Using human pluripotent stem cell differentiated endothelial cells to study the differential regulation in PAH

Kezhou Qin
Department of Cell Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College

Jun Yang (yang_jun@zju.edu.cn)
Zhejiang University School of Medicine  https://orcid.org/0000-0001-9715-8100

Research

Keywords: stem cell differentiation, endothelial cell, endothelial progenitor cell, pulmonary arterial hypertension

Posted Date: February 14th, 2020

DOI: https://doi.org/10.21203/rs.2.23516/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Aims

Endothelial cells (ECs) have been applied in clinic to treat pulmonary arterial hypertension (PAH), a disease characterized by disordered pulmonary vasculature. However, lack of enough transplantable cells before the deterioration of patient condition is current limitation to apply cell therapy in cardiovascular diseases. So, we thought it necessary to continue to differentiate embryo stem cells (ESCs)/induced-pluripotent stem cells (iPSCs) into endothelial cells (ECs) and identify their characteristics.

Methods and results

Comparing previous reported methods of human pluripotent cell differentiation toward vascular cells/Hemogenic Endothelial cells/ endothelial colony-forming cells, we established a highly efficient differentiation protocol to get cells that match phenotype of isolated ECs from health donors. This protocol, including two stages, early mesoderm endothelial progenitor stage and EC marker expression stage. In the first stage, Rock inhibitor Y27632 and DMSO plays an important role in inducing-APLNR + mesoderm and promotes EC differentiation potential; later on SB431542 and BMP4 drives cells toward EC lineage. Meanwhile, an improved protocol with chemically-defined medium (CDM) has similar differentiation efficiency, again demonstrating that a large number of clinically needed cells could be obtained with simple factors. ESC/iPSC-ECs, normal EPCs and IPAH-EPCs have the characteristics of early EPCs marked by CD133 and mesenchymal stem cells. Microarray analysis further revealed IPAH-derived EPCs features of rapid proliferation and weak immune regulation. At last, a model Zebrfish xenograft was utilized to assess the functionality of differentiated ESC/iPSC-ECs.

Conclusions

We established a highly efficient differentiation protocol to get ESC/iPSC-ECs with characteristics of phenotype and molecular matched with early-EPCs from health donors, and revealed the molecular pathogenesis in PAH.

1. Introduction

During hypoxia environment, endothelial cell (ECs) have an ability to modulate vascular tone and induce vascular remodeling and angiogenesis[1], and endothelial progenitor cells (EPCs) have been demonstrated that it can promote ischemic tissue angiogenesis [2], so they both are capable of facilitating vascular repair in different ischaemic tissues, such as acute myocardial infarction, unstable angina, stroke, diabetic micro vasculopathies, pulmonary arterial hypertension, atherosclerosis, and ischaemic retinopathies[3–7]. ECs and EPCs, which have limited expansion potential, are rare in peripheral or umbilical cord blood, comparing with other blood cells, so it's a huge challenge to repair vascular in clinical treatment[8–10].
Human ESCs/iPSCs can be induced to produce scalable endothelial cells and endothelial progenitor cells for vascular modeling [9, 11–16], and now there have been two methods of inducing ESCs or iPSCs differentiate to vascular cell, which are embryoid body formation [12, 17] and monolayer-directed differentiation[18, 19]. In the former method, the cells need to be transferred into ultra-low-attachment plates to get embryoid bodies (EBs) and produce various types of cells, the cost is large and the efficiency is low [7, 12, 20, 21]; besides, embryoid body differentiation is often time consuming[22]. Monolayer differentiation methods have a higher efficiency [15], but it is necessary to explore further to have a better understanding of complicated factors.

Endothelial dysfunction has been thought to be the main cause of PAH/IPAH. Some articles have reported that numbers of CD133$^+$ cells in peripheral blood increased in PAH/IPAH patients as compared with controls[23, 24]. Toshner et al also demonstrated PAH patient-derived endothelial progenitor cells have a hyperproliferative phenotype and a reduced capacity to form vascular networks[24]. But further investigation is necessary to continue at gene expression profile level.

In this study, we aim to explore the factors affecting vascular cell differentiation and to establish a chemical definition and cost-effective system of generating human ESC/iPSC-ECs through monolayer-directed differentiation, which is more efficient comparing with three other protocols [15, 25, 26]; meanwhile, we want to reveal the molecular features of ESC/iPSC-ECs, IPAH-derived EPCs and normal EPCs. We found that Rock inhibitor Y27632 and DMSO markedly accelerated vascular mesoderm generation from ESCs/iPSCs and improved the differentiation efficiency. Then, the functions of isolated cells have been jointly tested through tube formation assay, LDL uptake assay, cell transplantation assay, Microarray and zebrafish assay. Thus, the improved system can offer a simple, cost-effective platform to produce ESC/iPSC-ECs from ESCs/iPSCs for vascularization research and clinical application.

2. Methods

2.1 EPCs from adult peripheral blood samples

EPCs were isolated from human peripheral blood (PB). Fresh human PB (20 mL) was obtained under full ethical approval; then mononuclear cells (MNCs) were isolated from PB by density gradient centrifugation and cultured in EGM2(Lonza) supplemented with 16% FBS.

2.2 uptake of acetylated LDL

ESC/iPSC-ECs were incubated with 10 g/mL Dil-Ac-LDL (Molecular Probes) in serum-free EBM-2 (Lonza), respectively.

2.3 Cell Maintenance

Human ESCs/iPSCs were cultured in Essential 8 (E8) medium or mTeSR™1 Complete Kit (Catalog #85850) or hPSC-CDM™(Cauliscell Inc. #400105) supplemented with hPSC-CDM™ supplement (Cauliscell
Inc. #600301) on Matrigel-coated (BD Biosciences, #356230) 6-well plates and were passaged with 500 µM EDTA for 3–5 min. EPCs/ECFCs were maintained in EGM2 + 16%FBS (HyClone) [27].

2.4 Endothelial Cell Differentiation, Purification, and Culture

When human ESCs/iPSCs grew to 80%-90% confluency, they were dissociated with Accutase (Gibco, #A11105-01) and plated about 3 x 10^4 cells/well in vitronectin-coated (Cauliscell Inc. #500125) 12-well plates, ESCs/iPSCs were differentiated into mesoderm cells by culturing for 72 h in E8 medium (Gibco, A1516901) supplemented with 25 ng/mL Actin A (R&D, Catalog Number: 338-AC), 10 µM Y27632 (Sigma) and 10 ng/mL BMP4 (R&D, Catalog Number: 314-BP) for three days. Mesoderm cells were then cultured for 4 days in E6 medium (Gibco, A1516401) supplemented with FGF2 100 ng/ml (R&D, Catalog Number: 233-FB), VEGF 50 ng/ml (R&D, Catalog Number: 293-VE), BMP4 50 ng/mL and SB431542 5 µM (Sigma-Aldrich, CAS 301836-41-9-Calbiochem) to generate endothelial cells, and the whole differentiation process continues 7 days. Cells were counted and the cell suspension was prepared for the isolation of endothelial cells. Endothelial cells were isolated by using CD31^+ MicroBeads (Miltenyi Biotec, Order no.130-091-935) according to manufacturer's instructions and cultured in EGM2 with 16% FBS (HyClone).

2.5 Flow cytometry

At day 3 or 7 of differentiation, adherent cells were harvested using 0.25% TrypleE with EDTA and made into a single-cell suspension in PBS with 0.2% BSA. Mouse Anti-Human APJ APC-conjugated Antibody (R&D, Catalog Number: FAB8561A) was used as a ratio of 1: 50, Mouse Anti-human CD31 (CD31-FITC, Material Number: 555824, BD Pharmingen), Mouse Anti-human CD34 (CD34-APC, Material Number: 560940, BD Pharmingen), Mouse Anti-human CD43 (CD43-APC, Material Number: 560198, BD Pharmingen), Mouse Anti-human KDR (KDR-PE, FAB357P, R&D) and Mouse Anti-Human NRP-1 (NRP-1-PE, Material Number: 565951, BD) antibodies were used at a ratio of 1:20. Single-cell suspensions were subsequently incubated with antibody or antibodies at 4°C for about 40 min. Flow cytometric detection of the cell surface antigens were performed on a BD Accuri™ C6 Plus personal flow cytometer (Becton Dickinson). Compensation was set by single-positive controls.

2.5 In vitro capillary network formation assay on Matrigel

Endothelial cells were trypsinized and resuspended in EGM-2 media with 16% FBS. Cells were plated at a density of 1.0 x 10^4 cells per well in triplicate in 96-well plates coated with 50 µl of growth factor-reduced Matrigel (BD Biosciences). Plates were incubated overnight at 37 °C. After 6 h of incubation, photomicrographs were taken from each well at 10x magnification using an Olympus CKX41 microscope with a 10x objective.

2.7 Immunofluorescence assay

ESC/iPSC-ECs and EPCs were fixed with 4% (w/v) paraformaldehyde for 10 min and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min. After blocking with 10% (v/v) donkey serum for 30 min, cells were incubated with primary following antibodies; anti-EPHB4 (ABclonal, A3293, 1:100), anti-EFNB2 (ABclonal, A5669, 1:100), anti-CD133 (ABclonal, 1155750301, 1:100) and anti-CD146 (Abcam, ab75769, ABclonal, A5669, 1:100), anti-CD133 (ABclonal, 1155750301, 1:100) and anti-CD146 (Abcam, ab75769,
Cells were washed with PBS, then incubated with secondary antibodies conjugated with Alexa-488 or Alexa-594 (Molecular Probe) and visualized by confocal microscopy. The confocal images were obtained with a Zeiss confocal microscope. All the images were taken at room temperature and images were analyzed using a ZEN 2.6 (blue edition).

2.8 RNA extraction, cDNA synthesis and RT–qPCR

Total RNA from human cell lines was extracted with Trizol (Life Technologies). RNA yield was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA (1 µg) was converted to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA). Quantitative PCR (qPCR) was done using TransStart Tip Green qPCR SuperMix (TransGen Biotech) and detection was achieved using the CFX Connect™ Real-Time System (BIO-RAD). Primer sequence are listed in Additional file 1: Table S1. Expression of target genes was normalized to reference gene GAPDH.

2.9 Cell transplantation and therapy in Zebrafish

Zebrafish, the transgenic line Tg (Flk: GFP) were maintained according to standard procedures in compliance with local approval. After 48 hours post fertilization (hpf), embryos were used to inject ESC/iPSC-ECs stained with CM-Dil at approximately 60 µm above the ventral end of the duct of Cuvier, then the embryos were maintained at 30 °C for 48 hours. Fluorescent image acquisition was performed using a Leica MZ16FA stereo-microscope. Zebrafish embryos were treated with sugen5416 for 24 hours at 4 hpf, then 20–30 fish were respectively therapied by ESC/iPSC-ECs /medium (EGM2 + 16%FBS) and maintained at 30 °C for 48 hours, the number of normal zebrafish were recorded. The experiments were repeated three times. Euthanasia of all zebrafish was performed by exposure to bleach or rapid freezing followed by maceration.

2.10 Microarray analysis

Samples including IPAH-EPC1, IPAH-EPC2, IPAH-EPC3, normal EPCs (Con1, Con2, Con3) and ESC/iPSC-ECs (H7EC, H9EC and 202EC) were used to do microarray analysis with HG-U133 Plus_2. Microarray data were normalized by R package affy with rma method, differentially expressed genes (log FoldChange <-2 or > 2, and p-Value < 0.05) were clustered by R package clusterProfiler [28].

2.11 Statistical analysis

Statistical analyses were performed using Student's t-test and data were reported as mean ± s.d. or standard error of the mean, Figure legends show the number of biological repeats for each experiment (n), the experiments were repeated three or more than three times. Statistical significance was assumed when P < 0.05.

3. Results
3.1 A modified protocol for generating ECs from human ESCs or iPSCs

A high-efficiency protocol for deriving ECs from ESCs or iPSCs has been published[26], we optimized the protocol according to other protocols [15, 25, 29], we seeded about $3 \times 10^4$ cells/well in 12-well plate using 10 µM Y27632 and 10 ng/mL BMP4 for the three days (Fig. 1a, b and c). H1 ESCs generated 92.17% ± 0.42% endothelial cells after 7 days of differentiation (Fig. 1d). Mesoderm cells treated with ROCK inhibitor is efficient to differentiate into skeletal myocytes [30], so we predicted that Y27632 in the process of EC differentiation plays the same role as their protocol. In order to further explore the inhibitor(Y27632)’s function, we designed a serial concentration gradient experiment (Fig. 2c). The result is that a small doses of Y27632 treatment can improve differentiation efficiency in the three days and the differentiation rate increased with the increase of the inhibitor’s concentration, so we predicted that H1 ESC line is very sensitive to Y27632, then, we first built the highly efficient differentiation system.

In addition, thinking of using Y27632 to improve cell survival, we treated cells by Y27632 for one day or three days, and proved that it has a higher differentiation rate for the three days than for the one day (Fig. 2a and b). Then, we repeated the experiment with Y27632 treatment for one day or three days in 202-iPSC line, and the differentiation rate for the three days (41.9% ± 4.78%) is higher than with Y27632 treatment for one day (6.37% ± 1.07%) (Additional file 1: Figure S1a and b). Our results demonstrated that Y27632 has some effect on ESC differentiation, but the efficiency of EC differentiation is still not high as same as the above, so we predicted that there are other influencing factors.

3.2 Optimizing conditions to increase the ESC/iPSC-EC differentiation efficiency

Then we only utilized the protocol[26] and further explore how to promote the efficiency. Firstly, we designed a serial cell density experiment and found that the differentiation efficiency decreased with the increase of the cell density in H1-ESCs (Additional file 1: Figure S2a). Secondly, thinking of the function of two factors: SB431542 which inhibits TGF-β pathway to repress smad signal and BMP4 which promotes smad signal, we chose H9-ESC line to do the next experiments. With the increase of SB431542 concentration (5uM, 10uM and 20uM) in the second stage, the differentiation efficiency increased (Additional file 1: Figure S2b and d). BMP4 has been used both stages (first stage and second stage), here we checked whether adding BMP4 or not affected ESC/iPSC-EC differentiation efficiency in the second stage, the result is that there was a higher differentiation efficiency (Additional file 1: Figure S2c) when we plated 30,000 cells with BMP4; this result is as same as that BMP4 promotes generation of CD31+CD34+ cells[31]. From these results above, we drew a conclusion that seeding cell number has some effect on differentiation, BMP4 and SB431542 in the second stage promote ESC/iPSC-EC differentiation. But, we still didn’t solve the problem of low differentiation efficiency.
3.3 DMSO also plays an important role in Hemogenic Endothelial (HE) and ESC/iPSC-EC differentiation

We also improved the Hemogenic Endothelial differentiation protocol [25]. When we added the inhibitor Y27632 dissolved in DMSO for three days, we found that there is a higher differentiation efficiency in the H1-ESC line: CD31⁺CD34⁺ cells occupied 37.70%±1.55% versus 2.39% ± 0.17%, CD43⁺ cells occupied 21.85%±6.31% versus 9.17% ± 0.65%. It again proved that Y27632 dissolved in DMSO plays an important role in the ESCs-derived HE differentiation (Fig. 3a and b), and the efficiency of differentiation is lower with Y27632 dissolved in ddH2O (Fig. 3c and d). So here, we proved that DMSO also plays an important role in the HE differentiation system.

In order to understand well the function of DMSO, we found several papers referring to ESC differentiation. The efficiency of hPSC differentiation is improved even if hPSCs are treated with DMSO by short time [32]; addition of DMSO also downregulates expression of stemness-related genes such as OCT4 and NANOG, and increases the proficiency of hepatic differentiation [33]; DMSO at 0.01% and 0.1% concentration can act as an inducing agent for the formation of mesodermal phenotypes [34].

Under the condition with adding DMSO in the first step, we confirmed the highly efficient differentiation system using improved protocol again with Y27632 for one day or three days (Fig. 3e and f), and obtained the same result even if we changed another chemically-defined medium (CDM) (Additional file 1: Figure S3). So, the result is that DMSO is an important factor for ESC/iPSC-EC differentiation. From the above, we checked factors of affecting EC differentiation including seeding cell number, BMP4, SB431542, DMSO, Y27632 and treatment time, then we figured out the reason why we can't repeat our improved protocol for a while.

3.4 ESC/iPSC-ECs, normal EPCs and IPAH-EPCs have characteristics of early EPCs

Several articles [16, 35] has reported that they can induce ESCs/iPSCs into ECFCs (also called late EPCs) with CD31⁺NRP1⁺ and maintain these ECFCs in complete endothelial cell medium called EGM2 medium (Lonza). Because peripheral blood-derived EPCs were cultured in EGM2 with 16% FBS [27], ESC/iPSC-ECs also were maintained in the same conditions. To make sure whether ESC/iPSC-ECs belongs to ECFCs or not, we compared cell morphology of ESC/iPSC-ECs with peripheral blood-derived EPCs (Fig. 4a) and detected the elevation of NRP1 gene expression in the differentiation process (Fig. 4e); then, we checked several markers such as CD31(PECAM1), KDR, NRP1 and CD34 through flow cytometry, the result also showed cells with NRP1 or KDR from ESC/iPSC-ECs and EPCs from IPAH patient occupied higher percentage than EPCs from normal persons (Fig. 4b). In addition, we also proved that H1-derived ECs have the ability of tube formation (Fig. 4c) and can uptake acetylated LDL (Fig. 4d). Thus, these results suggest ESC/iPSC-ECs should be like EPCs.
EPCs have two distinct EPC subtypes which have been named as early EPCs and late EPCs which are also known as endothelial colony-forming cells (ECFCs) [35, 36], which can be identified through qRT-PCR by three marker genes containing HLA-DRA, lysozyme (LYZ), and CD14 for the early EPCs, and three marker genes containing caveolin1 (CAV1), VE-cadherin (VE-CAD), and VWF for the late EPCs [9]. Next, we tried to isolate the two types of EPCs from 202-iPSC-derived ECs which were induced by day 5 or day 7, and ECs were maintained in complete EGM2 medium with 16% FBS for 2 to 4 passages. Results showed ECs on day 7 with higher expression level of LYZ and lower expression level of VE-CAD have some characteristics of early EPCs (Fig. 5a). In order to further make sure whether ECs belongs to early EPCs or not, we also checked the expression level of an early EPC marker gene CD133 (PROM1) [37] and another two marker gene EFN B2 for arterial endothelium and EPHB4 for vein endothelium by qRT-PCR (Fig. 5b). CD133 and EFN B2 have a higher expression level in 202-iPSC-induced ECs isolated from on day 7, and results of immunofluorescence assay also showed all the three markers have a certain expression level (Fig. 5c). Besides, endothelial cell marker CD146 (MCAM) has an expression level (Fig. 5d). Meanwhile, we conducted bioinformatics analysis on datasets of ESC/iPSC-ECs, normal EPCs, idiopathic pulmonary arterial hypertension (IPAH)-derived EPCs (Fig. 5f) and GSE93511 (2D_MG_H1EC and 2D_MG_HUVEC) (Fig. 5e) [26], these genes including CD43 (SPN), CD45 (PTPRC) and PROM1 which express in early-EPC have some expression. Thus, we think conservatively that normal EPCs, IPAH-EPCs and ESC/iPSC-ECs we obtained have characteristics of early EPCs.

3.5 ESC/iPSC-ECs, normal EPCs and IPAH-EPCs have characteristics of mesenchymal stem cells for homing

A lot of homing-related genes have been identified, such as CXCR4 [38], IGF2/IGF2R, CXCL12 [39–41]. Besides, mesenchymal stem cells have the ability of homing, whose protein markers including CD73 (NT5E), CD44, CD90 (THY1), CD105 (ENG) [42, 43]. In addition, insulin-like growth factor–binding protein 3 (IGFBP3) may improve vessel repair [44, 45]. Based on these literature reports, we checked all above genes which of all has some expression level in ESC/iPSC-ECs through bioinformatics analysis using datasets of our microarray (Fig. 6a) and 2D_MG_H1EC and 2D_MG_HUVEC from GSE93511 [26] (Fig. 6b). From the above, we predicted ESC/iPSC-ECs, normal EPCs and IPAH-EPCs have characteristics of mesenchymal stem cells for homing.

In order to further demonstrate that ESC/iPSC-ECs have the ability of homing, we examined the engraftment potential of ESC/iPSC–ECs in vivo. when ESC/iPSC-ECs were injected into the blood stream of 48 hpf Zebrafish embryos, ESC/iPSC-ECs were capable of integrating into the vascular system that had already developed (Fig. 6c). Besides, we performed cell therapy after sugen5416 treatment, the result is that percentage of zebrafish returning to normal is higher (26.71 ± 5.86) % treated by ESC/iPSC-ECs than (12.06 ± 4.49) % only by medium (EGM2 + 16% FBS) (Fig. 6d). So, ESC/iPSC-ECs we obtained have huge potential applications.

3.6 Microarray analysis reveals dysfunction of IPAH-EPCs
Next, we analyzed the correlation between ESC/iPSC-ECs and normal EPCs (CON-EPCs), and the result showed that the correlation was up to more than 90% (Fig. 7a). In the process of cell culture, we found IPAH-EPCs proliferated faster than ESC/iPSC-ECs, and ESC/iPSC-ECs proliferated a little faster than normal EPCs (data not shown), and from microarray data the relative expression level of MKi67, the cell proliferation marker gene, is the highest in IPAH-EPCs, then in ESC/iPSC-ECs the average of MKi67 expression level is higher than that of CON-EPC (Fig. 7b). In view of the high similarity between ESC/iPSC-ECs and normal EPCs, we performed the Gene ontology biological process (GOBP) analyses of ESC/iPSC-ECs and IPAH-EPCs relative to normal EPCs. Of TOP20 pathways results show that all up-regulated genes of IPAH-EPCs are enriched on the pathways related to cell division, while part genes of ESC/iPSC-ECs are clustered on the pathways related to cell division, extracellular matrix, differentiation and development pathways, etc (Fig. 7c and d). This result again tells us that IPAH-EPCs have a higher proliferation rate, which is consistent with the previous analysis results. Besides, in order to better understand the molecular characteristics of ESC/iPSC-ECs, we continued to do the GOBP analysis of ESC/iPSC-ECs and normal EPCs relative to IPAH-EPCs. Of the TOP20 pathways results showed that up-regulated genes of ESC/iPSC-ECs are enriched in the immune-related pathways, top four of which are related to neutrophils (Fig. 7e); parts of pathways of normal EPCs also were related to immunity (Fig. 7f). This reveals that IPAH-EPCs dismiss immune-related molecular characteristics and primarily enhance proliferation capacity, and suggests that ESC/iPSC-ECs, with early EPC characteristics, have greater cellular therapeutic potential.

4. Discussion

As a matter of fact, DMSO plays a role in the whole ESC/iPSC-EC differentiation, which we just ignored, because normally small chemical molecules are dissolved in DMSO, and in our study, we used two chemical molecules Y27632 and SB431542; some research used CHIR99021 to promote EC differentiation[14, 26]. According to our experience, DMSO treatment need a separate 37°C and 5% CO2 incubator, which avoids to induce the differentiation of other cells, so we guess that DMSO is very powerful, and suggest that we should use DMSO with care.

The role of the inhibitor Y27632 was usually neglected in the process of ESC/iPSC-EC and HE differentiation, because robust differentiation protocols using single cell have to make cell survive by the inhibitor Y27632 which can phosphorylate and activate the myosin II pathway and inhibit an E-cadherin-dependent apoptotic pathway [46]. Some articles have reported that Y27632 enhances differentiation of human term placenta-derived trophoblasts in vitro [47], differentiation induction [48] and mesendodermal differentiation [49], and in another article prolonged Tbx6 expression-induced mesoderm differentiated into skeletal myocytes with the Rock inhibitor Y27632 [30]. Besides, the extension of treatment time to 3 days in the first stage for mesoderm induction, consistent with another research[14], may be able to produce mesodermal cells substantially.

ESC/iPSC-ECs have the characteristics of EPCs or ECFCs. Hematopoietic and endothelial progenitor cells share a number of cell-surface markers, because they may originate from a common precursor, the hemangioblast [50]. It has been reported that CD34+ CD133+ VEGFR2+ cells are haematopoietic and may
not actually be true EPCs [9], so we chose the endothelial marker CD31+ to isolate CD31+ cells, then identified their characteristics as ESC/iPSC-ECs through GOBP analysis of microarray data and so on; moreover, the expansion ability of isolated CD31+ cells is as the same as normal person's EPCs, which of both are less proliferative than EPCs from IPAH-patients. At last, gene expression analysis from our microarray data showed that ESC/iPSC-ECs expressing CD40, CD90 and CD105 are similar to mesenchymal stem cells, and the experiment of zebrafish transplantation proves their potential application value.

In conclusion, to verify the system and reduce the cost, we tried another CDM medium and repeated the protocol, the results proved that’s good. On the one hand, the function of each factor in the differentiation process is better to understand, and on the other hand, it lays a foundation for future application. ESC/iPSC-ECs, normal EPCs and IPAH-EPCs have characteristics of early-EPCs and mesenchymal stem cells for homing. Meanwhile, we demonstrate IPAH-EPCs have a higher proliferation again than normal EPC, and further reveal IPAH-EPCs with lack of immune-related genes.

**Abbreviations**

APLNR: Apelin Receptor; BMP4: bone morphogenetic protein-4; BSA: Bovine serum albumin; CAV1: Caveolin1; CD105: ENG, endoglin; CD133: PROM1, prominin 1; CD133: prominin-1; CD14: CD14 molecule, Monocyte Differentiation Antigen; CD146: MCAM, melanoma cell adhesion molecule; CD31: PECAM1, Platelet endothelial cell adhesion molecule-1; CD34: Cell differentiation antigen; CD43: Sialophorin; CD44: CD44 Molecule (Indian Blood Group); CD45: PTPRC, protein tyrosine phosphatase, receptor type, C; CD73: NT5E, 5'-nucleotidase, ecto (CD73); CD90: THY1, Thy-1 cell surface antigen; CDM: Chemically-defined medium; CM-Dil: A kind of cell membrane fluorescent dye; CON-EPCs: Normal EPCs, control endothelial progenitor cells; CXCL12: Chemokine (C-X-C motif) ligand 12; CXCR4: C-X-C chemokine receptor type 4; Dil-Ac-LDL: DiI-Acetylated Low Density Lipoprotein; DMSO: dimethyl sulfoxide; E8: Essential 8; EBs: Embryoid bodies; ECFC: Endothelial colony-forming cells; ECs: Endothelial cells; EFN2: Ephrin-B2; EPCs: Endothelial progenitor cells; EPHB4: EPH receptor B4; ESCs: Embryo stem cells; FBS: Fetal bovine serum; FGF2: Fibroblast growth factor 2; Flk: GFP: Protein-tyrosine kinase receptor(flk)-green fluorescent protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GOBP: Gene ontology biological process; H1EC: H1 embryo stem cell-derived endothelial cell; HE: Hemogenic Endothelial; HLA-DRA: Major histocompatibility complex, class II, DR alpha; hPSCs: Human pluripotent stem cells; HUVEC: Human umbilical vein endothelial cells; IGF2/IGF2R: Insulin-like growth factor 2 (somatomedin A)/insulin-like growth factor 2 receptor; IGFBP3: Insulin-like growth factor–binding protein 3; IPAH: Idiopathic pulmonary arterial hypertension; iPSCs: Induced-pluripotent stem cells; KDR: Kinase insert domain receptor (a type III receptor tyrosine kinase); LYZ: Lysozyme; MKi67: Marker of proliferation Ki-67; MNCs: Mononuclear cells; MSCs: Mesenchymal stem cells; NANOG: Homeobox Transcription Factor; NRP-1: Neuropilin 1; OCT4: POU Class 5 Homeobox 1; PAH: Pulmonary arterial hypertension; PB: Peripheral blood; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; ROCK: Rho-associated, coiled-coil containing protein kinase; SB431542: TGF-beta/Smad inhibitor; Tbx6: T-Box Transcription Factor; Tg:
Transgene; TGF-β: Transforming growth factor β; VE: Vascular endothelial cells; VE-CAD: VE-cadherin; VEGF: Vascular endothelial growth factor; VEGFR2: KDR, kinase insert domain receptor (a type III receptor tyrosine kinase), Vascular endothelial growth factor; vWF: Von Willebrand factor; Y27632: Rock inhibitor.

Declarations

Acknowledgements

The authors thank Hongtao Wang and Mengge Wang from State Key Laboratory of Experimental Hematology, Institute of Hematology & Blood Diseases Hospital, Tianjin 300020, China for their guidance with Hemogenic Endothelial (HE) differentiation.

Authors’ contributions

K.Z.Q. and J.Y. participated in the research design, K.Z.Q. conducted the experiments and performed the data analysis, K.Z.Q., J.Y. wrote the manuscript. All authors reviewed and revised the final version and approved manuscript submission.

Funding

This research was supported by Grants from National Key Research and Development Program of China—stem cell and translational research (No: 2016YFA0102300), CAMS Innovation Fund for Medical Sciences (CIFMS 2016-I2M-4-003), China National Thousand (Young) Talents Program of Jun Yang.

Availability of data and materials

All data are included in the text and supplementary information.

Ethics approval and consent to participate

All experiments were performed in accordance with the principles of the China Zebrafish Resource Center and approved by the Research Ethics Committee of Peking Union Medical College. All animal procedures were carried out in the Zebrafish Laboratory of State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. All experimental studies using human samples comply with the Declaration of Helsinki and were approved by the local ethics committee with reference number 2018028 (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College). All persons gave informed consent before the study.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details

1 Department of Cell Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, 5 Dong Dan Santiao, Beijing, China 100005

2 Research Center of Molecular Medicine, Zhejiang University School of Medicine. Zhejiang, China 310058.

References

1. Niskanen, H., et al., Endothelial cell differentiation is encompassed by changes in long range interactions between inactive chromatin regions. Nucleic Acids Res, 2018. 46(4): p. 1724-1740.

2. Zhao, W., et al., Early risk assessment of circulating endothelial progenitor cells and plasma stromal cell-derived factor-1 for nondisabling ischemic cerebrovascular events. BMC Neurol, 2019. 19(1): p. 22.

3. Jung, K.-H. and J.-K. Roh, Circulating Endothelial Progenitor Cells in Cerebrovascular Disease. Journal of Clinical Neurology, 2008. 4(4): p. 139-147.

4. Rafii, S. and D. Lyden, Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nature Medicine, 2003. 9(6): p. 702-712.

5. Sekiguchi, H., M. Il, and D.W. Losordo, The Relative Potency and Safety of Endothelial Progenitor Cells and Unselected Mononuclear Cells for Recovery from Myocardial Infarction and Ischemia. Journal of Cellular Physiology, 2009. 219(2): p. 235-242.

6. Tateishi-Yuyama, E., et al., Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet, 2002. 360(9331): p. 427-435.

7. Ward, M.R., D.J. Stewart, and M.J.B. Kutryk, Endothelial progenitor cell therapy for the treatment of coronary disease, acute MI, and pulmonary arterial hypertension: Current perspectives. Catheterization and Cardiovascular Interventions, 2007. 70(7): p. 983-998.

8. Medina, R.J., et al., Endothelial progenitors as tools to study vascular disease. Stem Cells Int, 2012. 2012: p. 346735.

9. Medina, R.J., et al., Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. BMC Med Genomics, 2010. 3: p. 18.

10. Zhang, J., et al., Functional characterization of human pluripotent stem cell-derived arterial endothelial cells. Proc Natl Acad Sci U S A, 2017. 114(30): p. E6072-E6078.

11. Belair, D.G., et al., Human vascular tissue models formed from human induced pluripotent stem cell derived endothelial cells. Stem Cell Rev Rep, 2015. 11(3): p. 511-25.
12. James, D., et al., Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGF beta inhibition is Id1 dependent. Nature Biotechnology, 2010. 28(2): p. 161-U15.

13. Lian, X., et al., Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem Cell Reports, 2014. 3(5): p. 804-16.

14. Nguyen, M.T.X., et al., Differentiation of Human Embryonic Stem Cells to Endothelial Progenitor Cells on Laminins in Defined and Xeno-free Systems. Stem Cell Reports, 2016. 7(4): p. 802-816.

15. Patsch, C., et al., Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. Nat Cell Biol, 2015. 17(8): p. 994-1003.

16. Prasain, N., et al., Differentiation of human pluripotent stem cells to cells similar to cord-blood endothelial colony-forming cells. Nat Biotechnol, 2014. 32(11): p. 1151-1157.

17. Levenberg, S., et al., Endothelial cells derived from human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America, 2002. 99(7): p. 4391-4396.

18. Kane, N.M., et al., Derivation of Endothelial Cells From Human Embryonic Stem Cells by Directed Differentiation Analysis of MicroRNA and Angiogenesis In Vitro and In Vivo. Arteriosclerosis Thrombosis and Vascular Biology, 2010. 30(7): p. 1389-1399.

19. Vodyanik, M.A., et al., Human embryonic stem cell-derived CD34(+) cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood, 2005. 105(2): p. 617-626.

20. Li, Z., et al., Comparison of reporter gene and iron particle labeling for tracking fate of human embryonic stem cells and differentiated endothelial cells in living subjects. Stem Cells, 2008. 26(4): p. 864-873.

21. Wang, H., et al., Gene expression profile signatures indicate a role for wnt signaling in endothelial commitment from embryonic stem cells. Circulation Research, 2006. 98(10): p. 1331-1339.

22. Levenberg, S., et al., Endothelial potential of human embryonic stem cells. Blood, 2007. 110(3): p. 806-814.

23. Foris, V., et al., CD133+ cells in pulmonary arterial hypertension. Eur Respir J, 2016. 48(2): p. 459-69.

24. Toshner, M., et al., Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. Am J Respir Crit Care Med, 2009. 180(8): p. 780-7.

25. Wang, H., et al., MEIS1 Regulates Hemogenic Endothelial Generation, Megakaryopoiesis, and Thrombopoiesis in Human Pluripotent Stem Cells by Targeting TAL1 and FLI1. Stem Cell Reports, 2018. 10(2): p. 447-460.

26. Zhang, J., et al., A Genome-wide Analysis of Human Pluripotent Stem Cell-Derived Endothelial Cells in 2D or 3D Culture. Stem Cell Reports, 2017. 8(4): p. 907-918.

27. Oramiston, M.L., et al., Generation and Culture of Blood Outgrowth Endothelial Cells from Human Peripheral Blood. J Vis Exp, 2015(106): p. e53384.

28. Yu, G., et al., clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS, 2012. 16(5): p. 284-7.
29. Duan, F., et al., *Biphasic modulation of insulin signaling enables highly efficient hematopoietic differentiation from human pluripotent stem cells.* Stem Cell Res Ther, 2018. **9**(1): p. 205.

30. Sadahiro, T., et al., *Tbx6 Induces Nascent Mesoderm from Pluripotent Stem Cells and Temporally Controls Cardiac versus Somite Lineage Diversification.* Cell Stem Cell, 2018. **23**(3): p. 382-395 e5.

31. Bai, H., et al., *BMP4 regulates vascular progenitor development in human embryonic stem cells through a Smad-dependent pathway.* J Cell Biochem, 2010. **109**(2): p. 363-74.

32. Li, J., et al., *A transient DMSO treatment increases the differentiation potential of human pluripotent stem cells through the Rb family.* PLoS One, 2018. **13**(12): p. e0208110.

33. Czysz, K., S. Minger, and N. Thomas, *DMSO efficiently down regulates pluripotency genes in human embryonic stem cells during definitive endoderm derivation and increases the proficiency of hepatic differentiation.* PLoS One, 2015. **10**(2): p. e0117689.

34. Pal, R., et al., *Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells.* Arch Toxicol, 2012. **86**(4): p. 651-61.

35. Yoder, M.C., et al., *Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals.* Blood, 2007. **109**(5): p. 1801-9.

36. Hur, J., et al., *Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis.* Arteriosclerosis Thrombosis and Vascular Biology, 2004. **24**(2): p. 288-293.

37. Kanayasu-Toyoda, T., et al., *Cell-Surface MMP-9 Protein Is a Novel Functional Marker to Identify and Separate Proangiogenic Cells from Early Endothelial Progenitor Cells Derived from CD133(+) Cells.* Stem Cells, 2016. **34**(5): p. 1251-62.

38. Yuan, Z., et al., *17beta-estradiol promotes recovery after myocardial infarction by enhancing homing and angiogenic capacity of bone marrow-derived endothelial progenitor cells through ERAlpha-SDF-1/CXCR4 crosstalking.* Acta Biochim Biophys Sin (Shanghai), 2018. **50**(12): p. 1247-1256.

39. Zhuang, Y., et al., *Chemokine stromal cell-derived factor 1/CXCL12 increases homing of mesenchymal stem cells to injured myocardium and neovascularization following myocardial infarction.* Chin Med J (Engl), 2009. **122**(2): p. 183-7.

40. Xiaowei, C., et al., *Overexpression of CXCL12 chemokine up-regulates connexin and integrin expression in mesenchymal stem cells through PI3K/Akt pathway.* Cell Commun Adhes, 2013. **20**(3-4): p. 67-72.

41. Ferrari, D., et al., *Purinergic stimulation of human mesenchymal stem cells potentiates their chemotactic response to CXCL12 and increases the homing capacity and production of proinflammatory cytokines.* Exp Hematol, 2011. **39**(3): p. 360-74, 374 e1-5.

42. Caplan, A.I., *Adult mesenchymal stem cells and women's health.* Menopause, 2015. **22**(2): p. 131-5.

43. Jiang, B., et al., *Concise Review: Mesenchymal Stem Cells Derived from Human Pluripotent Cells, an Unlimited and Quality-Controllable Source for Therapeutic Applications.* Stem Cells, 2019. **37**(5): p. 572-581.
44. Lofqvist, C., et al., IGFBP3 suppresses retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth. Proc Natl Acad Sci U S A, 2007. 104(25): p. 10589-94.
45. Chang, K.H., et al., IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. Proc Natl Acad Sci U S A, 2007. 104(25): p. 10595-600.
46. Vernardis, S.I., et al., Human embryonic and induced pluripotent stem cells maintain phenotype but alter their metabolism after exposure to ROCK inhibitor. Sci Rep, 2017. 7: p. 42138.
47. Motomura, K., et al., A Rho-associated coiled-coil containing kinases (ROCK) inhibitor, Y-27632, enhances adhesion, viability and differentiation of human term placenta-derived trophoblasts in vitro. PLoS One, 2017. 12(5): p. e0177994.
48. Kurosawa, H., Application of Rho-associated protein kinase (ROCK) inhibitor to human pluripotent stem cells. J Biosci Bioeng, 2012. 114(6): p. 577-81.
49. Maldonado, M., et al., ROCK inhibitor primes human induced pluripotent stem cells to selectively differentiate towards mesendodermal lineage via epithelial-mesenchymal transition-like modulation. Stem Cell Res, 2016. 17(2): p. 222-227.
50. Ingram, D.A., et al., Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood, 2004. 104(9): p. 2752-60.

Figures
Figure 1

Improved Protocol for the highly efficient differentiation rate of hESCs toward ECs. a Human PSCs were differentiated into endothelial cells via a mesoderm intermediate. b Representative yields (APLNR+ cells) by flow cytometric analysis for a mesoderm intermediate. c Representative yields (CD34+CD31+ cells or CD43+cells) by flow cytometric analysis for ESC-derived endothelial cells (H1-ECs) after 7 days of differentiation. d Statistics of CD31+CD34+ and CD43+ cells. Data are represented as mean ± SD, n=3, the experiments were repeated three independent times.

Figure 2

Y27632 maybe improve the efficiency of ESC/iPSC-ECs in dose and time dependent manner. a Series content (containing 1.67uM, 5uM, 7.5uM and 10uM) of Y27632 was used in the first stem of differentiation by flow cytometric analysis. Representative results are shown. b In the first step of EC differentiation, Y27632 was used for one day or three day by flow cytometric analysis. c Statistics of CD31+CD34+, CD31+, CD34+ and CD43+ cells. Data are represented as mean ± SD, n=3-4, the experiments were repeated more than three independent times. Student’s t test (***p < 0.001).

Figure 3

Changed conditions improved HE differentiation efficiency. a Improved Protocol for the HE differentiation of hESCs, and representative yields (APLNR+ cells, CD31+CD34+ cells and CD43+ cells) with DMSO
treatment for the first step by flow cytometric analysis. c Representative yields (APLNR+ cells, CD31+CD34+ cells and CD43+ cells) without DMSO treatment for the first step by flow cytometric analysis. e H1-EC differentiation plus DMSO and Y27632 in the first step. Repeated the EC differentiation plus DMSO and Y27632 for one day or three days. b, d and f Statistics of APLNR+, CD31+CD34+, CD31+, CD34+ and CD43+ cells. Data are represented as mean ± SD, n=3-4, the experiments were repeated more than three times. Student’s t test (**p < 0.01, ***p < 0.001).

Figure 4

Characteristics of ESC/IPSC-ECs (H7EC, 202EC, H1EC) and the comparation with peripheral blood derived EPCs. a Comparation of cell morphology with peripheral blood derived EPCs (scale bars 50um). b Using Selected markers to identify cell characters by flow cytometric analysis. c Uptake assay of Dil-acetylated LDL (scale bars 50um). d Tube formation assay of H1-derived EPCs (scale bars 500um). e qRT-PCR of NRP1 which was reported promotes ECFC proliferation. Gapdh was used as an internal control. Data are represented as mean ± SD, n=3, the experiments were repeated three times. Student’s t test (*p < 0.05, ***p < 0.001).
Figure 5

ESC/IPSC-ECs have similar expression level of early EPC marker genes. a and c ECs from DAY 5 or DAY7 was analyzed by several makers including CD14, DRA, LYZ, vWF, VE-CAD, CAV1 and CD133, EFNB2, EPHB4. Gapdh was used as an internal control. Data are represented as mean ± SD, n=3, the experiments were repeated three times. Student’s t test (**)p < 0.01, ***p < 0.001). b and d Immunofluorescence assay (scale bars: 20um). e and f Heatmap of EC-related genes from dataset of 2D_MG_H1EC and 2D_MG_HUVEC from GSE93511 and our microarray, IPAH-EPCs(IPAH1, IPAH2 and IPAH3), normal EPCs (Con1, Con2, Con3) and ESC/iPSC-ECs (H7EC, H9EC, 202EC, H1EC).
Figure 6

Vascular competence of ESC/iPSC-ECs in zebrafish xenograft model. a Heatmap of genes for homing from dataset of our microarray, IPAH-EPCs (IPAH1, IPAH2 and IPAH3), normal EPCs (Con1, Con2, Con3) and ESC/iPSC-ECs (H7EC, H9EC, 202EC). b Heatmap of genes for homing from dataset of 2D_MG_H1EC and 2D_MG_HUVEC from GSE93511. c A representative image of ESC/iPSC-ECs-derived vessel-type structures (in red) within the embryonic zebrafish (Flk: GFP; in green) 2 days after implantation, with magnification of the vessel. Scale bars are 300 μm. d Using zebrafish model for gene therapy. Data are represented as mean ± SD, n=3, the experiments were repeated three times and 20-30 fish per condition. Student’s t test (*p < 0.05).
Figure 7

Characteristics of normal EPCs, IPAH-EPCs and ESC/iPSC-ECs based on microarray data. a correlation analysis between normal EPCs (Con1, Con2, Con3) and ESC/iPSC-ECs (H7EC, H9EC, 202EC). b MKi67 relative expression from microarray data. Data are represented as mean ± SD. n=3, Student’s t test (*p < 0.05) . c and d GOBP analysis (TOP20) of ESC/iPSC-ECs and IPAH-EPCs relative to normal EPCs. e and f GOBP analysis (TOP20) of ESC/iPSC-ECs and normal EPCs relative to IPAH-EPCs.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1Qinekzhou20200201.docx