Stem Cell Reports
Article

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Efficient Differentiation of Human Pluripotent Stem Cells to Endothelial Progenitors via Small-Molecule Activation of WNT Signaling

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http://dx.doi.org/10.1016/j.stemcr.2014.09.005

SUMMARY

Human pluripotent stem cell (hPSC)-derived endothelial cells and their progenitors may provide the means for vascularization of tissue-engineered constructs and can serve as models to study vascular development and disease. Here, we report a method to efficiently produce endothelial cells from hPSCs via GSK3 inhibition and culture in defined media to direct hPSC differentiation to $CD34^+CD31^+$ endothelial progenitors. Exogenous vascular endothelial growth factor (VEGF) treatment was dispensable, and endothelial progenitor differentiation was β-catenin dependent. Furthermore, by clonal analysis, we showed that $CD34^+CD31^+CD117^+TIE\text{-}2^+$ endothelial progenitors were multipotent, capable of differentiating into calponin-expressing smooth muscle cells and $CD31^+CD144^+vWF^+I\text{-}CAM1^+$ endothelial cells. These endothelial cells were capable of 20 population doublings, formed tube-like structures, imported acetylated low-density lipoprotein, and maintained a dynamic barrier function. This study provides a rapid and efficient method for production of hPSC-derived endothelial progenitors and endothelial cells and identifies WNT/β-catenin signaling as a primary regulator for generating vascular cells from hPSCs.

INTRODUCTION

Human pluripotent stem cells (hPSCs) offer unprecedented opportunities to study the earliest stages of human development in vitro, to model human disease, to perform drug tests in culture, and to develop unlimited new sources of cells for possible therapeutic applications. To realize this potential, it is essential to be able to control hPSC differentiation to somatic lineages with high efficiency and reproducibility in a scalable and inexpensive manner (Ashton et al., 2011; Burridge et al., 2012; Kinney et al., 2014; Murry and Keller, 2008).

Functional human endothelial cells differentiated from hPSCs could be beneficial for many potential clinical applications (Burridge et al., 2012; Kaupisch et al., 2012; Levenberg et al., 2002; van der Meer et al., 2013), including engineering new blood vessels, endothelial cell transplantation into the heart for myocardial regeneration (Robey et al., 2008), and induction of angiogenesis for treatment of regional ischemia (Liu et al., 2014). Endothelial cell dysfunction is also associated with many diseases, including Alzheimer's disease, stroke, multiple sclerosis, and atherosclerosis (Boyle et al., 1997; Weiss et al., 2009). hPSC-derived endothelial progenitors and endothelial cells may provide building blocks for the establishment of in vitro disease models for screening and development of drugs to treat these diseases. Functionality of hPSC-derived endothelial cells has been shown using in vitro cell culture platforms and in vivo animal models (Adams et al., 2013; Kusuma et al., 2013; Orlova et al., 2014; Samuel et al., 2013; Wang et al., 2007). Similar to other somatic cells derived from hPSCs, differentiated $CD31^+$ endothelial cells exhibited functional heterogeneity (Rufaihah et al., 2013). Previously reported studies of hPSC differentiation to endothelial cells have demonstrated that Activin/Nodal/transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), vascular endothelial growth factor (VEGF), and microRNA-21 signaling promote this differentiation (Di Bernardini et al., 2013; James et al., 2010; Kane et al., 2010; Lu et al., 2007; Marchand et al., 2014; Rufaihah et al., 2011; Wang et al., 2004; Zambidis et al., 2005). In addition, mechanical sheer stress also promoted embryonic stem cell-derived endothelial phenotypes (Wolfe and Ahsan, 2013).

During murine embryogenesis, hemangioblasts, which can differentiate into multipotent hematopoietic stem cells and endothelial progenitors, are derived from a subpopulation of mesoderm that coexpresses brachyury and KDR (Huber et al., 2004). Similar blast colony-forming cells were also isolated from mouse embryonic stem cell aggregates in the presence of cytokines (Kennedy et al., 1997). When cocultured with OP9 stromal cells, hPSCs differentiated to mesodermal progenitors with the capacity to form blast or hemangioblast colonies in response to fibroblast growth factor 2 (FGF2) (Vodyanik et al., 2010). As another approach, hPSCs cultured as embryoid bodies were exposed to a growth factor cocktail containing activin A, BMP4, FGF2, and VEGF to induce differentiation to

$CD34^+CD31^+$ endothelial progenitors (Costa et al., 2013; Levenberg et al., 2002; Song et al., 2013). The $CD34^+CD31^+$ vascular progenitor population generated endothelial cells and smooth muscle cells in the proper culture environments (Bai et al., 2010). TGF-β signaling enhanced smooth muscle cell differentiation from these endothelial progenitors, whereas the TGF-β signaling inhibitor SB431542 promoted endothelial cell generation and expansion (James et al., 2010). Global gene transcription analysis demonstrated low variability between endothelial cells (ECs) differentiated from multiple lines of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) in the presence of these cytokines (White et al., 2013). Although prior studies have demonstrated differentiation of hPSCs to endothelial progenitors, and subsequently to ECs and smooth muscle cells, by applying growth factors from different signaling pathways, it is largely unknown whether these distinct differentiation protocols produce identical endothelial cells and their progenitors, and which developmental signaling mechanisms are necessary and sufficient to specify these differentiation fates.

Here, we describe a simple and efficient method for the conversion of hPSCs to $CD34^+CD31^+$ endothelial progenitors. Appropriate temporal activation of regulators of WNT signaling alone, in the absence of exogenous FGF2 and VEGF signaling, was sufficient to drive multiple hPSC lines to differentiate to greater than 50% $CD34^+CD31^+$ endothelial progenitors. However, endogenous MEK signaling was required for hPSC differentiation to endothelial progenitors because MEK inhibitor treatment substantially diminished the yield of $CD34^+CD31^+$ cells. These hPSC-derived endothelial progenitors were further enriched to 99% purity with a single step of CD34-based magnetic separation. Single-cell clonal differentiation assays revealed that $CD34^+CD31^+$ endothelial progenitors generated by WNT pathway activation were bipotent and could differentiate to functional endothelial cells and smooth muscle cells.

RESULTS

GSK3 Inhibition Is Sufficient to Induce hPSC Differentiation to Endothelial Progenitors in the Absence of Exogenous Growth Factors

We previously demonstrated that temporal modulation of canonical WNT signaling is sufficient to generate functional cardiomyocytes at high yield and purity (Lian et al., 2012, 2013b). In that study, a virtually pure population of $brachyury^+$ cells was generated within 24 hr after treatment of undifferentiated hPSCs with a GSK3 inhibitor. In order to direct these $brachyury^+$ cells to a cardiomyocyte fate, we then inhibited canonical WNT signaling by either expression of β-catenin small hairpin RNA (shRNA) or treatment with small-molecule inhibitors of WNT signaling at the proper corresponding time points. To assess whether these GSK3 inhibitor-induced $brachyury^+$ mesendoderm progenitors also have the potential to differentiate to endothelial progenitors, we treated 19-9-11 iPSCs with the GSK3 inhibitor CHIR99021 and subsequently cultured the cells in StemPro-34 medium supplemented with VEGF, both of which have been reported to stimulate vascular cell differentiation of hPSCs (Grigoriadis et al., 2010) (Figure 1A). After optimizing the concentrations and timing of application of various components, the differentiation protocol consisted of two phases. Phase I generated $brachyury^+$ cells by treatment of undifferentiated hPSCs with CHIR990211 for 2 days, whereas phase II directed the $brachyury^+$ progenitors to $CD34^+CD31^+$ cells by culture in StemPro-34 supplemented with VEGF for 3 days. We found that without CHIR99021 treatment, very few $CD34^+CD31^+$ cells were generated from human iPSCs, even in the presence of VEGF (Figure 1B). However, treatment of the human iPSCs with CHIR99021 generated more than 20% $CD34^+CD31^+$ cells. Interestingly, VEGF treatment did not significantly increase the yield of $CD34^+CD31^+$ cells in this differentiation system (Figure 1B). However, in the presence of sunitinib, which inhibits several receptor tyrosine kinases including VEGFR, very few $CD34^+CD31^+$ cells were generated, suggesting that endogenous VEGF signaling is required for endothelial progenitor differentiation stimulated by CHIR99021 treatment (Figure 1C). Because of these observations, we did not include exogenous VEGF in subsequent differentiation experiments. In addition, this differentiation protocol primarily generated $CD34^+CD31^+$ cells and produced very few $CD34^-CD31^+$ or $CD34^+CD31^-$ cells (Figure 1B).

We further tested the concentration dependence of CHIR99021 for inducing $CD31^+CD34^+$ endothelial progenitor differentiation and found that 6–10 μM generated 25% endothelial progenitors (Figure 1D). Similar results were obtained in the H1 hESC line (Figure S1 available online). These $CD34^+CD31^+$ endothelial progenitors also expressed CD144 (VE-cadherin), but not CD45, indicating these cells were not in hematopoietic lineages (Figure S1). These results illustrate that induction of WNT signaling via the GSK3 inhibitor CHIR99021 permitted subsequent differentiation of hPSCs to $CD34^+CD31^+$ cells, and that exogenous VEGF was not required.

Endothelial Progenitor Differentiation Induced by GSK3 Inhibitors in hPSCs Is β-Catenin Dependent

Selectivity is a general concern with the use of chemical inhibitors. We tested other GSK3 inhibitors, BIO-acetoxime and CHIR98014, and found that both also effectively induced endothelial progenitor differentiation to a similar

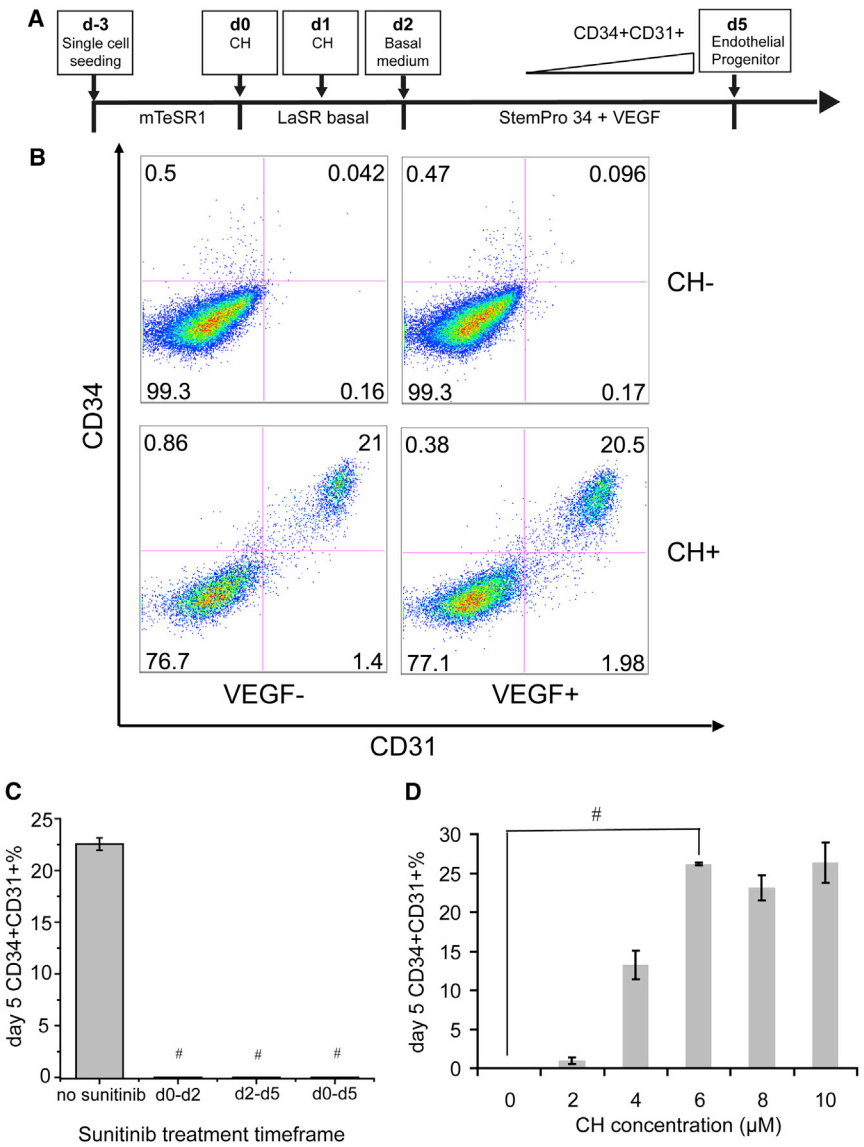

Figure 1. Derivation of CD34$^+$CD31$^+$ Cells from hPSCs via GSK3 Inhibitor Treatment

(A) Schematic of the protocol for differentiation of hPSCs to CD34$^+$CD31$^+$ cells via treatment with a GSK3 inhibitor.

(B) 19-9-11 iPSCs were cultured on Matrigel for 2 days in LaSR basal medium with or without 6 μM CH followed by another 3 days in StemPro-34 medium with or without VEGF.

(C) 19-9-11 iPSCs were cultured on Matrigel in LaSR basal medium with 6 μM CH for 2 days followed by another 3 days in StemPro-34 medium with 1 μM sunitinib at indicated times.

(D) 19-9-11 iPSCs were cultured on Matrigel in LaSR basal medium with different concentrations of CH for 2 days followed by another 3 days in StemPro-34 medium. All flow cytometric analyses of CD34 and CD31 expression were performed after 5 days of differentiation. Data are represented as mean ± SEM of at least three independent replicates.

See also Figure S1.

extent as CHIR99021 in the absence of VEGF treatment (Figure 2A). These small molecule compounds have distinct chemical structures, reducing the likelihood of shared off-target effects. However, GSK3 inhibition can affect multiple signaling pathways, including the desired activation of canonical WNT signaling by stabilizing β-catenin protein. In order to evaluate the role of β-catenin in GSK3 inhibitor-induced hPSC endothelial differentiation, we generated an iPSC line (19-9-11 ishcat-1) expressing β-catenin shRNA under the control of a tet-regulated inducible promoter. Upon doxycycline (dox) addition, the shRNA efficiently downregulated *β-catenin* expression (Figure 2B). We used this cell line to examine the stage-specific roles of β-catenin during monolayer endothelial differentiation stimulated by GSK3 inhibition. Undifferentiated 19-9-11 ishcat-1 iPSCs were treated with 6 μM CHIR99021 for 48 hr followed by another 3 days culture in StemPro-34 medium. Dox was added at various time points between day 0 and day 4, and endothelial progenitor differentiation was assessed at day 5 as the percentage of CD34$^+$CD31$^+$cells. We found that WNT/β-catenin signaling was essential for endothelial induction by CHIR99021 because *β-catenin* knockdown at day 0 did not generate CD34$^+$CD31$^+$ cells. We previously showed that β-catenin is essential for *brachyury* expression after hPSC treatment with GSK3 inhibitors (Lian et al., 2012). We reasoned that abrogation of the CD34$^+$CD31$^+$ population by *β-catenin* knockdown at day 0 was due to blocking differentiation of hPSCs to brachyury-expressing mesendoderm progenitors. We profiled the expression of brachyury

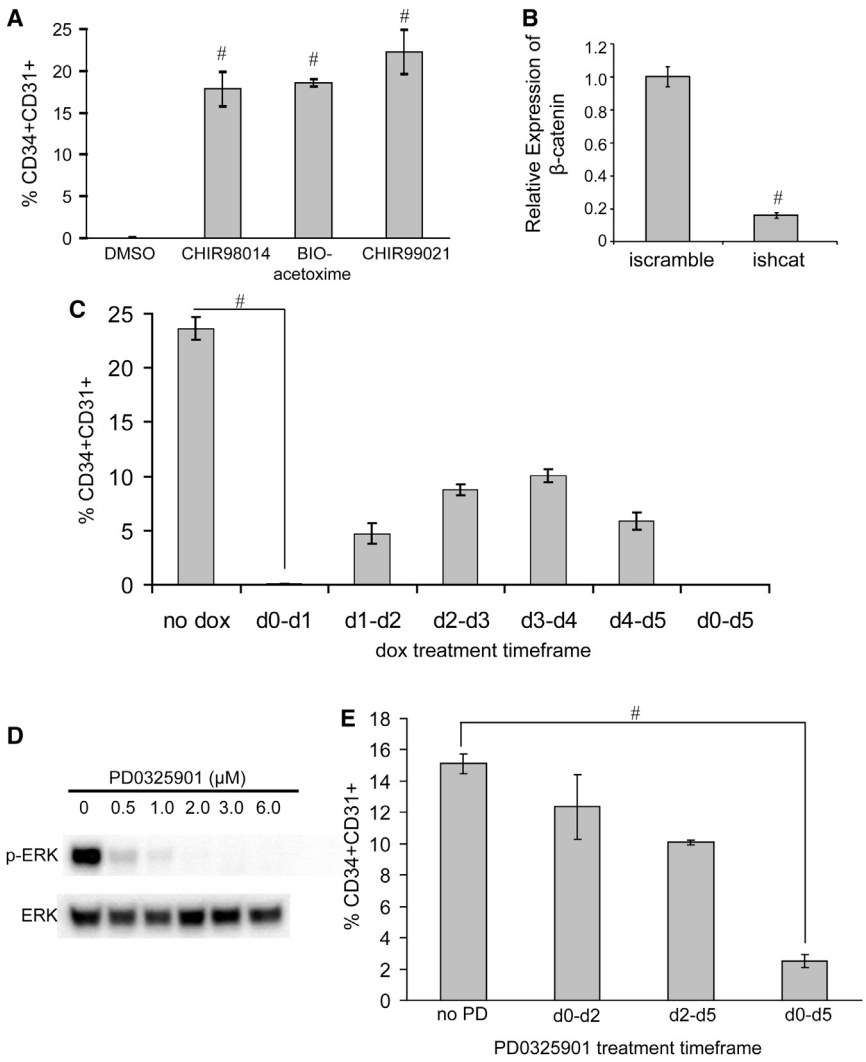

Figure 2. CD34$^+$CD31$^+$ Endothelial Progenitor Differentiation Induced by GSK3 Inhibitors in hPSCs Is β-Catenin Dependent

(A) 19-9-11 iPSCs were cultured on Matrigel in LaSR basal medium containing DMSO, 0.6 μM CHIR98014, 0.6 μM BIO-acetoxime, or 6 μM CHIR99021 for 2 days before culture in StemPro-34 medium for 3 days. At day 5, the percentage of CD34$^+$CD31$^+$ cells in culture was assessed by flow cytometry.

(B) 19-9-11 iscramble and 19-9-11 ishcat cells were cultured in mTeSR1 containing 2 μg/ml doxycycline. After 3 days, mRNA was collected and *β-catenin* expression evaluated by qPCR. #$p < 0.005$, ishcat versus iscramble; t test.

(C) 19-9-11 ishcat cells were cultured in LaSR basal medium containing 6 μM CHIR99021 for 2 days before exposure to StemPro-34 medium for 3 days, with 2 μg/ml dox addition at the indicated times. Five days after initiation of differentiation, cells were analyzed for CD34 and CD31 expression by flow cytometry.

(D) Western blot analysis of phospho-ERK (p-ERK) and ERK in 19-9-11 cells after 24 hr in LaSR basal medium supplemented with PD0325901 at the indicated concentrations.

(E) 19-9-11 cells were cultured in LaSR basal medium containing 6 μM CHIR99021 for 2 days before exposure to StemPro-34 medium for 3 days, with 1 μM PD0325901 addition at the indicated times. Five days after initiation of differentiation, cells were analyzed for CD34 and CD31 expression by flow cytometry. Data are represented as mean ± SEM of at least three independent replicates.

See also Figure S2.

during the 5 day differentiation and found a virtually pure population of brachyury$^+$ cells generated by day 2 in the absence of dox. The presence of dox treatment, however, abolished brachyury expression (Figure S2). Importantly, depletion of *β-catenin* expression at time points after brachyury expression was detected also reduced the percentage of CD34$^+$CD31$^+$ cells generated (Figure 2C), indicating β-catenin might also be involved in differentiation of mesendoderm to CD34$^+$CD31$^+$ cells. Because of the important role of FGF2 signaling on brachyury$^+$ cell differentiation from hPSCs (Yu et al., 2011) and the mesodermal origin of endothelial progenitors, we used an MEK inhibitor to study the role of MEK/ERK signaling on CD34$^+$CD31$^+$ cell differentiation. We found that 1 μM of MEK inhibitor PD0325901, which effectively blocked ERK phosphorylation (Figure 2D), substantially diminished CD34$^+$CD31$^+$ cell differentiation, suggesting that endogenous MEK/ERK signaling promotes endothelial progenitor differentiation (Figure 2E).

Differentiation Medium and Cell Density Dependence of GSK3 Inhibitor-Induced Endothelial Progenitor Differentiation

We have shown that hPSCs can be differentiated to CD34$^+$CD31$^+$ endothelial progenitors by 2 days (phase I) of culture in an optimized defined medium supplemented with GSK3 inhibitors and another 3 days (phase II) in StemPro-34 medium. In order to streamline the differentiation process, we tested whether a single basal medium could support endothelial progenitor differentiation

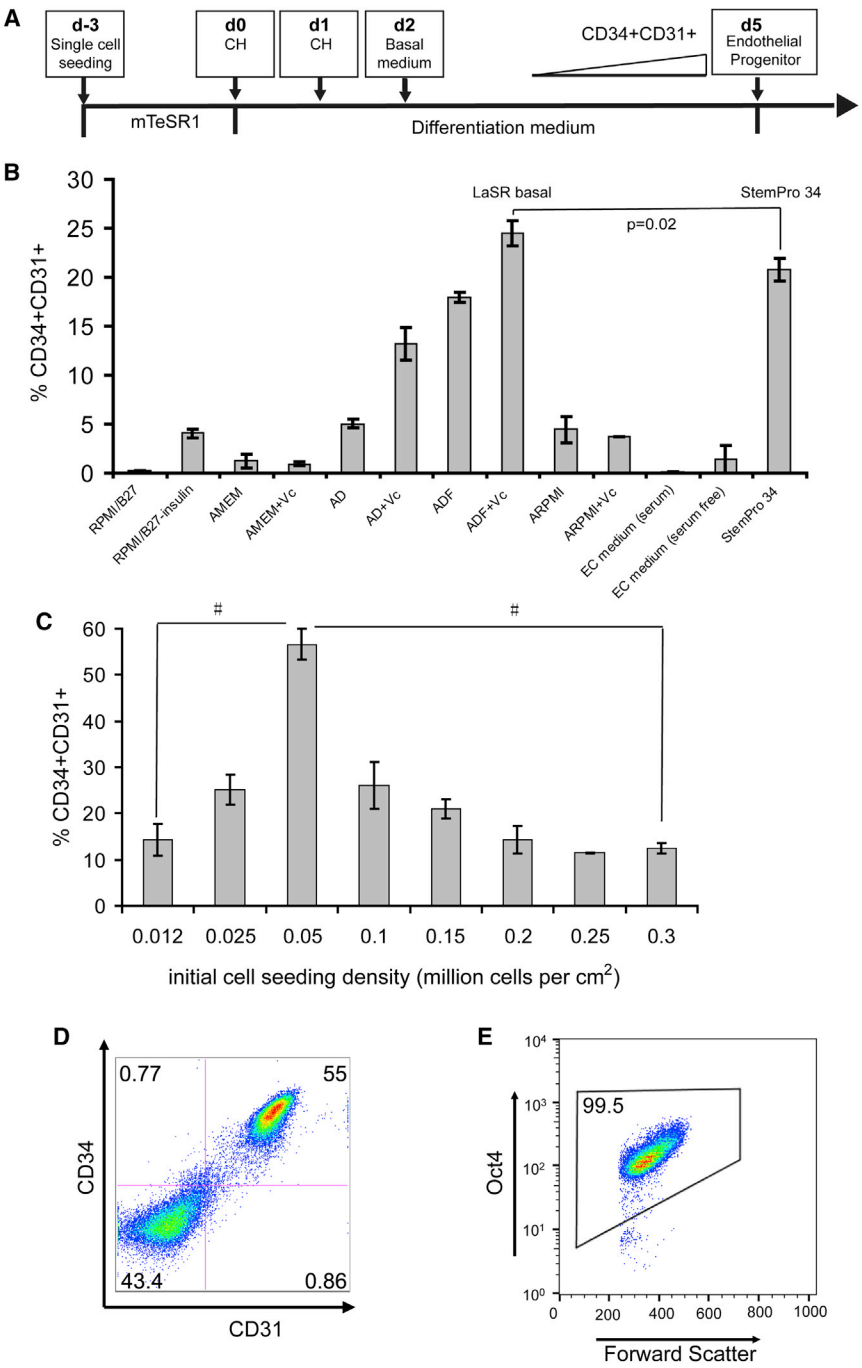

Figure 3. Differentiation Medium and Cell Density Dependence of GSK3 Inhibitor-Induced Endothelial Progenitor Differentiation

(A) Schematic of the protocol for defined, growth factor-free differentiation of hPSCs to endothelial progenitors in a single differentiation medium.

(B) 19-9-11 iPSCs were cultured as indicated in (A), with different basal differentiation media. At day 5, cells were analyzed for CD34 and CD31 expression by flow cytometry. p = 0.02, LaSR basal versus StemPro 34; t test.

(C–E) 19-9-11 iPSCs were differentiated as described in (A) using LaSR basal medium, with different day −3 cell seeding densities. At day 5, cells were analyzed for CD34 and CD31 expression by flow cytometry (C). Data are represented as mean ± SEM of at least three independent replicates. (D) shows a representative CD34/CD31 flow plot from a seeding density of 0.05 million cells/cm^2, and (E) shows a representative flow cytometry analysis of OCT4 expression in 19-9-11 cells at day 0 after expansion in mTeSR for 3 days.

(Figure 3A). We examined 13 different media supplemented with CHIR99021 for 2 days (phase I) followed by 3 additional days in the same basal medium lacking exogenous growth factors or chemical inhibitors (phase II). We found that advanced DMEM/F12 supplemented with ascorbic acid, which we termed LaSR basal medium, was the most efficient for endothelial progenitor differentiation, resulting in 25% CD34$^+$CD31$^+$ cells in 5 days (Figure 3B). These results indicated the importance of basal medium composition in endothelial progenitor differentiation, with some media (e.g., RPMI/B27 and EC medium containing serum) not supporting differentiation to any detectable CD34$^+$CD31$^+$ cells (Figure 3B).

On the basis of the knowledge that initial cell density affects hPSC differentiation fates to lineages such as cardiomyocytes, epithelial progenitors, and pancreatic cells

(Gage et al., 2013; Lian et al., 2013a; Selekman et al., 2013; Takizawa-Shirasawa et al., 2013), we quantitatively assessed the effect of initial cell seeding density on endothelial progenitor differentiation efficiency. Singularized hPSCs were seeded at densities ranging from 12,000 to 300,000 cells per cm^2 and then expanded in mTeSR1 for 3 days before transition to LaSR basal medium supplemented with CHIR99021 for 2 days and another 3 days in LaSR basal medium lacking CHIR99021. The optimal seeding density was 50,000 cells per cm^2 at day −3, which resulted in 325,000 cells per cm^2 at day 0 (Figure 3C). At day 5, 55% of cells seeded at this density expressed CD34 and CD31 (Figures 3C and 3D). We performed fluorescence-activated cell sorting (FACS) analysis of OCT4 expression of day 0 cells and found that more than 99.5% of the cells were $OCT4^+$, suggesting that hPSCs were pluripotent before addition of CH (Figure 3E).

$CD34^+CD31^+$ Endothelial Progenitors Are Multipotent

Molecular analysis of endothelial progenitor differentiation from hPSCs revealed dynamic changes in gene expression, with downregulation of the pluripotency markers *SOX2* and *OCT4*, and induction of the primitive streak-like genes *T* (Nakanishi et al., 2009) and *MIXL1* (Davis et al., 2008) in the first 24 hr after CH addition (Figure 4A). Expression of the endothelial progenitor markers *CD34* and *CD31* was detected at day 4 and increased at day 5 (Figure 4A). Western blot analysis of protein expression revealed decreasing OCT4 expression during the first 2 days of differentiation, and brachyury induction on days 1 and 2 followed by downregulation on day 3. The endothelial marker VE-cadherin was only detected after 5 days of differentiation (Figure 4B). Immunofluorescent analysis revealed the expression of CD34, CD31, and VE-cadherin on day 5 (Figure 4C). Ten days after differentiation without passaging, however, the expression of CD34 diminished, whereas the cells retained expression of $CD31^+$ and VE-cadherin (Figure 4C).

To study the multipotent nature of the transient population of $CD34^+$ cells, we first enriched the day 5 differentiation culture to 99% purity of $CD34^+$ cells by magnetic-activated cell sorting (MACS) (Figure 5A). We also enriched the day 5 differentiation cultures of additional hESC lines (H13, H14) and human iPSC lines (19-9-7 and 6-9-9) to more than 99% purity of $CD34^+$ cells (Figures S3A–S3D**)**. The sorted $CD34^+$ putative endothelial progenitors were plated on Matrigel-coated 48-well plates at a density of one cell per well in a combined medium (50% smooth muscle and 50% endothelial medium). After 10 days of culture, the differentiation populations from ten different single-cell clones contained both VE-cadherin$^+$ von Willebrand factor $(vWF)^+$ cells and Calponin$^+$ SMMHC$^+$ (Smooth muscle myosin heavy chain) cells, including some cells expressing both endothelial and smooth muscle markers (Figure 5B; Figures S3E and S3F), thereby demonstrating that single $CD34^+$ endothelial progenitors generated from hPSCs were multipotent. In addition, when the sorted $CD34^+$ clones were cultured in endothelial or smooth muscle medium rather than the mixed medium, they generated relatively pure populations of endothelial cells and smooth muscle cells, respectively (Figures 5C and 5D). The hPSC-derived endothelial progenitors expressed C-KIT (CD117), KDR (VEGFR2), TIE-2, CD31, CD34, CD144, but did not express vWF or intercellular adhesion molecule 1 (ICAM-1) (Figure 6). We also quantified TIE-2, CD117, KDR, and ICAM-1 expression with flow cytometry (Figure S4), and results were consistent with immunostaining experiments. The day 15 endothelial cells expressed CD31, CD144, vWF, ICAM-1, and KDR, but did not express CD117, CD34, or TIE-2 (Figure 6).

Characterization of Endothelial Cells Derived from hPSCs

To study the intrinsic properties of endothelial cells differentiated from hPSCs by GSK3 inhibition and culture in LaSR basal medium, we cultivated the purified $CD34^+$ cells in endothelial medium on laminin-coated plates and found that the resulting $CD34^+CD31^+vWF^+CD144^+$ endothelial cells were capable of approximately 20 population doublings over 2 months, generating more than 1 million cells from a single endothelial cell (Figure 7A). After 2 months in culture, the cells still expressed CD31 and VE-cadherin based on immunostaining (Figure 7B). Flow cytometry analysis also showed that more than 99% of cells expressed CD31 and VE-cadherin after 60 days in culture (Figure S5). Furthermore, upon treatment with exogenous VEGF, the $CD144^+$ cells formed vascular tubes in Matrigel matrix (Figure 7C) and were capable of acetylated low-density lipoprotein (AcLDL) uptake, indicative of endothelial function (Figure 7D). The endothelium also responds to inflammatory mediators, such as tumor necrosis factor α (TNF-α), by upregulating adhesion molecules including ICAM-1, which has been implicated in the capture of circulating leukocytes. Although unstimulated endothelial cells expressed low levels of ICAM-1, TNF-α treatment greatly increased the expression of ICAM-1 in the hPSC-derived endothelial cells (Figure 7E).

To characterize the barrier phenotype of the hPSC-derived endothelial cells, we measured the transendothelial electrical resistance (TEER) and transport of fluorescent 40 kDa dextran across a monolayer of differentiated endothelial cells. VEGF treatment induced a sustained decrease in TEER and an increase in dextran permeability. However, treatment of endothelial cells with the cAMP analog 8-pCPT-2′O-Me-cAMP (o-Me) decreased dextran permeability and increased TEER (Figures 7F and 7G).

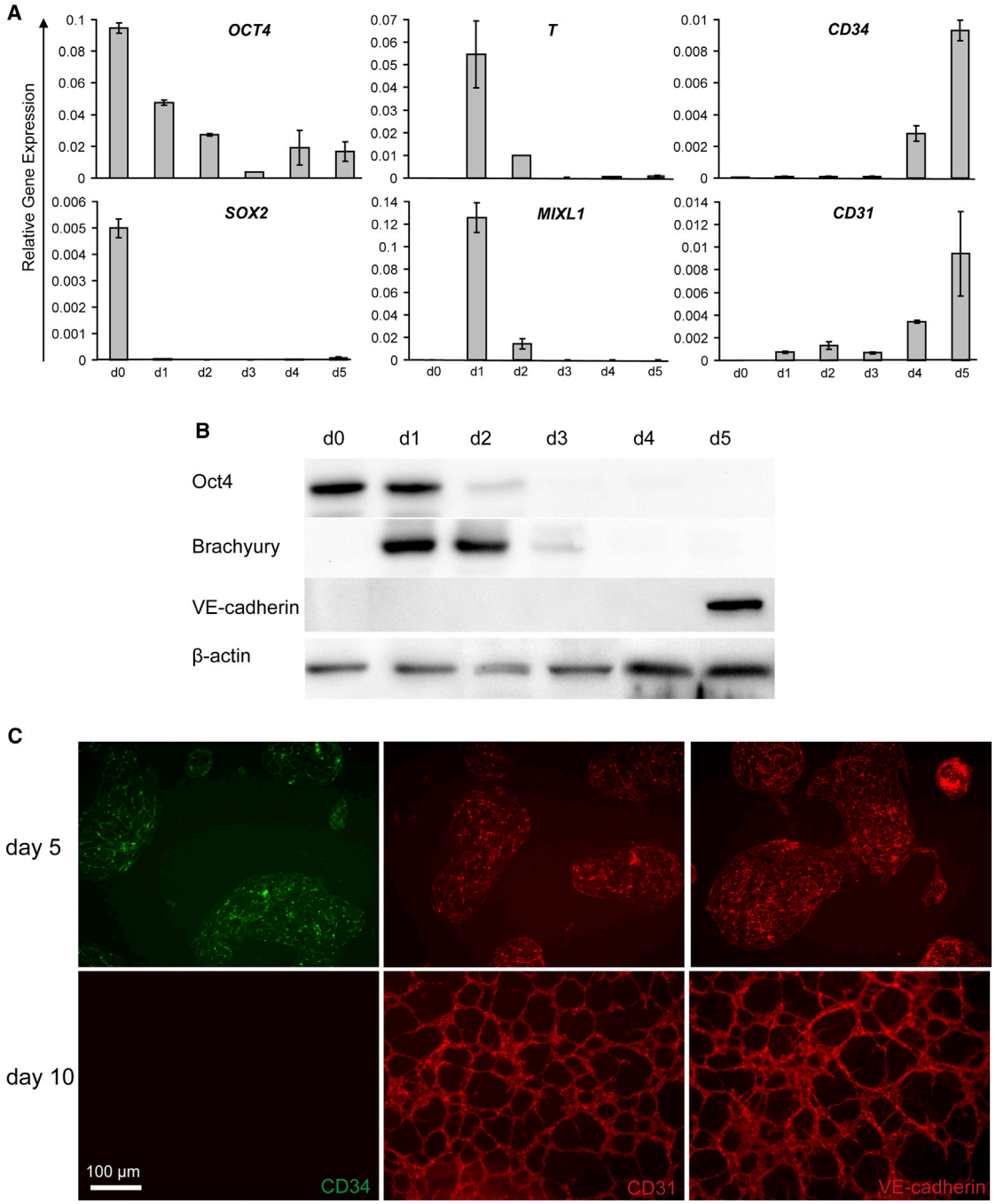

Figure 4. Molecular Analysis of Endothelial Progenitors Differentiated from hPSCs

19-9-11 iPSCs were differentiated as illustrated in Figure 3A using LaSR basal medium. At different time points, developmental gene or protein expression was assessed by quantitative RT-PCR (A) or western blot (B). Data are represented as mean ± SEM of at least three independent replicates. At 5 days or 10 days postdifferentiation, immunostaining for CD34, CD31, and VE-cadherin was performed (C).

DISCUSSION

Prior reports of differentiating hPSCs to endothelial cells required the addition of expensive growth factors and/or undefined serum to direct endothelial development, and these methods generated heterogeneous cell mixtures typically containing less than 10% endothelial cells. Here, we show that small-molecule activation of canonical WNT

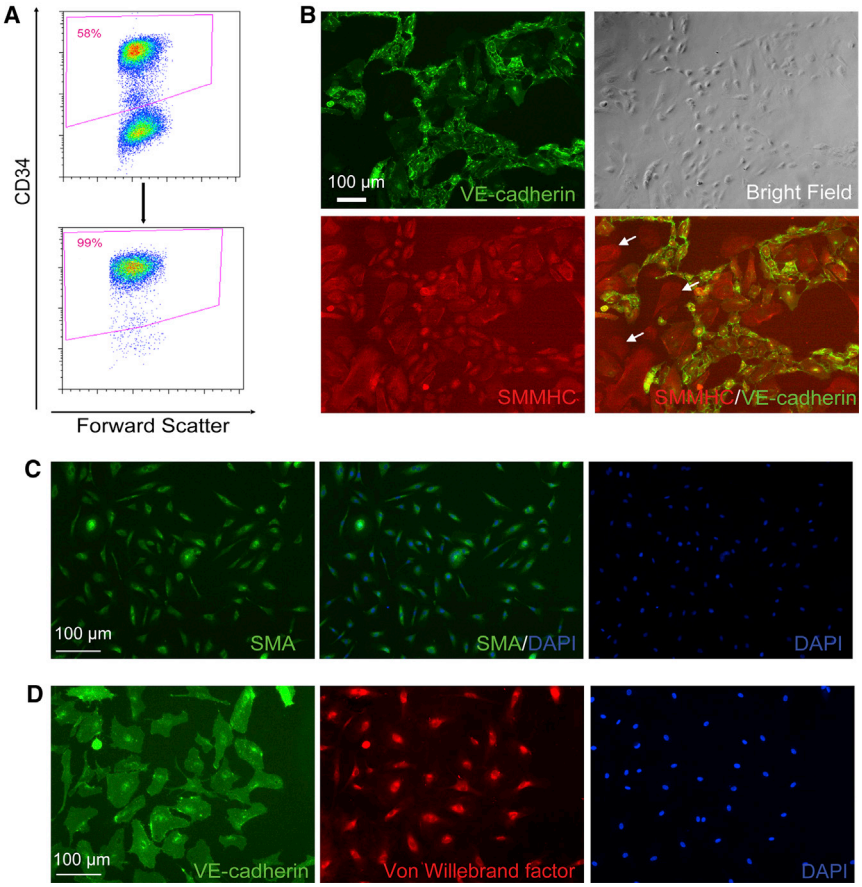

Figure 5. CD34$^+$CD31$^+$ Endothelial Progenitors Are Multipotent

19-9-11 iPSCs were differentiated as illustrated in Figure 3A using LaSR basal medium.

(A) At day 5, CD34$^+$ cells were enriched with the EasySep Human CD34 Positive Selection Kit and purification quantified by flow cytometry for CD34 expression.

(B–D) Sorted CD34$^+$ cells were cultured in a combined medium (B), a smooth muscle medium (C), or an endothelial medium (D) at a density of one cell per well in a 48-well plate for another 10 days. Sample immunofluorescence images for smooth muscle and endothelial markers were shown. In (B), arrows point to SMMHC$^+$VE-cadherin$^-$ cells. See also Figure S3.

signaling via GSK3 inhibition, in the absence of exogenous growth factors, is sufficient to generate high yields of CD34$^+$CD31$^+$ endothelial progenitors from hPSCs. Prior to this study, endothelial progenitors have not been isolated from hPSCs under defined conditions, and the mechanisms by which developmental signaling pathways regulate endothelial progenitor commitment remained largely unknown (Slukvin, 2013). With a doxycycline-inducible shRNA knockdown of the WNT pathway modulator *β-catenin*, we identified that β-catenin is required for CD34$^+$CD31$^+$ endothelial progenitor generation from hPSCs following GSK3 inhibition. Furthermore, although previous reports required more than 10 days to generate CD34$^+$CD31$^+$ cells, this small molecule differentiation approach accelerates developmental timing and converts hPSCs to greater than 50% CD34$^+$CD31$^+$ cells in 19-9-11 iPSCs in 5 days. This protocol produced more than 99% pure populations of CD34$^+$CD31$^+$ cells from six different hPSC lines following a single magnetic affinity separation.

GSK3 inhibition has been shown to be a potent inducer of mesendoderm lineage commitment in hPSCs (Tan et al., 2013). The WNT pathway exhibits crosstalk with a variety of other developmental signaling networks, including TGF-β superfamily (Cai et al., 2013), FGF (Stulberg et al., 2012), Notch (Wang et al., 2013), Hippo (Hergovich and Hemmings, 2010), and retinoic acid signaling (Chanda et al., 2013). The necessity of β-catenin in generating CD34$^+$CD31$^+$ cells indicates a direct role of canonical WNT signaling in endothelial progenitor differentiation, whereas the diminished yield of CD34$^+$CD31$^+$ cells in the presence of MEK and receptor tyrosine kinase inhibitors suggests endogenous pathways also mediate endothelial progenitor differentiation. Thus, canonical WNT signaling is not the only pathway required for endothelial progenitor specification, but it may act as a master regulator to orchestrate signaling that leads to endothelial progenitor differentiation.

We have previously reported a method to differentiate hPSCs to a relatively pure population of cardiomyocytes by dynamic modulation of canonical WNT signaling using small-molecule GSK3 inhibitors and Porcupine inhibitors in a RPMI/B27 medium lacking insulin (Lian et al., 2012, 2013a). This protocol uses WNT pathway activation to direct mesendoderm differentiation, followed by WNT pathway inhibition to specify cardiac mesoderm and cardiomyocyte fates. Although the small molecule cardiomyocyte and endothelial progenitor differentiation protocols

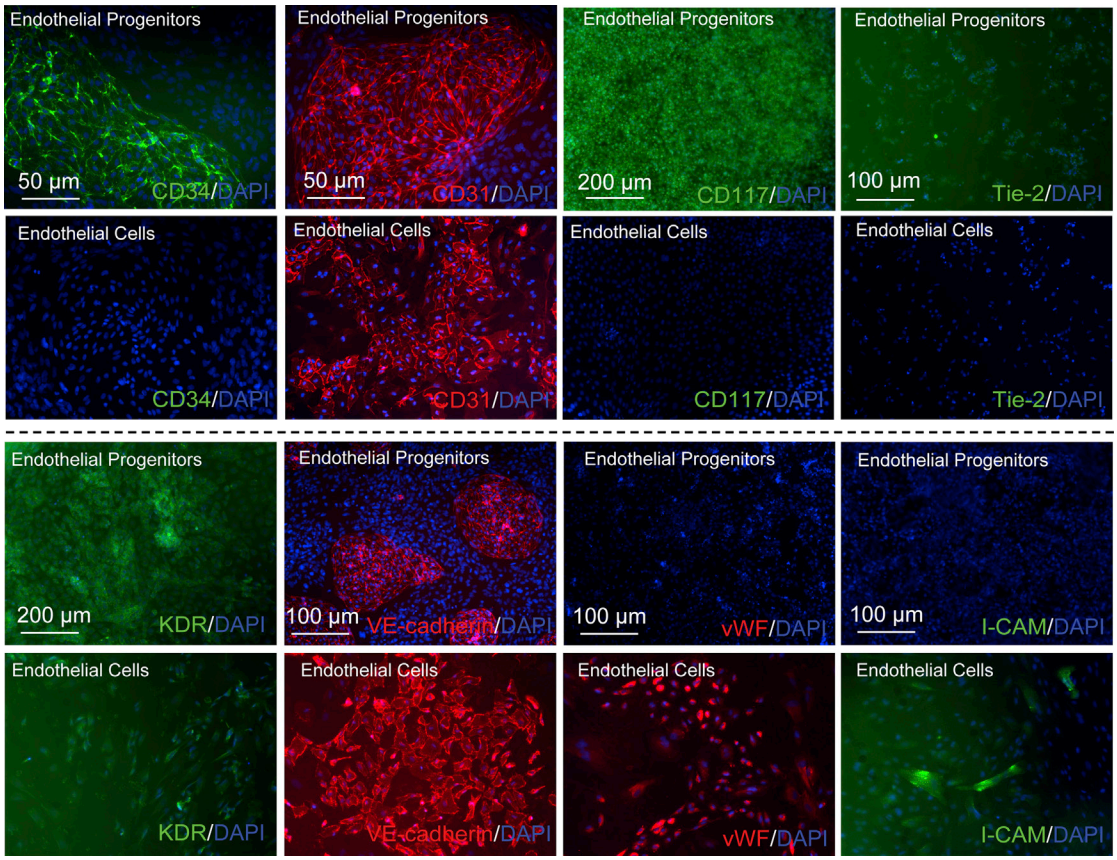

Figure 6. Day 5 Endothelial Progenitors Differ from Day 15 Endothelial Cells in Marker Expression
19-9-11 iPSCs were differentiated as illustrated in Figure 3A using LaSR basal medium, and unsorted day 5 progenitor cells and day 15 endothelial cells were immunostained for developmental markers as indicated. See also Figure S4.

result in optimal cell yields in distinct defined basal media, both progress through mesodermal progenitors via GSK3 inhibition. These mesodermal progenitors can be directed to spontaneously contracting cardiomyocytes via WNT inhibition, while permitting endogenous canonical WNT signaling yields a population enriched in endothelial progenitors. Together, these results demonstrate the concept of chemically guiding differentiation to distinct cell types via different patterns of modulation of master regulators of cell fates. This paradigm may represent a promising general approach to efficiently produce cells and tissues from stem cell sources in a defined manner.

Interestingly, the WNT pathway-induced endothelial progenitor differentiation protocol primarily generates CD34 and CD31 double-positive cells, as opposed to other methods using BMP4, which produce a mixture of $CD34^+CD31^-$, $CD34^+CD31^+$, $CD34^-CD31^+$ cells (Bai et al., 2010; Song et al., 2013). The $CD34^+CD31^+$ endothelial progenitors may be more amenable to large-scale expansion. Furthermore, these endothelial progenitors can be further directed to endothelial cells or smooth muscle cells by culture in the appropriate inductive media. Purified $CD31^+$VE-cadherin$^+$vWF$^+CD34^-$ endothelial cells were expanded for 20 doublings over 2 months and maintained expression of endothelial cell markers ($CD31^+$VE-cadherin$^+CD34^-$). These cells exhibited uptake of acetylated low-density lipoprotein and formed tube-like structures when cultured on Matrigel. These endothelial cells also responded to TNF-α treatment by increasing ICAM-1 expression and maintained a dynamic barrier, responding to VEGF and cAMP analog by increasing or decreasing dextran permeability, respectively. Our findings demonstrate efficient and reproducible endothelial progenitor and endothelial differentiation of hPSCs by GSK3 inhibition in the absence of exogenous growth factor stimulation.

EXPERIMENTAL PROCEDURES

Maintenance of hPSCs

Transgene and vector free human iPSCs (19-9-11, 19-9-7, 6-9-9) (Yu et al., 2009) and hESCs (H1, H13, H14) (Thomson et al., 1998) were

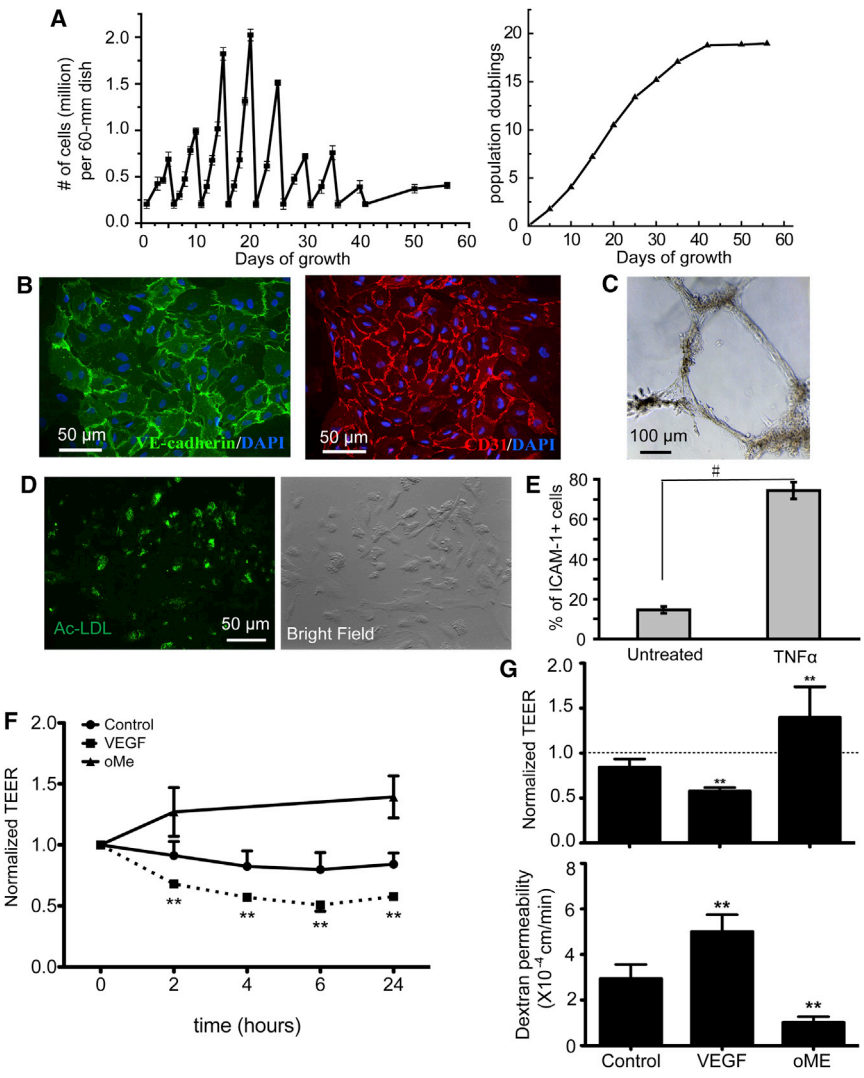

Figure 7. Characterization of Endothelial Cells Derived from hPSCs

(A) 19-9-11 iPSCs were differentiated as illustrated in Figure 3A using LaSR basal medium. At day 5, $CD34^+$ cells were enriched and cultured in endothelial medium on laminin-coated plates. At different time points, the cell numbers were counted.

(B–D) The purified day 60 endothelial cells were immunostained with VE-cadherin and CD31 (B) and tested for tube-forming ability upon VEGF treatment (C) and the ability to uptake AcLDL (D).

(E) Flow cytometry analysis of surface protein expression of ICAM-1 of untreated cells or cells treated with 10 ng/ml TNF-α for 16 hr.

(F and G) Barrier phenotype of the hPSC-derived endothelial cells. Endothelial cells were differentiated as illustrated in Figure 3A using LaSR basal medium and then $CD34^+$ cells were enriched by MACS and cultured on laminin in endothelial cell medium. After 10 days in culture, the endothelial cells were seeded on collagen/fibronectin coated transwell filters and maintained in endothelial SFM. Barrier function was assessed by measuring TEER 48 hr after seeding. Next, cells were treated with endothelial SFM containing 100 ng/ml VEGF or 10 μM oME. In (F), TEER was measured as a function of time, and normalized to the initial TEER value (36.7 ± 4.3 Ωcm^2. In (G), TEER and fluorescent dextran permeability were measured 24 hr after plating. **p < 0.005, VEGF (or oME) versus control; t test.

Data are represented as mean ± SEM of at least three independent replicates. See also Figure S5.

maintained on Matrigel (BD Biosciences)-coated plates (Corning) in mTeSR1 medium (STEMCELL Technologies) according to a previously published method (Lian et al., 2013a).

Endothelial Progenitor Differentiation via Modulation of Canonical WNT Signaling

hPSCs maintained on a Matrigel-coated surface in mTeSR1 were dissociated into single cells with Accutase (Life Technologies) at 37°C for 5 min and then seeded onto a Matrigel-coated cell culture dish at 50,000 cell/cm^2 in mTeSR1 supplemented with 5 μM ROCK inhibitor Y-27632 (Selleckchem) (day −3) for 24 hr. Cells were then cultured in mTeSR1, changed daily. At day 0, cells were treated with 6–10 μM CHIR99021 (Selleckchem) for 2 days in LaSR basal medium, which consists of Advanced DMEM/F12, 2.5 mM GlutaMAX, and 60 μg/ml ascorbic acid (Sigma, A8960). After 2 days, CHIR99021-containing medium was aspirated and cells were maintained in LaSR basal medium without CHIR99021 for 3–4 additional days. PD0325901 was purchased from Tocris Bioscience.

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 a 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) and split every 4–5 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 a 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 4–5 days with Accutase.

Vascular Tube Formation Assay

To assess the formation of capillary structures, 1×10^5 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 precoated with 250 μl Matrigel (BD Bioscience). Tube formation was observed by light microscopy after 24 hr of incubation.

RT-PCR and Quantitative RT-PCR

Total RNA was prepared with the RNeasy mini kit (QIAGEN) and treated with DNase (QIAGEN). One microgram 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 the Supplemental Experimental Procedures.

Flow Cytometry

Cells were dissociated into single cells 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 Experimental Procedures) 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. FACS gating was based on the corresponding isotype antibody control.

Immunostaining

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

Western Blot Analysis

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce) in the presence of Halt Protease and Phosphatase Inhibitor Cocktail (Pierce). Proteins were separated by 10% Tris-Glycine SDS/PAGE (Invitrogen) under denaturing conditions and transferred to a nitrocellulose membrane. After blocking with 5% dried milk in TBST, the membrane was incubated with primary antibody overnight at 4°C. The membrane was then washed, incubated with an anti-mouse/rabbit peroxidase-conjugated secondary antibody (Supplemental Experimental Procedures) at room temperature for 1 hr, and developed by SuperSignal chemiluminescence (Pierce).

Monolayer Integrity

Purified $CD31^+$ endothelial cells were seeded on 12-well plate size 0.4 μm porous polystyrene membrane inserts (Transwells, Corning) coated with a 0.4 mg/ml collagen IV and 0.1 mg/ml fibronectin mixture at a density of 250,000 cells/insert. Cells were maintained in endothelial cell serum-free medium (SFM) (Life Technologies) for 48 hr prior to addition 0.5 ml and 1.5 ml of endothelial SFM at the apical and basolateral chambers respectively. Barrier tightness was assessed by measurement of transendothelial electrical resistance (TEER) using aEVOM STX2 electrode system (World Precision Instruments) before medium replacement. Baseline resistance values served to normalize subsequent TEER measurements. Cells were treated with either 100 ng/ml vascular endothelial growth factor (VEGF) or 10 μM 8-(4-chlorophenylthio)-2′-O-Me-cAMP (oMe). TEER measurements were acquired every 2 hr, with the exception of oME-treated cells. After 24 hr of treatment, vascular permeability was assessed by replacing the medium in the top chamber with 500 μl of 1 μM 40 kDa fluorescein isothiocyanate-dextran (Sigma-Aldrich) dissolved in endothelium SFM. At 15, 30, 45, and 60 min, 150 μl aliquots were taken from the basolateral chamber and replaced with warm endothelial SFM. Cell permeability was calculated following the *Pe* clearance method (Calabria et al., 2006; Jones and Shusta, 2007).

Statistics

Data are presented as mean ± SEM. Statistical significance was determined by Student's t test (two-tail) between two groups. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.09.005.

AUTHOR CONTRIBUTIONS

X.L. and X.B. conceived, performed, and interpreted most of the experiments and wrote the manuscript; J.L., Y.W., W.D., and K.K.D. assisted in endothelial differentiation and characterization experiments; A.A. performed and interpreted endothelial characterization experiments; E.V.S. provided experimental advice and funding support; S.P.P. conceived experiments, provided experimental advice, wrote the manuscript, and provided funding support.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 EB007534 (S.P.P.) and R21 NS085351 (S.P.P. and E.V.S.) and by funding from Takeda Pharmaceuticals (S.P.P. and E.V.S.). K.K.D. was funded in part by an NIH Chemistry Biology Interface Training Grant (NIGMS T32 GM008505).

Received: March 9, 2014
Revised: September 5, 2014
Accepted: September 8, 2014
Published: October 9, 2014

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