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Arterial specification of endothelial cells derived from human induced pluripotent stem cells in a biomimetic flow bioreactor

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ABSTRACT

Endothelial cells (ECs) exist in different microenvironments *in vivo*, including under different levels of shear stress in arteries versus veins. Standard stem cell differentiation protocols to derive ECs and EC-subtypes from human induced pluripotent stem cells (hiPSCs) generally use growth factors or other soluble factors in an effort to specify cell fate. In this study, a biomimetic flow bioreactor was used to subject hiPSC-derived ECs (hiPSC-ECs) to shear stress to determine the impacts on phenotype and upregulation of markers associated with an anti-thrombotic, anti-inflammatory, arterial-like phenotype. The *in vitro* bioreactor system was able to efficiently mature hiPSC-ECs into arterial-like cells in 24 h, as demonstrated by qRT-PCR for arterial markers EphrinB2, CXCR4, Conexin40 and Notch1, as well protein-level expression of Notch1 intracellular domain (NICD). Furthermore, the exogenous addition of soluble factors was not able to fully recapitulate this phenotype that was imparted by shear stress exposure. The induction of these phenotypic changes was biomechanically mediated in the shear stress bioreactor. This biomimetic flow bioreactor is an effective means for the differentiation of hiPSC-ECs toward an arterial-like phenotype, and is amenable to scale-up for culturing large quantities of cells for tissue engineering applications.

1. Introduction

The vasculature is one of the earliest organ systems to develop in the embryo. Endothelial cells (ECs) line the entire vasculature and have critically important roles in maintaining a dynamic barrier between blood and interstitial compartments, maintaining vasomotor tone, regulating inflammation and thrombosis, and maintaining overall vascular homeostasis. ECs exhibit significant functional and phenotypic heterogeneity [1], and various stimuli, including biomechanical shear stress, can modulate their phenotype.

In order to establish a cellular platform for studying endothelial biology and generating functional vascularized tissue suitable for transplantation, a reliable EC source is needed. Currently, isolated endothelial cells from human tissue are the main cell source used to study basic endothelial biology. However, these primary cells rapidly lose phenotypic marker expression *in vitro*, and isolation of large numbers of cells for expansion is difficult. The advent of human induced pluripotent stem cells (hiPSCs) [2], which can be derived from a person's own somatic cells and differentiated into virtually every cell type in the body, constitutes a powerful tool for vascular tissue regeneration. In addition, ECs derived from hiPSCs are unique in that they have never been exposed to blood flow, which makes them different from tissue-derived ECs, which are invariably exposed to flow during the lifetime of the tissue donor. Thus, immature and non-lineage-committed endothelial cells, derived from hiPSCs, may possess greater inherent plasticity as compared to primary ECs, such as human umbilical cord vein endothelial cells (HUVECs) or human aortic endothelial cells (HAECs).

Abbreviations: hiPSC, human induced pluripotent stem cells; hiPSC-EC, human iPSC-derived endothelial cells; HUVEC, human umbilical cord vein endothelial cells; HAEC, human aortic endothelial cells.

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To date, multiple groups have described the differentiation of ECs from hiPSCs [3–6]. These cells display both specific functions characteristic of primary ECs [3] and have been demonstrated to have therapeutic potential by promoting perfusion of ischemic tissues in a mouse model of peripheral arterial disease [7]. However, ECs were isolated from static culture based on general EC marker expression, including CD31 (platelet endothelial cell adhesion molecule-1 or PECAM-1), KDR (vascular endothelial growth factor receptor-2 or VEGFR2) and VE-cadherin (CD144), which are present on arterial, venous, and lymphatic subtypes of endothelium. This means that endothelium derived from hiPSCs may be heterogeneous in terms of their precise lineage [5]. We sought to determine the impact of shear using a biomimetic flow bioreactor on the lineage specification of ECs derived from hiPSCs, in order to gain a better understanding of differentiation pathways and to potentially enhance the suitability of cells for various research and therapeutic applications.

Studies have shown that shear stress, or the adrenomedullin/cAMP pathway [8], can activate Notch signaling [9], thereby causing endothelial maturation and full differentiation into the arterial phenotype. Further, shear stress regulates EC gene expression for proliferation and survival [10] as well as vasoactive and anti-thrombotic substances such as nitric oxide (NO), prostacyclin [11], and thrombomodulin [12]. Shear pre-conditioned ECs have been shown to maintain their anti-thrombotic surface in arterial tissue engineered vascular grafts (TEVGs) *in vivo*, possibly by releasing NO [13]. Thus, shear stress caused by hemodynamic fluid flow is a crucial regulator of vascular homeostasis and normal EC function.

Arterio-venous fate determination occurs concurrently with the onset of blood flow [14]. Distinct molecular markers signify the differences between arterial and venous ECs during normal vascular patterning [15]. Nevertheless, the vascular endothelium is plastic in nature, and shear stress caused by blood flow can modulate the expression of arterial and venous-specific genes [16]. However, this phenotypic plasticity is present only to a certain degree in mature primary (adult) ECs. It has been shown that venous markers on vein grafts are lost after placement in the arterial environment, but that arterial identity is not induced, suggesting an incomplete adaptation to the high-flow arterial environment [17]. However, ECs derived from stem cells (hESCs) have much more plasticity as compared to adult ECs, as they are able to effectively upregulate markers associated with an arterial phenotype [9].

In this study, we evaluated the impact of shear stress on the expression of venous and arterial markers in ECs that were derived from hiPSCs. We generated ECs from hiPSCs using a directed differentiation approach, and examined the impact of shear stress on the maturation of hiPSC-ECs toward a venous- or arterial-like phenotype using our flow bioreactor. We cultured hiPSC-ECs on a porous mesh inside a biomimetic bioreactor system that mimics blood flow through a vessel, imparting “arterial” or “venous” levels of shear stress on the cells. The activation of vasoprotective, anti-inflammatory markers KLF2 and KLF4 was assessed, as well as the angiogenic potential of hiPSC-EC that were cultured in the bioreactor as compared to human umbilical cord vein endothelial cells (HUVECs) and human arterial endothelial cells (HAECs) We then compared the effect of the addition of soluble factors that have been shown to impact arterial specification on the expression of these same markers. Our results showed that physiological levels of shear stress upregulates markers associated with a vasoprotective, arterial-like phenotype significantly better than soluble factors, thus demonstrating the importance of biomechanical flow on EC subtype specification.

2. Materials and methods

2.1. Cultivation of human iPS cells (hiPSCs)

Previously described human iPSC (hiPSC) lines were utilized for all experiments [18,19] and were maintained on Matrigel as described in prior publications [2,19]. All hiPSCs expressed Oct4, Sox2, and Nanog as assessed by immunostaining (data not shown). These cells have normal karyotypes, express cell surface markers and genes that characterize pluripotent human ES cells, and maintain the developmental potential to differentiate into advanced derivatives of all three primary germ layers. Briefly, hiPSCs were propagated on hESC-qualified Matrigel (BD Bioscience) from passages 25–40 and maintained in mTeSR medium (Stemcell Technologies). Medium was replaced daily and hiPSC colonies were routinely passaged every 5–7 days by mechanical dissociation using dispase (Stemcell technologies). The hiPSC line C2 (neonatal foreskin) utilized here was provided by Dr. James A Thomson, Department of Anatomy, University of Wisconsin-Madison, Madison, WI and p-hiPSC line (human newborn fibroblasts) was provided by Dr. Yibing Qyang, Department of Medicine, Section of Cardiovascular Medicine, Yale University, New Haven, CT.

2.2. In vitro differentiation and isolation of endothelial cells from hiPSCs (hiPSC-ECs)

hiPSCs were differentiated into ECs via embryoid body formation using directed differentiation (Fig. 1A, top) in a manner similar to previously published protocols [5,6]. Briefly, embryoid bodies (EBs) were formed using dispase on hiPSC colonies for 15 min, until colonies lifted off plate, and were carefully collected into a 15 mL conical tube. After washing twice with phosphate buffered saline (PBS), EBs were plated at high density into ultra-low attachment 6-well plates (Corning, Inc.) and first differentiated to mesoderm using 20 ng/mL BMP-4 (R&D Systems) for 4 days in human embryoid medium (hEB), containing knockout DMEM (KO-DMEM, Life technologies) with 20% FBS (Hyclone), 1% NEAA, 1 mM L-glutamine and 0.5 mM 2-mercaptoethanol. At then end of 4 days, EB's were attached to 0.67% gelatin-coated plates (1 well to 1 well ratio) and were cultured for an additional 10 days in the differentiation medium containing Vasculife VEGF medium (Lifeline Technologies) supplemented with 10% FBS and 50 ng/mL VEGF (Fig. 1A–D) to specify vascular fate with medium changes every other day.

Isolation of hiPSC-ECs: After 14 days of differentiation, cells were harvested using a CD31+ magnetic bead isolation kit to obtain a homogeneous population of hiPSC-ECs. Briefly, medium was aspirated, cells were washed with PBS and collagenase A/B 2 mg/mL was added for 30 min at 37 °C. Cells were pipeted vigorously to break up clusters and were filtered through a 40 μm nylon mesh and centrifuged at 1000 RPM for 5 min. Then, the supernatant was aspirated and 5 mL of accutase (Stemcell technologies) was added for an additional 5 min to ensure a single cell suspension. To isolate ECs, a CD31 magnetic bead (Dynabeads, Invitrogen) isolation kit was used according to manufacturer's protocol. Briefly, approximately 2 million cells (per 6-well plate) were incubated with 20 μL of Dynabeads in PBS + 0.1% bovine serum albumin (BSA) for 20 min at 4 °C with rocking, followed by placing tube in magnet and washing to remove non-CD31+ cells. After isolation, CD31+ hiPSC-ECs (passage 0, P0) were plated on fibronectin-coated plates (3 μg/cm^2) in expansion medium containing Vasculife VEGF medium + 2% FBS + 5 ng/mL VEGF. Once confluent, cells were passaged routinely and plated at 10,000 cells/cm^2 (Fig. 1F–G). These isolated and expanded homogeneous hiPSC-ECs were used for all subsequent bioreactor seeding/shear experiments. Cells were routinely passaged and maintained CD31 and VE-cadherin marker expression as assessed by qPCR (Fig. 3H). Doubling time for cells was approximately 24 h and cells up to P4 were used for experiments.

2.3. Primary human endothelial cell culture

Pooled primary human umbilical cord vein endothelial cells (HUVECs) were obtained from the Yale University Vascular Biology and Therapeutics Tissue Culture Core Facility. Human aortic endothelial cells (HAECs) were purchased from PromoCell. Both cell lines were maintained in Vasculife VEGF medium (Lifeline Technologies). All cell lines were routinely passaged at 80% confluence every 3–4 days and used between passages 2–6.

2.4. Bioreactor design and assembly

The bioreactor used in this study is designed to simulate the flow of blood through a blood vessel lumen. The housing was custom-made (Yale University glass blower, Fig. 2C) and consists of a borosilicate glass chamber 51 mm OD, 43 mm ID, capped with a threaded white PTFE cap (Figs. 2B and 2C). Three holes, 7 mm, were bored through the cap, two for silicon tubing and one for a sterile air filter attachment port. A medium outlet port, 5 mm, is incorporated at the base. For closed-loop flow, Master-Flex L/S 16 tubing (Cole-Parmer ZW-06508-16) was used to attach to this base via a sealing luer-lock (Cole-Parmer ZW-06464-90) to male connectors on the cap. The lumens were 3 cc luer-lock syringes (BD 209657) cut at the 1.5 cc mark and each bioreactor housed two lumens. A peristaltic pump was used for flow (Cole Parmer).

Millipore's Biopore Membrane (BGCM00010) was used as a cell support inside the lumens of the vessel chambers that were placed inside the bioreactors (Fig. 2A, arrow). This membrane has been shown to be highly supportive of cell growth [20]. This membrane is bi-layer with the upper being composed of 50 μm thick polytetrafluoroethylene (PTFE) with 0.4 μm pores for cell attachment and ingrowth. The lower layer is