Erythroid differentiation of human induced pluripotent stem cells is independent of donor cell type of origin

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ABSTRACT

Epigenetic memory in induced pluripotent stem cells, which is related to the somatic cell type of origin of the stem cells, might lead to variations in the differentiation capacities of the pluripotent stem cells. In this context, induced pluripotent stem cells from human CD34+ hematopoietic stem cells might be more suitable for hematopoietic differentiation than the commonly used fibroblast-derived induced pluripotent stem cells. To investigate the influence of an epigenetic memory on the *ex vivo* expansion of induced pluripotent stem cells into erythroid cells, we compared induced pluripotent stem cells from human neural stem cells and human cord blood-derived CD34+ hematopoietic stem cells and evaluated their potential for differentiation into hematopoietic progenitor and mature red blood cells. Although genome-wide DNA methylation profiling at all promoter regions demonstrates that the epigenetic memory of induced pluripotent stem cells is influenced by the somatic cell type of origin of the stem cells, we found a similar hematopoietic induction potential and erythroid differentiation pattern of induced pluripotent stem cells of different somatic cell origin. All human induced pluripotent stem cell lines showed terminal maturation into normoblasts and enucleated reticulocytes, producing predominantly fetal hemoglobin. Differences were only observed in the growth rate of erythroid cells, which was slightly higher in the induced pluripotent stem cells derived from CD34+ hematopoietic stem cells. More detailed methylation analysis of the hematopoietic and erythroid promoters identified similar CpG methylation levels in the induced pluripotent stem cell lines derived from CD34+ cells and those derived from neural stem cells, which confirms their comparable erythroid differentiation potential.

Introduction

During the last years, enormous progress has been made in the *ex vivo* manufacture of human red blood cells (RBC). Using human hematopoietic stem cells (HSC) from cord blood (CB) or bone marrow as the primary source, expansion rates higher than 10^5-fold,[1-6] accompanied by fully terminal maturation into enucleated reticulocytes,[1-4] have been achieved. Recently, the first proof-of-principle experiment was performed by transfusing a small sample of manufactured RBC into a human recipient.[7] However, despite this achievement, the large-scale expansion of RBC for transfusion purposes (1 RBC unit contains 10^{12} RBC) remains problematic, as human HSC are a limited source. Up to now, protocols for the *ex vivo* expansion of multipotent HSC are not available.

One promising alternative might be the generation of RBC from human pluripotent stem cells, a theoretically unlimited source characterized by properties of self-renewal. Until recently, the *ex vivo* generation of RBC from human embryonic stem cells (hESC) was limited by ethical concerns. Furthermore, it is unknown whether any of the hESC lines approved in the USA and produced under good manufacturing practice conditions have the universal O Rhesus negative phenotype.[8] These limitations were overcome by the discovery of induced pluripotent stem cells (iPSC). Human iPSC, which resemble hESC *in vitro* and *in vivo*, were initially generated from fibroblasts through the ectopic expression of a combination of the transcription factors OCT4, SOX2, KLF4, c-MYC as well as NANOG and LIN28.[9-11] Since these first studies, iPSC have been derived from various tissues.[12-16] Our group reported the generation of iPSC from human neural stem cells (NSC) by reprogramming with only OCT4 due to the cells' endogenous expression of pluripotency-associated genes.[15]

More recently, first protocols were established to induce RBC development from human fibroblast-derived iPSC.[17-22] However, *in vitro* recapitulation of physiological erythropoiesis

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in its entirety, which includes mesoderm induction, generation of HSC, erythroid maturation, hemoglobin switching and enucleation, remains a challenge. Compared to the established protocols for the adult system, RBC generation from iPSC is less efficient. In addition to a poor expansion rate of erythroid cells, the terminal differentiation of cells generated *ex vivo* from iPSC fails, particularly with regards to enucleation and switching from embryonic to fetal and finally to adult hemoglobin.

Increasing evidence from murine[23,24] and human systems[25,26] indicates that iPSC exhibit an epigenetic memory related to their donor cell type of origin. Although iPSC show characteristics and behaviors of ESC, incomplete removal of tissue-specific methylation or aberrant *de novo* methylation has been observed, which might influence their differentiation behavior. Due to this potential epigenetic memory and its influence on hematopoietic differentiation, iPSC from CD34$^+$ HSC may be more suitable for erythroid differentiation than the commonly used fibroblast-derived iPSC.

To investigate the influence of an epigenetic memory on the *ex vivo* expansion of iPSC into hematopoietic and erythroid cells, we generated iPSC lines from human CB-derived CD34$^+$ HSC and human NSC.[15] We evaluated their global gene methylation status and their potential to differentiate into hematopoietic progenitors and mature RBC under *ex vivo* conditions. Whereas CD34$^+$ HSC are the physiological source for RBC in humans and are of mesodermal origin, NSC are derived from the ectodermal germ layer. For the sake of completeness, fibroblast-derived iPSC[27] and hESC H1 were included in our study as "controls".

Methods

Generation of human cord blood CD34$^+$ induced pluripotent stem cells

CD34$^+$ HSC were isolated from human CB, using MACS sorting (Miltenyi Biotec, Germany). Informed consent was obtained from the donating mothers, and the investigation was approved by the Ethics Committee of Heinrich-Heine-University Düsseldorf Medical School. CD34$^+$ cells were stimulated with stem cell factor (SCF), thrombopoietin (TPO), fms-related tyrosine kinase 3 ligand (FLT3-L) and interleukin 6 (IL-6) as described elsewhere[28] and reprogrammed with either OCT4, SOX2, KLF4 and c-MYC or only OCT4 and SOX2. Lentiviral vectors encoding the human cDNA of OCT4, SOX2, KLF4 and c-MYC under the control of the SFFV promoter[29,30] were produced as previously described.[15,31] Infected CD34$^+$ cells were replated on irradiated mouse embryonic fibroblast cells in ESC medium. Approximately 25 days after transduction, iPSC colonies were selected for further expansion on the basis of their morphology. Established CD34$^+$ iPSC lines were characterized as described elsewhere.[32] The generation and characterization of iPSC from human NSC with OCT4 and KLF4 (NSC-2F-iPSC) or only OCT4 (NSC-1F-iPSC) by our group has already been published.[15] Likewise, the generation of iPSC from human skin fibroblasts (Fib-iPSC) with OCT4, SOX2, KLF4 and c-MYC has been described.[27] H1-hESC were obtained from WiCell (WI, USA).

Global gene expression analysis

RNA samples for microarray analysis were prepared using RNeasy columns (Qiagen, Germany). The input in the linear amplification protocol (Ambion) was 300 ng of total RNA per sample, with 12 h of *in vitro* transcription incorporating the biotin-labeled nucleotides. Purified and labeled cRNA was hybridized onto HumanHT-12 v4 expression BeadChips (Illumina, USA) following the manufacturer's instructions. The chips were stained with streptavidin-Cy3 (GE Healthcare) and scanned using the iScan reader (Illumina). Methods for the microarray data processing are available in the *Online Supplement*.

Global DNA methylation analysis

Genomic DNA was extracted from human iPSC (passage ≥15) and the corresponding somatic cell populations. The methylation status of all promoter-associated CpG islands was analyzed. Detailed methods are available in the *Online Supplement*.

Hematopoietic and erythroid differentiation

Human ESC H1 (n=6) and iPSC (n=4 respectively n=3 for NSC-2F-iPSC, passages ≥15) were differentiated into hematopoietic cells using the embryoid body method, as previously described[18] (Figure 1, Table 1). Embryoid bodies were stimulated for 20 days with 100 ng/mL SCF, 100 ng/mL TPO, 100 ng/mL Flt3-L, 5 ng/mL IL-3, 5 ng/mL IL-6, 5 ng/mL vascular endothelial growth factor 165, BMP4 (20 ng/mL until day 9, then 10 ng/mL) (all from Peprotech, USA) and 3 U/mL erythropoietin (EPO, Erypo®, Janssen-Cilag, Germany). Day 21 embryoid bodies were dissociated into single cells using collagenase B (0.4 U/mL; Roche Diagnostics, Germany).[33] For erythroid differentiation, single cells were cultured for up to 25 days in Iscove liquid medium (Biochrom, Germany) containing 10% human plasma (Octapharm, Germany), 10 μg/mL insulin (Sigma Aldrich, Germany) and 330 μg/mL human holotransferrin (Spicac, UK). Cells were stimulated with the following cytokines: day 0 to day 8: 100 ng/mL SCF, 5 ng/mL IL-3 and 3 U/mL EPO; day 8 to day 11: 100 ng/mL SCF and 3 U/mL EPO; day 11 to day 25: 3 U/mL EPO.

Hematopoietic and erythroid development was monitored by flow cytometry, colony formation in semisolid media and microscopic evaluation of cell morphology. The hemoglobin composition was analyzed by high performance liquid chromatography and polymerase chain reaction analysis. Detailed methods are available in the *Online Supplement.*

Results

Induction of pluripotency in human cord blood CD34$^+$ hematopoietic stem cells

CD34$^+$ HSC from human CB were reprogrammed to pluripotency using lentiviral vectors expressing either OCT4 and SOX2 alone (CD34-2F-iPSC) or expressing OCT4, SOX2, KLF4 and c-MYC (CD34-4F-iPSC) as a polycistronic unit under the control of the retroviral SFFV promoter.[29,30] The transduction efficiency of the CD34$^+$ HSC was approximately 70% and yielded different quantities of ESC-like colonies, which were further propagated (*Online Supplementary Figure S1A*). Immunofluorescence staining was performed to detect the expression of the pluripotency markers OCT4, NANOG, SSEA4, Tra1-60 and Tra1-81 (*Online Supplementary Figure S1C,D*) as well as alkaline phosphatase staining (*Online Supplementary Figure S1B*). Silencing of the SFFV promoter after reprogramming was demonstrated by the absence of red fluorescent protein expression in the iPSC colonies. This was further validated using quantitative reverse transcriptase polymerase chain reaction with vector-specific primers (*Online Supplementary Figure S1F*). To evaluate their *in vivo* pluripotency, CD34$^+$ iPSC were subcutaneously transplanted into mice with severe combined immunodeficiency. Approximately 8

weeks after injection, the CD34+ iPSC gave rise to teratomas containing derivatives of all three germ layers, including neural rosettes (ectoderm), smooth muscle and chondrocytic elements (mesoderm) and gut-like structures (endoderm) (*Online Supplementary Figure S1E*).

Global gene expression profiling of human cord blood CD34+ induced pluripotent stem cells

To demonstrate successful reprogramming to pluripotency, global gene expression of CD34+ iPSC was compared to the expression profile of hESC and the parental CD34+ population. The canonical pluripotency factors POU5F1/OCT4, SOX2, LIN28, KLF4 and cMYC line up on the diagonal in the pairwise scatter blot comparing the CD34+ iPSC and H1-hESC (Figure 2A). It was found that 98.1% of the transcripts were similarly expressed (using a 2-fold threshold in $\log_2$ scale) in the CD34+ iPSC and H1-hESC. The distance between the populations was reduced to 0.019, becoming more than 3-fold closer relative to the parental CD34+ population, which demonstrates successful reprogramming to pluripotency. Hierarchical clustering analysis showed that CD34+ iPSC clustered closely with hESCs (H1 and H9) and were distinct from parental CB CD34+ HSC (Figure 2B). Similarly, principal component analysis demonstrated that all CD34+ iPSCs clustered within the same 1st principal component as the hESC and were distinct from their respective parental CD34+ populations (Figure 2C).

Genome-wide DNA methylation profiling of human induced pluripotent stem cells

To evaluate epigenetic remodeling during reprogramming, quantitative analysis of the methylation status of all gene promoter-associated CpG islands was performed in CB CD34+ iPSC, NSC iPSC,[15] CB-derived unrestricted somatic stem cells, iPSC and CB mesenchymal stromal cell iPSC,[32,34] as well as their respective starting cell populations.

Table 1. Used cell lines, their origin and reprogramming factors.

Name	Origin	Reprogramming
H1-hESC		
CD34-4F-iPS	CB CD34+ cells	OCT4 SOX2 KLF4 C-MYC
CD34-2F-iPS	CB CD34+ cells	OCT4 SOX2
NSC-2F-iPS[15]	NSC	OCT4 KLF4
NSC-1F-iPS[15]	NSC	OCT4
Fib-iPS[27]	skin fibroblast	OCT4 SOX2 KLF4 C-MYC

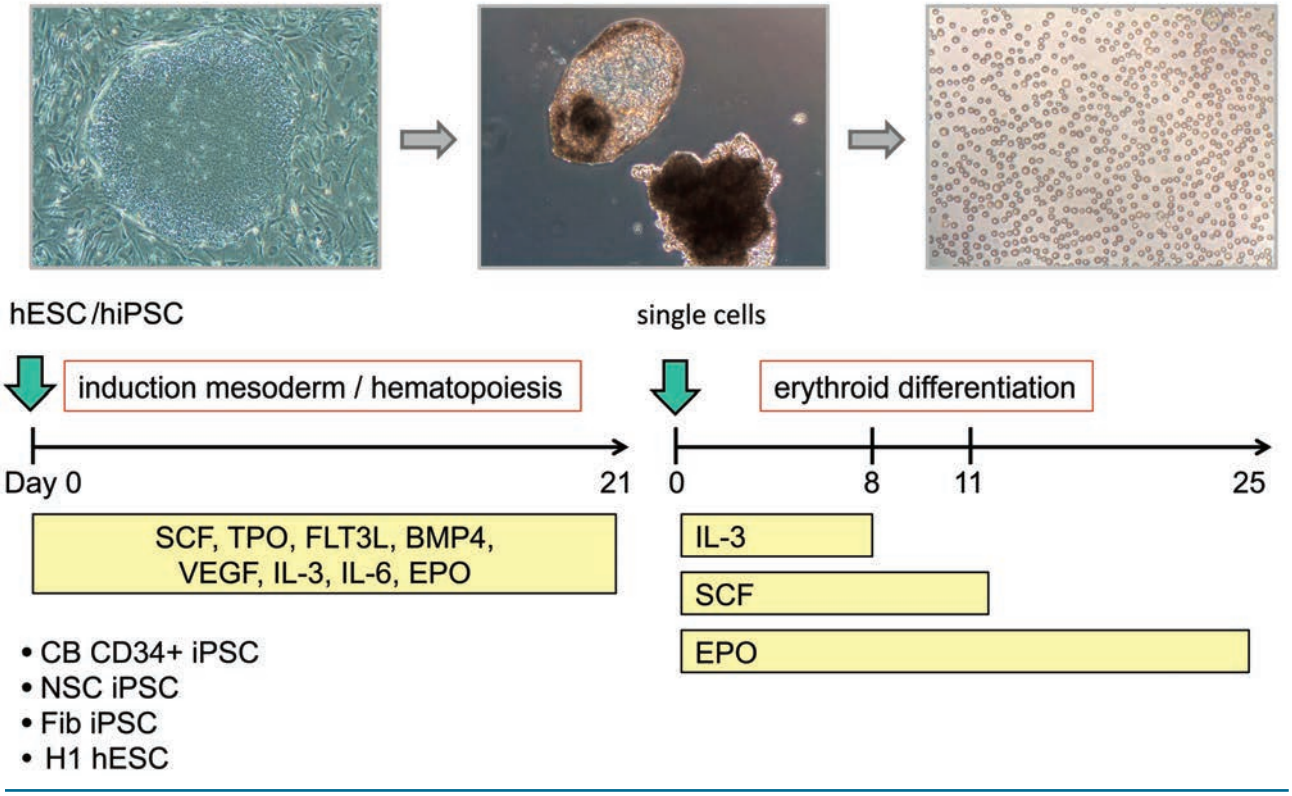

Figure 1. Experimental protocol for the *ex vivo* generation of hematopoietic and erythroid cells from human ESC and iPSC lines. To induce hematopoietic commitment, undifferentiated hESC/iPSC were allowed to form embryoid bodies, which were stimulated for 21 days with a cytokine cocktail, as indicated. For erythroid differentiation, day 21 embryoid bodies were digested into a single cell suspension and applied to a three-phase liquid culture in the presence of growth factors for an additional 18 – 25 days.

A representative scatter plot comparing CD34+ iPSC with their corresponding CD34+ HSC showed a similar distribution of the genes with higher and lower global methylation patterns (Figure 2D). The hierarchical clustering analysis of the global methylation patterns and the principal component analysis revealed a clustering of all these iPSC with their respective donor cell type of origin (Figure 2E,F). This finding points to an epigenetic memory at the level of promoter-associated CpG methylation of these iPSC. To focus in more detail on hematopoietic gene loci, we evaluated the CpG promoter methylation profiles for the hematopoietic and erythrocyte development-associated genes CD34, TAL1, KDR and T (*Online Supplementary Figure S2*), EPO, GATA1, GATA2 (*Online Supplementary Figure S3*), the hemoglobin clusters on chromosomes 11 and 16 (*Online Supplementary Figures S4* and *S5*), and HOXB4, CDX4 and RUNX1 (*Online Supplementary Figure S6*). As a general trend, we could not detect higher methylation levels for the NSC iPSC compared with the CD34+ iPSC at the hematopoietic loci analyzed. At the EPO and GATA2 loci the methylation levels were lower in CD34+ iPSC than in NSC iPSC (red *versus* blue bars in *Online Supplementary Figure S3*).

Hematopoietic differentiation of human cord blood CD34+ and neural stem cell induced pluripotent stem cells

Hematopoietic differentiation of iPSC (n=4 respectively n=3 for NSC-2F-iPSC, passages ≥15) and H1-hESC (n=6) was induced by embryoid body formation under cytokine stimulation for 21 days. Flow cytometry analysis after embryoid body digestion on day 21 (Figure 3A) demonstrated the presence of 23%±17.3% CD43+ hematopoietic cells from CD34-4F-iPSC and 18.6%±10.3% CD43+ cells from NSC-2F-iPSC. The major fraction of these CD43+ cells consisted of CD45+/CD34- myeloid precursors (CD34-4F-iPSC: 13.9%±6.7%; NSC-2F-iPSC: 14.6%±12.8%) expressing CD11, CD14 or CD15. Only a minor subset consisted of CD45+/CD34+ hematopoietic stem or progenitor cells (CD34-4F-iPSC 2.1%±0.9%; NSC-

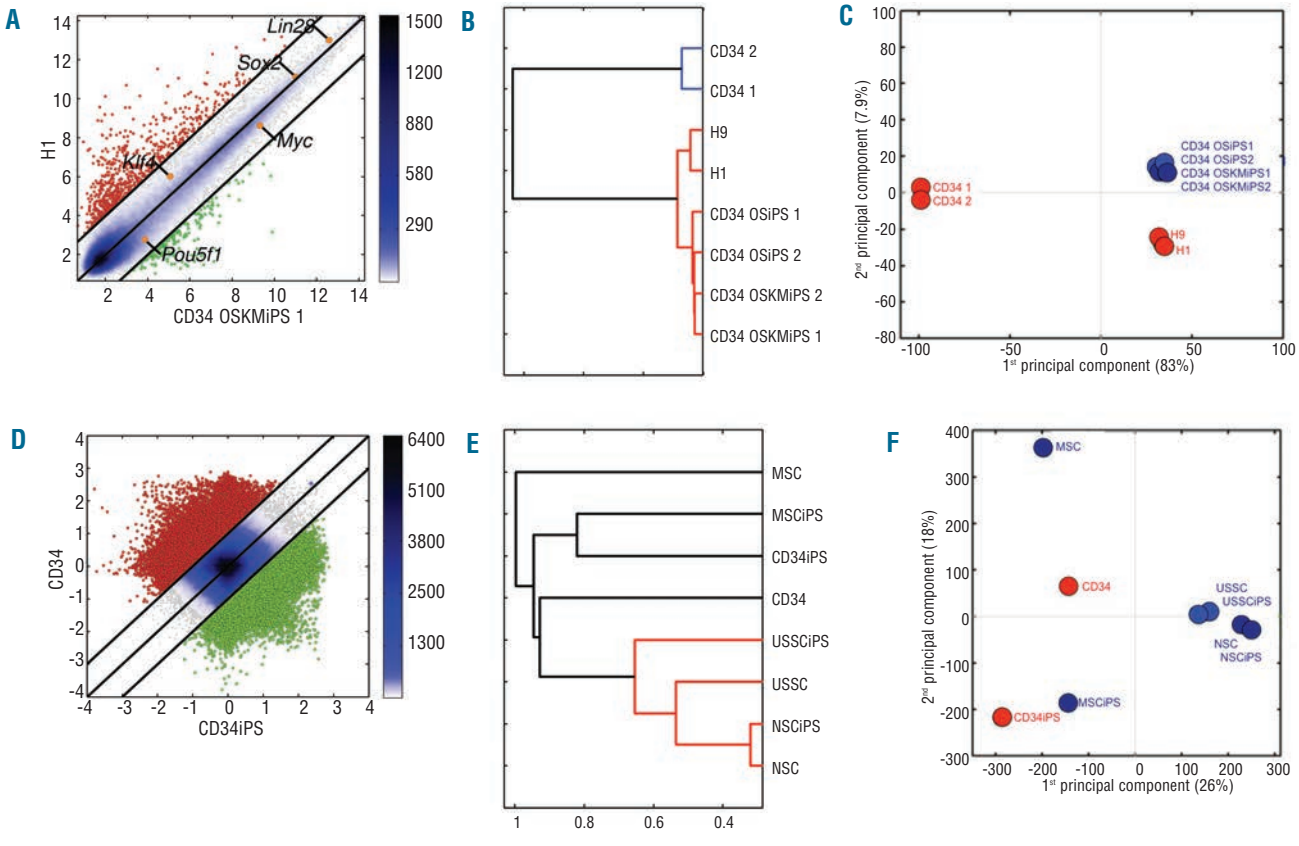

Figure 2. Global gene expression and epigenetic profiling of CB CD34+ iPSC and NSC iPSC. (A) Pairwise scatter plot of global gene expression profile data comparing CD34+ iPSC and hESC (H1). Black lines indicate 2-fold changes in the gene expression levels between the paired cell types. The positions of the pluripotent markers POU5F1/OCT4, SOX2, LIN28, KLF4 and cMYC are shown as orange circles. The gene expression levels are $\log_2$ scaled. (B) Hierarchical clustering of global gene expression comparing CD34+ iPSC reprogrammed with OCT4, SOX2 (OS) or OCT4, SOX2, KLF4 and cMYC (OSKM) and hESC (H9 and H1), as well as the CD34+ reprogramming starting populations. (C) Principal component analysis of the global gene expression of CD34+ iPSC, their respective CD34+ HSC and hESC (H1 and H9). The first and second principal components capture 83% and 7.9% of the sample population variability, respectively. (D) Pairwise scatter plot of the global methylation levels comparing CD34+ iPSC and CD34+ HSC. (E) Hierarchical clustering analysis of the global methylation patterns at all gene promoter-associated CpG islands of CD34+ iPSC, NSC iPSC, USSC iPSC and MSC iPSC as well as their respective somatic donor cell types of origin including CD34+ cells, NSC, USSC (cord blood unrestricted somatic stem cells) and MSC (cord blood mesenchymal stromal cells). (F) Principal component analysis of the global methylation patterns at all gene promoter associated CpG islands of CD34+ iPSC, NSC iPSC, USSC iPSC, and MSC iPSC, as well as their respective donor cell types of origin CD34+ cells, NSC, USSC and MSC. The first and second principal components capture 26% and 18% of the sample population variability, respectively.

2F-iPSC: 2.7%±1%). Furthermore, a small population of already differentiated GPA+/CD45- erythroid cells was observed (CD34-4F-iPSC: 7.1%±10%; NSC-2F-iPSC: 3.6%±0.8%). Morphologically, these GPA+ cells were already differentiated normoblasts and reticulocytes and, therefore, without further impact in the erythroid differentiation assay. In addition to GPA, the expression of CD36 (CD34-4F-iPSC: 11%±12.2%; NSC-2F-iPSC 6.3%±4%) and CD71 was measured to identify more primitive erythroid cells (Figure 4C, day 0 of the erythroid differentiation protocol). However, neither marker is specific for the erythroid lineage.[35,36] Independently of their hematopoietic nature (measured by CD43), the majority of cells stained positive for CD71 (CD34-4F-iPSC 63.4%±1.7%; NSC-2F-iPSC: 62.5%±10.1%), indicating a high percentage of proliferating cells with iron uptake, rather than of erythroid progenitors. Comparable results regarding the hematopoietic marker expression were observed for the CD34-2F-iPSC, NSC-1F-iPSC, Fib-iPSC and H1-hESC (Figure 3A,B). Colony formation assays demonstrated a similar colony potential in the CD34-4F-iPS, NSC iPS and Fib-iPS cell lines (1 colony/370 seeded cells), although the frequency of erythroid colonies was slightly higher in the CD34+ iPSC. CD34-2F-iPSC gave rise to a lower total number of colonies (Figure 3C,D).

Erythroid differentiation of human cord blood CD34+ and neural stem cell induced pluripotent stem cells

To address the terminal differentiation potential of iPSC into mature RBC, unselected cells from dissociated day 21 embryoid bodies were cultured for an additional 21 to 25 days in an erythropoiesis assay (n=3 respectively n=4 for CD34-4F-iPSC and NSC-1F-iPSC). During the first culture days, non-hematopoietic cells continuously disappeared, and by day 8, all of the cells were hematopoietic in nature. The major fraction consisted of erythroid precursors as determined by CD36 (CD34-4F-iPSC: 54.8%±14.7%; NSC-2F-iPSC: 62.1%±1.6%) and GPA expression (CD34-4F-iPSC: 66.4%±17.4%; NSC-2F-iPSC: 69.9%±17.5%) (Figure 4C). The remaining cells were mainly CD45+/GPA- myeloid cells. At this time, independently of the iPSC/hESC line, the majority of the erythroid cells showed morphological characteristics of basophilic and polychromatic erythroblasts. Until day 25, the cells further developed into a homogenous population of erythroid cells (CD34-4F-iPSC: 99%±0.6%; NSC-2F-iPSC: 98.6%±0.8%), showing characteristics of normoblasts with small condensed nuclei and enucleated reticulocytes (CD34-4F-iPSC: 27.3%±4.9%; NSC-2F-iPSC: 28.4±10.9%) (Figure 4B). In line with terminal differentiation, CD36 expression decreased to 19.9%±9.5% in the CD34-4F-iPSC and 19.7%±9.8% in the NSC-2F-iPSC. The expression of CD71, the transferrin receptor, remained high during the whole culture period. Comparable results were achieved for erythroid differentiation of CD34-2F-iPSC, NSC-1F-iPSC and Fib-iPSC. The terminal maturation and enucleation rates were similar among all iPSC lines (Figure 4B,C). Scanning electron microcopy showed reticulocytes with lobular phenotypes and occasionally mature RBC with a biconcave shape (approximately 1:10-20 cells) (Figure 4D). Interestingly, hESC differentiated more homogenously into erythroid cells (day 8: 87.6%±3.5% GPA+ erythroid cells), but the cells did not terminally

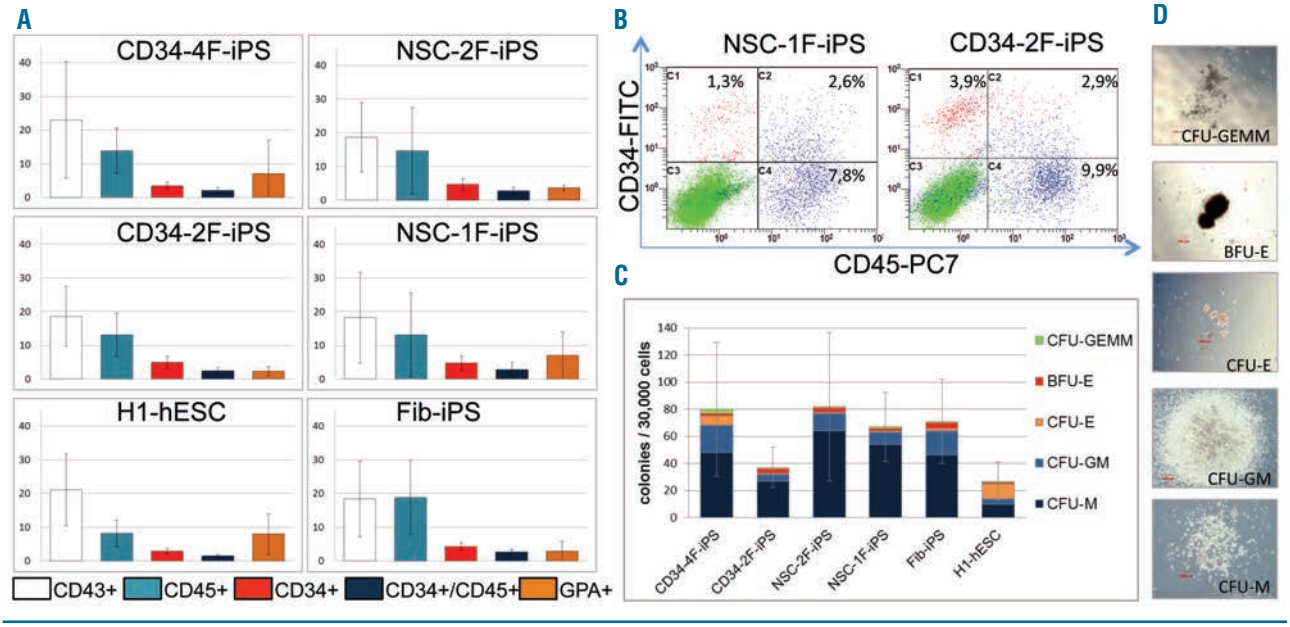

Figure 3. Hematopoietic differentiation of human CB CD34+ iPSC and NSC iPSC. (**A**) Expression of hematopoietic markers on single cells from dissociated day 21 embryoid bodies, as measured by flow cytometry (H1-hESC n=6, NSC-2F-iPSC n=3, NSC-1F-iPSC, CD34-2F-iPSC, CD34-4F-iPSC and Fib-iPSC n=4). The bars represent the percentages (mean ± SD) of positive cells. (**B**) Representative flow cytometry analyses for CD34 and CD45 expression on digested day 21 embryoid bodies from CD34-2F-iPSC that were reprogrammed with OCT4 and SOX2 and from NSC-1F-iPSC that were reprogrammed with OCT4. (**C**) Hematopoietic colony formation in semisolid media after digestion of day 21 embryoid bodies. The bars represent the mean number of erythroid and myeloid colonies that arose after 14 days from 30,000 digested, unselected single cells. Additionally, the standard deviation (SD), with regards to the total number of colonies, is indicated in each cell line bar. (**D**) Representative pictures for erythroid (BFU-E/CFU-E), myeloid (CFU-M/CFU-GM) and immature mixed (CFU-GEMM) colonies that arose from CD34+-derived iPSC, scored after 14 days (original magnification 50-fold, bars represent 100 μm).

mature at the same rate. Although CD36 expression decreased to the low level of 12.8%±10.9%, the percentage of enucleated reticulocytes was only 7.9%±5.3%. Morphological analysis showed larger cells with larger nuclei and less compact chromatin (Figure 4B).

Expansion of the erythroid cells reached its maximum between day 15 and 18. Prolonged culturing resulted in the death of the differentiated cells, associated with a decline in cell number. The highest cell expansion rate was observed in CD34-4F-iPSC with 1,368±461-fold expansion calculated from the initial seeded $CD34^+/CD45^+$ cells; the NSC-2F-iPSC underwent 635±165-fold expansion. Considering that $CD34^-$ precursors might contribute to erythroid development, we also calculated the number of manufactured erythroid cells per 1,000 initially seeded unselected single cells from day-21 embryoid bodies. The highest erythroid outcome was observed in the CD34-4F-iPSC with an average of $33x10^3$ erythroid cells/10^3 seeded cells of which approximately $9x10^3$ were enucleated. This was followed by the 2F-NSC-iPSC with an average of $18x10^3$ erythroid cells/10^3 seeded cells of which $5x10^3$ were enucleated (Figure 4E).

To determine the hemoglobin composition of the RBC that were generated, high performance liquid chromatography was performed during the final days of culture. Independently of their somatic origin, all iPSC-derived RBC contained mainly fetal hemoglobin (CD34-4F-iPSC: 89.4%±4.8%; NSC-2F-iPSC: 91%±1.8%; Fib-iPSC: 85.4%±7.7%) and to a lesser extent embryonic hemoglobin (CD34-4F-iPSC: 6.9%±4.8%; NSC-2F-iPSC: 6.7%±1.9%; Fib-iPSC: 6.0%±0.9%) (Figure 5A,B). Only a small amount of adult hemoglobin was detected (2%-7%). In contrast, H1-hESC-derived RBC expressed equal amounts of embryonic (49.1%±19.4%) and fetal (50.2%±20.3%) hemoglobin. Adult hemoglobin was barely detectable (<1%). The high performance liquid chro-

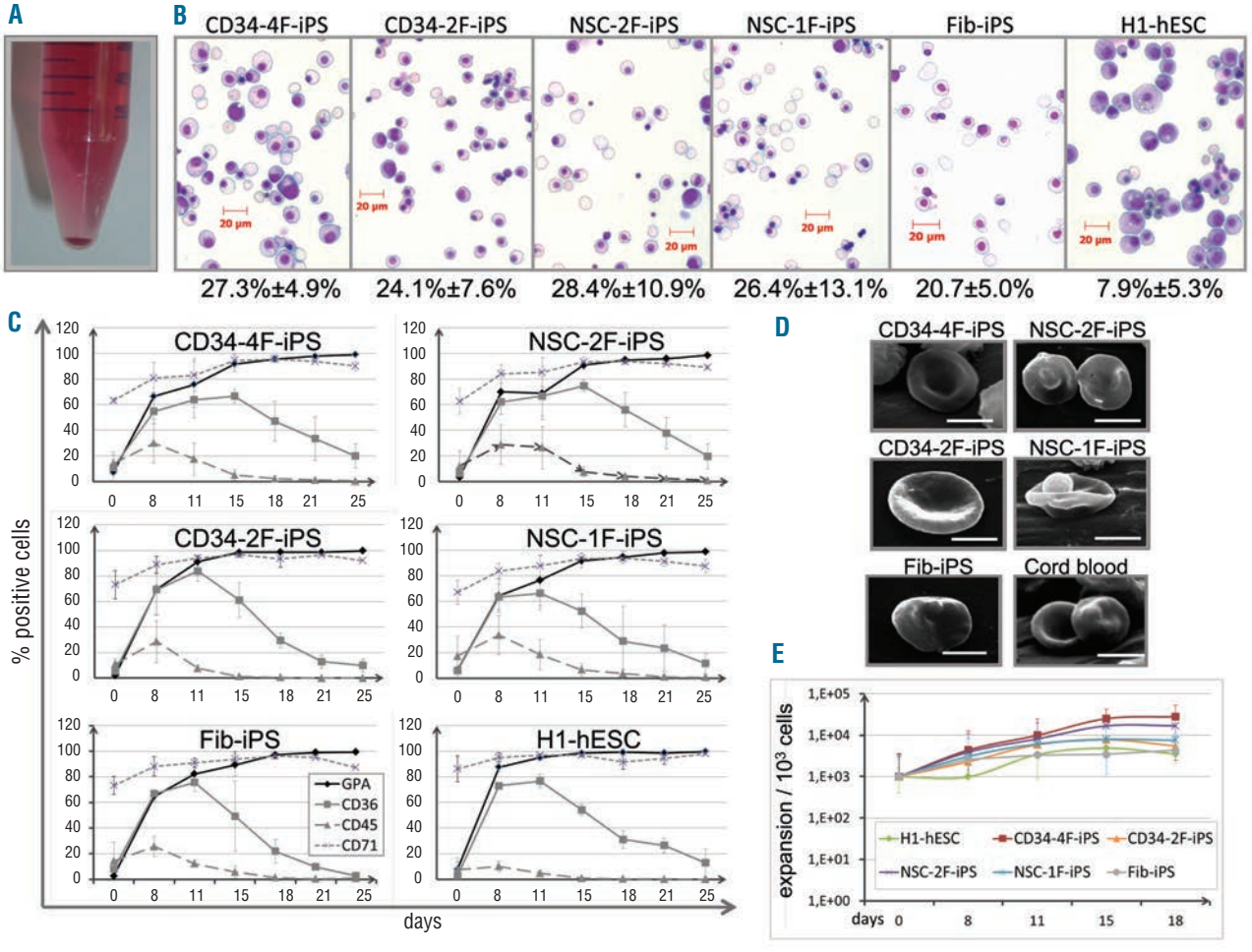

Figure 4. Erythroid differentiation of human CB $CD34^+$ and NSC iPSC. (A) Pellet of *ex vivo*-generated erythroid cells from $CD34^+$ iPSC, photographed on day 11 of the erythroid differentiation assay. (B) Representative pictures of cytospin preparations, prepared on day 18 of the erythroid differentiation assay (original magnification 400-fold, scale bars represent 20 µm). For each cell line, the percentage of enucleated RBC (mean ± SD) is indicated below the corresponding picture (n=3 respectively n=4 for CD34-4F-iPSC and NSC-1F-iPSC). (C) Cell surface expression of CD45, CD36, CD71 and GPA during the erythroid differentiation protocol (additional 25 days after embryoid body digestion), as measured by flow cytometry. The graphs represent the percentages (mean ± SD) of positive cells. (D) Scanning electron microscopy of *ex vivo*-generated reticulocytes and biconcave shaped RBC from $CD34^+$ iPSC, NSC iPSC and Fib-iPSC. The NSC-1F-iPSC-derived RBC is in contact with an expelled nucleus. As a control, reticulocytes from healthy human cord blood, purified by selection of $CD71^+$ cells, are shown (original magnification 1,500-fold, scale bar represents 5 µm). (E) Cumulative number of erythroid cells (mean ± SD) generated during the erythroid differentiation phase from 1,000 unselected single cells of digested day 21 embryoid bodies.

matography results were confirmed by semiquantitative polymerase chain reaction analyses for embryonic ε, fetal γ and adult β-chains (Figure 5C).

Discussion

We report the successful generation of iPSC from human CB CD34⁺ HSC and their differentiation into hematopoietic and erythroid cells. So far, protocols established for the *ex vivo* manufacture of RBC from human fibroblast-derived iPSC have failed to provide adequate expansion and terminal differentiation, compared to the protocols for adult HSC. Recent studies conducted with murine and human systems indicated that iPSC have an epigenetic memory of their somatic cell origin, which resulted in an impaired hematopoietic differentiation potential of fibroblast, NSC and keratinocyte-derived iPSC.[23-26] This impairment in differentiation was pronounced in early-passage iPSC lines (<passage 10) and was attenuated at later passages (>passage 15).[25] As a consequence of this potential epigenetic memory and its influence on hematopoietic differentiation pathways, CD34⁺ HSC may be more suitable than the commonly used fibroblasts as a reprogramming starting population to induce RBC generation from human iPSC.

To examine this issue further, we compared the hematopoietic and erythroid development of two newly established CD34⁺ iPSC lines with NSC-derived iPSC, which were recently described by our group.[15] Both populations are of fetal origin and can be classified as multipotent, somatic stem cells. Whereas CD34⁺ HSC are the physiological progenitors of RBC and are of mesodermal origin, NSC are derived from the ectodermal germ layer. To make our results more comparable with those of other publications, we also included fibroblast-derived iPSC in our study.[27] Previously we demonstrated that multipotent NSC are more amenable than unipotent fibroblasts to reprogramming to iPSC.[37] This observation was further val-

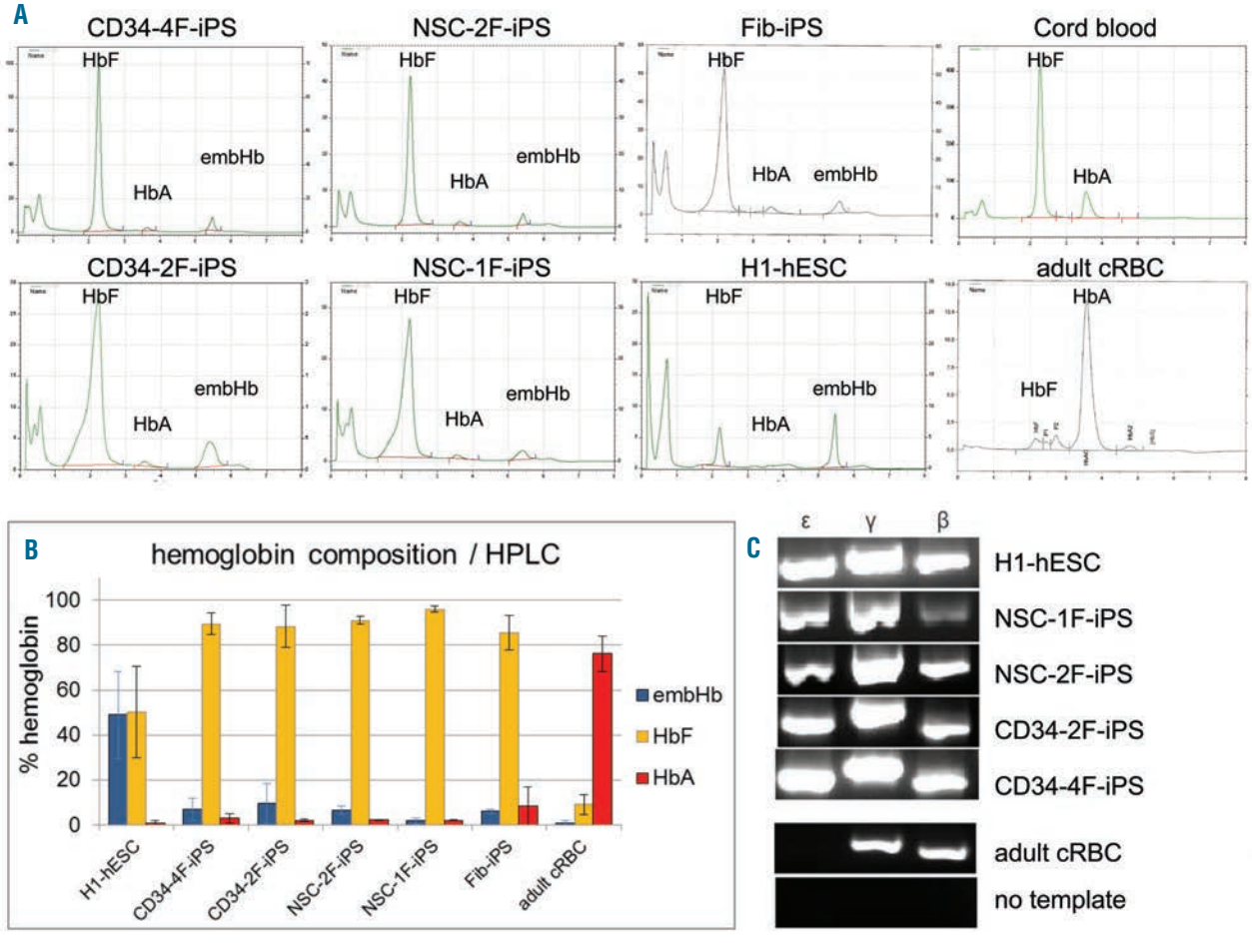

Figure 5. Hemoglobin composition of *ex vivo*-generated erythroid cells. (A) Representative high performance liquid chromatographic (HPLC) analyses, showing the tetrameric hemoglobin protein fractions in the *ex vivo*-generated erythroid cells from CD34⁺-iPSC, NSC iPSC, Fib iPSC, H1-hESC, (day 18 of the erythroid differentiation phase) and in healthy human cord blood. Additionally, HPLC analysis of *ex vivo*-generated erythroid cells from human adult CD34⁺ HSC, manufactured under the same culture conditions, is given (adult cRBC). The areas under the curve represent the percentages of embryonic (embHb), fetal (HbF) and adult (HbA) hemoglobin with respect to the total amount of hemoglobin. (B) Percentages (mean ± SD) of embryonic, fetal and adult hemoglobin obtained from HPLC analyses of H1-hESC, CD34⁺ iPSC, NSC iPSC and Fib iPSC-derived erythroid cells (day 18 of the erythroid culture assay). Additionally, the hemoglobin composition of erythroid cells generated from human adult CD34⁺ HSC is given (adult cRBC). (C) Semiquantitative polymerase chain reaction analyses for the embryonic (ε), fetal (γ) and adult (β) hemoglobin chains in *ex vivo*-generated erythroid cells from H1-hESC, NSC iPSC and CD34⁺ iPSC, as well as in erythroid cells generated from adult CD34⁺ HSC (adult cRBC).

idated with hematopoietic[38] and endodermal[39] cells.

To analyze the epigenetic marks of the reprogrammed cells, we performed genome-wide DNA methylation analysis of various iPSC lines (all at passages >15) derived from different sources (CD34+ HSC, NSC, mesenchymal stromal cells and unrestricted somatic stem cells) being aware that DNA methylation is just one parameter leading to variations among iPSC lines.[40] Our results indicate that all iPSC have an epigenetic memory, as shown by their global methylation profile at promoter regions. This was confirmed by hierarchical clustering and principal component analyses, demonstrating that all iPSC cluster together with their corresponding somatic stem cells.

For hematopoietic and erythroid differentiation, human CD34+ HSC-, NSC- and fibroblast-derived iPSC (passages >15) were applied to an embryoid body-based culture system described recently.[18] Despite the observed epigenetic memory in global gene methylation, all iPSC lines performed similarly in the initial hematopoietic induction, independently of their somatic origin. The hematopoietic commitment, analyzed by CD43 expression after 21 days of cytokine-stimulated embryoid body formation, averaged 20%, with some passage-dependent variations in each cell line. This is consistent with the suggestions of Bock *et al.*,[41] who postulated, based on quantitative embryoid body differentiation assays, variations in differentiation among iPSC lines within the normal spectrum of ESC variation. CD43 was recently identified as a hematopoiesis-specific marker that is expressed in early hematopoietic progenitors and persists in differentiating precursor cells.[42] In line with others, we observed only a minor population of CD45+/CD34+ or CD43+/CD34+ hematopoietic progenitors (~2%).[17-22,43] The vast majority of the CD43 hematopoietic cells were already committed myeloid, megakaryocyte and erythroid precursors. Additionally, the colony-forming potential of the digested day 21 embryoid body cells was not apparently different between the CD34+ iPSC, NSC iPSC and Fib iPSC; only the frequency of erythroid colonies was slightly higher in the CD34-4F-iPSC.

iPSC from all sources gave rise to a homogeneous population of erythroid cells at terminal stages of differentiation (>96% GPA+ cells), exhibiting phenotypes of terminally matured normoblasts, enucleated reticulocytes and, as demonstrated by electron microscopy, occasionally biconcave-shaped RBC. The percentage of enucleated cells (21%-29%) and the preferential synthesis of fetal hemoglobin (89%-96%) did not differ between the CB CD34+ iPSC, NSC iPSC and Fib-iPSC. However, although all the iPSC lines were similar in their ability to terminally mature into RBC, the expansion and final outcome of the erythroid cells was slightly higher in the CD34+ iPSC (1,370-fold) than in the NSC-iPSC (640-fold). This is in line with the higher observed frequency of erythroid colony formation in the CD34-4F-iPSC and might argue for a greater intrinsic erythroid potential of these cells.

Our results obtained from the iPSC contrast with those observed for RBC generation from adult human HSC. For comparison, the mean expansion of adult CD34+/CD45+ HSC (n=8), cultured under the same conditions, is 89,000-fold, with homogeneous differentiation into enucleated RBC (85.2%±4.8%) that express mainly adult hemoglobin (*unpublished data*). When using CD34+ cells from CB, established protocols with expansion rates of up to $1.96x10^6$ fold will theoretically allow for the generation of more than $3x10^{12}$ RBC from one CB unit.[1] However, the erythroid differentiation potential of the human iPSC described here is similar to that in recent studies using fibroblast and mesenchymal stromal cell-derived iPSC in embryoid body[18,21] or stromal layer-based culture systems.[17,20] Although using different culture conditions, all studies show a severely limited expansion of the erythroid cells compared to that from adult HSC, a low enucleation rate (<10%) and the expression of mainly fetal and embryonic hemoglobin. Compared to these results, we achieved a higher enucleation rate (21-29% *versus* <10%[17-22]), which might be the consequence of differences in the culture techniques or the intrinsic characteristics of our iPSC cell lines.

Because of the similar functionality of NSC and CB CD34+-derived iPSC in hematopoietic and erythroid differentiation, we focused the initial DNA methylation analysis in more detail on promoter regions associated with hematopoietic and erythroid differentiation pathways. We did not detect higher methylation levels at many of the hematopoietic-specific promoters in the NSC iPSC compared to the CD34+ iPSC. A higher methylation level in NSC iPSC, indicating impaired transcription of these regions, could be considered a prerequisite for the preferred differentiation of CD34+ iPSC towards hematopoietic lineages. These results do, therefore, support the observed similarity in hematopoietic and erythroid differentiation in NSC iPSC and CD34+ iPSC. In addition to our results, it would be very interesting to investigate the hematopoietic potential and epigenetic marks of reprogrammed erythroid progenitors further. The successful reprogramming of erythroid progenitors derived from patients with polycythemia vera has been described recently.[44]

In a recent study our iPSC derived from mouse NSC with OCT4 alone showed reduced neuronal and hemato-endothelial differentiation compared to iPSC reprogrammed with all four factors or ESC.[45] This might be the result of some epigenetic instability, as shown in the DNA methylation pattern of the pluripotency associated promoters, although their gene expression profile was very similar to that of ESC.[46] We can confirm these slight differences in the differentiation efficiency in human cells depending on the number of reprogramming factors. Although OCT4-derived (NSC) and OCT4/SOX2-derived (CD34+ HSC) iPSC gave rise to similar percentages of hematopoietic precursors compared to their corresponding iPSC lines that were reprogrammed with more factors, colony growth was impaired in CD34+ iPSC reprogrammed with only OCT4/SOX2. Furthermore, the outcome of the erythroid cells in the more specific erythropoiesis assays was likewise impaired in OCT4-induced NSC iPSC and OCT4/SOX2-induced CD34+ iPSC. Nevertheless, all iPSC lines gave rise to erythroid cells at terminally mature stages, and the expansion rates were at least as high as in H1-hESC.

Although we can confirm our initial hypothesis of an epigenetic memory in CD34+ iPSC at higher passages on a genome-wide level, this memory only marginally improved the expansion and differentiation of RBC under *ex vivo* conditions. RBC generation from iPSC does, therefore, still remain a challenge, and major hurdles must be overcome before iPSC-derived RBC will enter into clinical use. One key issue for transfusion purposes will be large-scale amplification. Strategies to improve expansion rates might focus on increased induction of early hematopoietic and erythroid progenitors from human iPSC and the maximization

of their subsequent expansion (e.g. by controlled expression of erythroid-specific genes, introduction of proliferation-associated transcription factors,[47,48] modification of growth factor combinations or mimicry of endogenous niches). Once all these difficulties have been successfully overcome, *ex vivo* manufactured RBC from human iPSC might be used for personalized transfusion medicine, preferentially applied to allo-immunized patients or those with rare blood groups. For these purposes, using CB CD34$^+$ HSC as the starting cell population, as described here, will have several potential advantages: (i) CB CD34$^+$ cells are of fetal origin and have not been challenged by environmental influences that lead to nuclear and mitochondrial mutations;[49,50] (ii) CD34$^+$ HSC can be easily isolated from CB collected in an already established worldwide network of CB banks, without any donor risk;[50] (iii) CB derived from CB banks will be well characterized for immunological and infectious markers; and (iv) CB-derived iPSC would allow the formation of CB iPSC banks to procure blood group-compatible RBC units and, on demand, histocompatible cell transplants. Furthermore, in newborns with congenital diseases, autologous CB might be readily available for the generation of autologous cell products, disease modeling or genetic modifications.

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