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ABSTRACT

Forced expression of the four transcription factors Oct4, Sox2, c-Myc, and Klf4 is sufficient to confer a pluripotent state upon the murine fibroblast genome, generating induced pluripotent stem (iPS) cells. Although the differentiation potential of these cells is thought to be equivalent to that of embryonic stem (ES) cells, it has not been rigorously determined. In this study, we sought to identify the capacity of iPS cells to differentiate into Flk1-positive progenitors and their mesodermal progeny, including cells of the cardiovascular and hematopoietic lineages. Immunostaining of tissues from iPS cell-derived chimeric mice demonstrated that iPS cells could contribute in vivo to cardiomyocytes, smooth muscle cells, endothelial cells, and hematopoietic cells. To compare the in vitro differentiation potential of murine ES and iPS cells, we either induced embryoid body (EB) formation of each cell type or cultured the cells on collagen type IV (ColIV), an extracellular matrix protein that had been reported to direct murine ES cell differentiation to mesodermal lineages. EB formation and exposure to ColIV both induced iPS cell differentiation into cells that expressed cardiovascular and hematopoietic markers. To determine whether ColIV-differentiated iPS cells contained a progenitor cell with cardiovascular and hematopoietic differentiation potential, Flk1-positive cells were isolated by magnetic cell sorting and exposed to specific differentiation conditions, which induced differentiation into functional cardiomyocytes, smooth muscle cells, endothelial cells, and hematopoietic cells. Our data demonstrate that murine iPS cells, like ES cells, can differentiate into cells of the cardiovascular and hematopoietic lineages and therefore may represent a valuable cell source for applications in regenerative medicine. STEM CELLS *2008;26:1537–1546*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Differentiation of the cells of the inner cell mass into the specialized cells required for the formation of the complex tissues that compose living organisms has traditionally been viewed as a unidirectional process, with cells in the embryo gradually becoming committed to a specific cell type. However, somatic cell nuclear transfer experiments have demonstrated that the oocyte can return the nucleus of an adult somatic cell to a pluripotent embryonic-like state [1, 2]. Although little is known about the factors in the oocyte that induce this process, several recent reports have described the ability of four transcription factors whose retroviral overexpression enabled the induction of a pluripotent, embryonic stem (ES) cell-like state in murine fibroblasts. Simultaneous overexpression of the pluripotency-associated POU domain class 5 transcription factor 1 (Oct4), SRY-box containing gene 2 (Sox2), proto-oncogene c-Myc, and Kruppel-like factor 4 (Klf4) led to the generation of induced pluripotent stem (iPS) cells that exhibited morphology and growth properties similar to those of ES cells and expressed ES cell marker genes [3]. A significant improvement of this in vitro reprogramming approach was then demonstrated by using different strategies for selecting the reprogrammed cells, generating murine iPS cells that functionally resembled ES cells and that were competent for formation of germline chimera [4–6].

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More recently, investigators have created iPS cells from adult human cells using either a combination of Oct4, Sox2, c-Myc, and Klf4, similar to the mouse system [7, 8], or Oct4, Sox2, Nanog homeobox (Nanog), and lin-28 homolog (LIN28) [9]. These human iPS cells have normal karyotypes; express telomerase activity, cell surface markers, and genes that typify human ES cells; and maintain the developmental potential to differentiate into advanced derivatives of all three primary germ layers [7–9]. The successful reprogramming of human somatic cells into a pluripotent ES cell-like state could provide a method to generate customized, patient-specific pluripotent cells for regenerative medicine efforts. However, this assumes that iPS cells possess a differentiation potential similar to that of ES cells, and critical study of the differentiation behavior of iPS cells will be essential for iPS cell-based therapies to become clinical reality.

In this study, we sought to characterize the differentiation potential of murine 2D4 iPS cells [4] and compare it with that of murine D3 ES cells. Immunostaining of tissues from iPS cell-derived chimeric mice [4] demonstrated that iPS cells differentiated in vivo into cardiomyocytes, smooth muscle cells (SMC), endothelial cells (EC), and hematopoietic cells, suggesting that they might also possess this potential in vitro. We used two alternate approaches to initiate differentiation in ES and iPS cells: embryoid body (EB) formation and exposure to collagen type IV (ColIV), an extracellular matrix protein that had been reported to direct ES cell differentiation to mesodermal lineages, including SMC, EC, and hematopoietic cells in both mouse [10–13] and human [14] cultures. Similar to results seen in murine ES cell differentiation experiments, iPS cell-derived EBs, as well as ColIV-differentiated iPS cells, demonstrated upregulation of mesodermal genes associated with cardiac, cardiovascular, and hematopoietic cells. It has been reported that a multipotential cardiovascular progenitor cell can be isolated from ES cell-derived EBs; this progenitor cell is characterized by the expression of kinase insert domain protein receptor (Flk1/Kdr) and the mesodermal marker brachyury and can give rise to cells of the cardiovascular lineage [15]. Likewise, presumed derivatives of this cell, which several groups have described as Flk1-, Kit oncogene (c-kit/CD117)-, NK2 transcription factor-related, locus 5 (Nkx2.5)-positive cardiovascular progenitor cells, were isolated from EBs and determined to be able to differentiate into cardiomyocytes, SMC, and EC [16, 17]. Fluorescence-activated cell sorter (FACS) analysis of EB- and ColIV-differentiated iPS cells revealed the presence of Flk1-positive progenitor cells. When isolated by magnetic cell sorting, these ColIV-differentiated iPS cell-derived Flk1-positive progenitor cells had the potential to differentiate into functional SMC, EC, and spontaneously beating cardiomyocytes. Moreover, when cocultured on green fluorescent protein (GFP)-expressing OP9 (OP9-GFP) stroma supplemented with a hematopoietic cytokine cocktail, the Flk1-positive progenitors differentiated into hematopoietic progenitor cells possessing multilineage myeloerythroid differentiation potential.

Our results suggest that murine iPS cells can be differentiated into cells of the cardiovascular and hematopoietic lineages and possess a differentiation potential equivalent to that of ES cells, at least with respect to these lineages. Furthermore, it is possible to isolate an Flk1-positive progenitor cell from differentiating iPS cells with the potential to differentiate into hematopoietic cells and all three cell types of the cardiovascular lineage. Thus, iPS cells could prove a valuable cell source for applications in regenerative medicine.

Materials and Methods

Murine ES and iPS Cell Cultures

Mouse embryonic fibroblasts (MEF) carrying a GFP-internal ribosome entry site-puro cassette in the endogenous Nanog locus were retrovirally reprogrammed into iPS cells with cDNAs encoding Oct4, Sox2, c-Myc, and Klf4 and were reported previously [4] (supplemental online Fig. 1). Specifically, the iPS cell line 2D4 described previously [4] was used in the present study. D3 ES cells (CRL-1934; American Type Culture Collection, Manassas, VA, http://www.atcc.org) and the Nanog-selected 2D4 iPS cells were maintained in an undifferentiated state on mitomycin-C-treated (M0503; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), primary MEF in leukemia inhibitory factor (LIF) medium (Knockout Dulbecco's modified Eagle's medium [Invitrogen, Carlsbad, CA, http://www.invitrogen.com] supplemented with 15% ES cell-qualified fetal calf serum [ES-FCS; Invitrogen], 0.1 mM β-mercaptoethanol [Sigma-Aldrich], 2 mM glutamine [Invitrogen], 0.1 mM nonessential amino acids [Invitrogen], and 1,000 U/ml recombinant LIF [Chemicon, Temecula, CA, http://www.chemicon.com]) at 37°C, 5% CO_2. All cells were passaged every other day using 0.05% trypsin-EDTA (Invitrogen).

In Vitro Differentiation Assays

For differentiation assays, murine D3 ES and 2D4 iPS cells were either introduced into a dynamic suspension culture system for generating EBs or cultured on collagen type IV-coated plates and flasks. Briefly, for EB formation, cells were dissociated, resuspended in α-minimum essential medium (Invitrogen) supplemented with 10% ES-FCS, 0.1 mM β-mercaptoethanol, 2 mM glutamine, and 0.1 mM nonessential amino acids, without LIF (α-MEM), transferred in 60-mm ultralow-attachment dishes (4×10^5 cells per dish; Corning Life Sciences, Acton, MA, http://www.corning.com/lifesciences), placed onto an orbital rotary shaker (Stovall Belly Button; ATR, Laurel, MD, http://www.atrbiotech.com), and cultured under continuous shaking at approximately 45 rpm for up to 14 days, followed by RNA isolation or FACS analysis. For morphometric analysis, phase-contrast images of ES and iPS cell-derived EBs were acquired every other day during the course of culture, and the diameters of at least 50 EBs from three replicate cultures were measured using a Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, http://www.zeiss.com).

For the ColIV cultures, ES and iPS cells were trypsinized and transferred to collagen type IV-coated plates or flasks (BD Biocoat; BD Biosciences, Bedford, MA, http://www.bdbiosciences.com) as described before [13]. After 4 days either the cells were harvested for RNA isolation and FACS analysis, or they were trypsinized and the Flk1-positive cells were isolated by indirect magnetic cell sorting using a purified rat anti-mouse Flk1 antibody (550549 [1:200]; BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml) and magnetic microbeads (Miltenyi Biotec, Auburn, CA, http://www.miltenyibiotec.com). The Flk1-positive cells were then used for RNA isolation, cocultured on OP9-GFP stromal cells (a kind gift of Juan Carlos Zúñiga-Pflücker), or plated on fibronectin-coated culture slides (BD Biosciences) in either α-MEM for cardiac differentiation, platelet-derived growth factor-BB (PDGF-BB) medium (created by supplementing smooth muscle growth medium [SMGM-2; Lonza, Walkersville, MD, http://www.lonza.com] with 10 ng/ml PDGF-BB medium [R&D Systems Inc., Minneapolis, http://www.rndsystems.com]) for SMC differentiation [11, 13]), or vascular endothelial growth factor (VEGF) medium (created by supplementing endothelial growth medium [EGM-2; Lonza] with 50 ng/ml VEGF [R&D Systems]) (for EC differentiation [11, 13]) for up to 12 days at 37°C, 5% CO_2. To expand the ES and iPS cell-derived SMC or EC, cells were grown to >80% confluence in either SMGM-2 or EGM-2 and passaged into gelatin-coated plates with a 1:2 to 1:3 ratio every 2–3 days. Bright-field images and movies of undifferentiated and differentiated ES and iPS cells, as well as EBs, were acquired using the Zeiss Axiovert 200 microscope.

OP9 Cocultures and Colony-Forming Unit Assays

Flk1-positive ES and iPS cell-derived cells were cultured on OP9-GFP stromal cells in 24-well plates at a concentration of 2×10^4 cells per well in 1 ml of α-minimum essential medium containing 20% fetal bovine serum (HyClone, Logan, UT, http://www.hyclone.com) and 1% penicillin/streptomycin, supplemented with stem cell factor (SCF; 50 ng/ml), interleukin (IL)-3 (5 ng/ml), VEGF (10 ng/ml), granulocyte colony-stimulating factor (10 ng/ml), and human erythropoietin (EPO) (20 ng/ml) for up to 7 days (all from Peprotech, Rocky Hill, NJ, http://www.peprotech.com). Half of the medium with cytokines was replaced every 2 days. To determine the myeloerythroid potential of the Flk1-positive progenitors, cells were harvested after 5 days of OP9-GFP coculture and plated in 1.5 ml of methylcellulose that contained SCF, IL-6, IL-3, and EPO (MethoCult 3434; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). In addition, thrombopoietin (5 ng/ml) was added to the methylcellulose cultures. Colonies were scored 5 days later.

Immunochemistry

Immunofluorescence staining of cells and frozen tissue sections of newborn 2D4 iPS cell-derived chimeric mice [4] was performed as described before [13]. Detailed information on the primary and secondary antibodies that have been used in this study can be found in the supplemental online data. To visualize the F-actin cytoskeleton, cells were stained using Alexa Fluor 594 phalloidin (1:40; Molecular Probes, Eugene, OR, http://probes.invitrogen.com). For counterstaining of cell nuclei, 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) was added to the final phosphate-buffered saline (PBS) washing. Staining without primary antibodies served as controls. Fluorescence images were acquired using a confocal TCS SP2 AOBS laser scanning microscope system (Leica Microsystems Inc., Exton, PA, http://www.leica.com) with ×40 (1.3 numerical aperture [NA]) and ×63 (1.4 NA) oil-immersion objectives. Images were processed with Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, http://www.adobe.com).

Fluorescence-Activated Cell Sorter Analysis

Cells were pelleted by centrifugation, washed in PBS, and stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated monoclonal rat anti-mouse antibodies against Flk1 (PE or APC), CD31 (FITC), CD144 (PE), and CD34 (PE, APC) (all 1:200; BD Pharmingen and eBioscience Inc., San Diego, http://www.ebioscience.com). For detection of von Willebrand factor (vWF), cells were incubated with a rabbit anti-vWF antibody (vWF; ab6994 [1:400]; Abcam, Cambridge, MA, http://www.abcam.com), followed by incubation with an Alexa Fluor 647-conjugated goat-anti-rabbit antibody (1:250; Molecular Probes). OP9-differentiated Flk1-progenitor cells were stained with PE-, cyanine 5 (Cy5)-, and APC-conjugated monoclonal rat anti-mouse antibodies against c-kit (PE), CD41 (Cy5), and CD45 (APC) (all 1:200; BD Pharmingen). Nonspecific fluorochrome- and isotype-matched IgGs (BD Pharmingen) served as controls. Staining with 7-aminoactinomycin D (559925; BD Pharmingen) was performed to exclude dead cells according to the manufacturer's instructions. OP9-GFP stromal cells were omitted from the analysis on the basis of their GFP expression and side scatter exclusion. All analyses were performed using a BD LSR II flow cytometer (BD Biosciences). FCS files were exported and analyzed using FlowJo 8.6.3 software (Tree Star, Ashland, OR, http://www.treestar.com).

RNA Analysis

Total RNA was extracted from mouse hearts (positive control), as well as from harvested EBs and cells, and semiquantitative polymerase chain reaction (PCR) was performed as previously described [13]. The sequences of each specific primer set, including their annealing temperatures and cycles, are listed in supplemental online Table 1. The aortic smooth muscle actin (Acta2), caldesmon 1 (Cald1), calponin 2 (Cnn2), platelet/endothelial cell adhesion molecule 1 (Pecam1/CD31), cadherin 5 (Cdh5/CD144/VE-Cadherin), and von Willebrand factor homolog (vWF) reverse transcription (RT)-PCR primer sets were obtained from SuperArray Bioscience Corporation (Frederick, MD, http://www.superarray.com) and used with the ReactionReady HotStart PCR master mix (including an internal normalizer) following the manufacturer's instructions. The resultant PCR products were resolved on 2% agarose gels stained with ethidium bromide.

Intracellular Calcium (Ca^{2+}) Measurements

We used the calcium indicator fluo-3 acetoxymethyl (AM) to measure intracellular Ca^{2+} transients in iPS cell-derived cardiomyocytes as described before [18]. Briefly, iPS cell-derived cardiomyocytes were cultured on gelatin-coated 35-mm MatTek glass-bottomed dishes (MatTek Corp., Ashland, MA, http://www.glass-bottom-dishes.com/). Cells were incubated for 20 minutes in 30 μmol/l fluo-3/AM and 0.02% (wt/wt) Pluronic F-127 (both from Molecular Probes) in modified Tyrode's solution containing (in mmol/l): 136 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 10 HEPES, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 0.5 probenecid (pH 7.4). Cells were then washed three times for 5 minutes each in indicator-free Tyrode's solution, which was also the standard bath solution for Ca^{2+} imaging. The fluo-3/AM-loaded cells were paced externally using a Grass S9 stimulator (Grass Technologies, West Warwick, RI, http://www.grasstechnologies.com) and bipolar platinum electrodes immersed in the bath. Ca^{2+} transients of fluo-3/AM-loaded cells were measured by rapid line-scan confocal microscopy using a Zeiss Pascal 5 laser scanning confocal microscope fitted with a ×40 (1.2 NA) water immersion objective (Carl Zeiss MicroImaging). Line scans were performed at 2 ms per line, with an excitation wavelength of 488 nm and an emission wavelength of >505 nm. Background fluorescence of unloaded cells was negligible. Image analysis was performed using custom software programmed in IDL 6.1 (ITT Visual Information Solutions, Boulder, CO, http://www.ittvis.com).

Smooth Muscle and Endothelial Cell Functionality Assays

To assess cell functionality, SMC contractility, uptake of acetylated low-density lipoprotein (acLDL) in EC, and Matrigel (BD Biosciences) EC in vitro tube formation were determined as described previously [12, 19, 20]. A detailed description of the assays can be found in the supplemental online data. To serve as controls, primary murine vascular smooth muscle cells (mSMC), isolated from thoracic aortas of C57BL/6 mice, and human umbilical vein endothelial cells (HUVEC), obtained from Lonza, were cultured in either SMGM-2 or EGM-2 (Lonza).

Statistical Analysis

All data are presented as mean ± SEM. Statistical significance was assessed by Student's *t* test or analysis of variance with Tukey's multiple comparison test. p values <.05 were defined as statistically significant.

Results

iPS Cells Contribute In Vivo to Cells of the Cardiovascular and Hematopoietic Lineages

It has previously been reported that murine 2D4 iPS cells carrying a GFP transgene in the ubiquitously expressed R26 locus (supplemental online Fig. 1), upon blastocyst injection, give rise to viable high-degree newborn chimeras with contribution to multiple tissues, including the heart [4], although the exact cardiovascular cell types were not identified. To determine whether GFP-labeled 2D4 iPS cells contribute in vivo to all cell types of the cardiovascular lineage, we surveyed sections from the 2D4 iPS cell-derived chimeric mice [4] for expression of

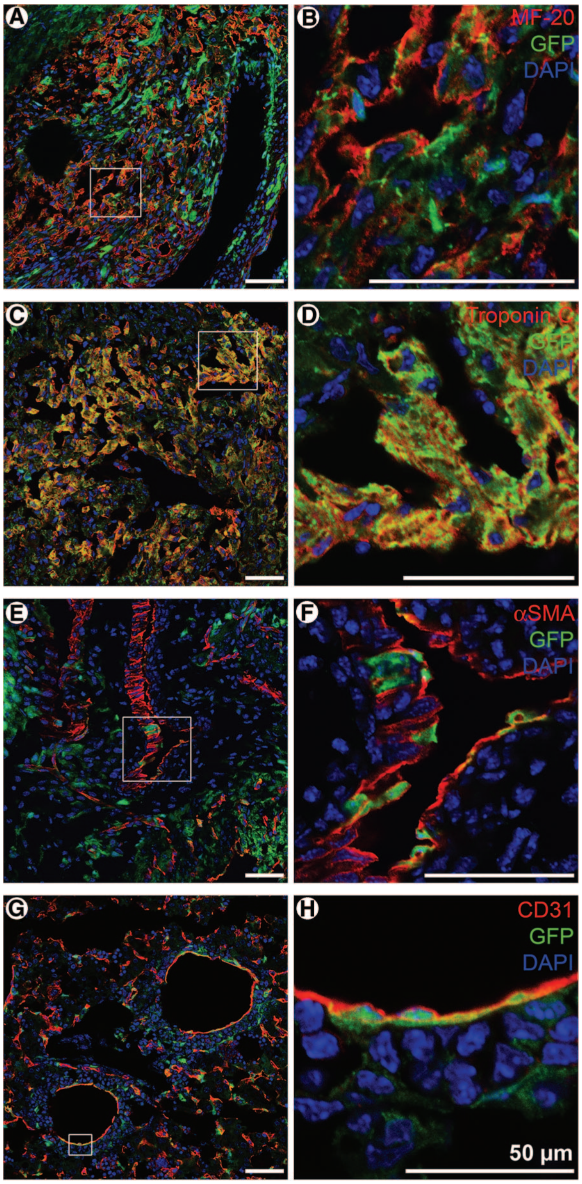

Figure 1. In vivo differentiation potential of murine induced pluripotent stem (iPS) cells. Immunohistochemical double staining of heart sections of newborn 2D4 iPS cell-derived chimera mice showed that GFP-expressing iPS cells (green) contribute to cells of the cardiovascular lineage, including cardiomyocytes (MF-20 **[A, B]**, red; troponin C **[C, D]**, red), smooth muscle cells (αSMA **[E, F]**, red), and endothelial cells (CD31 **[G, H]**, red). DAPI staining was performed to show cell nuclei (blue). Scale bars = 50 μm. Abbreviations: αSMA, α-smooth muscle actin; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; MF-20, sarcomeric myosin.

known cardiac (sarcomeric myosin heavy chain [MF-20] and troponin C), smooth muscle (α-smooth muscle actin [αSMA]), and endothelial (CD31) markers. Immunofluorescent staining demonstrated that GFP-expressing 2D4 iPS cells had the capacity to differentiate in vivo into mature cardiomyocytes (Fig. 1A–1D), vascular smooth muscle cells (Fig. 1E, 1F), and endothelial cells (Fig. 1G, 1H; supplemental online Fig. 2). In addition, in the previous study it was shown that between 18% and 28% of hematopoietic cells isolated from the spleen and thymus of newborn 2D4 iPS cell-derived chimeric mice were derived from iPS cells [4].

ES and iPS Cell-Derived EBs Express Markers of Early Mesodermal, Cardiovascular, and Hematopoietic Cells

Murine ES cells have been shown to be able to differentiate into cell lineages of all three embryonic germ layers (mesoderm, endoderm, and ectoderm) when allowed to aggregate in suspension and form three-dimensional (3D) colonies known as embryoid bodies [21]. To characterize the differentiation potential of iPS cells, EBs were formed from both D3 ES and 2D4 iPS cells. ES and iPS cells exhibited comparable EB growth characteristics with similar 3D morphology, although 2D4 iPS cell-derived EBs were initially (days 2 and 4 of EB culture) smaller in size compared with D3 ES cell-derived EBs (Fig. 2A; supplemental online Table 2). Gene expression profiling of developing ES and iPS cell-derived EBs demonstrated downregulated expression of markers of undifferentiated ES and iPS cells, including Oct4, Nanog, and GFP (undifferentiated iPS cells only; Fig. 2B), accompanied by an upregulation of the ectoderm marker nestin (Nes) and the endoderm marker α-fetoprotein (Afp), as well as mesodermal genes, including brachyury; T-cell acute lymphocytic leukemia 1 (Scl/Tal1); Flk1; FMS-like tyrosine kinase 1 (Flt1); endothelial-specific receptor tyrosine kinase (Tie2); GATA-binding protein 1 (Gata1); c-kit, lymphocyte antigen 6 complex, locus A (Sca1/Ly6a); ISL1 transcription factor; and LIM/homeodomain (Isl1), all of which have been reported to be mesodermal progenitor cell markers [17, 22] (Fig. 2C). FACS analysis demonstrated the presence of a Flk1-positive cell population in both ES and iPS cell-derived EBs, with highest expression levels between day 2 and day 4 (day 2, 3.06% $\pm$ 0.21% and 3.21% $\pm$ 0.49%; day 4, 1.95% $\pm$ 0.08% and 2.77% $\pm$ 0.18%) (Fig. 2D).

To further characterize the heterogeneous cell phenotypes present in ES and iPS cell-derived EBs, we examined total mRNA for cardiac, smooth muscle, endothelial, and hematopoietic markers using semiquantitative RT-PCR (supplemental online Fig. 3). There was comparable expression of cardiomyocyte-associated transcription factors (Nkx2.5, GATA-binding protein 4 [Gata4], myocyte enhancer factor 2C [Mef2c], and T-box 5 [Tbx5]) and genes (α-myosin heavy chain [αMHC]; βMHC; myosin, light polypeptide 7, regulatory [Mlc2a]; myosin, light polypeptide 2, regulatory, cardiac, slow [Mlc2v]; and natriuretic peptide precursor type A [Nppa]) in D3 ES and 2D4 iPS cell-derived EBs during differentiation (supplemental online Fig. 3A). Similar to ES cell-derived EBs, we found spontaneously beating iPS cell-derived EBs starting as early as day 10 (supplemental online Videos 1 [ES cell-derived EB] and 2 [iPS cell-derived EB]).

Likewise, SMC- and EC-associated gene expression patterns were seen in both ES and iPS cell-derived EBs (supplemental online Fig. 3B, 3C). Expression of Acta2, myocardin-related transcription factor a (Mrtf-a), myocardin-related transcription factor b (Mrtf-b), and Cald1 increased progressively in both ES and iPS cell-derived EBs (supplemental online Fig. 3B). Endothelial lineage-related genes, including Pecam1 (CD31), Cdh5 (CD144/VE-Cadherin), ephrin-B2 (Ephb2) (expressed predominantly on arterial EC [23]), and ephrin-B4 (Ephb4) (expressed predominantly on venous EC [23]), were comparably expressed in ES and iPS cell-derived EBs (supplemental online Fig. 3C). Similarly, the expression pattern of hematopoietic-associated markers, including homeobox protein B4 (Hoxb4); runt-related transcription factor 1 (Runx1); Notch gene homolog 1 (Notch1); transcription factor PU.1 (PU.1); core-binding factor, runt domain, α subunit 2, translocated to 3 (Eto2/ Cbfa2t3); and LIM domain only 2 (Lmo2), was indistinguishable between ES and iPS cell-derived EBs (supplemental online Fig. 3D). Taken together, D3 ES and 2D4 iPS cells

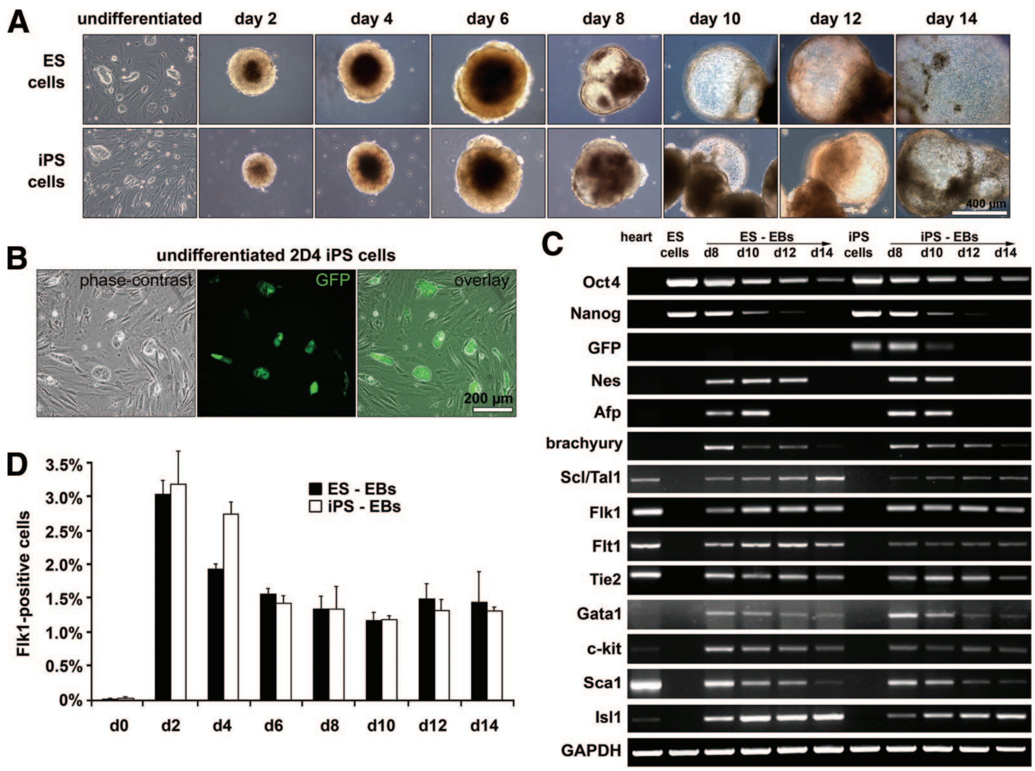

Figure 2. EB formation and differentiation profiles of murine ES and iPS cells. **(A):** Phase-contrast images show comparable growth characteristics of D3 ES and 2D4 iPS cell-derived EBs. Scale bar = 400 μm. **(B):** Nanog promoter-driven GFP expression in undifferentiated 2D4 iPS cells grown on mouse embryonic fibroblasts. Scale bar = 200 μm. **(C):** Reverse transcription-polymerase chain reaction analysis showing the downregulation of markers of undifferentiated ES and iPS cells, including Oct4, Nanog, and GFP, as well as upregulation of ectodermal, endodermal, and mesodermal genes. **(D):** Fluorescence-activated cell sorter analysis revealed the presence of Flk1-positive progenitor cells within ES and iPS cell-derived EB cultures. Abbreviations: d, day; ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; iPS, induced pluripotent stem.

exhibited a similar EB-forming ability and a comparable EB-induced cardiovascular and hematopoietic differentiation potential as measured by cell type-associated gene expression.

ColIV Induces Cardiovascular and Hematopoietic Differentiation of ES and iPS Cells

It has recently been demonstrated by us and others that culturing ES cells on collagen type IV is a simple in vitro system to differentiate these cells toward the cardiovascular and hematopoietic lineages [10–13]. To determine whether iPS cells have a similar differentiation potential, we cultured undifferentiated iPS cells for 4 days on ColIV. Similar to results seen in ColIV-differentiated ES cell cultures, ColIV-exposed iPS cells expressed mesodermal progenitor cell markers (Fig. 3A). FACS analysis revealed the presence of Flk1-expressing progenitor cells in both ColIV-differentiated ES cells and ColIV-differentiated iPS cells (2.2% $\pm$ 0.7% and 10.4% $\pm$ 1.4%). Semiquantitative RT-PCR analysis confirmed the presence of cardiac (Fig. 3B), vascular smooth muscle, and endothelial (Fig. 3C, 3D) genes, as well as hematopoietic-associated genes (Fig. 3E), within the heterogeneous population of ColIV-differentiated ES and iPS cells.

iPS Cell-Derived Flk1-Positive Progenitors Differentiate into Functional Cardiomyocytes, SMC, EC, and Hematopoietic Cells

To determine whether ColIV-differentiated iPS cell-derived Flk1-positive progenitor cells had the capacity to differentiate into cardiovascular cells, we isolated the Flk1-expressing cells and cultured them in media for cardiac, smooth muscle, and endothelial differentiation. Gene expression analysis of isolated undifferentiated Flk1-positive cells revealed the presence of genes associated with cardiovascular progenitor cells, including brachyury, Flk1, c-kit, and Nkx2.5, whereas no markers of differentiated cardiovascular cells were expressed (supplemental online Fig. 4) [15–17, 24]. However, when cultured in differentiation-promoting conditions, Flk1-positive cells differentiated into mature cardiovascular cells, as shown by the increased expression of cardiac, SMC, and EC markers, accompanied by the concomitant decrease in expression of stem and progenitor cell genes (supplemental online Fig. 4).

Flk1-positive cells differentiated in conditions to promote cardiac differentiation developed spontaneously beating cell clusters after 10–12 days of culture (supplemental online Video 3). Immunocytochemical staining of the spontaneously beating areas revealed the presence of cardiomyocytes with typical cross-striation that expressed cardiac markers, including sarcomeric myosin (MF-20) (Fig. 4Aa, 4Ab) and troponin C (Fig. 4Ac, 4Ad). Furthermore, iPS cell-derived cardiomyocytes loaded with the calcium indicator fluo-3/AM could be externally paced at frequencies of 0.5 Hz (Fig. 4Ba) and 1 Hz (Fig. 4Bb), generating characteristic Ca^{2+} transients.

Similar to ColIV-differentiated ES cell-derived Flk1-positive progenitors [11], the majority of the iPS cell-derived Flk1-expressing cells, cultured in PDGF-BB medium, differentiated into cells that expressed αSMA, calponin, and caldesmon (Fig. 5A). To assess their functional capacity, mSMC and iPS cell-derived SMC (iPS-SMC) were exposed to carbachol for 30 minutes. The two cell types showed similar contraction patterns

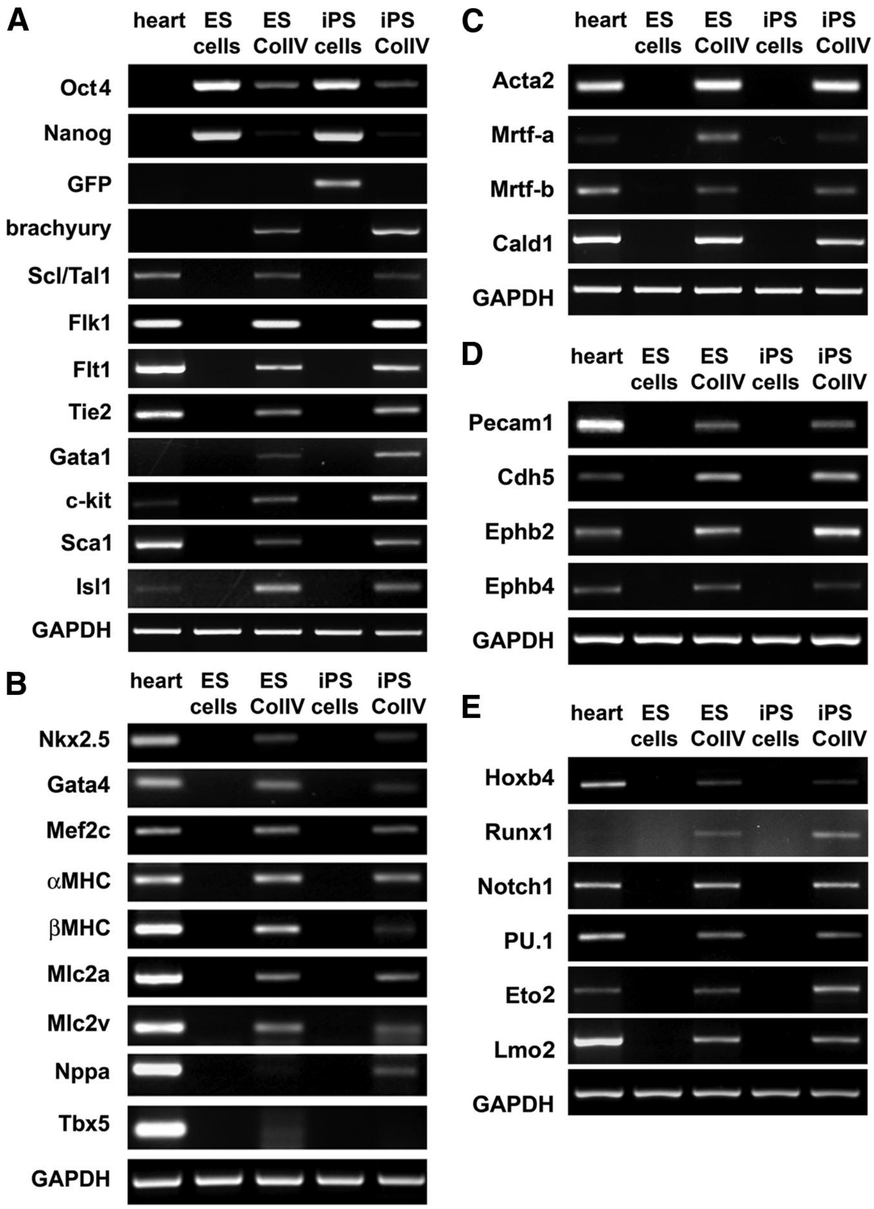

Figure 3. ColIV induces expression of early mesodermal, cardiovascular, and hematopoietic genes. Semiquantitative reverse transcription-polymerase chain reaction showed that ColIV-differentiated ES and iPS cells express markers of mesodermal progenitor cells **(A)**, cardiomyocytes **(B)**, and cells of the smooth muscle **(C)**, endothelial **(D)**, and hematopoietic **(E)** lineages. Abbreviations: ColIV, collagen type IV; ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; iPS, induced pluripotent stem.

(cell contraction, 66.9% ± 3.4% [mSMC] and 71.1% ± 2.4% [iPS-SMC]; $p = .16$) (Fig. 5B; supplemental online Fig. 5).

When treated with VEGF, ES and iPS cell-derived Flk1-positive progenitors differentiated into cells that expressed EC-associated markers, including CD31, CD144, Flk1, CD34, vWF, and endothelial nitric oxide synthase (Fig. 6A, 6B), and exhibited a comparable EC-typical cobblestone morphology (supplemental online Fig. 6). FACS analysis revealed that ES cell-derived EC (ES-EC) and iPS cell-derived EC (iPS-EC) exhibited similar EC marker expression; however, compared with mature human EC (HUVEC), both ES-EC and iPS-EC displayed slightly different cell surface marker expression profiles (HUVEC vs. ES-EC and iPS-EC: CD31, 99.8% ± 1.2% vs. 93.0% ± 3.5% and 93.3% ± 4.4%; CD144, 99.9% ± 2.1% vs. 78.7% ± 5.5% and 78.3% ± 5.1%; Flk1, 38.6% ± 3.2% vs. 80.1% ± 4.9% and 79.3% ± 4.6%; CD34, 10.6% ± 4.9% vs. 31.1% ± 5.1% and 29.9% ± 3.1%; vWF, 72.3% ± 1.8% vs. 54.0% ± 2.1% and 55.8% ± 2.8%) (Fig. 6A). ES-EC and iPS-EC but not MEF showed the ability to take up acetylated low-density lipoprotein. However, compared with HUVEC, ES-EC and iPS-EC incorporated lower amounts of acLDL (Fig. 6C; supplemental online Fig. 6); a phenomenon that had previously been reported in ES-EC [20]. Although ES-EC and iPS-EC exhibited different acLDL uptakes compared with mature human EC (HUVEC), they showed similar in vitro tube forming behavior when seeded on Matrigel for 24 hours, suggesting comparable angiogenic potentials (Fig. 6D).

To define the hematopoietic differentiation potential, iPS cell-derived Flk1-progenitor cells were cocultured on OP9-GFP stromal cells in media supplemented with hematopoietic cytokines. After 5–7 days of coculture, cells were harvested and analyzed for cell surface antigen expression by flow cytometry. Cells expressing both c-kit and CD41, which identifies nascent hematopoietic stem and progenitor cells, were seen (Fig. 7A) [25]. A subset of the cells expressed the pan-hematopoietic marker CD45 (Fig. 7A). After OP9-GFP coculture, cells were also plated in methylcellulose to determine the myeloerythroid differentiation potential. iPS and ES cell-derived Flk1-progenitor cells had similar frequencies of clonogenic progenitors (30 total colony-forming units [CFUs] per 20,000 ES cell-derived

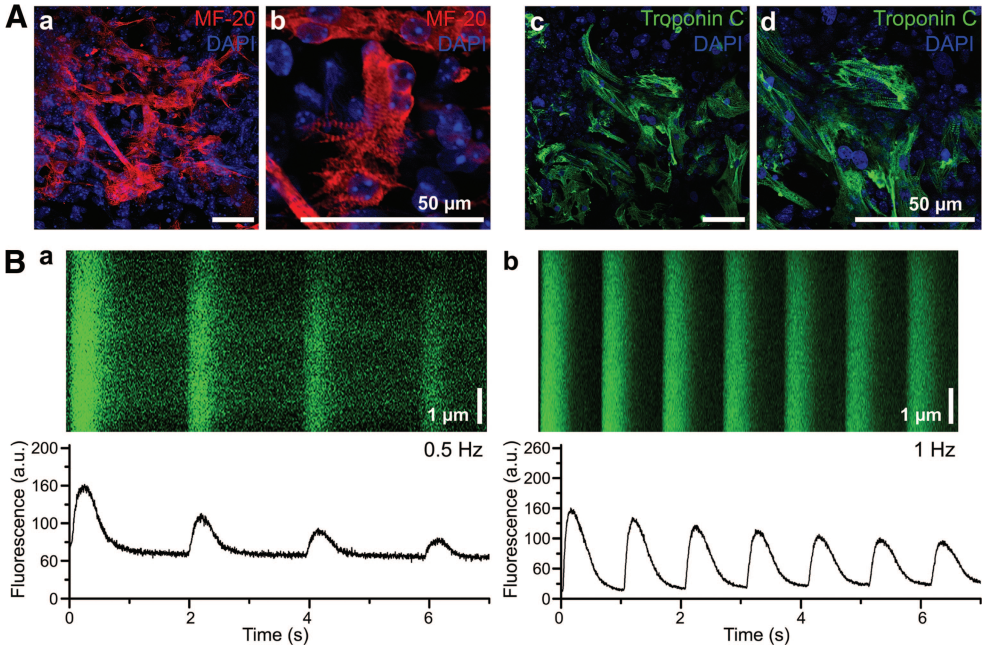

Figure 4. Induced pluripotent stem (iPS) cell-derived Flk1-positive progenitor cells differentiate into functional cardiomyocytes. **(A):** iPS cell-derived collagen type IV-differentiated Flk1-positive progenitor cells differentiate into MF-20-expressing (red) **(Aa, Ab)** and troponin C-expressing (green) **(Ac, Ad)** cardiomyocytes. Cell nuclei were stained with DAPI (blue). Scale bars = 50 μm. **(B):** Line-scan images (upper panels) and spatially averaged fluorescence transients (lower panels) from a single iPS cell-derived cardiomyocyte loaded with the Ca^{2+} indicator fluo-3/AM. Transients were evoked by pacing with platinum electrodes at a frequency of 0.5 Hz **(Ba)** and 1 Hz **(Bb)**. The spatially synchronous onset of each transient and the rapid upstroke of spatially averaged fluorescence traces indicate electrically triggered Ca^{2+} release. Fluorescence intensities are displayed in a.u. Scale bars = 1 μm. Abbreviations: a.u., arbitrary units; DAPI, 4′,6-diamidino-2-phenylindole; MF-20, sarcomeric myosin; s, seconds.

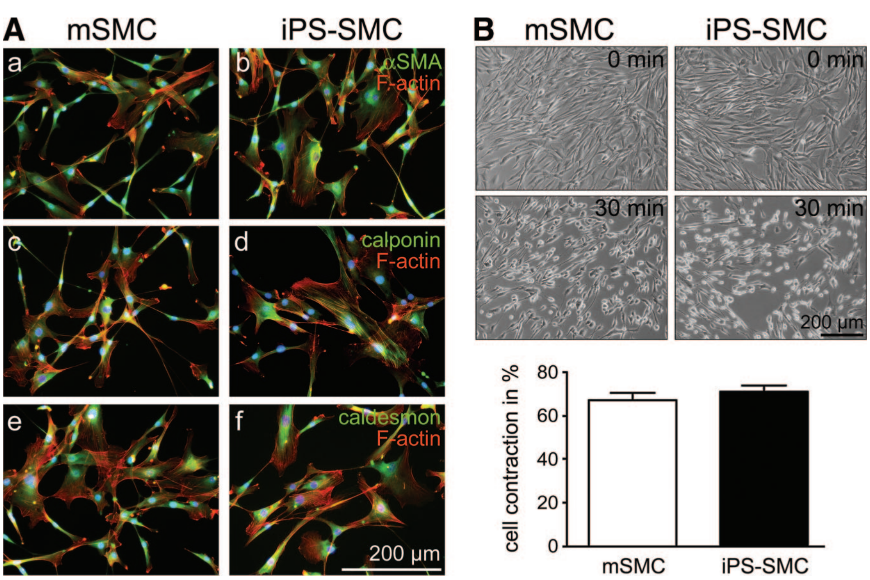

Figure 5. In vitro smooth muscle cell differentiation potential of induced pluripotent stem cell-derived Flk1-positive progenitors. **(A):** Immunocytochemical staining shows comparable expression of αSMA (green) **(Aa, Ab)**, calponin (green) **(Ac, Ad)**, and caldesmon (green) **(Ae, Af)** in mSMC and iPS-SMC. F-actin staining visualized the cell cytoskeleton (red). Cell nuclei are shown with 4′,6-diamidino-2-phenylindole (blue). Scale bar = 200 μm. **(B):** mSMC, as well as iPS cell-derived SMC, contract after 30 min of exposure to 10^{-5} M carbachol. Scale bar = 200 μm. Abbreviations: αSMA, α-smooth muscle actin; iPS-SMC, induced pluripotent stem-derived smooth muscle cells; min, minutes; mSMC, murine vascular smooth muscle cells.

Flk1-positive cells vs. 35 total CFUs per 20,000 iPS cell-derived Flk1-positive cells) and demonstrated an equivalent differentiation potential into erythroid, myeloid, and mixed colonies (Fig. 7B, 7C).

Discussion

The potential of ES cells, which have the capacity to differentiate into all somatic cell types, has attracted much interest in the field of regenerative medicine and has been a topic of intense research in recent years [26]. However, major obstacles to a successful clinical use of ES cell derivatives for tissue repair exist, including the immunological intolerance to allogeneic cells that can lead to rejection of mismatched cellular grafts and ethical concerns surrounding their use. In this study, we determined the cardiovascular and hematopoietic differentiation potential of murine iPS cells. Overall, only minor differences were seen in the growth and differentiation potential of 2D4 iPS cells compared with D3 ES cells. We observed that 2D4 iPS cells reproducibly formed spherical EBs with growth characteristics and gene expression profiles similar to those of D3 ES cell-derived EBs.

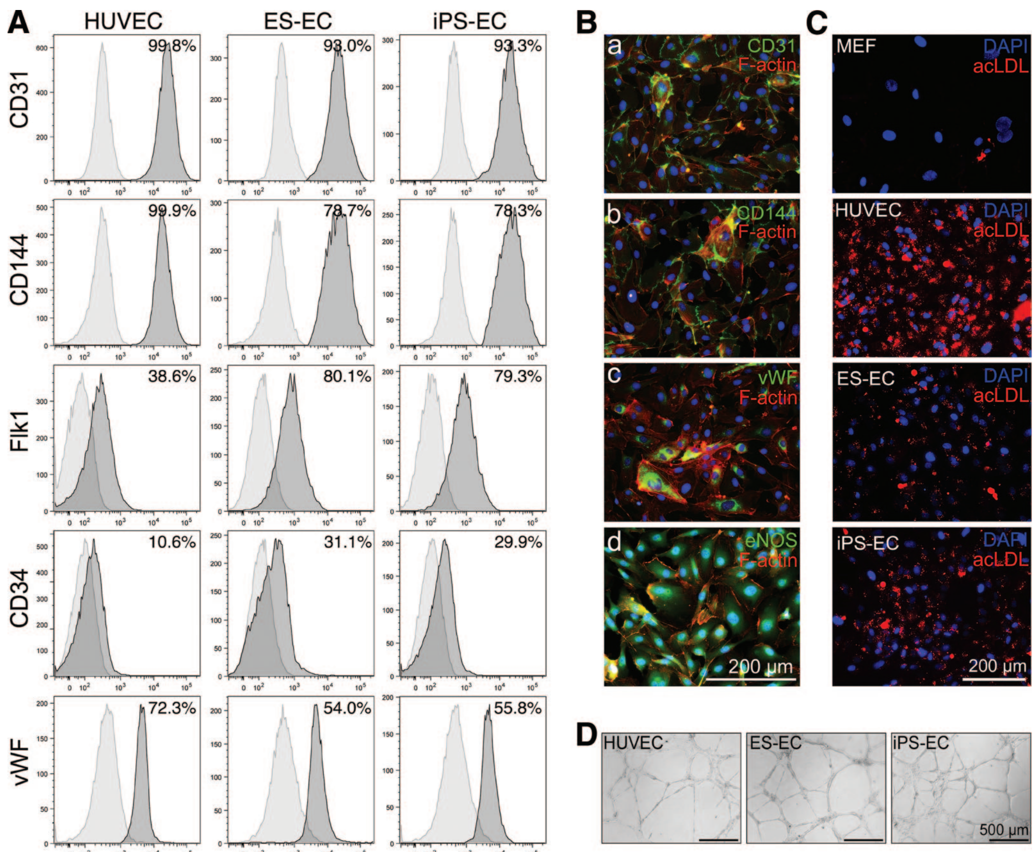

Figure 6. Embryonic stem (ES) and induced pluripotent stem (iPS) cell-derived Flk1-positive progenitor cells differentiate into functional endothelial cells (EC). **(A):** Phenotypic analysis of HUVEC, ES-EC, and iPS-EC. **(B):** Immunocytochemistry showed expression of EC-associated markers, including CD31 **(Ba)**, CD144 **(Bb)**, vWF **(Bc)**, and eNOS **(Bd)** (all green) in iPS-EC. F-actin stained the cell cytoskeleton (red). Cell nuclei are shown with DAPI (blue). Scale bar = 200 μm. **(C):** In contrast to mouse embryonic fibroblasts, HUVEC, ES-EC, and iPS-EC had the ability to uptake acLDL (red). Cell nuclei were stained with DAPI (blue). Scale bar = 200 μm. **(D):** HUVEC, ES-EC, and iPS-EC formed comparable capillary-like structures when cultured on Matrigel for 24 hours. Scale bars = 500 μm. Abbreviations: acLDL, acetylated low-density lipoprotein; DAPI, 4′,6-diamidino-2-phenylindole; eNOS, endothelial nitric oxide synthase; ES-EC, embryonic stem cell-derived endothelial cells; HUVEC, human umbilical vein endothelial cells; iPS-EC, induced pluripotent stem cell-derived endothelial cells; vWF, von Willebrand factor antibody.

When exposed to ColIV, ES and iPS cells differentiated into cells that showed expression of genes associated with early mesodermal, cardiovascular, and hematopoietic cells.

A cell capable of differentiating into all cardiovascular cell types has a theoretical advantage for more complete tissue regeneration over transplanting cardiomyocytes alone, as has been demonstrated for ES cell derivatives [26]. Conversely, partially differentiated cardiovascular progenitor cells, such as the Flk1-positive cells described here, will likely reduce the tumor formation seen when transplanting undifferentiated ES cells into the heart [27]. To this end, we were able to identify Flk1-positive progenitor cells in both iPS cell-derived EBs and ColIV-differentiated iPS cells, most likely representing a population of multipotent mesodermal progenitor cells [15–17, 28]. To confirm that those Flk1-expressing cells were capable of generating all cardiovascular cell types, we isolated ColIV-differentiated Flk1-positive cells and exposed them to cardiac, smooth muscle, and endothelial cell-specific differentiation conditions. This resulted in the production of spontaneously beating cell clusters and cells expressing cardiac markers as well as hallmark morphological characteristics of mature cardiomyocytes, including the typical cross-striation and generation of Ca^{2+} transients. Gene expression analysis, immunocytochemistry, contractility, and in vitro tube formation assays, as well as acLDL uptake tests, further revealed the successful differentiation of iPS cell-derived Flk1-progenitor cells into functional SMC and EC, findings that were similar to those previously reported for murine ES cells [10–12]. Although iPS cells contributed to mature cardiovascular cells in vivo as well, it will be important to determine in future experiments whether transplanted iPS cells can also integrate and differentiate into adult myocardium. In addition, coculture of the Flk1-positive cells with OP9-GFP stromal cells in hematopoietic cytokine-containing culture medium conferred differentiation into hematopoietic progenitor cells that expressed c-kit, CD41, and the pan-hematopoietic marker CD45 [25]. Furthermore, these ES and iPS cell-derived hematopoietic progenitors demonstrated a multilineage myeloerythroid differentiation potential.

Although ColIV-differentiated iPS cell-derived Flk1-positive progenitor cells had properties comparable to those of the ES cell-derived progenitors previously described, differences did exist [11, 12, 15–17]. Flk1-positive progenitor cells isolated from the ColIV-exposed cultures also possessed hematopoietic differentiation potential when cultured on OP9 stromal cells or in methylcellulose. In addition, Flk1-positive cells were more frequent in ColIV-differentiated iPS cell cultures compared with ES cell cultures, but whether this is a general property of iPS cells or is specific to the 2D4 iPS cell line will require further study and comparison of multiple lines. Nonetheless, the first successful therapeutic application of murine iPS cells to correct a mouse model of sickle cell anemia has been reported [29]. However, this required transducing the iPS cells with HoxB4 to

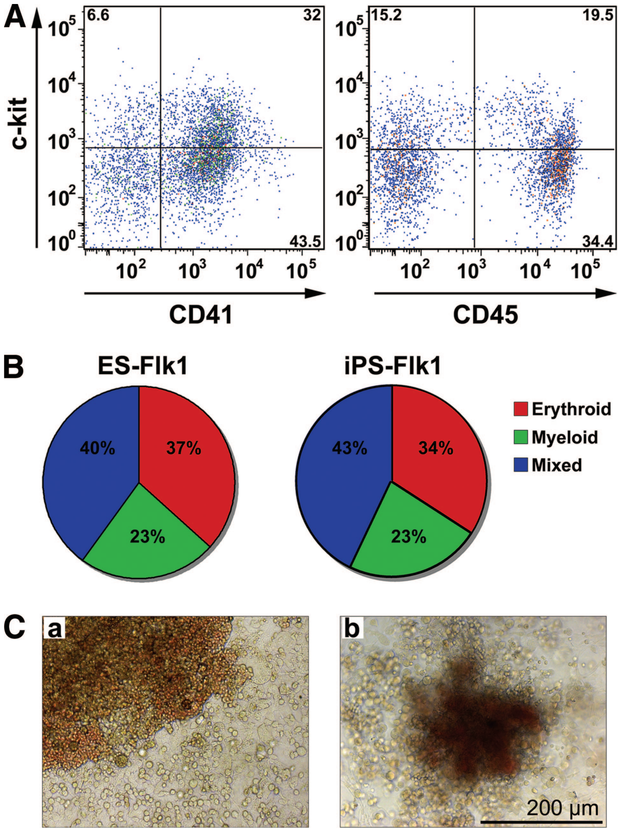

Figure 7. Hematopoietic differentiation potential of induced pluripotent stem (iPS) cell-derived Flk1-positive progenitor cells. **(A):** Fluorescence-activated cell sorter analysis of c-kit and CD41, as well as c-kit and CD45, on iPS cell-derived Flk1-progenitor cells grown on OP9 stroma for 5 days. **(B):** Pie charts depicting the variety of different hematopoietic colonies produced from both ES-Flk1 and iPS-Flk1 in methylcellulose. **(C):** Representative images of mixed colonies derived from ES-Flk1 **(Ca)** and iPS-Flk1 **(Cb)**. Scale bar = 200 μm. Abbreviations: ES-Flk1, embryonic stem cell-derived Flk1-positive progenitors; iPS-Flk1, induced pluripotent stem cell-derived Flk1-positive progenitors.

generate engraftable hematopoietic stem cells. Thus, an autologous hematopoietic progenitor cell would have significant therapeutic advantage if these ColIV-differentiated iPS cell-derived Flk1-positive progenitor cells could produce engraftable hematopoietic stem cells, which we are currently investigating.

Despite the similarities in growth and differentiation of ES and iPS cells, some issues remain with regard to the clinical translation of the population of iPS cell-derived Flk1-positive progenitor cells. Whether there is one cell within the Flk1-positive progenitor cell population that is capable of differentiating into both cardiovascular and hematopoietic lineages or whether these lineages differentiate from different subpopulations as has been suggested in the EB system will need to be determined [15]. Most importantly, whether a similar population can be isolated from human iPS cells is unknown at this time. Regardless of these uncertainties, direct reprogramming of somatic cells to generate patient-matched pluripotent stem cells has the potential to address many of the current limitations and could revolutionize the treatment of many diseases. The development of efficient, reliable, and easily reproducible differentiation protocols for generating human iPS cell-derived cardiovascular and hematopoietic progenitor cells will facilitate the development of patient-tailored cardiovascular and hematopoietic regenerative therapies.

Summary

Reprogrammed murine fibroblasts exhibit growth and differentiation characteristics comparable to those of murine ES cells. Given the appropriate extracellular signals, murine iPS cells differentiate with high efficiency into multipotent mesodermal progenitor cells that possess the potential to differentiate into functional cells of the cardiovascular and hematopoietic lineages. Ease of generation and lack of immunologic and ethical restrictions will likely make iPS cells a highly valuable cell source for applications in regenerative medicine.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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