Stem Cell Research 42 (2020) 101685

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Short report

Differentiation and expansion of endothelial cells requires pre-optimization of KDR+ expression kinetics

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ARTICLE INFO

Keywords:
Cell fate
Differentiation
Stem cells
Vascular progenitor cells
Endothelial cells

ABSTRACT

Human endothelial cells (ECs) are important tools in research and development of new therapies in the fields of angiogenesis, vasculogenesis, engineering organoids and multicellular tissues, drug discovery, and disease modeling. Efficient and robust induction of ECs from human pluripotent stem cells (hPSCs) serve as a renewable and indefinite cell sources. However, individual lines of embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are distinct and can often respond very differently to the same microenvironmental cues. Therefore, we set out to develop a differentiation methodology specifically designed for robustness across multiple human iPSC lines. In general, the key soluble signals remain consistent across cell lines, but because the differentiation and proliferation kinetics can differ slightly in hESC and iPSC cell lines, the time point for KDR+ cell sorting must be pre-determined for each cell line. This three-stage induction method uses three different chemically defined medium formulations and generates highly purified populations of actively proliferating and functional VE-cadherin+ ECs within 30 days.

1. Introduction

The human body requires high densities of functional vasculature in through almost every organ. Functional endothelial cells (ECs), lining the lumen of all vasculature, are essential cells in promoting vascularization, generating vascularized tissue-engineered materials, and studying mechanisms of vascular defects underlying various diseases including: ischemia, atherosclerosis and cancer.

Broadly accessible, human umbilical vein ECs (HUVECs) are the most exhaustively researched EC. Other primary sources of human ECs include aortic endothelial cells, microvascular endothelial cells harvested from various tissues (e.g. fat, heart, etc.), blood brain barrier ECs harvested from brain tissue, and endothelial progenitor cells (EPCs) harvested as endothelial colony-forming cells (ECFCs) from adult peripheral blood or cultured cord blood. These human ECs require isolations from primary human tissue and exhibit limited proliferation potential.

The advent of human embryonic stem cells (hESCs) (Thomson et al., 1998) and human induced PSCs (hiPSCs) (Takahashi et al., 2007) were major breakthroughs in the field of regenerative medicine. Human pluripotent stem cells (hPSCs) provide an unlimited source of autologous cells with broad differentiation ability to generate all cell types in the human body. Disease-specific hiPSCs are already serving as excellent sources in disease modeling and drug discovery (Karagiannis et al., 2019). A few protocols deriving human ECs from hESC and hiPSCs have been reported: first using highly inefficient (1–3%) embryoid bodies (EBs) (Levenberg et al., 2002), then chemically-defined mediums and feeder-free monolayer cultures (Kane et al., 2010; Glaser et al., 2016) and by sprouting endothelial progenitor cells (EPC) into 3D fibrin scaffolds (Zhang et al., 2014). Methods for EC and pericyte co-differentiation have also been developed (Orlova et al., 2014; Orlova et al., 2014), directing iPSCs in defined medium supplemented with BMP-4 (or Activin), VEGF, and the GSK3β-kinase inhibitor (CHIR) generating cultures containing 15–25% $CD31^+/CD34^+$ EPC and up to 50% PDGFR mesenchymal cells after 10 days. The incorporation of seeding density and GSK3-inhibition optimization generated over 50% $CD31^+/CD34^+$ EPC in one line of iPS cells with multipotent aptitude (Lian et al., 2014), but still required much longer times to generate mature VE-cadherin+ EC.

Although hiPSCs can differentiate into functional ECs, they still exhibit limited expansion potential compared with hESC-derived ECs (Li et al., 2011). More importantly, current stem cell derivation protocols are only optimized for one or two hESC or hiPSC lines, and are not easily applied across various cells lines (Kattman et al., 2011;

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https://doi.org/10.1016/j.scr.2019.101685
Received 26 June 2019; Received in revised form 5 December 2019; Accepted 9 December 2019
Available online 16 December 2019

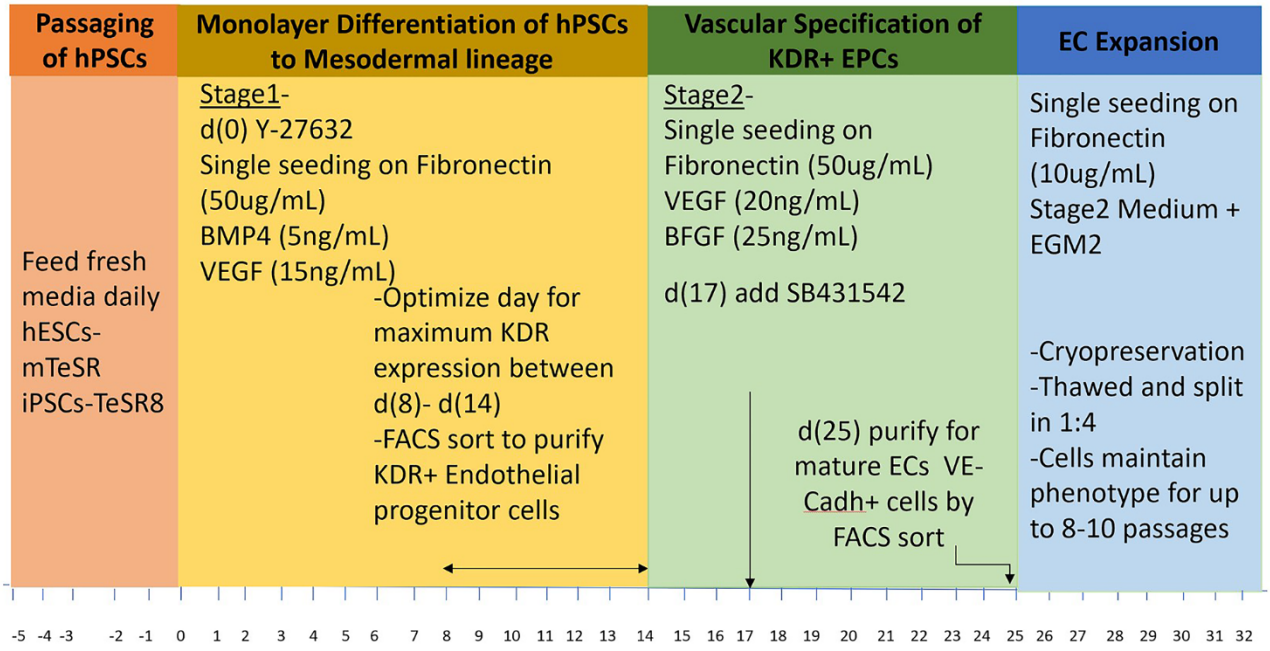

Fig. 1. Timeline and Growth Factors for EC differentiation of hPSCs.

Glaser et al., 2016). Therefore, we set out to develop a method for efficiently generating functional ECs that can be applied across multiple iPSC lines (**Fig 1**). The method is initiated with mesodermal induction which gives rise to an intermediate population of highly proliferative vascular progenitor cells (VPCs). This is followed by differentiation of VPCs into ECs that express multiple EC markers. In vitro-derived ECs can also be cryopreserved and expanded in culture at a later time up to 8–10 passages.

2. Reagents

1 Human Embryonic and Induced Pluripotent Stem Cell Lines: hESC-H9, 10196-10 ADRC iPSCs-75-Clone 5, 6160-7 ADRC iPSCs-12-Clone 1119-7, 7329-6 ADRC iPSCs-35-Clone 4, 7306-6 ADRC iPSCs-23-Clone 1
2 Matrigel hESC-Qualified Matrix (Corning® catalogue # 354277). Matrigel-coated plate: Prepare aliquots typically between 270-350 µl. Thaw Matrigel on ice and add one aliquot to 25 ml of cold DMEM/F-12 in a 50-ml tube using a cold pipette tip. Mix and add 1 ml of Matrigel per well of a six-well plate or approximately 20 µg/cm^2 with a cooled pipette. Seal the plates with Parafilm and store them at 4°C for up to 3 weeks. Warm the plates for 30 min at RT before use. Aspirate the remaining liquid from plates just before use, ensuring the pipet tips do not scratch the coated surface.
3 Versene Solution (Gibco™ catalogue # 15040066)
4 TrypLE Express Enzyme (1X), No Phenol Red (Gibco™ catalogue # 12604021)
5 Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco™ catalogue #11320033)
6 Dulbecco's Modified Eagle Medium: High glucose DMEM (Gibco™ catalogue # 11965092)
7 Minimum Essential Medium, Alpha 1X with Earl's salts, ribonucleosides, deoxyribonucleosides and L-glutamine (CORNING, catalogue # 10-022-CV)
8 mTeSR1 complete kit for hESC maintenance (StemCell Technologies, catalogue # 85850)
9 TeSR-E8 complete kit for hESC/hiPSC maintenance (StemCell Technologies, catalogue # 05990)
10 Bambanker HRM Cell Freezing Media for ES and iPS cells (Bulldog Bio catalogue # BBH01)
11 Penicillin/Streptomycin (5000 U/ml) (100-ml; Gibco™ catalogue # 15070 – 063).
12 L-Glutamine (200 mM) (100-ml; Gibco™ catalogue # 25030 – 081)
13 MEM Non-Essential Amino Acids Solution (100X) (Gibco™ catalogue #11140050)
14 Knockout Serum Replacement (500-ml; Gibco™ catalogue # 10828 – 028)
15 Nutridoma™-CS (Roche catalogue # 11363743001)
16 Bovine Serum Albumin powder, BioXtra (10 G, Sigma-Aldrich, catalogue # A3311). BSA, 0.5% (wt/vol) in PBS: Dissolve 1 g bovine albumin in 200 ml PBS. Sterilize the solution by filtration by using a 0.22-µm membrane filter, and store it for up to 4 weeks at 4°C.
17 Y-27632- RHO/ROCK pathway inhibitor; Inhibits ROCK1 and ROCK2 (StemCell Technologies, catalogue # 72304). Y-27632 (5 mM); Reconstitute 1 mg in 624 µl of PBS (pH 7.2) or water. Prepare aliquots and store them −20°C for up to 6 months. Aliquot into working volumes to avoid repeated freeze-thaw cycles.
18 Recombinant Human Bone Morphogenic Protein-4 (BMP4, (50 µg, Peprotech, catalogue # 120–05). BMP4 (50 µg ml^{-1} stock solution) Reconstitute the contents of the vial to a concentration of 50 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare 100 µl aliquots and store them for up to 1 year at −80°C.
19 Recombinant Human Vascular Endothelial Growth Factor ($VEGF_{165}$) (100 µg, Peprotech, catalogue # 100-20). VEGF (50 µg ml^{-1} stock solution): Reconstitute at 50 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare 50 µl aliquots and store them for up to 1 year at −80°C.
20 Recombinant Human Fibroblast Growth Factor (FGF-basic 146 a.a.) (50 µg, Peprotech, catalogue # 100–18 C), bFGF (25 µg ml^{-1} stock solution): Reconstitute the contents of the vial to a concentration of 25 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare 50 µl aliquots and store them for up to 1 year at −80°C.
21 β-Mercaptoethanol (25ML; Sigma- Aldrich, catalogue # M3148). Diluted β-Mercaptoethanol (100 mmol stock solution): Reconstitute 1 ml 14.3 M β -ME with 6.6 ml dH20.
22 TGF-β RI Kinase Inhibitor VI, SB431542- CAS 301836-41-9 – Calbiochem (5MG, Sigma- Aldrich, catalogue # 616461). SB431542 (10 mM): Reconstitute 5 mg in 1.3 ml of DMSO. Prepare aliquots

Table 1
Medium components for (A) Induction medium, (B) Endothelial specification medium, and (C) Endothelial expansion medium.

A		
Composition	Volume (100 ml)	Final concentration
Alpha MEM	77 ml	
20% KSR	20 ml	20%
Non- Essential Amino Acids	1 ml	1%
Penicillin-Streptomycin	1 ml	1%
L- glutamine (2 mM)	1 ml	1%
dBME (100 mM)	50 µl	0.05 mM
VEGF (5O µB/ml)	30 µl	15 ng/ml
BMP4 (50 µ&/ml)	10 µl	5 ng/ml

B		
Alpha MEM	64 ml	
High Glucose DMEM	28 ml	
Nutridoma	4 ml	4%
Non- Essential Amino Acids	1 ml	1%
L glutamine (2 mM)	1 ml	1%
Penicillin- Streptomycin	1 ml	1%
dBME (100 mM)	50 µl	0.05 µM
VEGF (50 µg/ml)	40 µl	20 ng/ml
bFGF (25 µg/ml)	100 µl	25 ng/ml
SB431542 (10 mM)*	200 µl<	20 µM

C		
Endothelial Specification Medium withSB431542	50 ml	50%
EGM2 w/o FBS	50 ml	50%

* added after 1 week in Vascular Specification Medium.

and store them indefinitely at −20°C. SB431542 does not have an expiration date.

23 Fetal Bovine Serum (FBS)-Heat Inactivated (500-ml; Gibco™ catalogue # 10082-147)
24 Normal Donkey Serum (5ML, Sigma-Aldrich catalogue # 566460)
25 Endothelial Cell Growth Medium BulletKit EGM™ (500-ml; LONZA catalogue #: CC-3124)
26 Fibronectin-coated plate: Human Fibronectin, 5 mg (Corning® catalogue # 356008). Reconstitute 5 mg in 5 ml of PBS (pH 7.2) to have a stock concentration of 1 mg ml^{-1}. Prepare aliquots and store them at −20°C for up to two weeks. Add 10 µl or 50 µl of fibronectin (1 mg ml^{-1}) to 1 ml of PBS, the final concentration is 10 µg ml^{-1} and 50 µg ml^{-1}, respectively. Add 4 ml of fibronectin solution per 100-mm plate or approximately 67 µl cm^{-2} of culture plate or coverslip. Leave the plate with fibronectin for at least 1 h at 37°C before use. Coated plates can be stored at 37°C for up to two weeks.
27 Dimethyl sulfoxide (DMSO) (100ML; Sigma-Aldrich, catalogue # 276855)
28 Triton X-100 (50ML; Sigma-Aldrich, catalogue # T8787). 0.5% Triton solution: Reconstitute 5 ml Triton in 1 L PBS and stir for 10 min.
29 Paraformaldehyde (PFA) (1 KG Millipore Sigma catalogue # 1040051000**).** 4% aqueous PFA: Reconstitute 4 g PFA in 100 ml PBS. Store for up to 1 mo in the dark at 4°C.
30 DAPI nucleic acid stain (10 MG; Invitrogen, catalogue # D1306
31 Fc Receptor Binding Inhibitor Polyclonal Antibody 1: 1000 (eBioscience, catalogue # 14-9161-73)
32 Fixable Viability Dye eFluor 780, 1: 1000 (eBioscience, catalogue # 65-0865-14)
33 Anti-human CD309 (VEGFR2) Antibody (PE) 1:100 (BioLegend, catalogue # 359903)
34 Anti-human CD144 (VE-Cadherin) Antibody (PE/Cy7) 1:100 (BioLegend, catalogue # 34851)
35 Anti–human von Willebrand Factor Domain (vWF)-A2 Domain Antibody 1:50 (R&D Systems, cat. no. RB01)
36 Anti–human ephrin-B4 (PE) 1:200 (LifeSpan BioSciences, Inc. catalogue # LS-C486174-25)
37 Anti–human ephrin-B2 (FITC) 1:100 (SANTA CRUZ BIOTECHNOLOGY, catalogue # sc-398735 FITC)
38 Anti–human CD184 (CXCR4) (PE) 1:100 (BioLegend, catalogue # 306505)
39 Anti–human CD31(PECAM-1)-allophycocyanin (APC) 1:100 (eBioscience, catalogue # 17-0319)
40 Image-iT FX Signal Enhancer (Invitrogen catalogue # I36933)
41 Low Density Lipoprotein, Human Plasma, Acetylated, Alexa Fluor™ 488 Conjugate (Alexa Fluor™ 488 AcLDL) 1: 100 (Invitrogen, catalogue # L23380)

3. Equipment

1 Incubator set to 5% CO_2
2 Inverted microscope with phase contrast
3 Fluorescence microscope
4 Confocal microscope
5 Sterile laminar flow hood
6 Centrifuge
7 Water or bead bath
8 LSRII (BD) or other flow cytometer

4. Procedure

4.1. Stem cell culture

1 Prewarm the Matrigel™-coated plates, Versene, PBS, 5 µM Y27632, and cell culture mediums: mTeSR1 for hESCs and TeSR8 for iPSCs to 37°C.
2 Thaw hESCs and iPSCs and resuspend in mTeSR1 or TeSR8, respectively.
3 Plate cells from each cryovial (~2.5 × 10^5) on 2 Matrigel™-coated wells of a 6-well plate with 2 ml of hESC or iPSC maintenance medium to each well and add 10 nM of Y27632. 37°C incubator and perform daily medium changes.

Note: Number of dishes plated may be modified based on how many cells were cryopreserved. Typically, each vial contains from 1 × 10^5 to 5 × 10^5 *cells. Generally higher number of cell aggregates need to be plated after thawing compared with routine passaging.*

1 Pass and split the cells before the colonies contact neighbors, this is generally every 4–7 days based on initial seeding density and the specific cell line.
2 Gently aspirate culture medium and wash cells with 2 ml of PBS twice per well.
3 Add 1 ml of Versene per well and incubate at room temperature (RT) for 6 min.
4 Aspirate the Versene and add 1 ml of culture medium. Gently scrape colonies off the plate with a cell scraper.
5 Transfer the detached cell aggregates to a 15 ml conical tube.
6 Break the cell aggregates by gently pipetting the cell suspension up and down until aggregate size is 100–500 µm.

Note: Individual hESC and iPSC lines behave differently, some break up more readily that the others. Aggregate size is a critical factor influencing cell viability and differentiation success. Smaller aggregates lead to increased cell death while larger aggregates decrease differentiation potential.

1 Plate the cell suspension on to pre-coated Matrigel. Cell aggregates from one well can be plated in 6 to 10 wells of a 6 well plate, based on how quickly the cells proliferate.

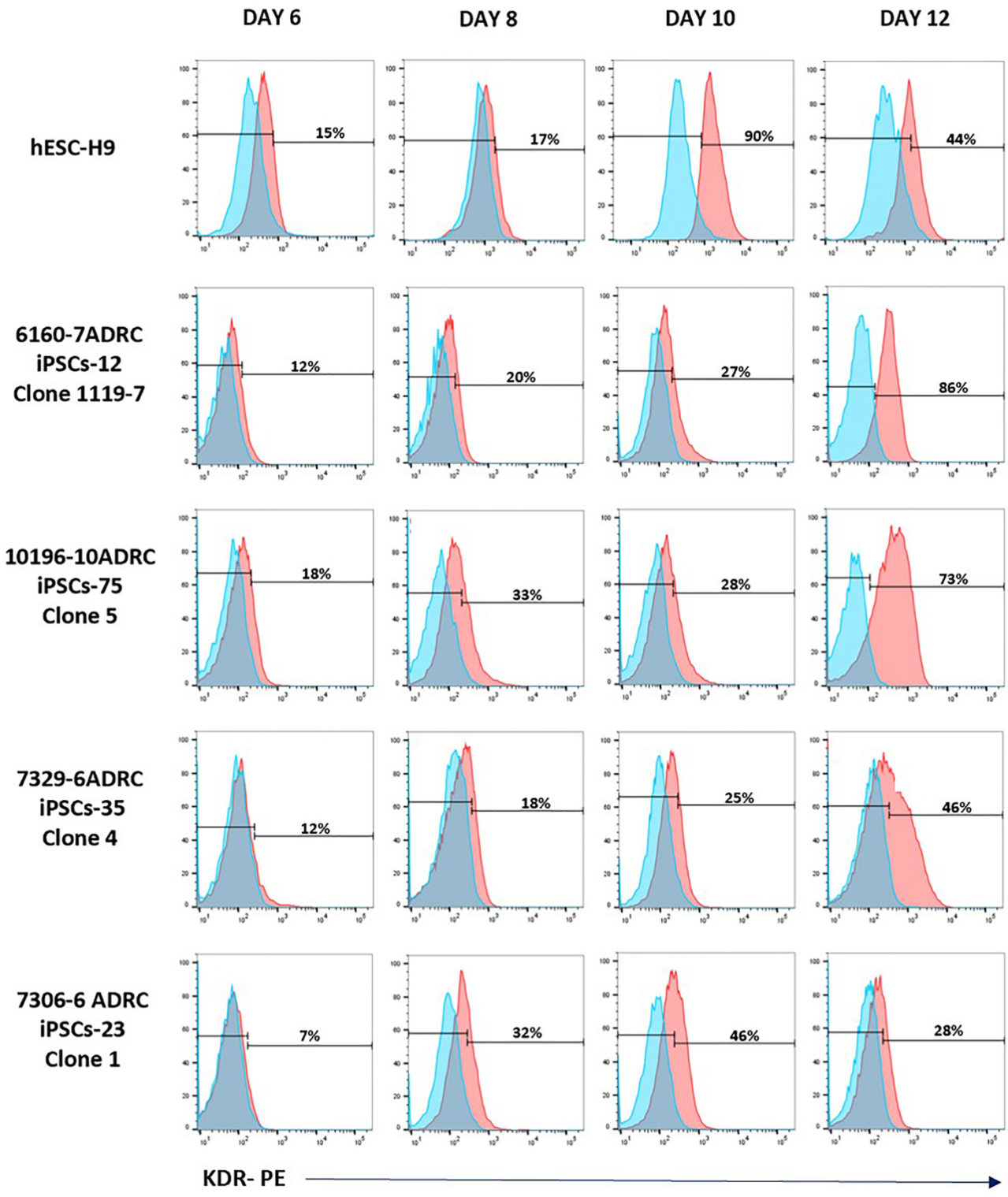

Fig. 2. *Representative histograms of the KDR+ expression* of cell lines: hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7, 10196-10ADRC iPSCs-75 Clone 5, 7329-6ADRC iPSCs-35 Clone 4 and, 7306-6 ADRC iPSCs-23 Clone as they change over time.

4.2. Mesoderm induction of hPSC

1 Replate cells for induction at ~20,000 cells/cm^2 on 50 μg/ml fibronectin-coated 100-mm plates, with 8 ml of stage 1 Induction Medium (Table 2A) and add 10 nM of Y27632. Cell aggregates from 2 wells of a 6-well plate is plated in a 100 mm culture dish.
2 *Note: Induction uses a greater density of cells compared with routine passaging.*
3 Incubate at 37°C and 5% CO_2 for 3 days. Do not change culture medium during first 3 days of induction.
4 On day 3 remove culture medium, wash cells twice with PBS and add fresh Induction Medium. Perform medium changes every two days.
5 Sort and collect KDR+ vascular progenitor cells (VPCs) on the day

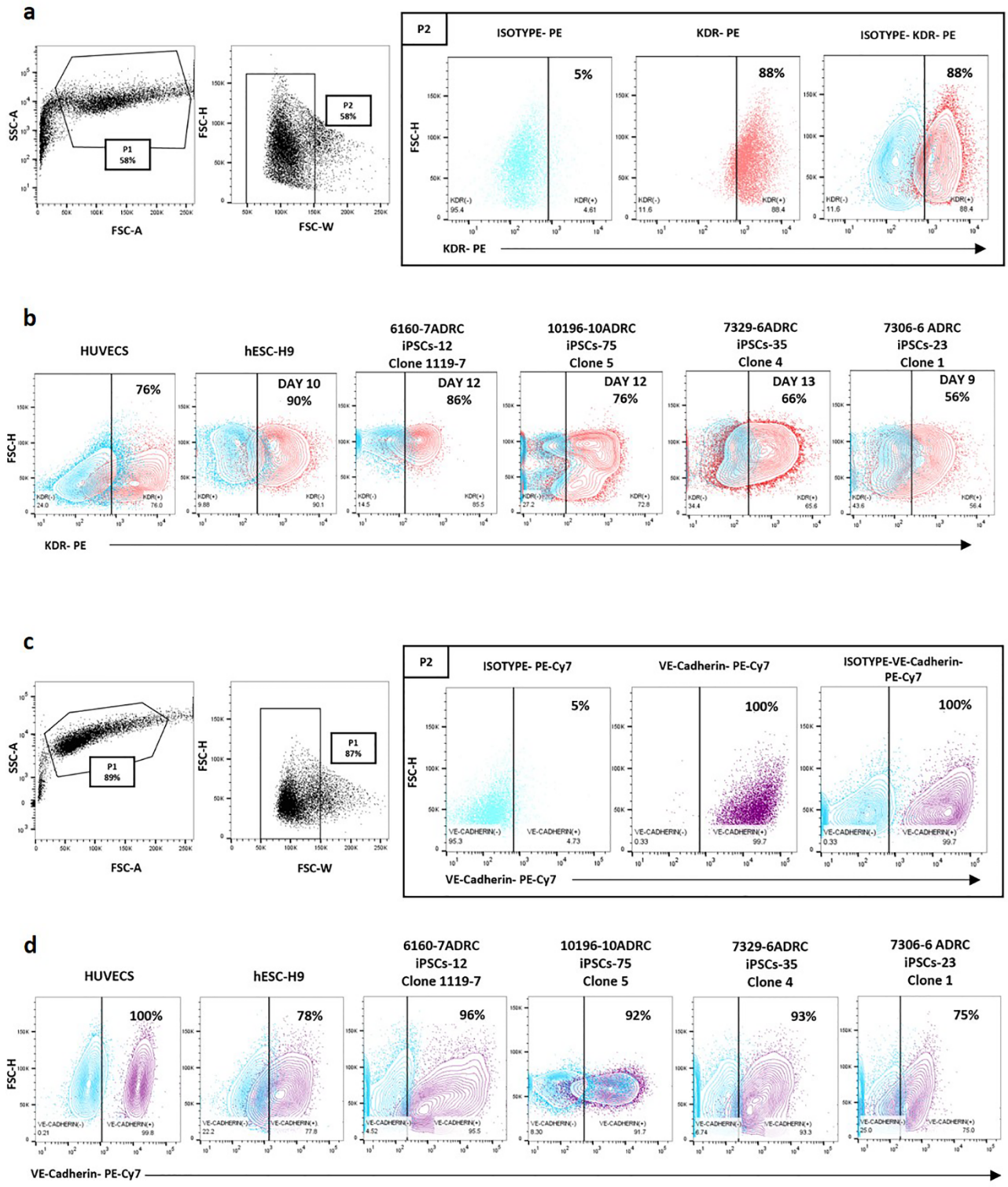

Fig. 3. *Flow cytometric analysis of KDR and VE-Cadherin expression for multiple hPSC lines.* (a) Representative dot plots of isotype-PE control and KDR-PE; P1 gated on live cells from side scatter (SSC) versus forward scatter (FSC) and P2 single cell populations of forward scatter height (FSC—H) versus forward scatter width (FSC-W). (b) Contour plots indicating percentage of KDR$^+$ cells for HUVEC controls and multiple hPSC lines. (c) Representative dot plots of isotype-PE-Cy7 and VE-Cadherin-PE-C7; P1 gated on live cells from SSC versus FSC and P2 single cell populations of FSC—H versus FSC-W. (d) Contour plots indicating the percentage VE-Cadherin$^+$ cells, of multiple hPSC lines. Percentages of positive cells were calculated based on the isotype controls (4%- 5%; blue population on the left). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in which cultures contain the greatest% of KDR + cells.

Note: During initial mesoderm induction, the hPSC will consist of a heterogeneous mixture of progenitor cells. When pluripotent stem cells start to differentiate, they will lose characteristic 3-D colony-like morphology and will grow as a cell monolayer. The cell mixture will have a population of VPCs and blood precursor cells expressing the KDR surface molecule. Peak KDR expression levels vary between different days of

Table 2
Summary of Stem Cell Line-Specific Endothelial Differentiations The table lists the specific stem cell expansion conditions, initial seeding density, pre-optimized time point and percentage of the presorted KDR+ VPCs, the final VE-cadherin+ EC purity and yield for each of the human pluripotent stem cell lines.

hPSC line	Stem Cell Passage Time/ Days	Seeding Density Stage1/ cm^2	Percentage of KDR+ cells	Percentage of VE-Cadherin+ cells Day 25	EC Yield
hESC-H9	4	20,000	Day 10~80–90%	~70–80%	~2.5 × 10^6
6160-7ADRC iPSCs-12 Clone 1119–7	5	20,000	Day 12~80–90%	~85–95%	~1.5 × 10^6
10196- 10ADRC iPSCs-75 Clone 5	3	20,000	Day 12~70–80%	~85–95%	~3.0 × 10^6
7329-6ADRC iPSCs-35 Clone 4	7	20,000	Day 13~60–70%	~85–95%	~6.5 × 10^5
7306-6 ADRC iPSCs-23 Clone 1	7	20,000	Day 9~50–60%	~75–85%	~6.0 × 10^5

differentiation for individual hPSC lines. Therefore, you need to first optimize the day in which maximum KDR expression is achieved. This is usually between days 8 to 14 of induction.

4.3. Isolation of KDR ± VPCs

1 Remove culture medium and wash cells thrice with 6 ml PBS per 100-mm dish.
2 Add 6 ml of TrypLE to each dish and allow cells to incubate for 3 to 5 min.
3 Pipet up and down multiple times with 6 ml of DMEM, washing over the bottom of the dish to remove all the cells. If some cells are still adhering to the bottom of the dish, then use a cell scraper to collect the attached cells.

Note: Individual hESC and iPSC lines also adhere differently to each other and the dish. It is critical to break the cells into single cells for flow cytometry.

1 Transfer cell suspension to a 15-ml centrifuge tube and centrifuge for 5 min at RT.

Note: Cell pellet from each 100-mm dish will consist about 1.5–2 × 10^6 cells.

1 Remove supernatant from the cell pellet. Resuspend the cell pellet in DMEM with 0.5% wt/vol BSA at a concentration of up to 1 × 10^6 cells/100 μl.
2 Incubate cell suspension with Human BD Fc Block™ at a concentration of 0.5 μg/ml of cell suspension, for 15 min at RT.
3 Add Fixable Viability Dye eFluor® 780 to cell suspension at a concentration of 1 μl/ml of cell suspension and incubate in dark for 10 min.
4 Aliquot 50 μl of cell suspension in another 15-ml centrifuge tube and label it "cells only". Aliquot 50 μl of cell suspension in another 15-ml centrifuge tube and label it "isotype control". Label the original cell suspension "KDR+ cells".
5 Add anti-human CD309/VEGFR2 PE monoclonal (Biolegend) at 1.33 μl/10^6 cells to the tube labeled KDR+ cell and vortex.
6 Add PE Mouse IgG1, κ Isotype Ctrl (FC) Antibody (Biolegend) at 1.33 μl/10^6 cells to the tube labeled isotype control and vortex.
7 Incubate all tubes on ice or at 4°C for 30 min, in the dark.
8 Add 4 ml of BSA buffer solution to all tubes and centrifuge at 200 × *g* for 4–5 min at 4°C.
9 Remove supernatant and repeat wash twice.
10 After final wash re-suspend the cell pellets in DMEM at a concentration of up to 1 × 10^6 cells/100 μl. Pipet up and down to evenly distribute the cells in the solution. Filter adherent and clumpy cell populations through a strainer to avoid instrument clogs during sorting.
11 Transfer the cell suspensions to labeled 5-ml round bottomed polystyrene FACS tube.
12 Fill another 15-ml centrifuge tube with 2 ml of Endothelial Specification Medium (Table 2B). This tube will serve as your "collection" tube for flow activated cell sorting (FACS).

Note: During cell sorting, the cell viability is often compromised. Collecting sorted cells in serum rich media such as 2 ml *of FBS can improve recovery, but we do not use it.*

1 Typically, there will be a discrete cell subpopulation expressing of KDR+ expressing cells. The percentage of this subpopulation varies from 50 − 70% of your total cell population. Collect these KDR+ expressing cells by sorting for the "brightest" population into the "collection" tube.

Note: To collect maximum percentage of KDR+ vascular progenitor cells you need to optimize the day for maximum KDR expression for each hPSC line.
Note: Because FACS provides greater cell purity compared to magnetic-activated cell separation (MACS), we prefer to use FACS. A MACS separation will also work, but multiple rounds of purification may be needed to eliminate all contaminating cell populations.

4.4. Generation of ECs from VPCs

1 Centrifuge the "collection" tube containing KDR+ cells at 200 × *g* for 5 min at RT.
2 Remove supernatant and re-suspend cells in 1 ml of Endothelial Specification Medium (stage 2) and plate in 50 μg/ml fibronectin-coated 100-mm dishes. Based on number of cell sorting events, calculate the number of cells such that they will be seeded at a density of 20,000–30,000 cells/cm^2.

Note: Cells need to be plated at a higher density after sorting than during routine passaging, since many cells die from FACS sorting procedures.

1 Add 8 ml of Endothelial Specification Medium to each 100-mm plates and place the cells in a 37°C incubator for 4 days.

Note: The cell sorting process is stressful so do not perform medium changes for the first 4 days and do not move the plate, this will provide the cells time to recover and adhere to the plate.

1 After 1 week, add 10 μmol/ml SB431542 to the KDR+ vascular progenitor cells.

Note: SB431542 is a transforming growth factor-β (TGF-β) pathway small-molecule inhibitor. SB431542 promotes the growth and proliferation of ECs by inhibiting activity of TGF-β activity. SB431542 works by interfering with activin receptor–like kinase-4, activin receptor–like kinase-5 and activin receptor–like kinase-7 that would normally activate type I receptors (ALK-4, ALK-5 and ALK-7, respectively) pathways.

1 Perform medium changes every 3 days with Endothelial

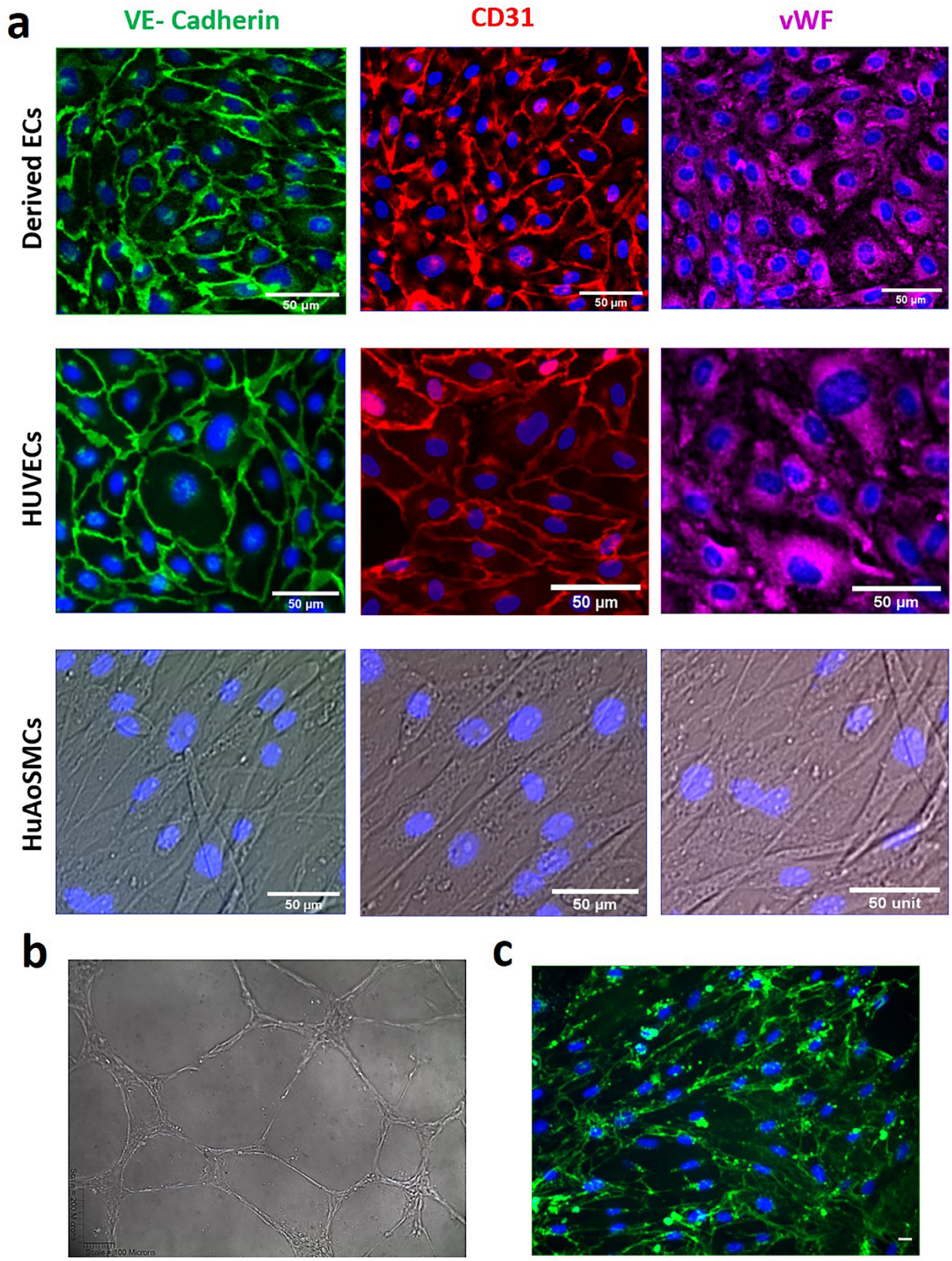

Fig. 4. *Characterization of hPSC-derived ECs.* (a) Confocal images of stem cell-derived ECs, HUVECs, and HuAoSMCs stained with**:** VE-cadherin (VE-cad, green), CD31 (red), and Von Willebrand (VWF, magenta), all counterstained with DAPI (blue). Functional assays included: (b) Matrigel tube-forming assay and ***(c)*** uptake of low density lipoprotein (LDL, green) counterstained with DAPI (blue). Scale bars = 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

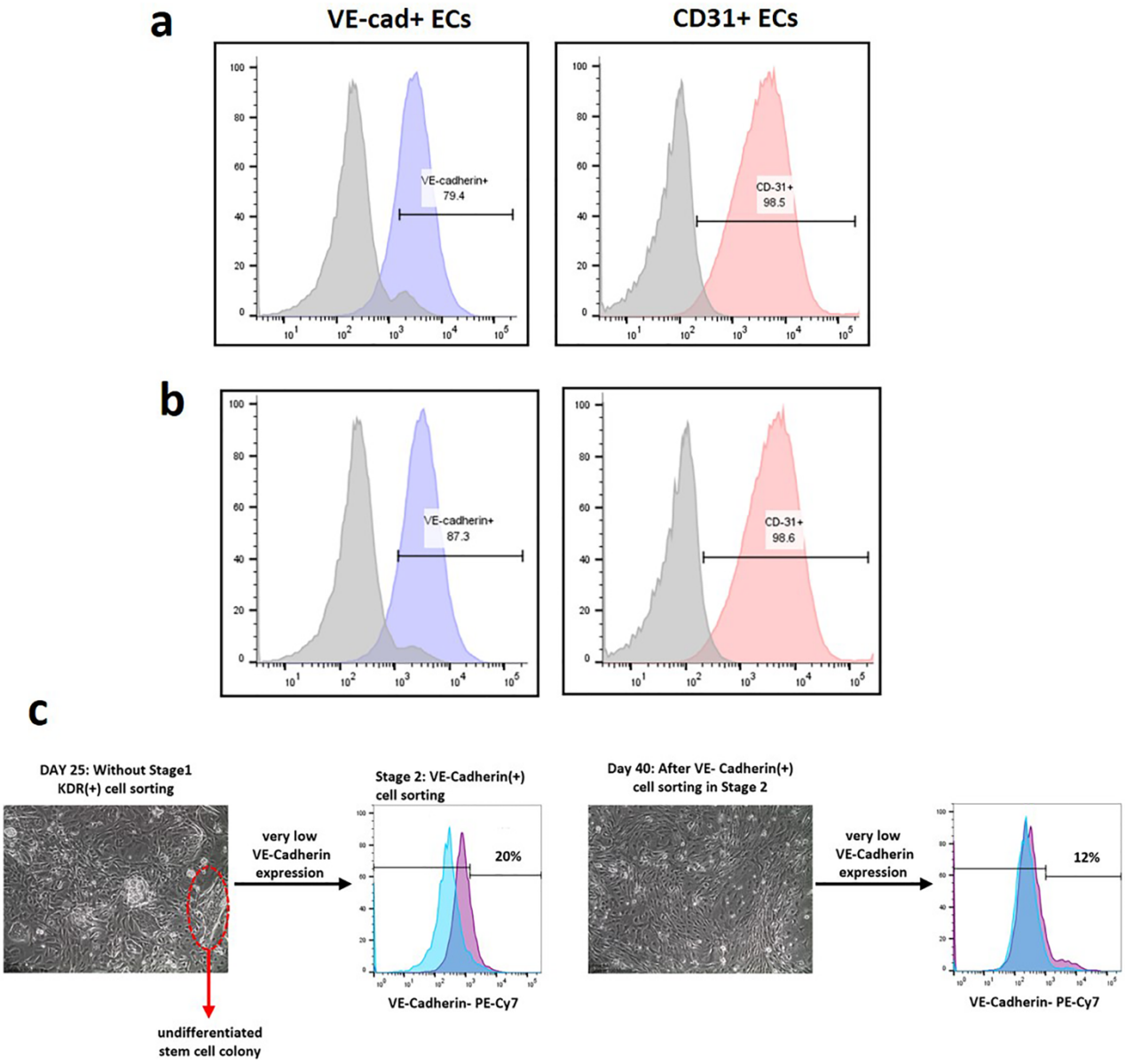

Fig. 5. *Characterization of EC after cryopreservation* Flow cytometric analysis of stem cell-derived ECs with histograms of VE-Cadherin and CD31 expression, before and after cryopreservation on (a) day 41 and (b) day 47, respectively. (c) Without the early KDR + sorting, the VE- cadherin expression might initially look fine, but will reduce in numbers over time. Histogram of VE-cadherin + cells at day 40 show that even if sorted for VE-cadherin + cells at day 25, the ECs are reduced by day 40.

Specification Medium supplemented with 10 μmol/ml of SB431542.

2 The cells should be passaged before complete confluence (~80%). Cells are passed using TrpLE and re-seeded at a density of 10,000 cells/cm^2.

Note: Cells become highly proliferative after addition of SB431542.

4.5. Purification of VE-Cadherin ± ECs

1 Purify VE-cadherin + ECs using flow cytometry between days 24 to 26 of total differentiation.

Note: By this time the hPSCs will have fully differentiated into mature ECs, but the culture may still contain a small number of smooth muscle cells (McCloskey et al., 2003) or stem cells. These contaminating cells will inhibit further EC proliferation and may even outgrow the ECs if not removed. Therefore, you will need to enrich the culture with cells only exhibiting EC markers.

1 Repeat steps (15–21).

2 Aliquot 50 μl of cell suspension in another 15-ml centrifuge tube and label it "cells only". Aliquot 50 μl of cell suspension in another 15-ml centrifuge tube and label it "isotype control". Label the original cell suspension "VE-cadherin + cell".

3 Add anti-human CD144/VE-cadherin PE-Cyanine7 monoclonal (eBioscience) at 1 μl/10^6 cells to the tube labeled VE-cadherin + cell and vortex.

4 Add PE-Cyanine7 Mouse IgG1, k Isotype Ctrl (eBioscience) at 1 μl/10^6 cells to the tube labeled isotype control and vortex.

5 Repeat steps (25–30).

6 Typically, a subpopulation of cells expressing VE-cadherin + will be present, typically 70 − 80% of your total cell population. Collect VE-cadherin + expressing cells by sorting for the "brightest" population into the "collection" tube containing Endothelial Expansion Medium.

Note: This enrichment step is necessary to collect highly purified populations of ECs, but can also lower initial cell viability and may be skipped if heterogeneous cell populations can be tolerated.

4.6. In vitro expansion of ECs

1 Centrifuge the "collection" tube containing VE-cadherin + cells for 5 min at RT.
2 Remove supernatant and re-suspend cells in 1 ml of Endothelial Expansion Medium (Table 1) and plate in 50 μg/ml fibronectin-coated T-75 flasks. Based on number of cell sorting events, calculate the number of cells such that they will be seeded at a density of 10,000 cells/cm^2.
3 Add 12 ml of EC medium to each T-75 flask and place the cells in a 37°C incubator and perform medium changes every 3 days.
4 Pass the cells when they reach 70–80% confluence. Cultures are passaged every 4–6 days.

Note: ECs are highly proliferative. At 100% confluence, when there is no area within the culture dish for the ECs to grow, the selection pressure may favor alternative cell types of other phenotypic changes including dedifferentiation.

1 Count the total number of cells and replate the cells in 10 μg/ml fibronectin-coated tissue culture flasks at a seeding density of 5000 cells/cm^2.
2 Subsequently, cells may be expanded in larger and larger containers (T-75-cm^2 flask, then T-175-cm^2 flask, and then multiple T-175-cm^2 flasks) or frozen.
3 Routinely analyze cells between 2 and 3 passages for EC markers such as VE-cadherin and KDR, to ensure that the cells continue to exhibit EC phenotype.

4.7. Microscopy

1 Plate 40,000 cells/cm^2 ECs on 10 μg/ml fibronectin-coated glass coverslips and feed with Endothelial Expansion Medium. Allow cells to grow to confluence.
2 Aspirate medium and wash with PBS.
3 Fix samples with 4% PFA solution for 5–10 min at RT. Rinse sample twice with PBS.

Note: Fixed coverslip samples can be stored in PBS until ready to stain for up to 1 week at 4 °C.

1 Add 0.5% Triton to cells and incubate for 3–5 min at RT to permeabilize the cell membrane.
2 Incubate samples with 0.5% BSA buffer containing 5–10% normal donkey serum for 1 h at RT to block the cells.
3 Dilute the endothelial- specific primary antibodies in 0.5% BSA buffer at concentrations listed above and add the staining solution to appropriate coverslips. Allow the samples to incubate overnight at 4°C.
4 Wash samples three times with PBS.
5 Dilute secondary antibody at a ratio of 1:200 in 0.5% BSA. Prepare coverslips with cell for negative control by adding only secondary antibody. Incubate samples at RT for 30 min.
6 Wash samples three times with PBS.
7 Add DAPI at a concentration of 1:1000 in PBS and incubate for 10 min at RT, to counterstain the cell nucleus.
8 Apply a drop of fluorescent mounting media to the coverslip samples so that the coverslips can adhere to glass slides, followed by overnight drying at 4°C.

Note: Stained coverslip cell samples can be stored in the dark at 4 °C and imaged for as long as 1 year.

4.8. EC tube-forming assay

1 Place 96 well culture plates on ice and, gently add 100 μl of cooled Corning Matrigel™ Matrix to each well, making sure to avoid any trapped air bubbles. In case of trapped air bubbles centrifuge the plate at 300xg for 10 min, in a precooled centrifuge at 4°C. Make sure to fully coat the surface of the plate.
2 Allow the Matrigel Matrix to solidify for 30 ± 60 min in the incubator. Carefully aspirate any remaining liquid.
3 Count 30,000 hPSC-derived ECs to each well in 200 μl of Endothelial Expansion Medium and culture in a 37°C incubator.
4 Image cells after 24 and 48 h.

Note: The vascular community, in general, does not favor using a simple Matrigel assay as an indicator of vascular assembly. Although vascular assembly within three-dimensional collagen gels is possible (McCloskey et al., 2005), co-culture with fibroblasts, mesenchymal stem cells, or pericytes is often needed (Sorrell et al., 2007; Whisler et al., 2014; Yamamoto et al., 2019). Moreover, because undifferentiated stem cells readily contribute to in vivo vasculature assembly (Blancas et al., 2013), cell contribution to in vivo vasculature is not at all indicative of a mature EC.

4.9. Low density lipoprotein (LDL)-uptake

1 Plate ECs at a density of 40,000 cells/cm^2 onto 10 μg/ml fibronectin-coated wells of a 24 well plate in 500 μl Endothelial Expansion Medium.
2 Culture in a 37°C incubator until about 80% confluent.
3 Aspirate medium and rinse with PBS. Add Alexa Fluor 488 acetylated low density lipoprotein (LDL; Invitrogen), diluted 1:100 in DMEM with high glucose (Invitrogen). Place the plate in a 37°C incubator for 5 h.
4 Wash samples three times with PBS.
5 Add DAPI at a concentration of 1:1000 in PBS and incubate for 10 min at RT, to counterstain the cell nucleus.
6 Fix samples with 4% PFA solution for 5–10 min at RT. Rinse sample twice with PBS.
7 Image cells with a fluorescent microscope.

4.10. Freezing thawing PCS-derived ECs

1 After characterizing ECs, cryopreserve ECs using freezing medium (50% FBS, 40% Endothelial Specification Medium, 10% DMSO) to maintain stocks of cells until they are needed. Freeze cells at a density 1–2 × 10^6 cells per ml of freezing medium.
2 Aspirate medium from cells in Step 49, rinse with PBS, add TrpLe to lift cells followed by neutralization with equal volumes of DMEM, and centrifuge for 5 min.
3 Remove the supernatant and re-suspend cell pellet in freezing medium. Add 1 ml of cell suspension into each cryovial.
4 Store the cryovials in −80°C for 24 h and then move to liquid N2 for long term storage.

Note: Cells can be stored for extensive periods of time under the conditions mentioned above.

1 Thaw frozen stocks of cultured cells carefully in a water or bead bath at 37°C.
2 Add 5 ml of chilled Endothelial Expansion Medium in a 15-ml tube and transfer thawed cell suspension to the tube.
3 Centrifuge cells 5 min at RT. Aspirating the supernatant carefully re-suspend the cell pellet in 1 ml of Endothelial Expansion Medium. Plate the cells from one cryovial into a 10 μg/ml fibronectin coated T-75 flask.
4 Expand cells as outlined in steps 48–51.

5 These derived ECs can be used for functional applications.

5. Expected outcomes

The protocol for generating relatively pure populations of proliferating ECs from hPSCs will take almost 30 days (**Fig 1**). Starting from hPSCs to mature ECs, the morphological changes can be observed (Supplemental Fig. S1). Between days 4 to 6 of mesodermal induction, the hPSC colonies transition from tightly packed 3D dome-like colonies into 2D monolayers of cells, shortly followed by the formation of islands of VPCs that comprise of VPCs around day 7. Between days 8 to 14 of differentiation, the hPSC consist of a heterogenous mixture of VPCs. Around this time, a large percentage of cells will express KDR (Figs. 2 and 3, Table 2) and a small percentage of the cells will begin expressing VE-cadherin. The day for optimal KDR expression will vary for each individual hPSC line, and this time point (Glaser et al., 2016) determines which day the cells should be sorted and replated in Endothelial Specification Medium . Purified VPCs are observed to grow into mature ECs with increasing percentages of the cells expressing VE-Cadherin surface molecule (Figs. 2 and 3).

At this time, the purified VE-cadherin+ ECs are extremely proliferative and express EC markers VE-cadherin (CD144), CD31 (PECAM-1), and Von Willebrand Factor comparable to HUVEC positive control cells (**Fig 4**a), as well as, form tubes on Matrigel (**Fig 4b**) and take up low density lipoprotein (**Fig 4c**). Negative control cells do not express any of these markers. Note that the VE-cadherin will localize at the cell-to-cell junctions when fully confluent.

Depending on cell line, between 6×10^5 and 3×10^6 total ECs can be derived per one 100-mm plate of hPSC, and 80- 90% of the ECs retain their viability and phenotype from thawing after cryopreservation (Fig 5a and b). The ECs are split to 1:4 ratio when they reach 80–90% confluence and maintain their EC phenotype up to 8–10 population doublings. However, if the differentiating cells are not purified for KDR after the first induction the, contaminating populations will inhibit EC proliferation (McCloskey et al., 2003) and will usually outgrow the ECs during further expansion (**Fig 5c**), even after VE-cadherin sorting.

CRediT authorship contribution statement

Basharat Jahan: Investigation, Data curation, Validation, Writing - original draft. **Kara E. McCloskey:** Supervision, Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interestsor personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funded through an NSF-Science and Technology center (STC) for the Emergent behavior of Integrated Biological Systems (EBICS) Award # 0939511.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101685.

References

Blancas, A.A., Wong, L.E., Glaser, D.E., McCloskey, K.E., 2013. Specialized tip/stalk-like and phalanx-like endothelial cells from embryonic stem cells. Stem Cells Dev. 22 (9), 1398–1407.

Glaser, D.E., Turner, W.S., Madfis, N., Wong, L., Zamora, J., White, N., Reyes, S., Burns, A.B., Gopinathan, A., McCloskey, K.E., 2016. Multifactorial optimizations for directing endothelial fate from stem cells. PLoS One 11 (12), e0166663.

Kane, N.M., Meloni, M., Spencer, H.L., Craig, M.A., Strehl, R., Milligan, G., Houslay, M.D., Mountford, J.C., Emanueli, C., Baker, A.H., 2010. Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microRNA and angiogenesis in vitro and in vivo. Arterioscler. Thromb. Vasc. Biol. 30 (7), 1389–1397.

Karagiannis, P., Takahashi, K., Saito, M., Yoshida, Y., Okita, K., Watanabe, A., Inoue, H., Yamashita, J.K., Todani, M., Nakagawa, M., Osawa, M., Yashiro, Y., Yamanaka, S., Osafune, K., 2019. "Induced pluripotent stem cells and their use in human models of disease and development. Physiol. Rev. 99 (1), 79–114.

Kattman, S.J., Witty, A.D., Gagliardi, M., Dubois, N.C., Niapour, M., Hotta, A., Ellis, J., Keller, G., 2011. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 8 (2), 228–240.

Levenberg, S., Golub, J.S., Amit, M., Itskovitz-Eldor, J., Langer, R., 2002. Endothelial cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. U S A 99 (7), 4391–4396.

Li, Z., Hu, S., Ghosh, Z., Han, Z., Wu, J.C., 2011. Functional characterization and expression profiling of human induced pluripotent stem cell- and embryonic stem cell-derived endothelial cells. Stem Cells Dev. 20 (10), 1701–1710.

Lian, X., Bao, X., Al-Ahmad, A., Liu, J., Wu, Y., Dong, W., Dunn, K.K., Shusta, E.V., Palecek, S.P., 2014. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem Cell Rep. 3 (5), 804–816.

McCloskey, K.E., Gilroy, M.E., Nerem, R.M., 2005. Use of embryonic stem cell-derived endothelial cells as a cell source to generate vessel structures in vitro. Tissue Eng. 11 (3–4), 497–505.

McCloskey, K.E., Lyons, I., Rao, R.R., Stice, S.L., Nerem, R.M., 2003. Purified and proliferating endothelial cells derived and expanded in vitro from embryonic stem cells. Endothelium 10 (6), 329–336.

Orlova, V.V., Drabsch, Y., Freund, C., Petrus-Reurer, S., van den Hil, F.E., Muenthaisong, S., Dijke, P.T., Mummery, C.L., 2014a. Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. Arterioscler. Thromb. Vasc. Biol. 34 (1), 177–186.

Orlova, V.V., van den Hil, F.E., Petrus-Reurer, S., Drabsch, Y., Ten Dijke, P., Mummery, C.L., 2014b. Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. Nat. Protoc. 9 (6), 1514–1531.

Sorrell, J.M., Baber, M.A., Caplan, A.I., 2007. A self-assembled fibroblast-endothelial cell co-culture system that supports in vitro vasculogenesis by both human umbilical vein endothelial cells and human dermal microvascular endothelial cells. Cells Tissues Organs 186 (3), 157–168.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861–872.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. "Embryonic stem cell lines derived from human blastocysts. Science 282 (5391), 1145–1147.

Whisler, J.A., Chen, M.B., Kamm, R.D., 2014. Control of perfusable microvascular network morphology using a multiculture microfluidic system. Tissue Eng. Part C Methods 20 (7), 543–552.

Yamamoto, K., Tanimura, K., Watanabe, M., Sano, H., Uwamori, H., Mabuchi, Y., Matsuzaki, Y., Chung, S., Kamm, R.D., Tanishita, K., Sudo, R., 2019. Construction of continuous capillary networks stabilized by pericyte-like perivascular cells. Tissue Eng. Part A 25 (5–6), 499–510.

Zhang, S., Dutton, J.R., Su, L., Zhang, J., Ye, L., 2014. The influence of a spatiotemporal 3D environment on endothelial cell differentiation of human induced pluripotent stem cells. Biomaterials 35 (12), 3786–3793.