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Differentiation of functional endothelial cells from human induced pluripotent stem cells: A novel, highly efficient and cost effective method

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ABSTRACT

Endothelial cells derived from human induced pluripotent stem cells (hiPSC- EC) are of significant value for research on human vascular development, *in vitro* disease models and drug screening. Here we report an alternative, highly efficient and cost-effective simple three step method (mesoderm induction, endothelial cell differentiation and endothelial cell expansion) to differentiate hiPSC directly into endothelial cells. We demonstrate that efficiency of described method to derive CD31+ and VE-Cadherin+ double positive cells is higher than 80% in 12 days. Most notably we established that hiPSC-EC differentiation efficacy depends on optimization of both mesoderm differentiation and endothelial cell differentiation steps.

1. Introduction

Endothelial cells line the entire circulatory system, from the heart to capillaries and lymphatics. Due to their widespread distribution and their vast array of biological functions, endothelial cells are indispensable for development and growth and they play central roles in numerous diseases, ranging from congenital heart disease, to cancer and diabetes mellitus (Hink et al., 2001; Moretti et al., 2013). Despite such prominent interest in endothelial cell biology, studies involving cultured endothelial cells are still challenging, because it is difficult to generate primary endothelial cells from adult organs in substantial quantities, because endothelial cell lines de-differentiate and do not lend themselves for representative cell assays and because the derived endothelial cells often do not represent the intricate organ- and vessel type-specific phenotype encountered *in vivo*. Such limitations are even more concerning when studying genetic diseases, where derivation of isogenic endothelial cells is essential for meaningful experiments.

In this regard differentiating induced pluripotent stem (iPS) cells *in vitro* holds promise to solve these limitations, because iPS cells could be amplified unlimitedly *in vitro* in a chemically defined system (Chen et al., 2011; Orlova et al., 2014). As for endothelial cell generation from human pluripotent stem cells, currently two different principles are commonly used: embryoid body (EB) formation and monolayer differentiation (Table 1) (Lian et al., 2014; Orlova et al., 2014; Patsch et al., 2015; Sahara et al., 2014; Tatsumi et al., 2011; Yang et al., 2008). All fore-mentioned groups developed their unique protocols to successfully generate endothelial cells, although with various time and cost efficiency.

Here, we report a highly efficient, consistent, and cost effective protocol for generation of cells with phenotypic and functional properties of endothelial cells from hiPSCs. We achieved this through optimization of cell density and colony size for mesodermal differentiation, and optimization of growth factors for mesodermal to endothelial differentiation.

2. Material and methods

2.1. Cell culture

Human induced pluripotent stem cells (hiPSCs) used here were previously established and characterized (Dudek et al., 2013;

Abbreviations: iPSC, induced pluripotent stem cell; hiPSC, human induced pluripotent stem cell; iPSC-EC, induced pluripotent stem cell-derived endothelial cell; EC, endothelial cell; HCAEC, human coronary artery endothelial cells; vWF, von Willebrand factor; Ac-LDL, acetylated-low density lipoprotein

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Table 1.
Comparison of different EC differentiation protocols.

Publications	Highest Efficiency	Enrichment needed	Duration	Reagents	Estimated cost[a]
Yang et al. (2008)	30% CD31+ Endothelial Progenitors	Indirect differentiation; Enrichment needed	14 days	BMP4 F12 bFGF Activin A StemPro34 VEGFA	~40 Euros
Tatsumi et al. (2011).	20% CD144+ VEGFR2+ Endothelial Progenitors	Indirect Differentiation; Enrichment Needed	5 days	ES medium DMEM/F12 N2/B27 BIO Stem pro34 VEGFA	~32 Euros
Orlova et al. (2014)	19.9% CD31+ CD144+ Endothelial cell	Direct Differentiation; No enrichment	10 days	BPEL/APEL Activin A BMP4 VEGF SB431542	~120 Euros
Sahara et al. (2014).	64.3% CD31+ CD144+ Endothelial cell	Direct Differentiation; No enrichment	7 days	ROCK inhibitor N2/B27 DMEM/F12 StemPro-34 BMP4 VEGFA	~44 Euros
Lian et al. (2014).	Protocol 1 20.55% Protocol 2 55% CD31+ CD34+ Endothelial Progenitors	Direct differentiation; No enrichment	5 days	Advanced DMEM/F12 Ascorbic acid GlutaMAX CHIR99021 StemPro-34 VEGFA	Protocol1 ~42 Euros Protocol2 ~12 Euros
Patsch et al. (2015)	69.2% CD144+ Endothelial Progenitors	Direct Differentiation; No enrichment	6 days	StemPro-34 200 ng ml−1 VEGF-A 2 μM forskolin N2/B27 8 μM CHIR 1 μM CP21 25 ng/ml BMP4	~100 Euros
Our protocol	81% CD31+ CD144+ Endothelial cell	Direct differentiation; No enrichment	10 days	DMEM/F12 CHIR EBM VEGFA bFGF EMV2	28 Euros

[a] Listed reagents and cost from different protocols compared to ours. The estimated cost is calculated from induction of one complete 6-well plate (2 ml medium per well) hiPSCs into desired cell types (endothelial progenitors or endothelial cells). Detailed information on the companies from which prices for reagents were used is provided in the supplementary information.

Streckfuss-Bomeke et al., 2013) (Table S6). hiPSC were maintained in Essential 8 basal medium (Gibco Life Technologies) supplemented with Essential 8 Supplement (Gibco Life Technologies), and Penicillin-Streptomycin (Sigma-Aldrich). hiPSCs were cultured on Geltrex Reduced Growth Factors (Millipore) coated tissue culture dishes, and treated with Versene solution (Life technology) for passaging. Human Coronary Artery Endothelial Cells (HCAEC) were cultured in HCAEC growth medium (GENLANTIS) on the attachment factor coated (GENLANTIS) flasks. All cultured cells were kept in standard physical growth conditions (37 °C, 5% CO_2).

2.2. In vitro ECs differentiation methods

hiPSCs were seeded onto Geltrex coated dishes and cultured with Essential 8 Medium supplemented with pro survival factor (Millipore), which is cell-permeable pyrrolidine, a small molecule which promotes single human iPS cell or stem cell survival. To determine the size of the hiPSC colonies, fully confluent hiPSC layers were examined under the microscope at day 0 and colony sized was measured by using the AxioVision microscope software (Carl Zeiss). The colonies were gently scraped off from the dish and seeded on to Matrigel coated dishes. When cells had attached after 24 h, medium was changed to DMEM/F12 supplemented with 4 μM Chir99021 (Millipore). After 2 further days, medium was replaced with Endothelial cell basal medium (Promocell) supplemented with growth factors bFGF (Peprotech) and VEGFA (R&D) or Activin A (Peprotech) and BMP4 (Peprotech). After four further days, medium was replaced with EMV2 (Promo cell) medium supplemented with VEGFA, and medium was changed every other day until cell analysis. Experimental set-up is summarized in Fig. 1(A).

2.3. Flow cytometry and Fluorescence-activated cell sorting (FACS)

hiPSC derived endothelial cell progenitors were harvested after trypsin-EDTA digestion and washed in cold PBS. Pellets were re-suspended in 2% v/v bovine serum albumin (BSA), cell suspensions were standardized to a concentration of 1×10^6 cells/ml. After cells were incubated at 37 °C incubator for 30 min to allow for recovery from trypsinization, directly-labeled antibodies were added to cell suspensions (PE conjugated CD 31 antibody (BD Pharmingen) and Alexa Fluor 647 conjugated VE-cadherin antibody (BD Pharmingen). After 1 h incubation, samples were centrifuged and supernatants decanted. Cells were then resuspended in ice-cold FACS buffer to final concentrations of 1×10^6 cells/ml. Cells were then filtered through 100 μm cell strainers and fixed in 4% PFA for 5 min at room temperature for FACS analysis. For cell sorting experiments, all samples were blocked in blocking buffer with 5% BSA for 1 h before antibody was added, and incubated at 37 °C for 2 h (for living cell sorting) or 4 °C overnight (only for fixed cells). After PBS washing for three times, cell concentration was adjusted to 1×10^6 cells/ml for FACS sorting.

2.4. Culture of FACS-isolated cell populations

FACS-sorted CD31+ and VE-cadherin+ double positive cells were plated onto tissue culture plates coated with attachment factor (GENLANTIS) in Endothelial Cell growth medium (EMV2) supplemented with 15 ng/ml VEGFA. Once settled, sorted cells were cultured in EMV2 growth medium until passage 3 for functional analysis.

2.5. Immunofluorescence labeling of adherent cells

Cells growing in chamber slides were fixed by 4% paraformaldehyde, permeabilized in 0.1% TritonX-100 for 10 min and blocked in 5% BSA for 30 min before antibodies were added.