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Hemangioblastic Derivatives from Human Induced Pluripotent Stem Cells Exhibit Limited Expansion and Early Senescence

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ABSTRACT

Human induced pluripotent stem cells (hiPSC) have been shown to differentiate into a variety of replacement cell types. Detailed evaluation and comparison to their human embryonic stem cell (hESC) counterparts is critical for assessment of their therapeutic potential. Using established methods, we demonstrate here that hiPSCs are capable of generating hemangioblasts/blast cells (BCs), endothelial cells and hematopoietic cells with phenotypic and morphological characteristics similar to those derived from hESCs, but with a dramatic decreased efficiency. Furthermore, in distinct contrast to the hESC derivatives, functional differences were observed in BCs derived from hiPSCs, including significantly increased apoptosis, severely limited growth and expansion capability, as well as a substantially decreased hematopoietic colony forming capability. After further differentiation into erythroid cells, >1000-fold difference in expansion capability was observed in hiPSC-BCs versus hESC-BCs. Although endothelial cells derived from hiPSCs were capable of taking up acetylated low-density-lipoprotein and forming capillary-vascular like structures on Matrigel, these cells also demonstrated early cellular senescence (the majority of endothelial cells senesced after one passage). Similarly, retinal pigmented epithelium cells derived from hiPSCs began senescing in the first passage. Before clinical application, it will be necessary to determine the cause and extent of such abnormalities, and whether they also occur in hiPSCs generated using different reprogramming methods.

INTRODUCTION

There is considerable excitement that human induced pluripotent stem cells (hiPSCs) can serve as a potentially safe and embryo-free source of patient-specific cells for regenerative medicine. Since 2007, when hiPSC lines were first generated by Yu et al[1] and Takahashi et al[2], a variety of methods have been reported for reprogramming somatic cells to

pluripotency[3-7]. The ability of hiPSCs to differentiate into derivatives of all three embryonic germ layers is well established, and rapid progress is being made towards controlled *in vitro* differentiation of hiPSCs into specific cell types, precursors as well as differentiated progenies representing various tissues, such as heart [8], pancreas [9], liver [10], eye including retinal pigment epithelium cells(RPE) [11-14], neuronal [15,16], and endothelial and hematopoietic lineages [17-20]. Although these studies clearly suggest a similar differentiation potential between hiPSCs and human embryonic stem cells (hESC), it is unclear whether they can be expanded into homogeneous cell populations suitable for use in drug discovery and clinical translation.

We have developed an efficient method to reproducibly generate large numbers of bipotential progenitors—known as hemangioblasts − from multiple hESC lines using an *in vitro* differentiation system [21,22]. These blast cells (BCs) expressed gene signatures characteristic of hemangioblasts, and could be differentiated into multiple hematopoietic lineages as well as into endothelial cells that could repair ischemic vasculature *in vivo*[21]. Using hESC-derived hemangioblasts/BCs as intermediates, we have also generated functional oxygen-carrying erythrocytes on a large scale from multiple hESC lines, demonstrating the robust expansion capability of these cells[23]. This system provides an excellent model for evaluation and comparison of hiPSC derivatives to their hESC counterparts. In the present study, we successfully generated BCs, endothelial cells, and hematopoietic cells from multiple well-characterized hiPSC lines. We further compared the functional characteristics of BCs derived from hESCs and hiPSCs, and found that hemangioblastic derivatives generated from these hiPSC lines display abnormal molecular and/or cellular processes compared to their corresponding hESC counterparts. Similarly, RPE cells derived from hiPSCs begin senescing in the first passage, indicating the observed

phenomenon is not limited to hemangioblastic lineages.

MATERIALS AND METHODS

Human ESC and iPSC cultures. The hESC lines used in this study were H1, H7, and H9 [24]; nine Harvard Stem Cell Institute hESC lines [25] and thirteen hESC lines derived at Advanced Cell Technology[26,27] (See Table1 and supplementary Table 1). The hiPSC lines used were iPS(IMR90)-1, iPS(Foreskin)1-1, iPS(Foreskin)4-1, and 4-3, which were generated using Thomson's four factors (Oct-4, Sox-2, Nanog and Lin28) with Lentiviral vector [1]; rv-hiPS01, rv-hiPS02, rv-hiPS03 and rv-hiPS04, which were derived by using Yamanaka's four factors (Oct-4, Sox-2, c-Myc and Klf4) with retroviral vector [6]. Cells were cultured on mitomycin-treated feeder MEFs in hESC medium supplemented with 20% Knockout Serum Replacement (Invitrogen), 10 ng/ml bFGF (Stemgent), β-mercaptoethanol (Sigma), NEAA, GlutaMax-I and penicillin/streptomycin(Invitrogen). Cells were fed with fresh medium daily and confluent hESC/hiPSC cultures were split using 1 mg/ml collagenase IV in DMEM/F12 (Invitrogen).

Embryoid body (EBs) formation. hESC/hiPSC cultures were incubated with collagenase IV at 37° C for up to 10 minutes, and the large clumps of cells were collected. The clumps were washed once with 5 ml hESC medium. To initiate differentiation, the cell clumps were resuspended in EB differentiation medium [Stemline II (Sigma) supplemented with 50 ng/ml BMP4 (R&D System) and VEGF (Invitrogen)], at a density of approximately 2×10^6 cells per well of a 6-well ultralow attachment plate (Corning).

Generation of hemangioblasts/BCs. Hemangioblasts/BCs were generated as previously described [21,22]. Briefly, EBs were cultured in differentiation medium for 48 hours, and then half of the medium replaced with medium containing BMP4 (50 ng/ml), VEGF (50 ng/ml) and bFGF (20 ng/ml). After 24-hour

incubation, the EBs were collected, washed once in 2 ml D-PBS (Invitrogen), and incubated with 1 ml of trypsin/EDTA (Invitrogen) at 37° C for 2-3 minutes. The EBs were dissociated by pipetting up and down 10 times using a 1000 µl pipette or until the cell suspension became homogenous with no visible clumps. An equal volume of MEF medium was added and the cells were centrifuged at 210g for 4 minutes. The cell pellet was resuspended in Stemline II medium to 1-2 × 10^6 cells/ml, 5 × 10^4 cells plated in 1- 2 ml of blast growth medium (BGM) [21,22], and then incubated for up to 6 days.

Characterization of hematopoietic colony forming capability. Day 6 hiPSC-BCs and hESC-BCs were purified from blast cell cultures. 1 × 10^3 blast cells were resuspended in 20 µl of Stemline II medium, mixed well with 0.5 ml of either CFC medium (Stem Cell Technologies, H4436 only) or BGM (H4436 plus cytokines) using 2 quick vortex pulses. Cells in semi-solid CFU assay medium were plated into wells of a 24-well Ultra-low attachment plate for CFU colony growth. Cells were incubated in a CO_2 incubator at 37°C for up to 15 days. The formation of hematopoietic colonies was monitored microscopically. On day 15, the number of hematopoietic colonies in each well as well as their morphology was recorded. For the CFU-MK assay, BCs were purified and plated according to the manufacturer's instructions using a MK CFU assay kit (Stem Cell Technologies, Cat 04973).

Differentiation of hiPSC-BCs and hESC-BCs into erythroid cells. Erythroid differentiation was carried out as previously described[23]. Briefly, day 5 blast cells from both hiPSCs and hESCs were purified and plated into 24-well plates (30,000 cells/ well) with BGM medium and allow to expand for 5-7 day or until the culture become confluent. At the end of each expansion, cells were collected from each well and counted. To expand further, equal numbers of cells from both hiPSC-BCs and hESC-BCs were replated in BGM medium. A total of three expansion cycles were performed in this study

(each expansion experiment was carried out in triplicate). To further differentiate toward erythroid cells, expanded BGM medium with cells was mixed at 1:1 ratio with Stemline II + EPO (3U/ml, Cell Sciences) and cultured for an additional 6-8 days. Additional Stemline II + EPO medium was added every 2-3 days. The erythrocytes were then spun onto glass slides and stained for benzidine/Giemsa (Sigma) and erythrocyte marker CD235a (DAKO) as described previously [23].

Differentiation of endothelial cells from hiPSC-BCs and hESC-BCs. Purified BCs from both hESCs and hiPSCs were plated into wells of fibronectin-coated 24-well plate (BD Biosciences) with EGM-2 medium (Lonza) at the density of 36,000 cells per well (n=4). Endothelial cells differentiated from hESCs and hiPSCs were sub-cultured when they reached confluence, and were continuously cultured for up to 30 days. The morphology and growth rate of endothelial cells were recorded. To test the endothelial clonogenic capability of BCs derived from hESCs and hiPSCs, individual blast colonies were handpicked and plated for endothelial cell colony formation.

Endothelial markers and cell senescence staining. The BC-derived endothelial cells were allowed to grow for 10 days in EGM-2 medium. The endothelial cells derived from hiPSCs were trypsinized and plated onto Matrigel (BD Biosciences) for network structure formation as described previously[21]. Expression of endothelial markers CD31(DAKO), VE-Cad (Cell Signaling Technology) and vWF (DAKO) on the hiPSC-derived endothelial cells was confirmed by immunofluorescence staining as described previously[21]. LDL uptake of hiPSC-endothelial cells was evaluated by culturing the cells in the presence of Alexa-Fluor 594 Ac-LDL (Invitrogen) at 10 µg/ml in EGM-2 for 8 hours, followed by washing with medium 3 times. The intracellular fluorescent LDL was examined using fluorescence microscopy. For detection of endothelial cell senescence, cells were fixed between 20 to 30 day days after initiation of

endothelial differentiation and then stained for endogenous β-galactosidase using a Cell Senescence Kit (Cell Signaling Technology) according to the manufacturer's manual.

Immunoflourescence staining. Blast colonies were handpicked using a fine glass pipette and immobilized onto positively charged glass slides through cytospin at 500 rpm. After fixation in a mixture of methanol and acetone (Fisher Scientific, v/v 1:3) for 20 minutes, the slides were incubated in 0.1% Triton X-100 in PBS for 10 minutes, and washed 3 times with PBS (5 minutes per wash). For RPE markers, cells were fixed for 10 minutes with 2% paraformaldehyde in PBS, permeabilized with 0.1% NP-40 substitute (Sigma) for 15 minutes and blocked for one hour with 10% Goat serum in PBS. For BC markers, antibodies against human CD71 (BD Pharmagen), CXCR4 (GeneTex), and TPO-receptor (R&D Systems), CD31, CD34 (Dako) and KDR (R&D Systems) were used. For RPE markers, antibodies to bestrophin (Novus Biologicals), MITF and CRALBP(Abcam), and Pax6 (Covance) were used . For apoptotic BC identification, antibody binding to a cleaved form of caspase-3 (Cell Signaling Technology) was used. Cells were incubated with primary antibodies in blocking solution over night at 4°C, washed with PBS (3x5 minutes), followed by incubation with flourochrome-conjugated secondary antibodies at room temperature for 1 hour. Cell nuclei were stained with DAPI (1 μg/ml) in PBS and the slides were mounted using Aquamount (PolySciences).

For RPE Generation, analysis and imaging, see supplementary methods.

RESULTS

HiPSCs are capable of differentiating into hemangioblasts/BCs, hematopoietic and endothelial cells *in vitro*.

A series of studies were carried out using well characterized hiPSCs lines generated by lentiviral expression of the transcription factors Oct-4, Sox-2, Nanog, and Lin28 (Thomson reprogramming method; cell lines IMR90-1, Foreskin-1-1, Foreskin-4-1 and Foreskin-4-3)[1] as well as retroviral expression of Oct-4, Sox-2, Klf4, and c-Myc (Yamanaka reprogramming method; cell lines rv-hiPS01, rv-hiPS02, rv-hiPS03 and rv-hiPS04)[6]. These hiPSC lines expressed the standard markers of pluripotency, and formed teratomas after inoculation in SCID mice as reported [1,6], and were all morphologically indistinguishable from hESCs (Figure 1A and 1B) after transferring and growing in our laboratories. Using our previously optimized methods[21,22], we first carried out a series of experiments to determine whether the hiPSC lines could generate BCs. hiPSCs were differentiated into EBs under conditions optimized for the development of BCs, and individual EB cells were plated in blast-growth/expansion medium (BGM) for the development of blast colony. EB cells from all hiPSC lines developed blast colonies six days after plating. Variable efficiencies were observed for the different hiPSC lines (Table 1), although the blast colonies that developed from all of the lines were much smaller in size, and the cells in the colonies were not as healthy as those generated from hESCs (Figure 1B, Supplementary Figure 1A). Nevertheless, as observed for BCs derived from hESCs (Figure 1A) [21], these BCs from hiPSCs expressed hemangioblast markers CD71, CXCR-4 and Tpo receptor (Figure 1B), but majority of these BCs did not express CD31, CD34 and KDR as demonstrated by immunocytochemical analyses. After replating in hematopoietic colony-forming media supplemented with a spectrum of cytokines for 10 to 14 days, erythroid (CFU-E), myeloid (CFU-G and CFU-GM), and macrophage (CFU-M, not shown) hematopoietic cell colonies developed (Figure 1C). BCs also formed CFU-MK with the potential to differentiate into megakaryocytes(Figure 1C- iv). To determine their endothelial potential, hiPSC-derived BCs were plated on fibronectin-coated plates and cultured in endothelial cell differentiation medium. The cells attached to the surface and grew as adherent cells (Figure 1D-i), which formed capillary-vascular like structures

on Matrigel (Figure 1D-ii), expressed high levels of vWF, PECAM-1 (Figure 1D-iii) and VE-cadherin (Figure 1D-iv), and took up acetylated low-density-lipoprotein (Ac-LDL, Figure 1D-iv). These results clearly demonstrate that hiPSCs generated with different combinations of reprogramming factors using different delivery systems (Lentivirus and Retrovirus) are capable of differentiating into hemangioblasts, hematopoietic and endothelial lineages under serum-free conditions, which is consistent with previous reports [17-20].

hiPSC-derived BCs exhibit apoptotic phenotype

As summarized in table-1, we observed that the efficiency of blast colony formation from all tested hiPSC lines was dramatically lower than that from all tested hESC lines. Even with the best cell line (IMR90-1) observed in our testing system, substantially fewer BCs developed compared to all tested hESC lines which display excellent reproducibility although some variations were observed among different hESC lines[21,22] . In contrast to hESC-derived BCs, their hiPSC counterparts displayed an apoptotic morphology (Figure 1A and 1B, Supplementary 1A). Caspase-3, the major protease involved for the execution of apoptosis, is produced as an inactive enzyme protein, and is cleaved by other initiation caspases such as caspase-8 and caspase-9 during the onset of the apoptosis [28]. The presence of the cleaved form of caspase-3 in the hiPSC-derived BCs is a clear indicator that the cells were undergoing apoptosis. Over 20% of the BCs generated from hiPSCs were positive for the cleaved form of caspase-3 (Figure 2A, green color) with fragmented nuclear morphology (Figure 2A(iii), arrow); in distinct contrast, less than 1% of BCs derived from hESCs stained positive for the cleaved form of caspase-3. The adjacent non-apoptotic BC (CD71+, red) has an intact nucleus. As previously reported [23], hESC-derived BCs always possess remarkable expansion capability in BGM. Figure 1A shows a typical blast colony generated from hESCs, which expands dramatically from day 6 to 10 (Supplementary Figure 1A), whereas blast colonies derived from hiPSCs (Figure 1B, supplementary 1A) are significantly smaller and expand slightly, if at all, during day 6 to 10.

Endothelial cells derived from hiPSCs senesce in early growth phase

Due to the very low efficiency for most hiPSC lines, we focused on the best performed IMR90-1 line for endothelial cell experiments. HiPSC (IMR90-1)-derived BCs were seeded at high density in fibronectin-coated plates with medium optimized for endothelial cell differentiation. The cells quickly attached to the plate (within 3-4 hr) and differentiated into endothelial cells (Figure 1D). However, the cells grew very slowly (Figure 2B), if at all, and the majority of them displayed a large, senescent-like phenotype (Figure 2B-v and 2B-vi]). Over 50% of the cells expressed β-galactosidase (Figure 2B-viii) which has been shown to be a reliable marker for cellular senescence [29]. In contrast, hESC-derived endothelial cells exhibited normal endothelial cell morphology and a robust growth rate (Figure 2B-i,ii and iii). Less than 5% of the hESC-derived cells stained positive for β-galactosidase (Figure 2B-iii).

HiPSC-derived BCs show dramatically reduced expansion and hematopoietic CFU-forming capability

Although BCs from hiPSCs were capable of forming multiple types of hematopoietic CFUs, the efficiency was also dramatically reduced compared to hESC-BCs (Figure 3A, i-vi). When 10^3 BCs from either hESCs or hiPSCs were plated for the development of hematopoietic CFUs, 181.3 ± 34.7 and 76.4 ± 12.3 versus 2.9 ± 1.5 CFUs formed after 14 days for BCs from MA01 and H1 hESCs, and IMR90-1 hiPSCs that is the most efficient cell line we observed, respectively (26-63-fold difference; Figure 3A-vii, $p < 0.001$). Furthermore, CFU-GEMM very rarely developed from cells derived from hiPSCs, and most CFUs were also much smaller than those generated from hESCs (Figures 1C and 3A, supplementary 1B, very rarely large

CFU-G developed from hiPSC-derived BCs as shown in Figure 1C).

We previously demonstrated that erythroid cells can be reproducibly generated from hESC-BCs with high efficiency [23], suggesting that hiPSCs could potentially serve as a source for generating O Rh(-) universal blood. Since most hiPSC lines showed very low efficiency of developing BCs, we than focused on the best performed hiPSC line IMR90-1 for erythroid expansion. Using these methods, 3×10^4 hiPSC(IMR90-1)- and hESC (MA01)-BCs were subjected to three cycles of erythroid cell expansion/differentiation. As shown in Figure 3B & 3C, a more than 5×10^3 -fold increase in number of cells was obtained from hESC-BCs; these cells morphologically resembled erythroblasts and stained positive for hemoglobin and CD235a (Figure 3B). Although positive expansion was obtained for the first cycle (initial 3-5 days), hiPSC-derived BCs showed negative expansion in both the second and third cycles of expansion (3-fold expansion after 15 days, Figure 3C).

RPEs derived from hiPSCs begin senescing in the first passage

In order to determine whether the observed phenomenon is limited to hemangioblastic lineages, we generated RPE cells from both hESCs and hiPSCs as previously described [30]. The pigmented clusters developed from IMR90-1 and iPS(Foreskin)1-1 cells were isolated manually and digested with collagenase, the cells were plated on gelatin-coated tissue culture plates in RPE-optimized media. Using this strategy, more than 100 RPE lines were established using over two dozen hESC lines, including WiCell-, Harvard-, and Advanced Cell Technology-derived lines (Table 1 and Supplementary Table 1). We observed that hiPSCs differentiated into RPE cells in a manner similar to hESCs initially (Figure 4). The cells isolated from these cultures showed the usual expression of RPE markers (Supplementary 2A and 2B) as demonstrated by immuno-staining and RT-PCR analysis. However, after RPE cells were isolated from the differentiating cultures,

noticeable differences between hESC- and hiPSC-RPEs were observed. The starting hESC-RPE cultures (p0) usually had very few cells, whereas the hESC-derived RPE cells grew robustly; one well of a four-well plate at P0 produced approximately 9-12 million cells at p2 (even if started from a few hundred cells isolated from differentiated hESC cultures). When RPE cells proliferate in culture, they lose their pigmented polygonal morphology, turning into spindle shaped cells. After the cells reach confluence, they regain their RPE phenotype. The hESC-derived cells were routinely passaged at least 2-3 times before observing the presence of a few abnormal, large and often fibroblast-like cells that still had RPE markers. Although abnormal looking cells began to appear at p3, the hESC-derived RPEs continued to proliferate and re-gain their RPE phenotype until at least passage 5 (Supplementary 2C). . In contrast, hiPSC-RPEs grew very slowly and often failed to fill the original well of the 4-well plate. In some cases, hiPSC-derived RPEs failed to regain normal RPE morphology at p1 (similar to that observed with hESC-RPEs at much later passages). In striking contrast to hESC-derived RPEs, abnormal senescent-like cells (large vacuolated cells with irregular morphology) were observed in all hiPSC-cultures at passage 1. β-Galactozidase staining confirmed that these cells were senescent (Figure 4B[iii]).

DISCUSSION

Consistent with previous published reports[12-14,17-20], we show that well established hiPSC lines are capable of generating hemangioblasts/BCs, endothelial and hematopoietic cells, as well as other lineages such as RPE cells, that share a high similarity to their hESC counterparts. A major hallmark of hESC-derived cells is the high recovery and proliferative capability of the cells, including, for instance, the ability of the latter to generate functional oxygen-carrying red blood cells on a large scale (10 to 100 billion cells/six-well plate hESCs)[23]. However, in contrast to hESC-derivatives, hemangioblasts/BCs and RPE

generated from hiPSCs displayed limited expansion capability and apoptosis morphology. When compared side by side, hiPSC-BCs demonstrated extremely limited hematopoietic colony forming and erythroid lineage expansion and differentiation capabilities; endothelial lineage-specific differentiation studies of hiPSC-BCs revealed reduced endothelial clonogenic capability, limited growth rate, and early senescence. Although many hESC lines studied showed consistent, reproducible BC-generation capability, limited success was observed with most hiPSC lines studied here with the exception of IMR90-1. We therefore believe that vigorous screening of all established hiPSC lines using well established differentiation protocol combined with phenotypical characterization of derivatives will ultimately help narrow down the list of true hiPSC lines, as well as providing powerful technical criteria for deriving new breed of hiPSC lines.

The molecular mechanisms responsible for these differences remain elusive. Further investigation will be necessary to determine the nature and extent of these abnormalities. The lineage-specific differentiation of hESCs has been well-studied, highly efficient and reproducible methodologies have been reported for differentiating hESCs toward all three germ layers [31]. Although hiPSC lines have been derived from a variety of somatic cell types using diverse reprogramming strategies, only limited reports on mesoderm, ectoderm and definitive endoderm lineage-specific differentiation are available. Using EB formation in the presence or absence of serum, Lengerke et al and Ye et al demonstrated hematopoietic development of hiPSCs derived by retroviral overexpression of reprogramming factors[19,20]. Similarly, using co-culture with BM stromal cell line OP9 and cell sorting, Choi et al reported successful hematopoietic/endothelial lineage-specific differentiation using hiPSC lines derived using lentiviral forced expression of Oct4, Sox2, Nanog and Lin28 in human fibroblasts [17]. However, none of these studies provided information on the proliferative capability of the

progenitor cells. . Choi et al [18] recently generated mature myelomonocytic cells through co-culture of hiPSCs with OP9 feeder cells, and the yield of differentiated cells from hiPSCs was found to be comparable to that obtained from H1 hESCs. However, only short term (2 days) expansion data was presented. Other hiPSC lineage-specific differentiation studies, including generation of endothelial cells [32], hepatocytes [10], pancreatic insulin-producing β-cells [9], adipocytes [33], and retinal pigmented epithelial cells [11,13,14], focused primarily on the characterization of the differentiated cell population rather than progenitor cells during early lineage specification. One study, however, did compare the proliferation of hESC- and hiPSC-derived cardiomyocytes (partially purified from contracting EBs) through BrdU incorporation. Reduced cell proliferation was indeed observed particularly in cardiomyocytes from late EBs [8].

There have been numerous reports, both in the scientific literature and media, suggesting iPSC may be identical to ESCs. Global gene expression profiles of hiPSCs and hESCs were compared for 32 266 transcripts [2]. Although the result showed a similar global gene expression profile, there were 1267 genes (approximately 4% of the total interrogated genes) with a greater than 5-fold difference in expression level between hiPSCs and hESCs. It is unclear whether this difference in gene expression will have any serious consequences in normal cellular function, or it is simply the result of cell culture conditions. A genome-wide study to compare mouse and human iPSCs with ESCs was published recently by Chin et al. [34]. This is by far the most comprehensive study using Comparative Genome Hybridization arrays to uncover sub-karyotypic genome alterations and gene expression changes; coding RNA and miRNA profiling, to determine changes in expression of small noncoding RNAs; and histone modification profiling, to determine whether epigenetic changes correlate with gene expression differences. Based on a vast amount of data, they concluded that early- and late

passage hiPSCs are not identical to their embryonic-derived counterparts [34]. A significant number of hiPSC lines were found to have abnormal expression of imprinted genes, which were found to be stable in hESCs[35]. Doi et al [36] recently showed that differential methylation of tissue-specific CpG island shores could distinguish hiPSCs, hESCs and fibroblasts, clearly demonstrating the differences between hiPSCs and hESCs. Furthermore, vaccination with hESCs generated a broad immunological and clinical response against cancer cells in a mice, whereas vaccination with hiPSCs failed to confer tumor protection in the animals[37]. These results suggest that there is, at least, a differential expression of oncofetal antigens between hESCs and hiPSCs. Although substantial evidence points toward the fact that hiPSCs and hESCs are not identical, several groups reported the generation of viable, fertile live-born progeny by tetraploid complementation using murine iPSCs, indicating these iPSCs maintain a pluripotent potential that is very close to murine ESCs [38-40]. Furthermore, by using a humanized sickle cell anemia mouse model, Hanna et al [41] showed that mice can be rescued after transplantation with hematopoietic progenitors obtained in vitro from autologous iPS cells. These studies clearly suggest the differences between iPS cells derived from human and mouse.

The mechanisms behind the slower growth and early cellular senescence observed here are unknown. However, recent studies clearly indicate the involvement of p53/p21 pathways in the somatic cell reprogramming process [42]. Lin28, one of the transcription factors used to generate the hiPSCs used in the present study, has also been shown to be tumorogenic [43,44] . Although the proviral insertion of transgenes of reprogramming transcription factors are reported to be silenced in the full reprogrammed cells [1,6], it remains to be seen whether they could be reactivated upon initiation of certain lineage differentiation protocols. We have observed the nuclear localization of LIN28 in hiPSC-derived BCs, which has not been observed in hESC-derived BCs (unpublished data). It will, therefore, be important to determine whether the abnormalities observed in the present study apply to hiPSCs generated using other transcription factors and/or reprogramming strategies, especially hiPSC lines derived without exogenous vector and transgene sequences[3-6].

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Figure 1. Differentiation of hiPSCs and hESCs toward hemangioblasts/blast cells and hematopoietic cells. (A) Hemangioblasts/blast cells (ii, x400) derived from hESCs (i, x100) were stained with CD71 (x1000), CXCR4 (x1000) and Tpo receptor (x1000). (B) Hemangioblasts/blast cells (ii, x400) derived from hiPSCs (i, x100) were stained with CD71 (x1000), CXCR4 (x1000) and Tpo receptor (x1000). (C) Multiple types of hematopoietic colony-forming units (CFU) developed from hiPSC-blast cells 14 days after replaing, x100 for CFU-E, CFU-GM and CFU-Mk, x40 for CFU-G. (D) Hemangioblast/blast cells derived from hiPSCs differentiated into endothelial cells (i, x200); hiPSC-derived endothelial cells formed vascular-like network on Matrigel (ii, x40), expressed vWF (red) and CD31 (iii, green, x400) as well as VE-Cadherin (iv, green) with LDL uptake capability (iv, red x400).
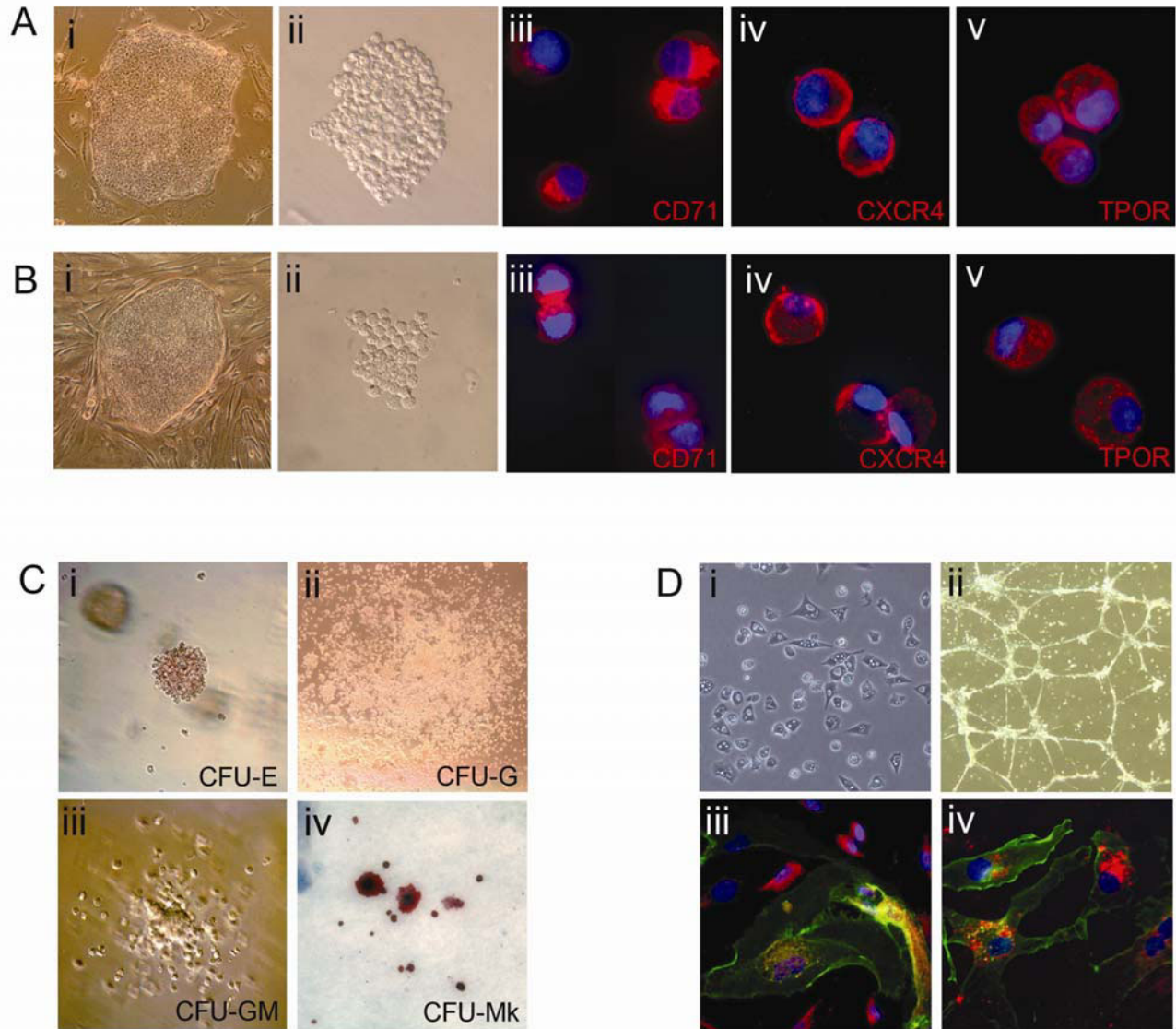
Figure 2. Apoptotic blast cells derived from hiPSCs and early senescent phenotype of endothelial cells from hiPSCs. (A), Blast cell stained for CD71 (i, red), apoptotic marker cleaved caspase-3 (ii, green), DAPI (iii, blue) and merged image (iv). Arrow indicates an apoptotic blast cell with fragmented nucleus, x1000. v: Percentage of apoptotic blast cells derived from hESCs and hiPSCs, $p < 0.01$. (B) Endothelial cells differentiated from hESCs (i, ii, and iii) and hiPSCs (iv, v, and vi) were examined for Ac-LDL up-take (i and iv, red, x200) and cell senescence marker β- galactosidase (blue, ii and v, x100, and iii and vi, x 200). (vii) Endothelial cells from hiPSCs exhibited limited growth/expansion capability, $p < 0.001$ (total endothelial cells derived from 36,000 BCs, and expanded for 30 days). (viii) Endothelial cells from hiPSCs committed early senescence, $p < 0.001$ (Percentage of senescent endothelial cells).
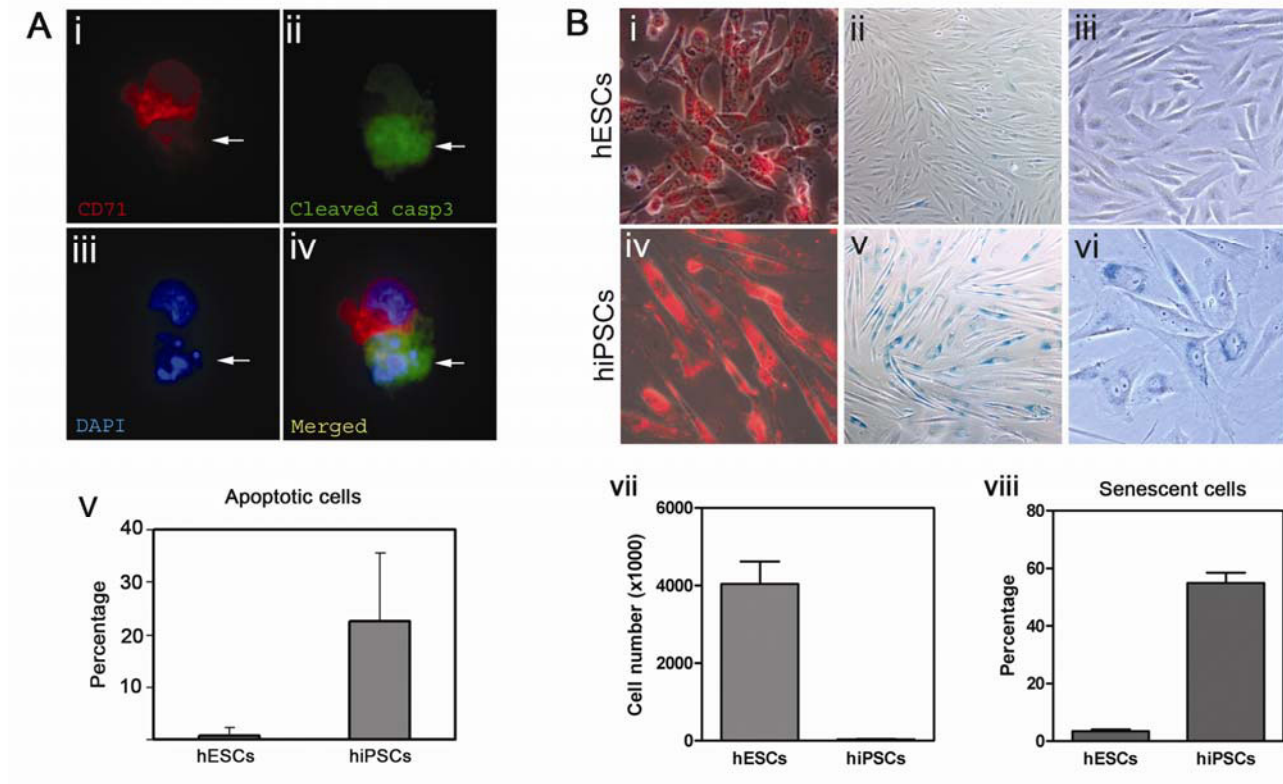
Figure 3. Reduced CFU formation, erythroid cell differentiation and expansion capability of hiPSC-blast cells. (A), A typical well of CFU assay from 1000 hESC-blast cells (i, ii, and iii) and hiPSC-blast cells (iv, v, vi). (vii) CFU development of blast cells derived from IMR90-1 hiPSCs (n = 10), H1 (n = 5) and MA01 (n = 5) hESCs, p < 0.001. (B), (i - v) Erythroid cells obtained from hESC-blast cells after three cycles (15 days) of differentiation and expansion. Erythroid cells were stained with benzidine for hemoglobin (brown) and Giemsa (blue, x200), and CD235a (red, x1000); (vi - x) Erythroid cells obtained from hiPSC-blast cells after three cycles (15 days) of differentiation and expansion. Erythroid cells were stained with benzidine for hemoglobin (brown) and Giemsa (blue, x200), and CD235a (red, x1000). (C), Quantitative results of erythrocyte expansion. Total fold increase: hiPSCs = 3.03 vs. hESCs =5,071.74.

	cycle 1 (n=3)	cycle 2 (n=3)	cycle 3 (n=3)
hESC-BCs	56.67 ± 9.07*	4.52 ± 0.62*	19.80 ± 2.55*
hiPSCs-BCs	6.45 ± 0.78	0.54 ± 0.11	0.87 ± 0.51

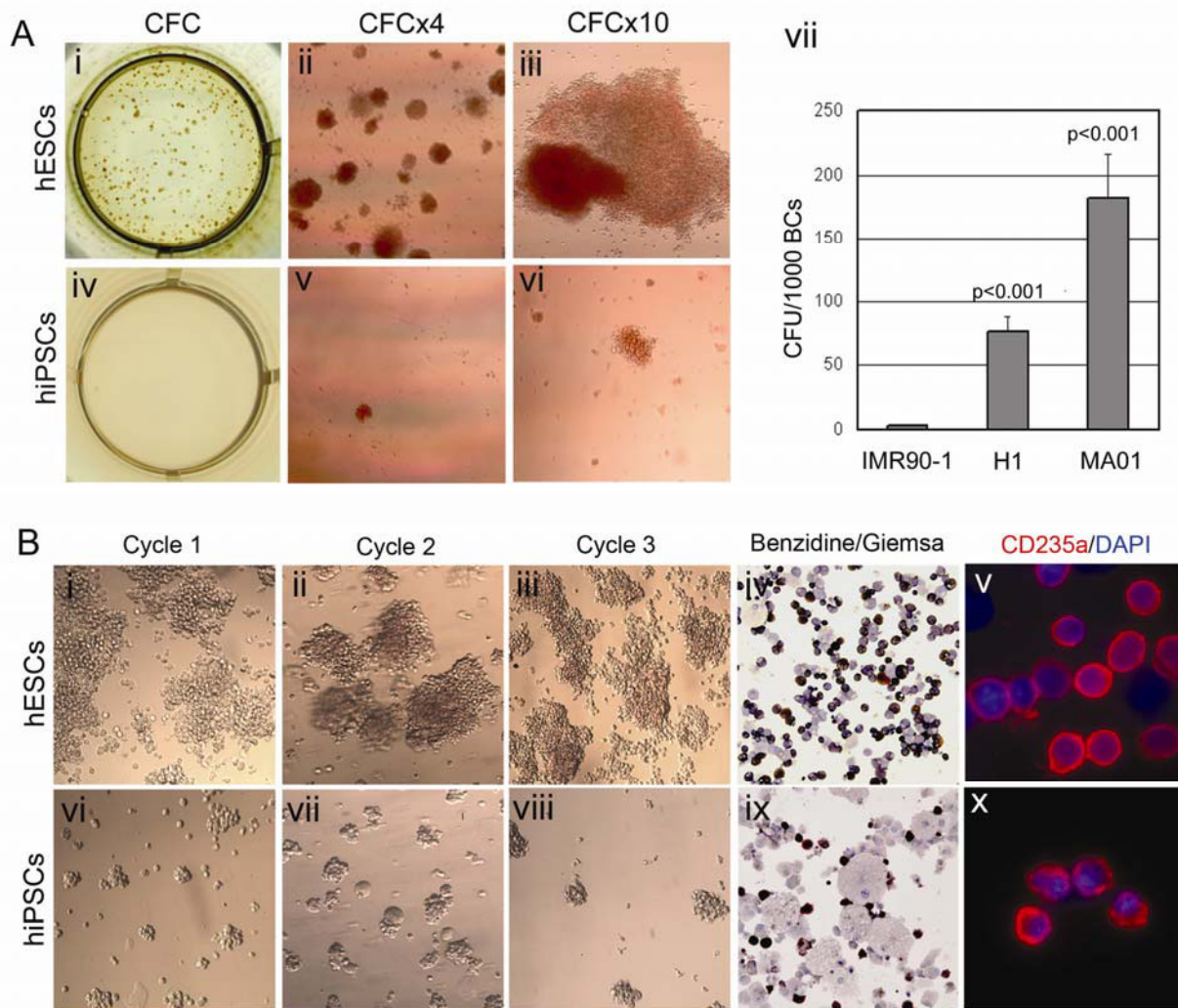Total fold increase: hiPSCs=3.03 vs. hESCs=5,071.74.
*p<0.001

Figure 4. Derivation and characterization of retinal pigment epithelium (RPE) cells from hiPSCs and hESCs.

A. RPE cells derived from hESCs: (i), differentiated RPE clusters before isolation, x40; (ii), RPE cells at passage 0, x200; (iii), RPE cells at passage 2 stained with β- galactosidase, x200. B. RPE cells derived from hiPSCs: (i), differentiated RPE clusters before isolation, x40; (ii), RPE cells at passage 0, x200; (iii), RPE cells at passage 2 stained with β- galactosidase (blue), x200.
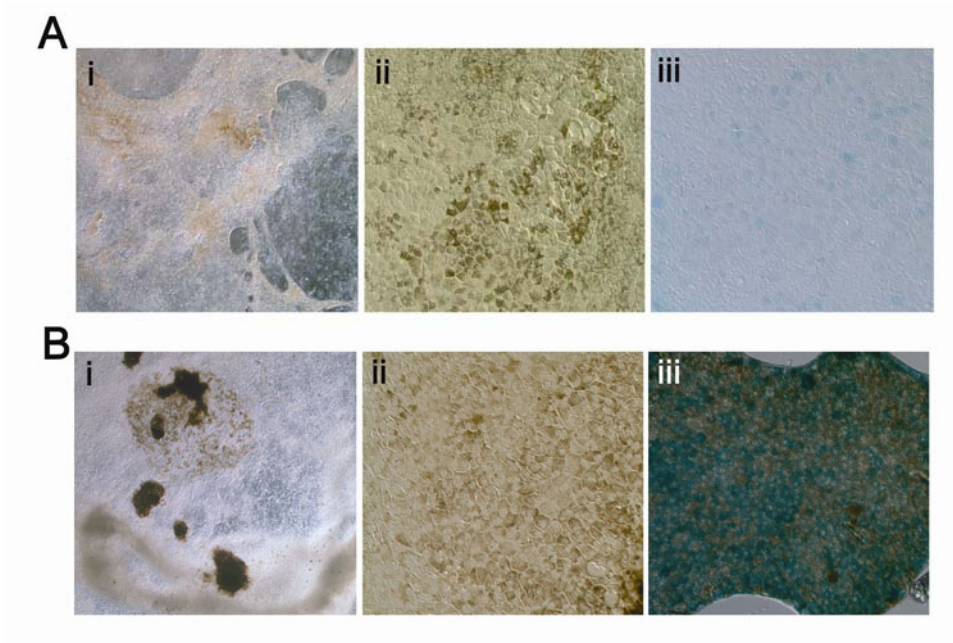
Table 1

Table 1: Differentiation efficiencies of human induced pluripotent stem cells (hiPSC) and human embryonic stem cells (hESC)

Sources	hiPSCs								hESCs													
	WiCell-Lentiviruses (Oct4, Sox2, Nanog, Lin28)				Harvard-Retroviruses (Oct4, Sox2, Klf4, c-Myc)				WiCell			Harvard Stem Cell Institute				Advanced Cell Technology						
Cell lines	IMR90-1	Foreskin			Rv-hiPS01	Rv-hiPS02	Rv-hiPS03	Rv-hiPS04	H1	H7	H9	HuES1	HuES3	HuES9	HuES16	MA01	MA03	MA09	MA40	MA99	NED1	NED2
		1-1	4-1	4-3																		
Blast colony efficiency	++*	±	±	±	±	±	±	±	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
CFU efficiency	+	±	±	±	±	±	±	±	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++

*Note: For every 100,000 EBs cells plated, (±) represent results of mostly 0 colonies or occasionally obtaining colonies under 10; (+) represent results of constantly obtaining low number of colonies (10 to 30); (++) Modest efficiency yielding up to 50 blast colonies; (+++++) represent normal efficiency yielding over 500 blast colonies.