF-iris-PDGF (VIP) transfected PES-nanofiber re-expanded cells via intra-ventricular delivery in 300 μl volume. At baseline, pre and post ligations, and on days 7, 14, 21, and 28, mice were assessed for functional recovery and blood flow. On day 28, the mice were sacrificed and both right and left gastrocnemius muscles were excised. Half of each sample was snap frozen and the other half was formalin fixed for further investigation.

2.11. Laser Doppler Perfusion Imaging

Mice were anesthetized and blood flow in the hind limbs was measured with a laser Doppler perfusion image analyzer (Moor Instruments Co Ltd, Devon, UK) at baseline, after ligation, and at days, 7, 14, 21, and 28 after stem cell transplantation. Briefly, blood flow at the hind limb surface was measured with red (634-nm) and NIR (810-nm) wavelengths of the laser-Doppler perfusion imager. The scanner head was placed 25 cm above the exposed hind limbs. Scanning speed was set at 10 ms/pixel, which gave a frequency bandwidth of 0.1–15 kHz. Flux and direct-current gains were adjusted to maximize signal strength while preventing saturation of the photodiode. The imagers were able to map tissue blood flow over areas from 5 cm $\times$ 5 cm up to 50 cm $\times$ 50 cm with 256 $\times$ 256 pixel resolution; - each pixel being an actual measurement. At high scan rates, over 64,000 measurements are made in less than 5 minutes. Perfusion values as measurement of blood flow in the microcirculation with an attached CCD camera were stored and presented as a color-coded matrix on a computer monitor.

2.12. Capillary staining and counting

To determine the capillary density in the border zone of the hind limb ischemia, tissues were dissected and snap-freeze in liquid nitrogen. Cryo-sections of frozen tissues were stained using an alkaline phosphatase kit (Sigma FAST™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium tablets), which were used for the detection of alkaline phosphatase activity and stained as a substrate precipitant. For quantification of positively stained vessels (predominantly endothelium), 4 sections within the necrosis border zone of each animal were analyzed by an investigator who was blinded with respect to the cell treatment. Capillaries were counted in 12 randomly chosen high-power fields (HPFs) in 4 sections per tissue and 5 animals per group. The results were expressed as capillaries per high power field.

2.13. Immunohistochemistry

Ischemic tissue samples were fixed in 10% neutral buffered formalin, rinsed in PBS, transferred to 30% sucrose in PBS at 4°C, and frozen in optimum cutting temperature compound. Immunohistochemistry was performed on frozen tissue sections using monoclonal antibody against ICAM-1, VCAM-1, VEGF, and CD31 with the Vectastain Elite ABC kit following the manufacturer's protocol. Sections were counterstained with hematoxylin. Briefly, tissue sections were pre-blocked with 10% goat serum at room temperature for 1 h; primary antibodies were added and incubated at 4 °C overnight. The next day sections were washed with PBS, and secondary antibodies were added and incubated for 30 min at room temperature (with the Vectastain Elite ABC kit). Sections were mounted with Vectashield mounting medium (Vector Labs) with or without DAPI and visualized under a phase contrast microscope (Nikon E800 with MetaMorph version 4.5 software, Universal Imaging Corp.).

2.14. Statistical analysis

All values were presented as mean $\pm$ standard error of the mean. One-way ANOVA with Scheffe's post hoc test for unequal sample sizes was used to compare numeric data between the four experimental groups. Datasets consisting of two groups only were compared by unpaired Student's t-test. A level of $p < 0.05$ was considered as significant difference.

3. Results

3.1. Repeated expansion of human umbilical cord blood-derived stem cells on PES-nanofibers

CD133+/CD34+ cells were isolated from freshly collected human umbilical cord blood samples from various donors using AutoMACs system and were expanded on PES nanofiber-coated 24-well plate for 10 days with a serum-free expansion medium containing growth factors. After 10 days of culture, cells collected from the PES nanofiber-coated culture plate showed 359-fold expansion. Expanded cells were reseeded on a PES nanofiber-coated culture plate for verification of their expansion potentials for at least four more times. At each round of expansion, cells were harvested and counted. After five rounds of culture,

a total 5.06 million-fold of expansion of human umbilical cord blood derived stem cells were achieved (Fig. 1).

3.2. Morphology of stem cells after repeated expansions

To confirm cellular adherence characteristics to the PES nanofiber and microstructure of re-expanded human umbilical cord blood-derived CD34+ cells were fixed and subjected to the scanning electron micrography and images were captured digitally at various magnification. Electron micrographic images confirm that re-expanded cells on PES nanofiber adhere loosely to the nanofiber and remain round in shape similar to the first time expanded cells (Fig. 2).

3.2. Stem cell phenotype after each round of expansion

We next investigated whether PES-nanofiber-mediated expansion preserves stem cell phenotype of the human umbilical cord blood derived stem cells. Flow cytometric analysis for markers including CD34, CXCR4 and CD133 showed that the expression level of CD34 and CXCR4 remained above 90% after each round of culture (Fig. 3). However, expression level of CD133 was around 10% after first round of expansion and remained unaltered after 5 rounds of expansions (Fig. 3).

3.3. Characterization of cells after five rounds of expansion

Flow cytometric analyses of five rounds of expanded cells showed positive expression for stem cell markers and hematopoietic cell markers, and show negative expression for T cell markers and activation markers (Fig. 4A). We have performed five independent flow cytometric analyses for five different samples and the results are graphically presented in a cumulative form (Fig. 4B).

3.5. Multipotential ability of re-expanded stem cells

To investigate whether multiple round of culture on PES nanofiber retain stemness of the expanded cells, five times nanofiber-expanded cells were induced for differentiation *in vitro* towards endothelial lineage and smooth muscle lineage for two weeks.

Immunocytochemical staining was performed to evaluate lineage specific differentiation using endothelial specific (CD31 and vWF) and smooth muscle specific (SM- α Actin and SM-Myosin heavy chain) using standard methods and images were captured using a fluorescence microscope (Nikon E800 with MetaMorph version 4.5 software, Universal Imaging Corp.) and images were captured digitally. Immunocytochemical analysis revealed that stem cells after multiple round of re-expansion on PES-nanofiber retain multipotential capabilities (Fig. 5).

3.6. Biological functionality of re-expanded stem cells

Biological functionality of five-times expanded cells were tested for revascularization in a murine hind limb ischemia model using without or with genetic modification with proangiogenic factors (VEGF plus PDGF). Doppler imaging was performed for the animals at various time points, such as, base line, before and after femoral artery ligation, and on days 7, 14, 21, and 28 after stem cell therapy on day of ligation. Doppler images show that

stem cell therapy enhances blood flow possibly due to vasculogenesis in the ligated limb, and the blood flow is accelerated upon stem cell therapy with genetic modification containing proangiogenic factors (VEGF plus PDGF) (Fig. 6A). Cumulative Doppler image analysis revealed that a significant enhancement of blood flow on day 28 after stem cell therapy compared to the vehicle treated controls. However, the significant accelerated blood flow was achieved at day 14 when animals were treated with genetic modification using proangiogenic factors (VEGF plus PDGF) and remained up to 28 days tested (Fig. 6B) compared to the same controls.

To address whether Doppler images of blood flow was due to the formation of new blood vessels, ischemic tissues were collected from animals of all three groups at day 28 after completion of study, and tissues were cryo-preserved and stained for neovessels using alkanine phosphatase staining. Sections were viewed under a phase contrast microscope, and images were captured digitally (Nikon E800 with MetaMorph version 4.5 software, Universal Imaging Corp.). Image analysis revealed that there was a significant increase in the number of capillaries in the animals received stem cell therapy; and the number was much higher in animals that received stem cells with genetic modification using proangiogenic factors (VEGF plus PDGF) compared to the vehicle-treated animals (Fig. 6C).

3.7. Angiogenic factors in ischemic tissues of mice

Ischemic frozen calf muscle tissues from animals of all three groups were cryo-sectioned and stained for angiogenic factors such as ICAM-1, VCAM-1, VEGF, and CD31 using immunohistochemical methods. Immunohistochemical staining revealed that the levels of angiogenic factors were enhanced in the animals received stem cell therapy. Their levels were much higher in animals that received stem cells with genetic modification using proangiogenic factors (VEGF plus PDGF) compared to animals received vehicle only (Fig. 7).

In summary, we have shown that PES nanofibers could be used as a substrate to promote robust expansion of functional human umbilical cord blood-derived stem cells, which retain progenitor cell phenotype. Additionally, we have shown that the repeatedly expanded cells retain multipotential abilities; such as differentiate into endothelial and smooth muscle lineages. Furthermore, re-expanded cells retain their biological functionality in mediating blood flow and neovascularization in a mouse model of hind limb ischemia. Moreover, re-expanded cells could be genetically modified with proangiogenic growth factors to enhance restoration of blood flow and neovascularization in murine hind limb ischemia.

4. Discussion

In spite of numerous efforts, a universally applicable optimal method to obtain sufficient numbers of biologically functional adult hematopoietic stem cells remains elusive. Various synthetic polymeric biomaterial substrates such as TCPS, polyethylene (PE), high-density PE (HDPE), polycarbonate (PC), PET, cellulose acetate, teflon fluorinated ethylene propylene (FEP), glass, polysulfone (PS), Teflon perfluoralkoxy (PFA), polymethylpentene (PMP), barex (polyacetonitrile-methylacrylate), polypropylene (PP), acetal

(polyformaldehyde), cellulose acetate, titanium, aluminum, and stainless-steel were investigated for expansion of HSCs with limited success [16, 17]. Some of the materials were shown to have protein adsorption effects or leaching of toxic chemicals, which resulted in membrane damage and subsequent cell death of HSCs. Among all these materials, barex, PMP, cellulose acetate, Teflon and titanium showed comparable but lesser proliferation effects compared to TCPS. However, modified PET showed higher HSC expansion [17] compared to unmodified version [16]. Clearly, the composition of base materials was critical for the expansion, and cytokines are always required for survival and proliferation of HSCs [30]. Thus, modifications of base materials with ECM molecules or chemical moieties and topographical patterns were among the choices for more effective expansion of HSCs.

Studies demonstrated that surface chemistry and topography play critical roles in the expansion and proliferation of HSCs [18–20]. We and others have previously shown that chemically modified PES substrates support *ex vivo* expansion of human UCB-derived CD34+ cells [15, 19, 20, 27, 28]. When the PES was chemically modified with either carboxylic, hydroxyl or amine group, it showed different patterns of focal adhesion [19]. Among all modifications, aminated PES substrates supported highest expansion of CD34+ cells compared to other chemically modified PES or unmodified PES. Positively charged amine groups could selectively adsorb protein components, such as, fibronectin from the culture medium that could lead to presentation of immobilized proteins similar to those in the bone marrow [19]. Other possible reason was a direct electrostatic interaction between negatively charged sialylated CD34 receptor with positively charged immobilized amine groups, and that was thought to enhance the self-renewal and inhibit the differentiation pathways [19]. However, CD34+ cell proliferation has been reported to be highest in aminated PES nanofiber compared to carboxylated or hydroxylated PES fibres [19]. Using aminated PES nanofiber-mediated expansion technology and subsequent re-expansion methods, we in this study show that we could achieve ~5 millionfold yields within 24 days of the initial seeding (Fig. 1). This method will potentially provide sufficient amount of HSCs for any future applications. Interestingly, the stem cell phenotype was preserved during the course of the multiple expansions, and indicates that the expansion technology is acceptable.

In addition, the high level of the stem cell homing receptor, CXCR4 was also expressed in the primary expansion cells, and was maintained throughout the course of re-expansions (Fig. 3). The CXCR4 receptor, which is highly expressed on both endothelial and hematopoietic progenitor cells [31], has been shown to be essentially involved in mobilization and homing of hematopoietic stem cells [32, 33] to the site of injury or ischemia where its ligand, stromal-derived factor (SDF)-1 is expressed. Importantly, this homing plays a critical role in postnatal vasculogenesis [34]. Studies have also shown that there is a close interaction between SDF1/CXCR4 and vascular endothelial growth factor-A (VEGF) in the development of neovascularization [35]. In the current study, we observed a significant increase in neovascularization due to nanofiber expanded and proangiogenic growth factor transfected cells (Fig. 6), thereby suggesting that the interaction between upregulated CXCR4 expressed by the expanded cells by VEGF is important. However, the mechanism involved in the upregulation of CXCR4 in nanofiber expanded progenitor

population is yet to be identified. We also demonstrated that biological functionality of the re-expanded cells was preserved in a murine hind limb ischemia model for revascularization (Fig. 6). Immunohistochemical evidences also support that the enhanced expression of angiogenic factors were responsible for increased number of vasculogenesis (Fig. 7). Transdifferentiation of HSCs might not play major role in healing of injured tissues [36]. Alternately, paracrine mechanisms such as release of VEGF and fibroblast growth factor (FGF) from the progenitor cells have been reported to mediate the therapeutic effects of HSCs in ischemia [37, 38]. However, by using an approach of combining angiogenic growth factors with PES re-expanded stem cells, we show significant increase of vasculogenesis and a better recovery of blood flow in murine limb ischemia compared to untreated controls (Fig. 6). It has been reported that in addition to pro-angiogenic effects of VEGF, platelet derived growth factor (PDGF) also facilitate improvement of neovascularization by maturation of vessels and thus increase survival and proliferation [39, 40].

Moreover, the major cause of vasculogenesis is hypoxia that triggers a series of molecular events, which lead to release of angiogenic growth factors such as VEGF [41]. Inflammatory cells release cytokines and chemokines in response to ischemia, which also play a prominent role in angiogenesis [42]. All these signals activate endothelial cells that line along blood vessels, and promote sprouting of new vessels [43]. Following ischemia, angiogenic factors such as VEGF was also up-regulated, which promoted migration of CD34+ cells to the site of injury to interact with its receptors expressed on these cells [44]. It has been shown that circulating progenitor cells were able to incorporate into the neovessels within the ischemic tissue and differentiate into endothelial cells, even though their contributions vary from organ to organ [45, 46]. This incorporation has been further shown to be proportional to the degree of tissue ischemia and the gradient of hypoxia is important in directing the progenitor cells to the site of injury [47], subsequently, contributing to angiogenesis during wound healing and limb ischemia [48–50]. Furthermore, progenitor cells can also secrete growth factors in the ischemic tissues to promote angiogenesis through paracrine effects. Progenitors from human peripheral blood have been shown to differentiate into early endothelial progenitor cells through paracrine effects to mediate vasculogenesis [51–53]. As for example, hematopoietic cells release angiogenic factors like VEGF-A, angiopoietins and matrix metalloproteinases (MMPs), which also facilitate the angiogenesis process [54, 55].

5. Conclusion

Our current results demonstrate that using a stem cell expansion method, we can achieve ~5 million-fold yields of human umbilical cord blood derived CD34+ stem cells within 24 days of the initial seeding. Interestingly, high level of the stem cell homing receptor, CXCR4 was expressed in the primary expansion cells, and was maintained throughout the course of repeated expansions. In addition, re-expanded cells preserved their multi-potential differential capabilities *in vitro* such as, endothelial and smooth muscle lineages. Moreover, biological functionality of the re-expanded cells was preserved *in vivo*. Re-expanded cells could also be genetically modified for enhanced vasculogenesis. Immunohistochemical evidences support enhanced expression of angiogenic factors responsible for this enhanced neovascularization. Our current study provides further evidences that nanofiber-based *ex-*

vivo expansion technology can generate significant numbers of biologically active stem cells for potential clinical applications.

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Figure 1. Re-expansion of human umbilical cord blood-derived stem cells on PES-nanofiber scaffolds

CD133+/CD34+ cells isolated from freshly collected human umbilical cord bloods (from five different samples) were expanded on PES-nanofiber scaffolds with a serum-free expansion media containing growth factors. Cells were re-expanded for four more times and fold expansions were shown after each round of culture.

Figure 2. Scanning electron micrographs of PES-nanofiber expanded stem cells

A scanning electron micrograph was performed to visualize cellular morphology and structures after five times expansion of cells on PES-nanofiber scaffold with various magnifications.

Figure 3. Assessment of stem cell-phenotype after each round of expansions

Flowcytometric analysis for the expression levels of CD34, CXCR4, and CD133 molecules after each round of expansion of stem cells on PES-nanofiber scaffold. Three representative samples were showed out of five samples studied.

Figure 4. Characterization of cells after five rounds of expansions
A. A representative flowcytometric analysis of five-times expanded cells for various markers related to the stem cells. **B.** Five independent flowcytometric analysis data is graphically presented in a cumulative form.

Figure 5. Multipotential differentiation of re-expanded stem cells

Five times PES-nanofiber-expanded cells were induced *in vitro* to differentiate into endothelial lineage (A) or smooth muscle lineage (B). Immunocytochemical staining was performed to evaluate lineage specific differentiation using various markers stated above.

Figure 6. Evaluation of biological functionality of five times expanded stem cells with or without genetic modification

Biological functionality of five times expanded cells were assessed for revascularization in a murine hind limb ischemia model using genetically modified cells with proangiogenic factors (VEGF plus PDGF) or without modification. **A.** Representative Doppler images were shown at various time points from animals of each group. **B.** Cumulative Doppler image analysis data graphically presented from 7–9 mice in each group tested. Statistical analysis (non-paired Students t-test) was performed with experimental group vs. media control group. Statistical significance were marked with (*) when p value was < 0.05 . **C.** Neovascularization was assessed in the ischemic tissues from animals of all study groups stated after alkaline phosphatase staining. Representative micrographs were depicted here. **D.** Total number of capillaries and total capillary area in each field was evaluated from all groups of animals using four tissue sections and three slides from each group of animals. Statistical analysis was performed and p values were stated above.

Figure 7. Immunohistochemical studies for pro-angiogenic molecular expressions in ischemic tissues of mice

Ischemic tissues of gastrocnimious muscles from animals of each group stated above were cryo-sectioned and stained for histochemical studies for proangiogenic factors using specific antibodies such as ICAM-1, VCAM-1, VEGF and CD31. Four animals from each group were studied and representative images were depicted above.