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Patient-specific cardiovascular progenitor cells derived from integration-free induced pluripotent stem cells for vascular tissue regeneration

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ABSTRACT

Tissue-engineered blood vessels (TEBVs) are promising in regenerating a live vascular replacement. However, the vascular cell source is limited, and it is crucial to develop a scaffold that accommodates new type of vascular progenitor cells and facilitates *in vivo* lineage specification of the cells into functional vascular smooth muscle cells (VSMCs) to regenerate vascular tissue. In the present study, integration-free human induced pluripotent stem cells (hiPSCs) were established from patient peripheral blood mononuclear cells through episomal vector nucleofection of reprogramming factors. The established hiPSCs were then induced into mesoderm-originated cardiovascular progenitor cells (CVPCs) with a highly efficient directed lineage specification method. The derived CVPCs were demonstrated to be able to differentiate into functional VSMCs. Subcutaneous implantation of CVPCs seeded on macroporous nanofibrous poly(L-lactide) scaffolds led to *in vivo* VSMC lineage specification and matrix deposition inside the scaffolds. In summary, we established integration-free patient-specific hiPSCs from peripheral blood mononuclear cells, derived CVPCs through directed lineage specification, and developed an advanced scaffold for these progenitor cells to further differentiate *in vivo* into VSMCs and regenerate vascular tissue in a subcutaneous implantation model. This study has established an efficient patient-specific approach towards *in vivo* regeneration of vascular tissue.

1. Introduction

Cardiovascular disease remains the leading cause of mortality and impaired quality of life worldwide [1]. Vascular transplantation or grafting has been widely used to treat cardiovascular disease including aortic aneurysm and peripheral vascular disease [2]. However, suitable autologous blood vessels are limited for many patients, mostly due to widespread atherosclerotic vascular disease, useful vessels having been exhausted in previous procedures

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or the availability of autografts with matched size. Synthetic vascular grafts, made of expanded polytetrafluoroethylene, polyethylene terephthalate or polyurethane, have been successfully used to replace diseased vessels with a diameter greater than 6 mm in a high-flow and low-resistance circulation [3]. However, the grafts smaller than 6 mm in diameter are susceptible to thrombosis and stenosis, leading to very poor patency rate. Coating the intimal surface with heparin and other anticoagulant materials has been attempted, but these approaches are less-than-optimal [4]. Another inherent deficiency of synthetic grafts is the lack of growth potential, which leads to multiple operations in pediatric patients as they grow [5].

Tissue-engineered blood vessels (TEBVs) are promising in regenerating a live vascular replacement, where vascular cells seeded in three-dimensional biodegradable scaffolds are induced to develop functional vascular tissue. However, the vascular cell source is limited [6]. The efforts to construct TEBVs using mature vascular smooth muscle cells (VSMCs) have been reported, especially at the early stage of TEBV development [7]. Unfortunately, mature VSMCs isolated from autologous explanted vascular segments suffered from both inadequate cell numbers and decreased functionality over extensive *in vitro* expansion. Adult mesenchymal stem cells, with potent proliferative capability and differentiation potential into vascular cells, have been used to regenerate vascular tissues [8–11]. However, recent studies revealed poor engraftment of mesenchymal stem cells in the *in situ* regenerated vascular tissues [12,13]. With the unlimited potential to differentiate into various cell types, pluripotent stem cells have become a promising cell source in tissue engineering [14]. In one of our previous studies [15], we successfully established human induced pluripotent stem cells (hiPSCs) from donor fibroblasts through lentiviral transfection of Yamanaka Factors and induced the established hiPSCs into functional SMCs through an embryonic body (EB)-mediated route. The derived SMCs demonstrated the capabilities of vascular tissue formation in a subcutaneous implantation model. However, many issues need to be further addressed. First, an integration-free reprogramming is necessary as viral integration may lead to tumorigenesis [16]. Second, a directed lineage specification method is critical as EB route is time-consuming and results in a heterogeneous population of SMCs from diverse germ layers, which may not reflect the physiological properties of VSMCs in native blood vessels [17]. To address these issues, we aim to develop a novel procedure to generate patient-specific, integration-free hiPSCs and their defined mesoderm-originated cardiovascular progenitor cell (CVPC) and VSMC derivatives. The capabilities of the derived CVPCs in lineage specification into VSMCs and vascular tissue formation in pre-designed three-dimensional biodegradable scaffolds were investigated.

2. Materials and methods

2.1. Generation of integration-free hiPSCs with episomal vectors nucelofection

The procedure is illustrated in Fig. 1A. The use of the peripheral blood was approved by the Institutional Review Board of The University of Michigan and written informed consent was obtained from patients with aortic aneurysm diseases. Peripheral blood mononuclear cells (PBMCs) from three patients were obtained by density gradient centrifugation with lymphocyte separation medium (MP Biomedicals, Santa Ana, CA, USA). PBMCs were cultured under PBMC culture medium for 4–6 days. To generate integration-free hiPSCs, 1×10^6 cells were nucleofected with 20 μg episomal vectors (EV) plasmid DNA using a Human CD34 Cell Nucleofector Kit (Lonza, Walkersville, MD, USA) following a previous report [18]. The mixture of EVs contained 10 μg EV SFFV-OS, 5 μg EV SFFV-BCL-XL and 5 μg EV SFFV-MK. Immediately after nucleofection, the cells were cultured in plates pretreated with Retronectin (Lonza, Walkersville, MD, USA). Then the cells were transferred onto mouse embryonic fibroblast layers the next day. The alkaline phosphatase-positive hiPSC colonies were observed at 3–4 wk after nucleofection. Some hiPSCs colonies were picked for long-term culture. The hiPSCs were manually passaged every 5–7 days. At least three hiPSC lines were established for each donor. For long-term feeder-free culture, the cells were cultured and maintained on Matrigel-coated surface with TeSR-E8 medium.

2.2. Characterization of hiPSCs

Alkaline Phosphatase Staining Kit II (Stemgent, Cambridge, MA, USA) was used to stain for alkaline phosphatase. Briefly, the cells were fixed at room temperature for 5 min, washed with phosphate buffered saline (PBS) with Tween 20, and incubated with Substrate Solution for 5–15 min before the reaction was stopped. Immunofluorescence staining was applied to characterize marker expression. Briefly, hiPSCs or their derivatives were fixed in ice-cold 4% paraformaldehyde for 20 min, permeabilized in 0.5% Triton X-100, and blocked with 5% normal goat serum. The cells were then incubated overnight with a panel of primary antibodies: rabbit anti-OCT4, rabbit anti-NANOG, rabbit anti-SOX2, mouse anti-TRA-1-81, mouse anti-TRA-1-60 (1:100, Stemgent, Cambridge, MA, USA); rat anti-SSEA3, mouse anti-SSEA4 (1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA); mouse anti-α-SMA, mouse anti-CNN1 (1:500, Sigma, St. Louis, MO, USA); rabbit anti-SM22α (1:500, Abcam, Cambridge, MA, USA); rabbit anti-MESP1 (1:100, Aviva Systems Biology, San Diego, CA, USA) and mouse anti-ISL1 (1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After washing with PBS, Alexa 488 or Alexa 594 conjugated secondary goat anti-mouse, rat or rabbit IgG or IgM antibodies were added and incubated for 1 h. The antibodies used were listed in Supplemental Table S1. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Grand Island, NY, USA). The samples were observed under an Olympus BX53 fluorescence microscope. The percentage of SMC marker positive cells was quantified from three independent cultures ($n = 3$).

2.3. Teratoma formation assay

NOD.CB17-Prkdcscid/J mice (Stock# 001303, Jackson Laboratory, Bar Harbor, ME, USA) were used for teratoma assay. 1×10^6 hiPSCs were suspended in 50 μl of PBS and mixed with 50 μl of ice-cold Matrigel [19]. The cell/Matrigel mixture was then subcutaneously injected intra-muscularly into hind limbs. After 8 wk, the formed teratoma was dissected and fixed with 4% paraformaldehyde for 24 h. Paraffin-embedded tissue samples were sliced and stained with hematoxylin and eosin (H-E). Images were captured with an Olympus IX71 microscope. The animal procedures were performed according to the protocol approved by the University of Michigan Committee for Use and Care of Laboratory Animals.

2.4. Lineage specification of hiPSCs into CVPCs and VSMCs

The procedure was illustrated in Fig. 2A. The hiPSCs were digested with Versene (Life Technologies, Grand Island, NY, USA) into single cells and plated onto Matrigel-coated culture dishes at a density of 5×10^4 cells/cm^2 in CVPC induction medium (CIM), which contained DMEM/F12, 1% penicillin/streptomycin, 1 × B27 supplement (without vitamin A), 400 μM 1-thioglycerol (Sigma, St. Louis, MO, USA), 50 μg/mL ascorbic acid (Sigma, St. Louis, MO, USA),