Engineering of the human vessel wall with hair follicle stem cells *in vitro*

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Abstract. Hair follicle stem cells (HFSCs) are increasingly used as a stem cell paradigm in vascular tissue engineering due to the fact that they are a rich source of easily accessible multipotent adult stem cells. Promising results have been demonstrated with small diameter (less than 6 mm) tissue engineered blood vessels under low blood pressure, however engineering large vessels (>6 mm in diameter) remains a challenge due to the fact it demands a higher number of seed cells and higher quality biomechanical properties. The aim of the current study was to engineer a large vessel (6 mm in diameter) with differentiated smooth muscle cells (SMCs) induced from human (h)HFSCs using transforming growth factor-β1 and platelet-derived growth factor BB in combination with low-serum culture medium. The cells were seeded onto polyglycolic acid and then wrapped around a silicone tube and further cultured *in vitro*. A round vessel wall was formed subsequent to 8 weeks of culture. Histological examination indicated that layers of smooth muscle-like cells and collagenous fibres were oriented in the induced group. In contrast, disorganised cells and collagenous fibres were apparent in the undifferentiated group. The approach developed in the current study demonstrated potential for constructing large muscular vessels with differentiated SMCs induced from hHFSCs.

Introduction

Cardiovascular diseases remain a significant health and socio-economic burden in developed countries (1). Surgical bypass with an autologous vein remains the primary treatment (2), however, a usable vascular graft is often absent due to limited sources and donor site morbidity. In addition, current widely used synthetic materials have several limitations including immunological and thrombotic complications. Furthermore, these vascular grafts are usually non-degradable and lack growth potential (3,4).

The development of tissue engineering technology is promising for potential improvement over currently used synthetic grafts. A blood vessel made of autologous cells and a biocompatible scaffold with the potential to remodel, repair and grow would be a major therapeutic advance (5). Promising results have been demonstrated with small diameter (<6 mm) tissue engineered blood vessels (TEBVs) under low blood pressure (6-8), however, few studies have focused on cases with larger vessels (>6 mm in diameter), which demand an increased number of seed cells and improved biomechanical properties.

However, tissue engineering approaches are limited by the large number of cells that must be obtained for regenerative medicine. Stem cells are promising cell sources with increased proliferation and broad differentiation capacity, making them suitable for the preparation of TEBVs (9-11). Although several types of stem and progenitor cells have been investigated for their potential as sources of seed cells in vascular tissue engineering (12-16), hair follicles are increasingly used for stem cell research due to the fact that they are a rich source of easily accessible multipotent adult stem cells. Hair follicle stem cells (HFSCs) have been demonstrated to possess osteogenic, adipogenic, chondrogenic, neurogenic and myogenic lineage differentiation potential (7,17-19).

In a previous study, human HFSCs (hHFSCs) were successfully induced to differentiate into functional smooth muscle cells (SMCs) by transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor BB (PDGF-BB) in combination with low-serum culture medium (20). The aim of the present study was to engineer a large vessel (6 mm in diameter) using induced hHFSCs and polyglycolic acid (PGA) with 8 weeks of *in vitro* culture. The rapid degradation of the PGA prevents the accumulation of degraded fragments *in vivo*. The culture system used indicated potential to construct large muscular vessels with differentiated SMCs induced from hHFSCs.

Materials and methods

Isolation and culture of hHFSCs. hHFSCs were obtained from human scalp tissue from healthy adult patients (average age,

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30 years) undergoing cosmetic plastic surgery, as described previously (20). All protocols for human tissue handling were approved by the Research Ethical Committee of Shanghai 9th People's Hospital, and written informed consent was obtained from the patients. hHFSCs at the second passage were used in the subsequent study. The hHFSCs were characterised by determining their CD marker profile (K15, K19 and integrin β1) and their ability to differentiate into osteogenic, adipogenic and chondrogenic lineages (data not shown), as reported previously (21,22).

Induction of SM differentiation. As previously reported (20), hHFSCs reaching subconfluence were cultured in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% foetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 5 ng/ml recombinant human TGF-β1 (R&D Systems, Inc., Minneapolis, MN, USA) and 10 ng/ml recombinant human PDGF-BB (R&D Systems, Inc.) with 1% FBS. DMEM supplemented with 1% FBS was defined as the basal medium (BM). Human umbilical artery SMCs (hUASMCs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and used as the positive control. The culture media were changed every 2 days. Cell characterisation and functional evaluation (data not shown) were performed subseqeuent to 8 days of culture as described previously (20).

Culture of hHFSC-PGA sheets in dishes. In the current study, 35 mg unwoven PGA fibres (Albany International Research Co., Albany, NY, USA) were constructed into an approximately 35x80x2 mm mesh. The scaffold was first soaked in 75% ethanol (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 2 h. Subsequently, it was washed three times with phosphate-buffered saline and incubated in DMEM for 10 min. The medium was removed, and the scaffold was incubated in an incubator (Binder GmbH, Tuttlingen, German) at 37˚C prior to use. Differentiated and undifferentiated hHFSCs ($6x10^7$) were each evenly seeded onto the PGA mesh in 100-mm culture dishes (Falcon; BD Biosciences, San Jose, CA, USA). To accomplish the complete adhesion of the hHFSCs to the fibres, the cell-scaffold constructs were then maintained in the incubator at 37˚C with 95% humidity and 5% CO_2 for approximately 4 h. Thereafter, sufficient induced culture medium or BM was added to the two dishes to cover the constructs. The cell-PGA sheets were incubated at 37˚C for another 5 days prior to use.

Induced culture in dishes. Subsequent to culture in dishes for 5 days, the cell-PGA sheets were wrapped around the silicone tubes and fixed by biodegradable sutures (Ethicon, Inc., Somerville, NJ, USA) for another 8 weeks of culture. The culture media was changed twice a week. The cell-PGA constructs cultured in BM were used as the controls.

Histological analysis. Subsequent to 8 weeks of culture, the engineered vessel walls were harvested, fixed in 10% formalin (Sigma-Aldrich; Merck Millipore) and embedded in paraffin (Sigma-Aldrich; Merck Millipore). Following this, they were sequentially cut into sections of 4 mm thickness. The sections were then tested with haematoxylin and eosin or Masson's trichrome and Gömöri staining (all stains were from Sigma-Aldrich; Merck Millipore).

Hydroxyproline assay. For hydroxyproline assessment, the vessel wall was dried and weighed. The total hydroxyproline content of each vessel was determined by a colorimetric assay described by Reddy and Ewemeka (23). In the current study, a Sigma-MAK008, Hydroxyproline Assay Kit (Sigma-Aldrich; Merck Millipore) and a Genesys 20 Spectrophotometer (Z376027; Sigma-Aldrich; Merck Millipore) were used. Normal human saphenous vein with a diameter of 4 mm served as the control. The veins were obtained from human adult patients undergoing cardiovascular surgery with autologous vein graft. The residual saphenous veins were contributed for future experimental studies, written informed consent was obtained from the patients. All protocols for human tissue handling were approved by the Research Ethical Committee of Shanghai 9th People's Hospital.

Statistical analysis. Each experiment was repeated a minimum of three times. The results were expressed as the mean ± standard deviation. Significant differences were measured using Student's t-test. $P<0.05$ was considered to indicate a statistically significant differences. All of the statistical analyses were performed using SPSS software, version 16 (SPSS, Inc., Chicago, IL, USA).

Results

Culture of hHFSC-PGA constructs in dishes. Subsequent to 24 h of culture, the cells began to spread and extended along the length of the fibres. Following an additional 5 days of culture in the dishes, a cell-PGA sheet had formed (Fig. 1A). Micrographs indicated that abundant hHFSCs had adhered to the PGA fibres, with secreted extracellular matrix (ECM) filling the spaces between the fibres (Fig. 1B).

Induced culture in dishes. The hHFSC-PGA constructs were incubated in culture dishes for 8 weeks subsequent to being wrapped around silicone tubes (Fig. 2). In the induced group, the constructs demonstrated a glossy and tubular structure with a round lumen 6 mm in diameter (Fig. 3A). In contrast, the vessel walls in the static culture group exhibited a collapsed lumen and rough surface (Fig. 3B).

Histological observation. Subsequent to 8 weeks of further induced culture *in vitro*, several smooth muscle-like cells and few collagenous fibres were observed by histological examination (Fig. 4A and B). The PGA fibres had degraded completely, and few elastic fibres were observed at this time (Fig. 4C). In contrast, disorganised cells, randomly collagenous fibres and small elastic fibres were observed in the undifferentiated group (Fig. 4D-F). The above results were further confirmed by immunohistochemical staining for smooth muscle α-actin and calponin (data not shown), using the hUASMCs as a positive control (Fig. 4G-I).

Hydroxyproline assay. The hydroxyproline content was significantly higher ($P<0.05$) in the induced group than in the

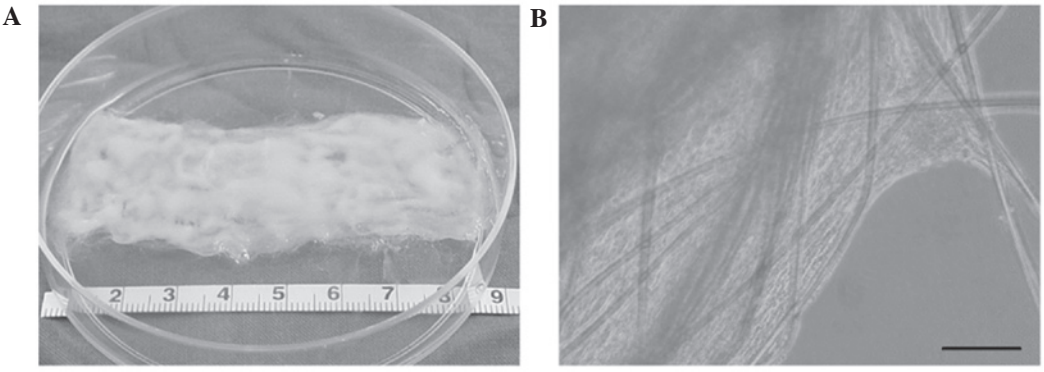

Figure 1. Cell-scaffold constructs cultured in dishes. (A) Gross view of the production of extracellular matrix by hHFSCs on PGA fibers. (B) At 5 days, microscopic observation demonstrated that sufficient hHFSCs had adhered to the PGA fibers with secreted extracellular matrix filling the space between the fibers. Scale bar, 100 μm. hHFSC, human hair follicle stem cell; PGA, polyglycolic acid.

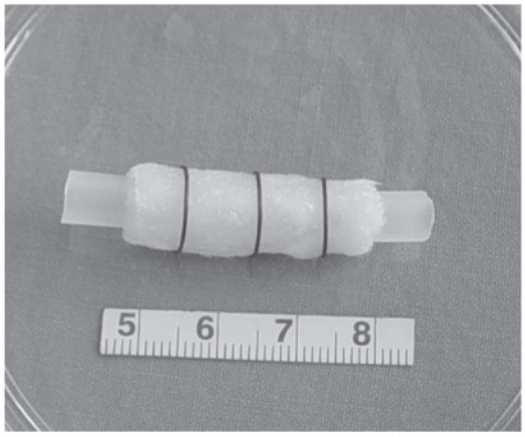

Figure 2. Gross view of the cell-polyglycolic acid sheet wrapped around a silicone tube in the culture dish, secured by biodegradable sutures.

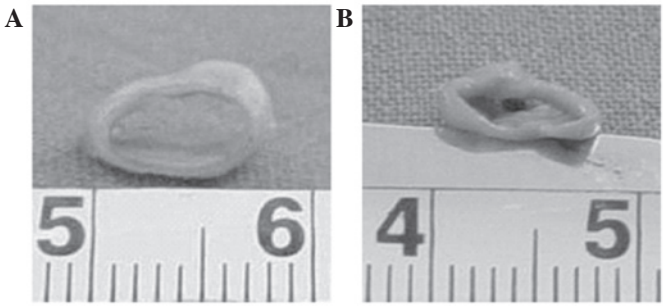

Figure 3. Gross view of the engineered vessels. (A) Vessel walls with round lumen (6 mm in diameter) are formed after 8 weeks of culture with induced media. (B) Vessel walls with a collapsed lumen and rough surface are observed after culture in basal medium.

control group at the same time points (Fig. 5). In addition, the hydroxyproline concentration in the induced group reached approximately 65% of that in the hUASMCs.

Discussion

hHFSCs have been previously successfully isolated from patients, expanded, differentiated and used to construct autologous tissue (22,24-26). This method eliminates the need for immunosuppressants and mitigates the risk of teratoma formation associated with embryonic stem cells and induced pluripotent stem cells (27). In a previous study, hHFSCs were induced to differentiate into functional SMCs by TGF-β1 and PDGF-BB in combination with low-serum culture medium (20). In the current study, a large diameter vessel wall was engineered using the aforementioned differentiated hHFSCs and PGA unwoven fibre mesh *in vitro*. Subsequent to culture, the newly formed tissues exhibited SM-like characteristics, including morphological performance, the expression of SM cell-specific markers (SM α-actin and calponin) and appropriate hydroxyproline content. These results demonstrated that hHFSCs may be utilised as a potential cell source for the tissue engineering of SMs, particularly that of the large diameter aorta.

Tissue engineering predominantly focuses on the incorporation of isolated cells with supporting scaffolds (28). An optimal scaffold degrades proportionally with tissue regeneration to be gradually replaced by newly formed functional tissue, and it supports cellular adhesion and collagenous matrix deposition (29). In vascular tissue engineering, the scaffold should reflect the biomechanical properties of blood vessels and serve as a platform for cell attachment and proliferation (30). It should be non-thrombogenic, non-immunogenic, biocompatible, haemocompatible, biodegradable and elastic (31,32). Furthermore, it should also control the extent and the strength of cell adhesion, proliferation, differentiation and maturation to achieve the desired phenotype and proper function (33). PGA is one of the most extensively used polymer scaffold materials in the engineering of numerous types of tissues, including blood vessels. It is a polyester that undergoes rapid degradation via hydrolysis of ester bonds, leaving behind glycolic acid and is further catabolised into water and carbon dioxide (34). The mechanical properties and degradation profile of PGA make it an attractive candidate for vascular tissue engineering (35). Numerous studies have demonstrated the successful use of PGA scaffolds for constructing vascular grafts (7,31,32,34,36). In the current study, cells in the experimental group were demonstrated to possess good proliferative ability and ECM secretion on PGA.

In the histological examination, it was identified that the content and distribution of SMCs and elastin in the experimental group were not as dense as those in normal

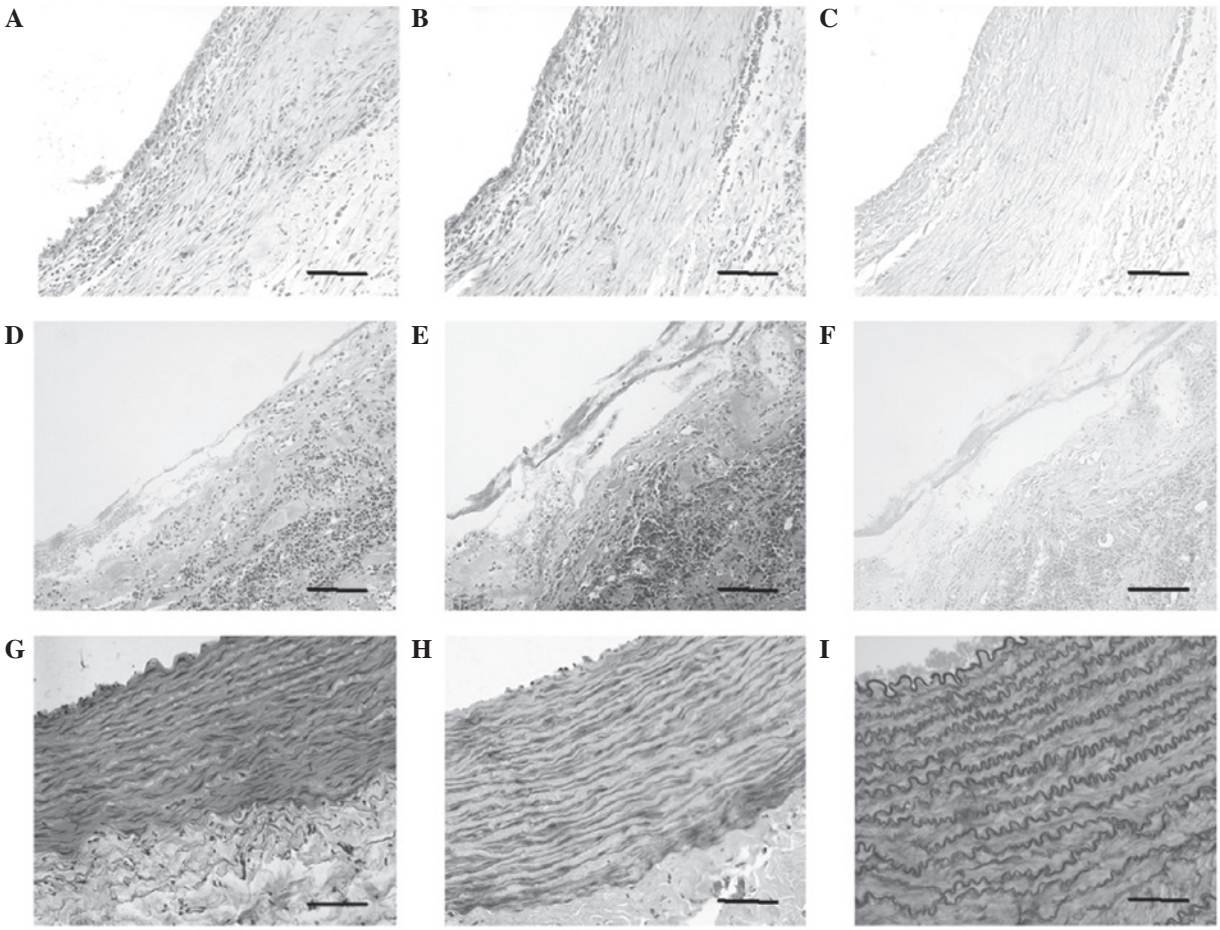

Figure 4. Histology of the engineered vessel walls after 8 weeks of culture with (A-C) or without (D-F) induced media. (A) Hematoxylin and eosin staining shows several layers of smooth muscle-like fibers in induced groups. Staining with (B) Masson's trichrome and (D) Gömöri trichrome indicates few collagen and elastic fibers in the experimental groups. By contrast, in the control group disorganized distributed cells were observed in (D) hematoxylin and eosin staining. Meanwhile, randomly collagenous fibers were found in (E) Masson's trichrome staining and few elastic fibers were shown in (F) Gömöri trichrome staining. Native canine abdominal arteries are presented for reference. It demonstrated layered and organized cells of the vessel walls in (G) hematoxylin and eosin staining. Dense collagenous fibers and much elastic fibers were shown in (H) Masson's trichrome staining and (I) Gömöri trichrome staining respectively.. Scale bars, 50 μm.

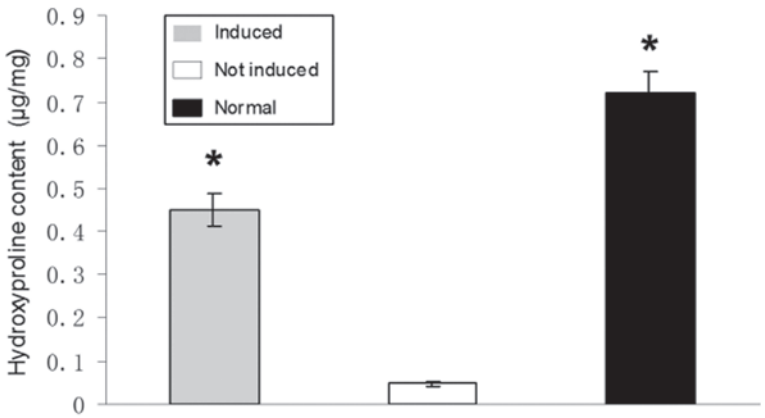

Figure 5. The amount of hydroxyproline in the induced group is higher than that in the control (non-induced) group and is approximately 65% of that of the normal vessel subsequent to 8 weeks of induced culture. Four samples from each group were measured in each of the above experiments. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group.

blood vessels. Normal blood vessels have been demonstrated to develop under the influence of the mechanical force of blood, which is an important physiological component of the environment experienced by cells: It promotes the circumferential orientation of the cells in addition to the deposition of the extracellular matrix, and it likely contributes to the

survival of implanted substitutes (36). It is suggested that dynamic culturing is important in vascular tissue engineering. *In vitro* investigations have demonstrated that low shear stress induces SMC proliferation (37-40) and promotes collagen alignment (41) and that cyclic stretching induces clear alterations in the SMC phenotype, function and gene expression (42,43). SMCs use multiple sensing mechanisms to perceive the mechanical stimulus generated from pulsatile stretching and transduce it into intracellular signals. This results in the modulation of gene expression and cellular functions including proliferation, apoptosis, migration and remodelling (44-49). Therefore, a bioreactor, which is currently is under development, may be used in future studies to mimic the physiological environment of the arterial vessel wall.

In conclusion, a large vessel (6 mm in diameter) was constructed using PGA seeded with hHFSCs *in vitro*. Induced culture constructs exhibited improved performance histologically and in hydroxyproline content when compared with constructs of the undifferentiated group. Further research focused on dynamic culturing of the constructs and further seeding of endothelial cells on the surface of the lumen to engineer composite vascular conduits is required, in order to progress in this area of bio-engineering.

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