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SHORT REPORT

Chemically-defined albumin-free differentiation of human pluripotent stem cells to endothelial progenitor cells

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Abstract
Human pluripotent stem cell (hPSC)-derived endothelial cells and their progenitors are important for vascular research and therapeutic revascularization. Here, we report a completely defined endothelial progenitor differentiation platform that uses a minimalistic medium consisting of Dulbecco's modified eagle medium and ascorbic acid, lacking of albumin and growth factors. Following hPSC treatment with a GSK-3β inhibitor and culture in this medium, this protocol generates more than 30% multipotent CD34+ CD31+ endothelial progenitors that can be purified to >95% CD34+ cells via magnetic activated cell sorting (MACS). These CD34+ progenitors are capable of differentiating into endothelial cells in serum-free inductive media. These hPSC-derived endothelial cells express key endothelial markers including CD31, VE-cadherin, and von Willebrand factor (vWF), exhibit endothelial-specific phenotypes and functions including tube formation and acetylated low-density lipoprotein (Ac-LDL) uptake. This fully defined platform should facilitate production of proliferative, xeno-free endothelial progenitor cells for both research and clinical applications.

Introduction

Human pluripotent stem cells (hPSCs) are increasingly used in vascular research, including disease modeling, drug screening, and development of regenerative therapies (Ashton et al., 2011; Bautch, 2011; Kinney et al., 2014; Kusuma et al., 2014; Murry and Keller, 2008; Segers and Lee, 2008). Recently, dramatic improvements in the efficiency of directed differentiation protocols to produce endothelial cells have been reported by stage-specific modulation of pathways including TGFβ superfamily (James et al., 2010; Rufaihah et al., 2011; Wang et al., 2007), VEGF (vascular endothelial growth factor) (Goldman et al., 2009; James et al., 2010; Rufaihah et al., 2011; Wang et al., 2007), and Notch signaling (Marcelo et

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al., 2013; Sahara et al., 2014). However, most of these approaches require animal cells, fetal bovine serum, or cytokines and growth factors, limiting their applications for large-scale endothelial cell production for research or therapeutic applications.

Recently, we reported a rapid and robust endothelial progenitor differentiation protocol under serum-free conditions, which only employs a Gsk-3β inhibitor in LaSR basal medium (Advanced DMEM/F12, 2.5 mM GlutaMAX and 60 μg/mL ascorbic acid) (Lian et al., 2014). The presence of bovine serum albumin (BSA) in this medium increases the cost, adds xenogenic components, and heightens lot-to-lot variability.

Toward developing a defined, xeno-free endothelial progenitor differentiation platform, we screened several commercially available basal media, supplemented with insulin and ascorbic acid, for the ability to generate CD34+ CD31+ cells after treatment with 5 μM CHIR99021. We found that DMEM supplemented with 100 μg/mL ascorbic acid generated 20–30% CD34+ CD31+ endothelial progenitors that were enriched to >95% CD34+ progenitors via MACS. This minimal, defined differentiation platform should facilitate generation of proliferative endothelial progenitor cells from hPSCs for both research and clinical applications.

Methods and materials

hPSC culture

hPSCs were maintained in E8 medium on Synthemax according to previously published methods (Chen et al., 2011; Lian et al., 2012, 2013a,2013b).

Endothelial progenitor differentiation via modulation of canonical Wnt signaling

hPSCs maintained on a Synthemax-coated surface in E8 were dissociated into single cells with Accutase (Life Technologies) at 37 °C for 5 min and then seeded onto a Synthemax-coated cell culture dish at 50,000 cell/cm^2 in E8 supplemented with 5 μM ROCK inhibitor Y-27632 (Selleckchem) (day −3) for 24 h. Cells were then cultured in E8, changed daily. At day 0, cells were treated with 3–9 μM CHIR99021 (Selleckchem) for 2 days in DMEM (Life Technologies, 11965) supplemented with 100 μg/mL ascorbic acid (Sigma, A8960) (DMEM/Vc). After 2 days, CHIR99021-containing medium was aspirated and cells were maintained in DMEM/Vc without CHIR99021 for 3 to 4 additional days.

Purification and cryopreservation of endothelial progenitors

Day 5 differentiated populations were dissociated with Accutase for 10 min and purified with an EasySep Magnet kit (STEMCELL Technologies) using a CD34 antibody (Miltenyi Biotec) according to the manufacturer's instructions. After purification, the total number of enriched CD34+ cells was counted and the yields were calculated as number of CD34+ endothelial progenitors generated per hPSC seeded at day −3. The endothelial progenitor cells were resuspended at a density of 2 × 10^6 cells per mL of endothelial freezing medium, which consists of 30% FBS (Life Technologies), 10% DMSO (Sigma), 60% EGM-2 (Lonza) and 5 μM Y-27632. 1 mL of the cell suspension was aliquoted into each cryovial and frozen in a Mr. Frosty™ freezing container at −80 °C. 24 h later, the cryovials were transferred to liquid nitrogen for long-term storage. For recovery, frozen cells were partially thawed in a 37 °C water bath, and were then transferred into a 15-mL conical tube containing 5 mL 10% FBS DMEM medium (Life Technologies). After centrifuging, cells were resuspended in EGM-2 medium (Lonza) or human endothelial-SFM supplemented with 20 ng/mL bFGF and 10 ng/mL EGF (Life Technologies) containing 5 μM Y27632 and plated into collagen IV-coated dishes (BD BioCoat) at a density of 0.05 million cells per cm^2. The next day, medium was replaced with fresh room temperature EGM-2 medium (Lonza) or human endothelial-SFM supplemented with 20 ng/mL bFGF and 10 ng/mL EGF (Life Technologies).

Differentiation of CD34+ cells to endothelial cells

Day 5 differentiated populations were dissociated with Accutase for 10 min and purified with an EasySep Magnet kit (STEMCELL Technologies) using an anti-CD34 antibody according to the manufacturer's instructions. The purified CD34+ cells were plated on collagen IV-coated dishes (BD BioCoat) in EGM-2 medium (Lonza) or human endothelial-SFM supplemented with 20 ng/mL bFGF and 10 ng/mL EGF (Life Technologies) and split every 3–4 days with Accutase.

Differentiation of CD34+ cells to smooth muscle cells

Day 5 differentiated populations were dissociated with Accutase for 10 min and purified with an EasySep Magnet kit (STEMCELL Technologies) using an anti-CD34 antibody according to the manufacturer's instructions. The purified CD34+ cells were plated on collagen IV-coated dishes (BD BioCoat) in SmGM-2 medium (Lonza) and split every 3–4 days with Accutase.

Vascular tube formation assay

To assess the formation of capillary structures, 1 × 10^5 day 15 endothelial cells in 0.4 mL EGM-2 medium (Lonza) supplemented with 50 ng/mL VEGF (R&D Systems) were plated into one well of 24-well tissue culture plate pre-coated with 250 μl Matrigel (BD Biosciences). Tube formation was observed by light microscopy after 24 h of incubation.

RT-PCR and quantitative RT-PCR

Total RNA was prepared with the RNeasy mini kit (QIAGEN) and treated with DNase (QIAGEN). 1 μg RNA was reverse transcribed into cDNA via Oligo (dT) with Superscript III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was done in triplicate with iQSYBR Green SuperMix (Bio-Rad). RT-PCR was performed with Gotaq Master Mix (Promega) and then subjected to 2% agarose gel electrophoresis. *ACTB* was

used as an endogenous housekeeping control. PCR primer sequences are provided in Supplementary Table 4.

Flow cytometry

Cells were singularized with Accutase for 10 min and then fixed with 1% paraformaldehyde for 20 min at room temperature and stained with primary and secondary antibodies (Supplemental Table 3) in PBS plus 0.1% Triton X-100 and 0.5% BSA. Data were collected on a FACSCaliber flow cytometer (Beckton Dickinson) and analyzed using FlowJo. For ICAM-1 expression, day 15 post-purified endothelial cells were treated with or without 10 ng/mL TNFα for 16 h prior to flow cytometry analysis.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with primary and secondary antibodies (Supplemental Table 3) in PBS plus 0.4% Triton X-100 and 5% non-fat dry milk (Bio-Rad). Nuclei were stained with Gold Anti-fade Reagent with DAPI (Invitrogen). An epifluorescence microscope (Leica DM IRB) with a QImaging® Retiga 4000R camera was used for imaging analysis.

Results

Albumin-free medium for endothelial progenitor differentiation

We previously demonstrated that activation of canonical Wnt signaling in hPSCs in LaSR basal medium generates functional CD34+/CD31+ endothelial progenitors in numerous hPSC lines (Lian et al., 2014). Figs. 1A and S1 show schematics of the endothelial differentiation and purification protocols. LaSR basal medium consists of advanced DMEM/F12 medium, which contains proteins including transferrin and BSA (AlbuMAX II) (Supplementary Table 1). To develop a defined, xeno-free medium for endothelial progenitor differentiation, we assessed the efficiency of endothelial progenitor differentiation induced in H13 human embryonic stem cells (hESCs) by 6 μM CHIR99021 treatment in 4 commercially available basal media supplemented with 10 μg/mL insulin and 60 μg/mL ascorbic acid, as these two factors were shown to enhance endothelial cell proliferation and differentiation (May and Harrison, 2013; Montecinos et al., 2007; Piecewicz et al., 2012; Zhao et al., 2011). Only DMEM generated more than 10% CD34+ CD31+ endothelial progenitors. Supplementing DMEM with ascorbic acid significantly increased the percentage of endothelial progenitors at day 5, while insulin diminished endothelial progenitor purity. Other basal media yielded few, if any, CD34+ CD31+ cells (Fig. 1B).

We optimized the concentrations of CHIR99021 (CH) and ascorbic acid in DMEM and found that 5 μM CH and 100 μg/mL ascorbic acid provided the greatest purity of endothelial progenitors (Fig. 1C, D). Next, we tested DMEM supplemented with ascorbic acid as an endothelial progenitor differentiation medium in multiple additional hESC (H1, H14) and iPSC (19-9-11, 6-9-9, 19-9-7) lines at passages between 20 and 100, and they all generated 20–30% CD34+ CD31+ cells (Fig. S2, Supplementary Table 2), comparable to the differentiation efficiencies reported in LaSR basal medium (Lian et al., 2014).

CD34+ CD31+ endothelial progenitors are multipotent

Molecular analysis during endothelial progenitor differentiation showed dynamic changes in gene expression, with downregulation of the pluripotency markers *NANOG*, *SOX2*, and *OCT4*, and induction of mesoderm genes *T*, *MIXL1* and *EOMES* in the first 24 h after CHIR99021 addition (Fig. 2A). Expression of the endothelial progenitor markers *KDR*, *CD34*, *CDH5* and *CD31* was detected at day 4 and increased at day 5 (Fig. 2A). Immunofluorescent analysis revealed robust surface expression of both CD34 and CD31 on day 5 (Fig. 2B). In addition, flow cytometry profiling during endothelial progenitor differentiation showed a population of cells expressing CD144, but not ICAM-1, appeared at day 5 (Fig. S3A), consistent with our previous report of hPSC differentiation to endothelial progenitors in albumin-containing medium (Lian et al., 2014). To further investigate the multipotent nature of these CD34+/CD31+ cells, single step MACS using an anti-CD34 antibody was performed on day 5 of differentiation, yielding 99% pure CD34+ cells (Fig. 2C). Additional cell lines were also enriched to >95% CD34+ populations with a yield of 4–5 CD34+ endothelial progenitors for every input hPSC (Fig. S2, Supplementary Table 2). The purified CD34+ cells were plated on Collagen IV-coated 96-well plates at a density of one cell per well in either endothelial or smooth muscle medium. After 10 days of culture, they generated relatively pure populations of cells expressing smooth muscle myosin heavy chain (SMMHC), smooth muscle actin (SMA) and calponin, or VE-cadherin, vWF and CD31, respectively (Fig. 2D and E), indicating their multipotency. In addition, we tested whether day 5 CD34+ cells exhibit hematopoietic potential in IMDM medium supplemented with growth factor cocktails (300 ng/mL stem cell factor (SCF), 300 ng/mL Flt-3, 50 ng/mL colony-stimulating factor 3 (CSF3), 10 ng/mL IL-3, and 10 ng/mL IL-6) shown to sustain human hematopoietic stem cells (Wang et al., 2004), but did not detect CD45+ cells after 7 days (Fig S3B).

Characterization of hPSC-derived endothelial cells

To further assess the intrinsic properties of endothelial cells differentiated from CD34+ cells generated in this defined platform, MACS-sorted CD34+ cells were cultured in commercial endothelial media (EGM2 and human endothelial SFM) on collagen IV-coated plates. The resulting cells exhibited morphological characteristics typical of primary endothelial cells (Fig. S1). These hPSC-derived endothelial cells proliferated actively and were capable of 20 population doublings over 2 months in serum-containing EGM2 (Fig. 3A). Flow cytometry and immunostaining analysis of cells differentiated in serum-free human endothelial SFM revealed robust expression of CD31, VE-cadherin and vWF, comparable

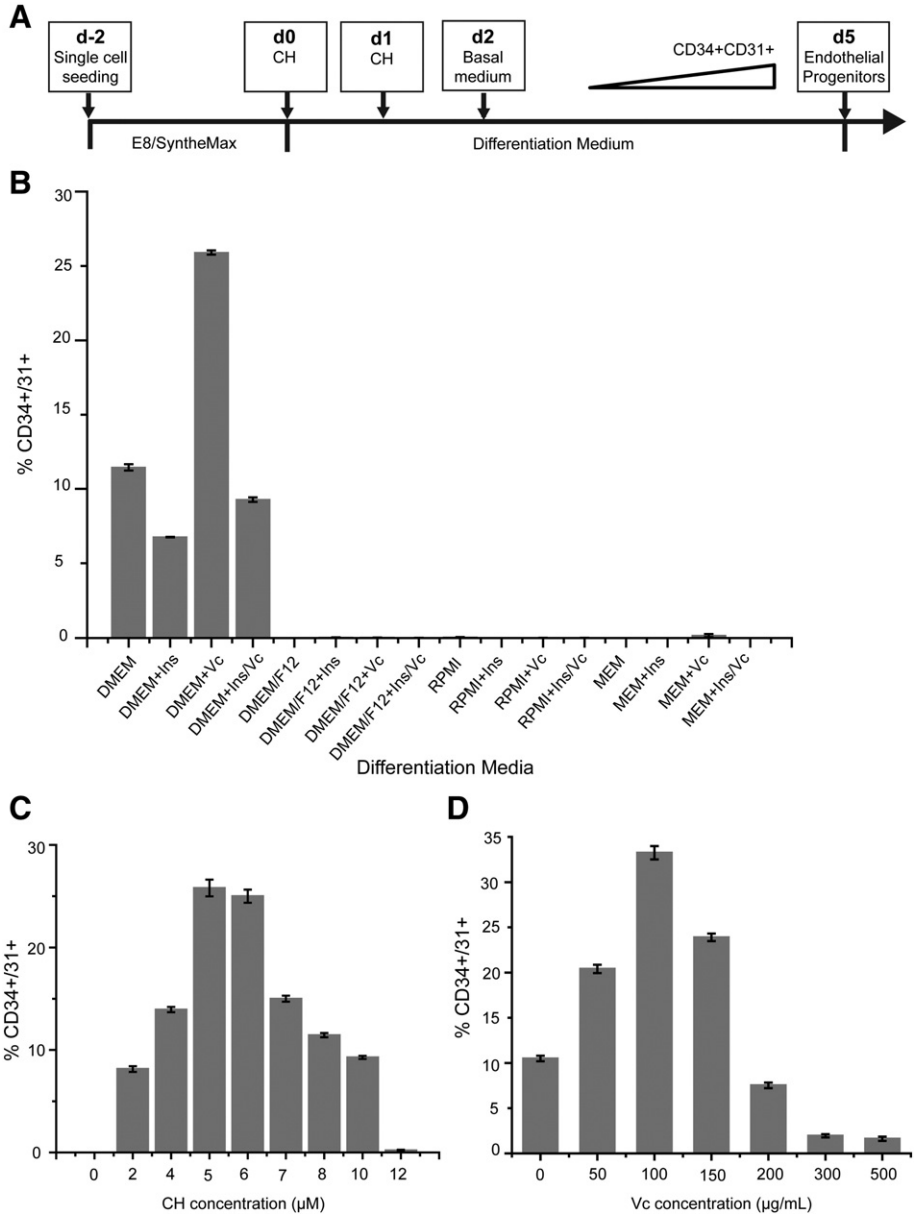

Figure 1 Defined, xeno-free medium for hPSC differentiation to CD34+ CD31+ endothelial progenitors via Gsk-3β inhibitor treatment. (A) Schematic of the protocol for defined, xeno-free differentiation of hPSCs to endothelial progenitors in a single albumin-free differentiation medium. (B) H13 hESCs were cultured as indicated in (A) in different differentiation media and the percentage of CD34+ CD31+ cells was determined by flow cytometry. (C) H13 hESCs were cultured on Synthemax in DMEM containing 60 μg/mL ascorbic acid and the indicated concentrations of CH for 2 days followed by another 3 days in the same medium and the percentage of CD34+ CD31+ cells was determined by flow cytometry. (D) H13 hESCs were cultured on Synthemax and treated with 5 μM CH for 2 days followed by another 3 days in DMEM medium supplemented with indicated concentration of ascorbic acid and the percentage of CD34+ CD31+ cells was determined by flow cytometry. All analyses of CD34 and CD31 expression were performed after 5 days of differentiation. Data are represented as mean ± s.e.m. of at least three independent replicates.

to primary human umbilical vein endothelial cells (HUVECs) (Fig. 3B, C).

Next, we assessed the endothelial nature of these hPSC-derived CD31+ cells differentiated in serum-free media by testing for tube formation and acetylated low-density lipoprotein (Ac-LDL) uptake. Upon treatment with VEGF, the cells organized into tube-like structures in Matrigel (Fig. 3D), and were able to take up Ac-LDL (Fig. 3E), demonstrating their endothelial function. In addition, these hPSC-derived endothelial cells upregulated expression of the adhesion molecule ICAM-1 upon TNF-α treatment (Fig. S3C), indicating their ability to respond to inflammatory mediators. Furthermore, they also maintained viability (Fig. S4A) and endothelial marker expression after storage in liquid nitrogen for a month (Fig. S4B, C), indicative of cryopreservation ability.

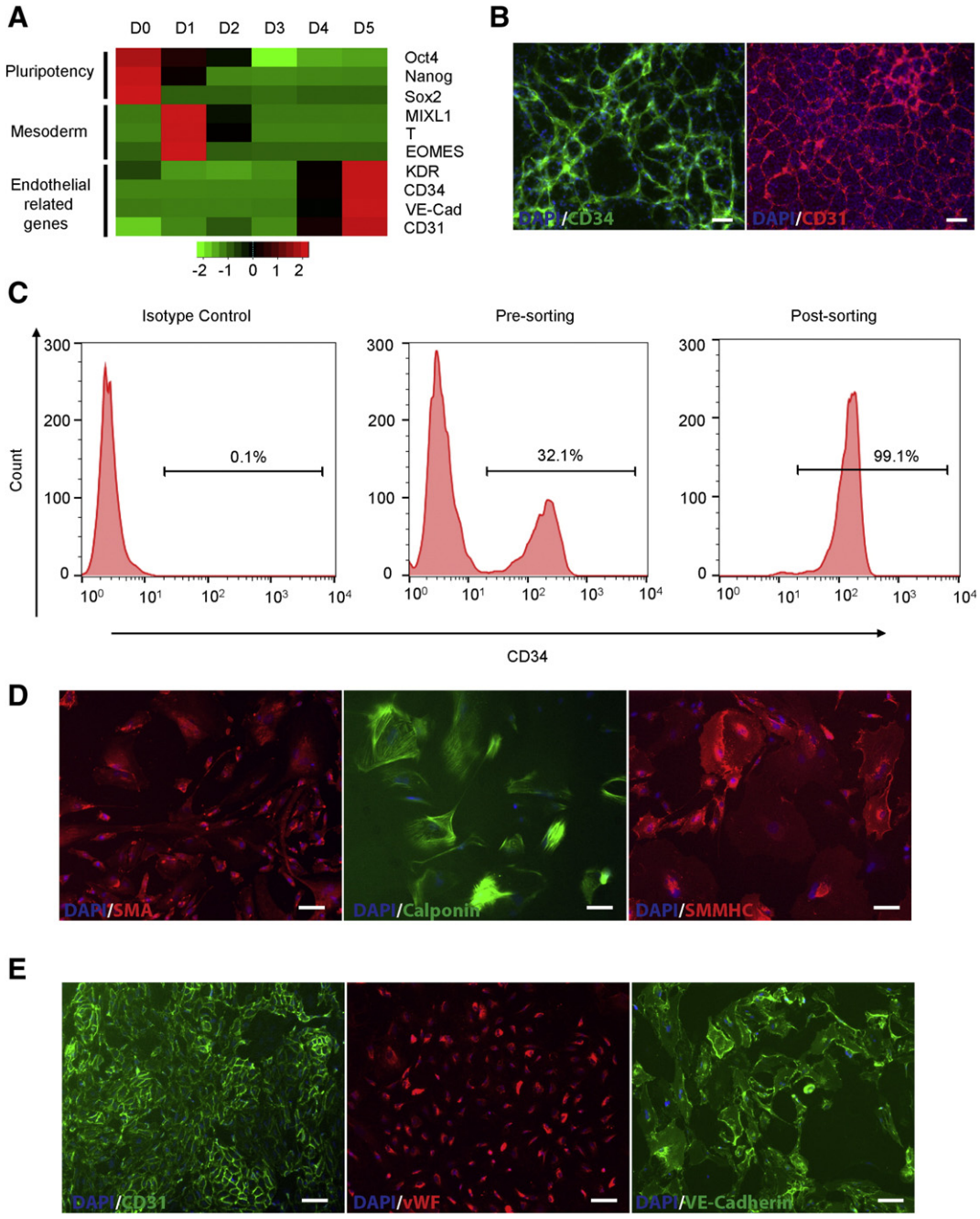

Figure 2 Molecular analysis of endothelial progenitors differentiated from hPSCs. (A-B) H13 hESCs were differentiated as illustrated in Fig. 1A using DMEM medium supplemented with 100 μg/mL ascorbic acid. At different time points, developmental gene expression was assessed by quantitative RT-PCR (A). Color key is in $\log_{10}$ scale. Day 5 cells were subjected to immunostaining analysis for CD34 and CD31 (B). (C-E) At day 5, CD34+ cells were enriched with the EasySep™ Human CD34 Positive Selection Kit and purification quantified by flow cytometry for CD34 expression (C). Sorted CD34+ cells were plated in (D) smooth muscle medium or (E) EGM2 endothelial cell medium at a density of one cell per well of 48-well plate and cultured for another 10 days. Sample immunofluorescence images for smooth muscle and endothelial markers were shown. Scale bars, 50 μm.

Discussion

Existing methods for hPSC differentiation to endothelial progenitors require the addition of growth factors and/or xenogenic components, limiting their application for large-scale production and therapeutic applications (Bautch, 2011; Wilson et al., 2014). Here, we report a defined, albumin-free, non-xenogenic differentiation system for directing hPSCs to endothelial progenitors. We showed that a completely defined medium, DMEM supplemented with 100 μg/mL ascorbic acid, is sufficient to efficiently generate CD34+ CD31+ endothelial progenitors from hPSCs following Gsk-3β inhibition. These

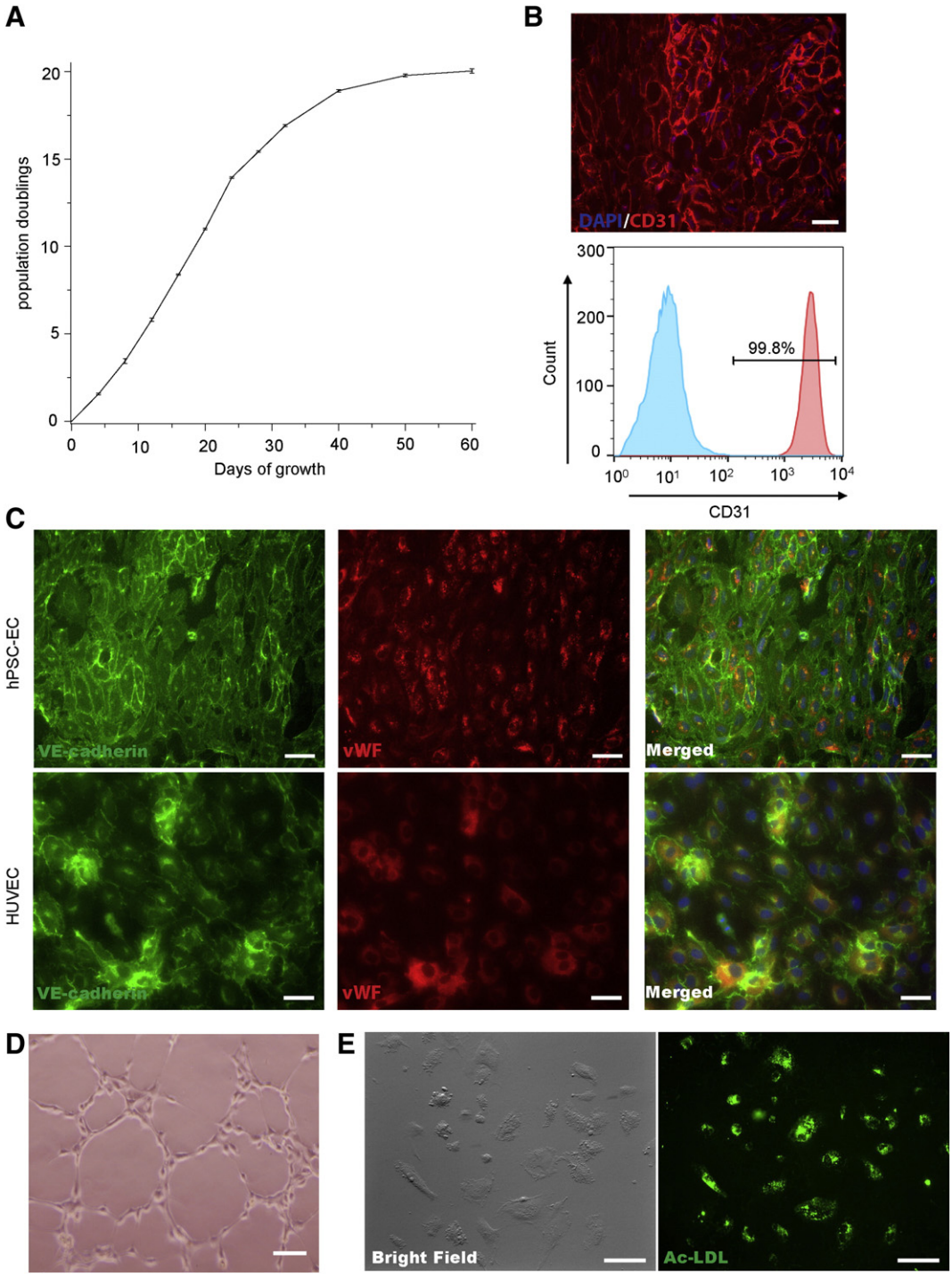

Figure 3 Characterization of endothelial cells differentiated from hPSCs. (A) H13 hESCs were differentiated as illustrated in Fig. 1A using DMEM/Vc medium. At day 5, CD34+ cells were enriched and cultured in endothelial medium on collagen IV-coated plates. (A) Cells cultured in EGM2 were passaged every five days. At different time points, the cell numbers were counted and the number of cumulative population doublings was calculated. Data are represented as mean ± standard deviation of three independent replicates. (B-E) CD34+ cells were cultured in serum-free human endothelial SFM. The purified day 15 endothelial cells were immunostained for CD31 (B), VE-cad and vWF (C), and tested for (D) tube-forming ability upon VEGF treatment and the ability to (E) uptake Ac-LDL. Data are represented as mean ± s.e.m. of at least three independent replicates. Scale bars, 50 μm.

hPSC-derived endothelial progenitors are multipotent and can be further directed into smooth muscle cells or endothelial cells upon subsequent culture in appropriate inductive media. CD31+/VE-cadherin+ endothelial cells differentiated under serum-free conditions exhibited uptake of acetylated low-density lipoprotein (Ac-LDL) and formed tube-like structures

when cultured on Matrigel in the presence of VEGF. However, long-term expansion of these cells required serum-containing medium.

Albumin has been reported to increase growth rate and overall cell health (Ashman et al., 2005; Zoellner et al., 1996). Here, however, we demonstrate that albumin is dispensable in endothelial progenitor differentiation. In spite of the greater simplicity of this new albumin free-medium, it supported endothelial progenitor induction of hPSCs comparably to LaSR basal medium. This simplified medium offers several advantages in both research and clinical applications of hPSC-derived endothelial progenitors. First, it eliminates batch-to-batch variability of albumin, likely increasing reproducibility of differentiation processes. Second, it provides a simpler chemical background for examining and screening factors regulating gene expression, differentiation, and proliferation. For example, albumin can bind and sequester lipids, proteins and small molecules (Garcia-Gonzalo and Izpisúa Belmonte, 2008). Third, it can reduce the risk of potential pathogen contamination and cell immunogenicity, facilitating therapeutic applications of hPSC-derived endothelial progenitor cells. Finally, this new system can significantly reduce reagent cost and simplify quality control for endothelial progenitor cell differentiation.

Conclusions

This study demonstrates that a completely defined, xeno-free medium can be used to efficiently derive functional endothelial progenitors from hPSCs in the absence of exogenous proteins. This is an important step toward the ultimate clinical application of hPSC-derived endothelial progenitors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.05.004.

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