Human induced pluripotent stem cells can reach complete terminal maturation: *in vivo* and *in vitro* evidence in the erythropoietic differentiation model

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ABSTRACT

Background

Human induced pluripotent stem cells offer perspectives for cell therapy and research models for diseases. We applied this approach to the normal and pathological erythroid differentiation model by establishing induced pluripotent stem cells from normal and homozygous sickle cell disease donors.

Design and Methods

We addressed the question as to whether these cells can reach complete erythroid terminal maturation notably with a complete switch from fetal to adult hemoglobin. Sickle cell disease induced pluripotent stem cells were differentiated *in vitro* into red blood cells and characterized for their terminal maturation in terms of hemoglobin content, oxygen transport capacity, deformability, sickling and adherence. Nucleated erythroblast populations generated from normal and pathological induced pluripotent stem cells were then injected into non-obese diabetic severe combined immunodeficiency mice to follow the *in vivo* hemoglobin maturation.

Results

We observed that *in vitro* erythroid differentiation results in predominance of fetal hemoglobin which rescues the functionality of red blood cells in the pathological model of sickle cell disease. We observed, *in vivo*, the switch from fetal to adult hemoglobin after infusion of nucleated erythroid precursors derived from either normal or pathological induced pluripotent stem cells into mice.

Conclusions

These results demonstrate that human induced pluripotent stem cells: i) can achieve complete terminal erythroid maturation, *in vitro* in terms of nucleus expulsion and *in vivo* in terms of hemoglobin maturation; and ii) open the way to generation of functionally corrected red blood cells from sickle cell disease induced pluripotent stem cells, without any genetic modification or drug treatment.

Key words: human induced pluripotent stem cells, terminal maturation, erythropoietic differentiation.

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The online version of this article has a Supplementary Appendix.

Introduction

Somatic cell reprogramming through overexpression of multipotency-related genes has led to the production of human induced pluripotent stem cells (hiPSC),[1,2] thus allowing the generation of hESC-like cells, i.e. cells which can indefinitely self-renew *in vitro* while maintaining their ability to differentiate into cells of all three germ layers. This technology provides a powerful new tool to investigate tissue differentiation[3-8] and to model genetic diseases in order to explore their physiopathology, providing new concepts of treatment.[9,10] It also opens the way to the production of patient-specific cells for cell therapy.[11]

As far as the hematopoietic lineage is concerned, hiPSC exhibit the same temporal course of development as embryonic stem cells (ESC)[12] with a similar variation in the potentials for erythroid and myeloid differentiation.[12] Hematopoietic progenitors have been generated from myeloproliferative disease hiPSC.[6,13,14] Our previous studies have demonstrated the possibility of generating enucleated erythrocytes containing functional fetal hemoglobin (HbF) from normal hiPSC.[5,15] We now report for the first time in a normal and a pathological erythropoietic differentiation models that hiPSC are intrinsically able to mature into adult hemoglobin synthesizing cells.

The concept of disease corrected hematopoietic progenitors obtained by genetic manipulation was reported in the murin sickle cell disease (SCD) iPSC model[16] and, more recently, in human iPSC from sickle cell patients by correction using homologous recombination strategy.[17,18] SCD is a genetic hemoglobinopathy due to a point mutation in the sixth codon of the beta-globin gene which triggers the synthesis of abnormal hemoglobin (HbS). The physiopathology of SCD may be explained by at least the polymerization of HbS under conditions of stress or hypoxia, which is responsible for cell sickling and the subsequent obstruction of post-capillary microvenules by rigidified and less deformable red blood cells (RBC).[19] A common therapeutic strategy is to attempt to induce the synthesis of HbF in SCD-RBC. In fact, it has been shown that HbF has an attenuating effect on the severity of the disease.[20] By inserting itself into the deoxyHbS polymer, it inhibits intra-erythrocyte HbS polymerization and interrupts its elongation. We show here that sickle cell disease could be modeled *in vivo* in NOD-SCID mice by the achievement of a complete maturation of erythrocyte with Hb S from the hiPSC line obtained from amniotic fluid cells harboring a homozygous HbS/S (hiPS^{SCD}) sickle cell disease. In contrast, the RBC produced *in vitro* only contained HbF that rescues the functionality of these RBCs.

Design and Methods

Generation and characterization of hiPSC

The normal human adult iPSC (hiPS^{n}) cell line was generated from human adult fibroblasts (FD-136) as previously extensively described.[5] Briefly, it was established from a healthy 25-year old woman who gave her informed consent to the procedure. Plasmids pSin-EF2-Oct4-Pur, pSin-EF2-Sox2-Pur, pSin-EF2-Nanog-Pur and pSin-EF2-Lin28-Pur13 were used for reprogramming.

The human sickle cell disease iPSC (hiPS^{SCD}) cell line was generated from human amniotic fluid cells (hAFC) obtained from pregnant women undergoing amniocentesis for diagnostic purposes (Gynecology Department of Beaujon Hospital, France). Briefly, hAFC from sickle cell anemia fetuses were collected with the informed consent of the parents and used for reprogramming to hiPSC. A homozygous HbS mutation in hAFC was confirmed by sequencing. hiPSC were generated according to the technique of Yu *et al.*[2] using lentiviral supernatants for the reprogramming genes Lin28, Nanog, Sox2 and Oct4 employing pSIN-EF2 plasmids[2] (purchased from Addgene). The hiPSC line from sickle cell disease is called PB04 and is recorded in the European registry (*www.hes-reg.eu*). Detailed information on the generation protocol is available in the *Online Supplementary Appendix*.

Erythroid induction and differentiation

The RBC generation protocol was adapted from that we developed for hESC and hiPSC derived from normal fetal and adult fibroblasts.[5] It is made up of two steps: 1) differentiation of hiPSC by formation of embryoid bodies (hEB) during 27 days in a liquid culture medium (LCM) on the basis of IMDM (Biochrom), 450 µg/mL holo-human transferrin (Scipac), 10 µg/mL recombinant human insulin (Incelligent SG; CellGen), 2 IU/mL heparin, and 5% human plasma in the presence of stem cell factor (SCF; 100 ng/mL), Thrombopoietin (TPO; 100 ng/mL), FLT3 ligand (FL;100 ng/mL), rhu bone morphogenetic protein 4 (BMP4; 10 ng/mL), rhu vascular endothelial growth factor (VEGF-A165; 5 ng/mL), interleukin-3 (IL-3; 5ng/mL, interleukin-6 (IL-6; 5 ng/mL) (Peprotech) and erythropoietin (Epo; 3 U/mL) (Eprex, kindly provided by Janssen-Cilag, France);[5] and 2) differentiation/maturation of Day 27 hEB into RBC in the presence of the following sequential combination of cytokines: i) Day 0 to Day 8, dissociated EB were plated at a density of 0.1-1x10^{6} cells/mL in LCM containing 5% human plasma, 2 IU/mL heparin, SCF (100 ng/mL), IL3 (5 ng/mL) and Epo (3 IU/mL); ii) Day 8 to Day 18, the cells were resuspended at 0.3-1x10^{6} cells/mL and cultured in fresh medium supplemented with 10% human plasma, 4 IU/mL heparin, SCF, IL3 and Epo; iii) Day 18 to Day 25, the cells were resuspended at 2-4x10^{6} cells/mL and cultured in fresh medium containing Epo. The cultures were maintained at 37°C in 5% CO_2 in air.

Cells were spun onto a glass slide by cytocentrifugation and stained with May-Grünwald-Giemsa (MGG) and new methylene blue reagents (Sigma) for morphological analyses. A Day-25 enucleated population purified by passage through a deleukocytation filter (Leucolab LCG2, Macopharma, Tourcoing, France) was used to determine the functionality of RBC.

Flow cytometric analysis

Samples were analyzed in a FACS Calibur flow cytometer (Becton Dickinson, San José, CA, USA). The following antibodies were used for flow cytometry: anti-SSEA4-phycoerythrin (PE) and anti-SSEA1-PE (Clinisciences, Montrouge, France); anti-Tra1-60, anti-Tra1-81, goat anti-mouse IgM-PE and goat anti-mouse IgG-PE (Chemicon, Saint-Quentin en Yvelines, France); anti-CD34-PE, anti-CD45-PE, anti-CD45-PC7, anti-CD71-PE (transferrin receptor), anti-CD36-FITC (thrombospondin receptor) and anti-CD235a-PE (glycophorin A) (Beckman Coulter-Immunotech, Marseille, France). FITC-coupled HESCA-1 antibody [clone 051007-4A5] was purchased from Millipore. A primary human anti-RhD antibody (Biotest, Seraclone® reagents for ABO blood typing) and a secondary PE-conjugated goat anti-human antibody (Beckman Coulter) were employed for RhD determination. Controls were native RBC from cord blood (CB-RBC) and SCD-RBC.

New methylene blue (NMB) staining

Cells (2-3x10^{5}) were washed in PBS (pH 7.4) and incubated with 2 µL of NMB in a glass tube for 10 min. The cells were spun onto a glass slide by cytocentrifugation for microscopic exami-

nation and cells with at least 2 granules were scored as reticulocytes.

Semisolid culture assays

BFU-E, CFU-E and CFU-GM progenitors were assayed as previously described.[21]

Composition of hiPSC RBC hemoglobin

hiPSC RBC and RBC from mouse blood samples were analyzed by cation exchange liquid chromatography (CE-LC) (Variant ITM, Beta-Thalassemia Short Program, Bio-Rad Laboratories, Hercules, CA, USA) and reverse phase liquid chromatography (RP-LC). RP-LC of globin chains was performed on a C4 Uptisphere column (4.6 x 250 mm, 5 μm particle size, Interchim, Montluçon, France). Globin chains were eluted with a 2-solvent system (solvent A, 200 mL/L acetonitrile and 0.5 mL/L trifluoroacetic acid (TFA) in water; solvent B, 550 mL/L acetonitrile and 0.5 mL/L TFA in water) and a 4-step RP-LC elution program with a linear gradient of 50-75% solvent B for 80 min, 75-90% solvent B for 10 min and 90-50% solvent B for 10 min, followed by re-equilibration with 50% solvent B for 10 min. The flow rate was 1 mL/min and eluates were detected by UV absorbance at 220 nm. The isolated globin chain fractions were identified by electrospray ionization mass spectrometry.[22] The percentages of the different hemoglobin fractions were determined by CE-HPLC using a Bio-Rad Variant II Hb analyzer (Bio-Rad Laboratories).[23]

Functionality of hiPSC RBC hemoglobin

Oxygen binding curves at equilibrium (OEC) were determined in a Hemox analyzer (TCS Scientific, New Hope, PA, USA) that ensures simultaneous measurement of the dissolved O_2 concentration using a Clark electrode and the optical properties of the RBC suspension linked to the fractional saturation of Hb with O_2. After equilibration under air atmosphere, the gas flow passing through the optical cuvette containing the RBC suspension was switched to N2 just before data acquisition. Deoxygenation was achieved by adding a few microliters of a concentrated sodium dithionite solution and the zero O_2 electrode current was then set accurately. An aliquot of the RBC suspension was taken after the OEC recording and centrifuged to confirm the absence of abnormal hemolysis. OEC measurements were carried out at 37°C in 50 mM Tris (pH 7.4) containing 140 mM NaCl and 5 mM glucose.

Enzyme activities

Digitonin (0.2%) was added to the erythrocytes obtained after leukocyte depletion and Hb was quantified by spectrophotometry using Drabkin's reagent. G6PD and PK activities were determined by measurement of the rate of increase in NADPH absorbance at 340 nm, using a Synchron CX4 Beckman spectrophotometer and reagents from Randox Laboratories (Crumlin, UK) and Roche Diagnostics, respectively. Results were expressed in units per gram of Hb.

Deformability measurements

Osmotic scan ektacytometry (Technicon, Bayer Corp., Diagnostics Division, Tarrytown, NY, USA) is a laser diffraction method that measures erythrocyte elongation in a laminar shear stress as a continuous function of the osmolarity of the medium. Application of an increasing osmotic gradient produces a signal called the deformability index (DI). Analysis of the DI curve provides a measure of the dynamic deformability of the cell membrane as a function of the osmolality at a constant applied shear stress of 170 dynes/cm^2. DImax, expressed in arbitrary units and defined as the maximum value of DI, is normally related to the mean surface area of the RBC. OHyper is the osmolality at which DI decreases to half the value of DImax in the hypertonic region of the curve, and is inversely related to the MCHC. Purified Day-25 hiPSC-RBC were resuspended in 4% polyvinylpyrrolidone solution and exposed to an increasing osmotic gradient (60 to 450 mOsm/kg). Normal adult DI curves were obtained by analyzing 70 normal blood samples.

Sickling test

The sickling test aims to induce HbS polymerization under hypoxia. Samples (10^6 cells) of hiPSC^{SCD}-RBC and native RBC from adult SCD patients (SCD-RBC) were exposed to an atmosphere poor in oxygen, under a stream of nitrogen for 2 h. The cells were then fixed in 2% paraformaldehyde, spun onto a glass slide by cytocentrifugation, and stained with May-Grünwald-Giemsa reagent (Sigma) for microscopic morphological analysis.

Adhesion assay

Cell adhesion to laminin 511/521 was measured under physiological flow conditions in a capillary flow chamber. Laminin 511/521 (5 μg, Sigma-Aldrich) from human placenta was immobilized on the internal surface of uncoated μL Luer$^{0.1}$ microslides (Ibidi, Munich, Germany) (internal channel dimensions: length 50 mm, width 5 mm, height 0.1 mm) at 4°C overnight. hiPSC^{SCD}-RBC and SCD-RBC were washed 3 times in 10 mL of PBS and resuspended in Hanks' buffer containing 0.4% BSA at a hematocrit of 0.25%. The cell suspensions were perfused through the microslides at a shear stress of 0.4 dynes/cm^2 for 5 min and washed out with Hanks' buffer at 0.5, 1, 1.5 and 2 dynes/cm^2, each for 5 min. After each wash, adherent cells were counted in 20 representative areas along the centerline of the microslides using an AxioObserver Z1 microscope and AxioVision 4 analysis software (Carl Zeiss, Le Pecq, France). Images of the same 20 areas were obtained throughout each experiment by applying the 'Mark and Find' module of AxioVision analysis software.[24]

Study in the NOD/SCID mouse model

Experiments were performed in parallel with both normal and SCD hiPSC-derived cells.

All experiments and procedures were performed in compliance with the French Ministry of Agriculture regulations for animal experimentation and approved by the institutional ethics committee.

NOD/SCID-LtSz-scid/scid (NOD/SCID) mice were raised under sterile conditions. Six to 8-week old mice were sublethally irradiated with 2.5 Grays from a ^{137}Cs source (2.115 Gy/min) before cell injection. To saturate the reticuloendothelial system, human type-O RBC were injected intraperitoneally (4-5x10^9 cells per mouse). After 24 h, Day 7 erythroid cells were washed, labeled with CFSE, and resuspended in PBS containing 0.1% BSA without cytokines, and then injected into the retro-orbital vein of the mice (10^8 cells/mouse). Controls received heparinized peripheral blood under the same conditions. The human cells were detected as CFSE-labeled by flow cytometry in 5 μL samples of heparinized blood drawn from the mice by retro-orbital vein puncture at various time points. Three animals were used at each time point. On Days 3 and 7 post-injection, the cells from peripheral blood were washed. CFSE positive cells labeled with an anti-CD235a antibody (Miltenyi) were sorted according to their human CD235a expression, and analyzed for the different globin chain fractions by capillary electrophoresis (CE-LC) and reverse phase (RP-LC) liquid chromatography techniques.

The low number of human RBC in the blood of NOD/SCID mice did not allow RBC enucleation to be analyzed. So we

focused on identifying the human globin chains among the murine globin chains by analyzing the total blood of the mice.

Results

Generation and characterization of hiPSC

Details of generation and characterization of the hiPSC^{SCD} are presented in the *Online Supplementary Appendix* and *Online Supplementary Figures S1, S2 and S3*. Characterization and erythroid differentiation of the normal human adult iPS cell line (hiPS^{nl}) have already been extensively described.[5]

Differentiation of hiPSC^{SCD} into EB conditioned for erythroid commitment

We used the conditions previously described by our group[5] to generate enucleated RBC from hiPSC *in vitro* (n=6).

Day 0 hiPSC^{SCD} expressed a high level of markers of undifferentiated human cells (TRA-1-60, TRA-1-81 and SSEA4). As expected, expression of SSEA1 was low and hematopoietic markers (CD45, CD34, CD36 and CD235a) were undetectable. CD71 was highly expressed, indicating the high proliferative capacity of the cells (*Online Supplementary Figure S4*). During EB differentiation, the expression of markers of undifferentiated human cells decreased significantly to remain weakly positive (1-2%). D27-EB significantly expressed the hematopoietic and erythroid markers CD45, CD34, CD45/CD34, CD71, CD36 and CD235a (*Online Supplementary Figures S4B and C, and S5*). Furthermore, the low number of progenitors (CFC) (15±3/10^5 cells) (n=3) demonstrates the low clonogenic potential.

Differentiation/maturation of EB to the erythroid pathway

Dissociated D27-EB were re-plated according to the liquid culture protocol for erythroblastic differentiation/maturation.[5,25] The erythroid commitment of EB was complete after four days of liquid culture with generation of up to 99% erythroblasts (n=6). On Day 25 of the erythroid phase, the population contained 20-26% RBC and 74-80% orthochromatic erythroblasts (Figure 1). The degree of maturity of the RBC was followed by flow cytometric analysis of membrane antigens during culture (Days 0–25). At the end of culture, the RBC strongly expressed CD235a and CD71 and weakly expressed CD36, which is consistent with a reticulocyte phenotype (*Online Supplementary Figure S6A and B*). Overall amplification potential was seen as: 1x10^6 hiPSC giving rise to 2-5.2 x10^6 D27-EB, which generated 15-28.3x10^8 mature erythroid cells.

Analysis of RBC derived from SCD patient-hiPSC (hiPSC^{SCD} -RBC) on Day 25

After filtration, the percentage of reticulocytes was 70±4% by new methylene blue staining (Figure 2A). Immunophenotypic characterization (CD235a, CD71 and CD36) of the enucleated cells demonstrated a reticulocyte profile (Figure 2B). The RBC had a glucose-6-phosphate dehydrogenase (G6PD) content of 99±8 units and a pyruvate kinase (PK) level of 135±23 units per gram of Hb (n=3), as expected from a young erythroid population.[25,26]

Hb analysis of erythroid cells on Day 25

To analyze the Hb type, we combined identification of the globin chains by RP-LC and mass spectrometry with a study of tetrameric Hb by CE-HPLC. We observed: i) 30±1.8% gamma-G, 46±1% alpha, 22±2% gamma-A+epsilon, and 2±1% zeta chains; with ii) a largely predominant synthesis of fetal chains (gamma-G + gamma-A: 52%). These results were confirmed by mass spectrometric identification of the fractions eluted by RP-LC (n=3) (Figure 3A). An analysis of tetrameric Hb by capillary electrophoresis high performance liquid chromatography (CE-HPLC) showed the synthesis of 86±5% HbF and 14±4% Hb Gower 2 (n=3) (Figure 3B), in agreement with the RP-LC and mass spectrometry data demonstrating the synthesis of HbF in its tetrameric form in hiPSC^{SCD} -RBC. The nearly exclusive synthesis of HbF (86±5%) suggested a homogeneous distribution of HbF in all RBC, since it is known that there is a close linear correlation between the percentage of F cells and the level of HbF.[27]

Functionality of hiPSC^{SCD} -RBC

Oxygen binding curves at equilibrium (OEC) were determined in a Hemox analyzer. RBC exhibited an oxyphoric function close to that of cord blood-RBC (CB-RBC) which also contain a high proportion of HbF. The co-operativity index (n_{50}Hill) was similar to that of CB-RBC, and we observed only a small increase in O_2 affinity ($P_{50}O_2$), possibly due to a slightly higher HbF content in hiPSC^{SCD} -RBC as compared to the CB-RBC control (n=3) (Figure 3C). Overall, hiPSC^{SCD}-RBC bound O_2 in a similar manner to CB-RBC, and obviously with a higher O_2 affinity than that of adult RBC.

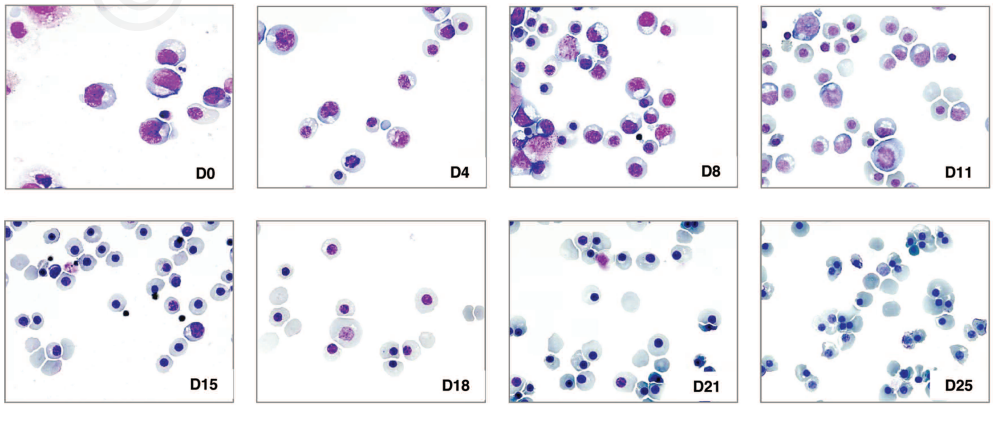

Figure 1. Morphology of the erythroid cells generated from hiPSC^{SCD}. Photographs of the cells on Days 0, 4, 8, 11, 15, 18, 21 and 25 of erythroid culture after MGG staining (magnification 630x). One representative experiment from n=6 experiments.

Deformability measurements

Osmotic scan ektacytometry measures erythrocyte elongation as a signal termed the deformability index (DI).[28] The DImax of hiPSC^{SCD}-RBC (0.496) was comparable to that of RBC from healthy donors (0.38-0.58), confirming their capacity to deform normally. The parameter OHyper determined under hyper osmolar conditions (349 mOsm/kg) confirmed the good hydration status of hiPSC^{SCD}-RBC as compared to a normal control (340-360 mOsm/kg). These results showed that the rheological properties of hiPSC^{SCD}-RBC were maintained (Figure 3D).

Sickling test

The sickling test aims to induce HbS polymerization under hypoxia. In an atmosphere poor in oxygen, hiPSC^{SCD}-RBC behaved like normal reticulocytes, displaying no or very little sickling (3±1%), unlike the SCD-RBC used as a positive control (63±3%) (n=3) (Figure 4A and B).

Adhesion assay

The glycoproteins Lu/BCAM (CD239) have been identified as the only erythroid receptors for laminin involved in the abnormal adhesion of SCD-RBC to the vascular endothelium.[24] Cell adhesion to laminin was measured under physiological flow conditions using a capillary flow chamber (n=3). hiPSC^{SCD}--RBC did not adhere to laminin, whereas SCD-RBC exhibited 80-120 fold enhanced adhesion at a shear stress of 2 dynes/cm^2 (Figure 4C).

Confirmation of the presence of the S mutation in erythroid cells

To confirm that the erythroid cells (Day 7 and Day 25) were genetically matched to the hiPSC^{SCD}- line, we performed sequence analyses to assess the presence of the S mutation in the first exon of the beta-globin gene (*Online Supplementary Figure S3B*). All erythroid cells carried the S mutation (*Online Supplementary Figure S3C and D*) com-

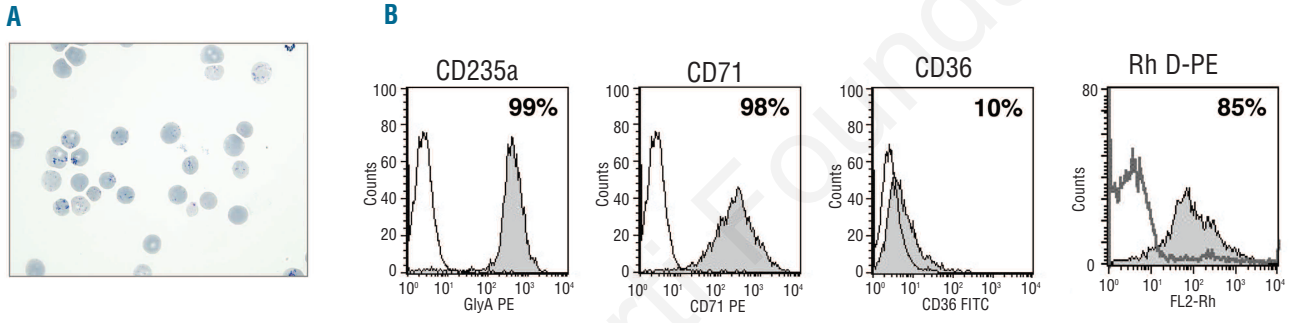

Figure 2. Characterization of hiPSC^{SCD} –RBC. (A) Photographs of reticulocytes stained with new methylene blue on Day 25 of culture (magnification 500x). (B) Flow cytometric analysis of erythroid markers (CD71, CD36 and CD235a) and RhD antigen expression in hiPSC^{SCD} -RBC on Day 25. Open histograms: negative controls. Solid histograms: cells stained with antibodies. One representative experiment from 6 experiments.

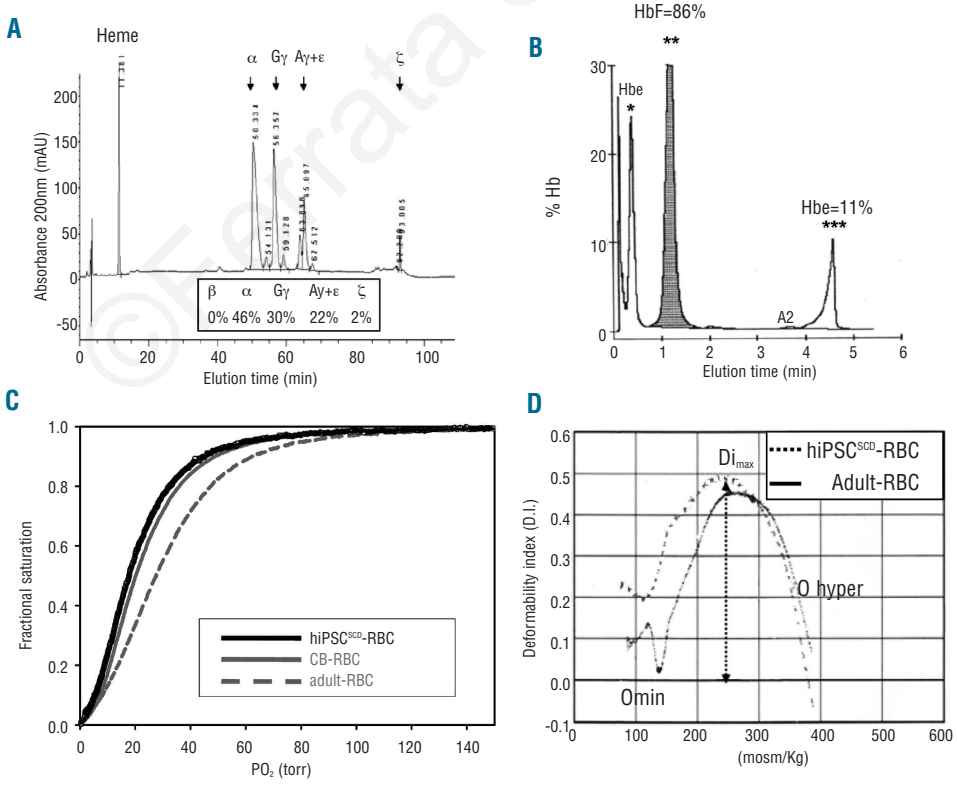

Figure 3. Hemoglobin analyses and functionality of erythroid cells generated from hiPSC^{SCD}. (A) Representative RP-LC profiles of the globin chains identified by mass spectrometry in Day 25 mature cultured RBC. (B) CE-HPLC analyses of the Hb in Day 25 mature cultured RBC. *Embryonic hemoglobins (Hbe) [Portland 1 (zeta-2, gamma-2), Portland 2 (zeta-2, beta-2) and Gower 1 (zeta-2, epsilon-2) ** fetal hemoglobin (HbF, alpha-2, gamma-2) and * embryonic hemoglobin (Hbe) [Gower 2 (alpha-2, epsilon-2) One representative experiment from 3 experiments. (C) Equilibrium oxygen binding of hiPSC^{SCD} -RBC (black curve) compared to fetal RBC from cord blood (CB-RBC) (gray curve) and adult-RBC (dashed curve). One representative experiment from 3 experiments. (D) Deformability profiles. The curves define a maximum deformability index (DImax) in an iso-osmolar medium. Profile for Day 25 hiPSC^{SCD}-RBC (dashed curve) as compared to adult-RBC (black curve).**

pared to the original sequence of human beta-globin as control (*Online Supplementary Figure S3A*).

Studies in the NOD/SCID mouse model

Characterization of the populations before their in vivo injection

Day 7 erythroid cells from both hiPCs cell lines were labeled with the intracellular fluorescent dye carboxyfluoroscein succinimidyl ester (CFSE)[29] and infused into sublethally irradiated NOD/SCID mice to follow their *in vivo* fate. The mice received 10^8cells/animal, consisting of 95±3% erythroblasts (Figure 5A and B). For each cell line, 3 animals were used at each time point and subsequently pooled for analyses. The population was CD36, CD235a and CD71 positive with a large number of late erythroid progenitors (*Online Supplementary Table S2*). Analysis of the globin chains of Day 7 erythroid cells from both hiPCs cell lines before their *in vivo* injection by RP-LC and mass spectrometry showed for: i) hiPSC^{nl}: 30% gamma-G, 46% alpha, 22% gamma-A+epsilon, and 2% zeta chains. Synthesis of fetal chains was largely predominant (52% gamma-G + gamma-A) (Figure 5C); and ii) for hiPSC^{SCD}: 29% gamma-G, 44% alpha, 15% gamma-A+epsilon, and 7% zeta chains. Synthesis of fetal chains was largely predominant (44% gamma-G + gamma-A) (Figure 5D).

Globin switching in vivo

To follow the *in vivo* fate of erythroid precursor cells and identify putative Hb modification, we infused 10^8 Day-7 erythroid cells, pre-labeled with the intracellular fluorescent dye carboxyfluoroscein succinimidyl ester (CFSE), into sublethally irradiated NOD/SCID mice. We took daily samples of blood from the mice by retro-orbital vein puncture and quantified the CFSE$^+$ cells. They were detectable for four days at levels between 0.3 and 0.7%.

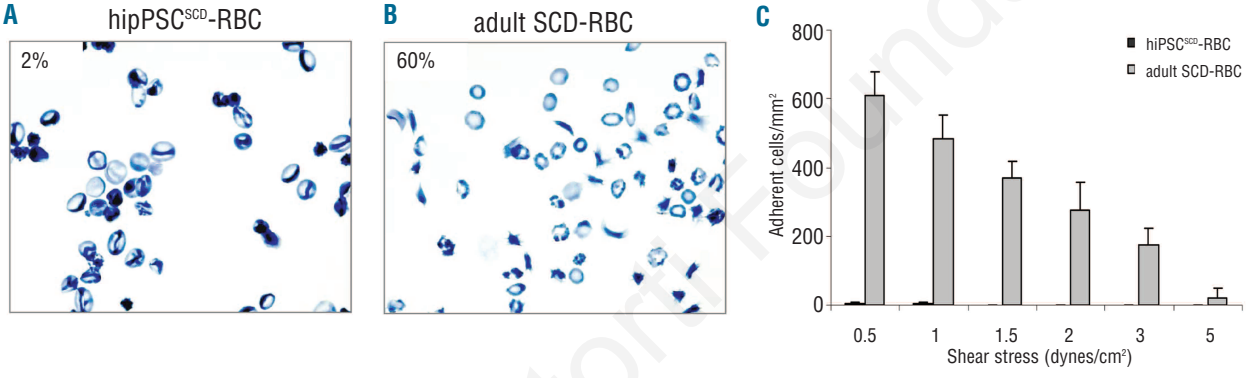

Figure 4. Characterization of hiPSC^{SCD} -RBC subjected to stress. (A and B) Morphology of erythroid cells exposed to an atmosphere poor in oxygen. Photographs of (A) hiPSC^{SCD}-RBC (Day 25) and (B) SCD-RBC (magnification 630x). (C) Number of RBC adhering to laminin under flow at different wall shear stresses. Results are the mean ± SEM of 3 independent experiments.

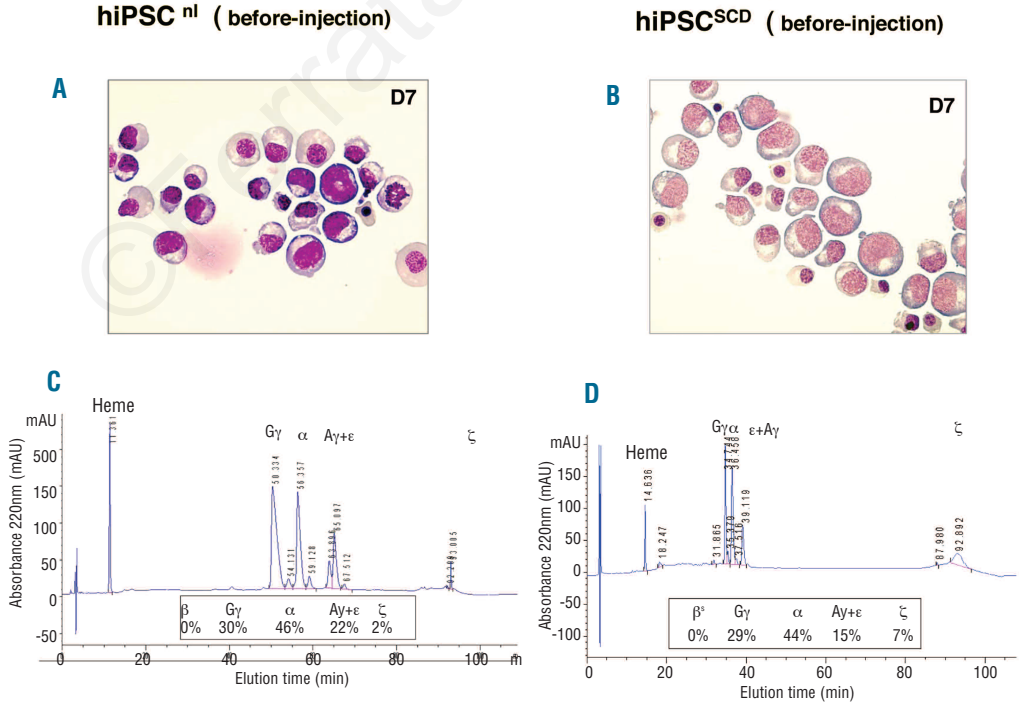

Figure 5. Characterization of the erythroid cells before injection into NOD/SCID mice. Photographs of the erythroid cells on Day 7 of culture (A) hiPSC^{nl} (B) hiPSC^{SCD}. Representative from 3 experiments of RP-LC profiles of the human globin chains identified by mass spectrometry on Day 7 before injection into NOD/SCID mice, (C) hiPSC^{nl}, (D) hiPSC^{SCD}.

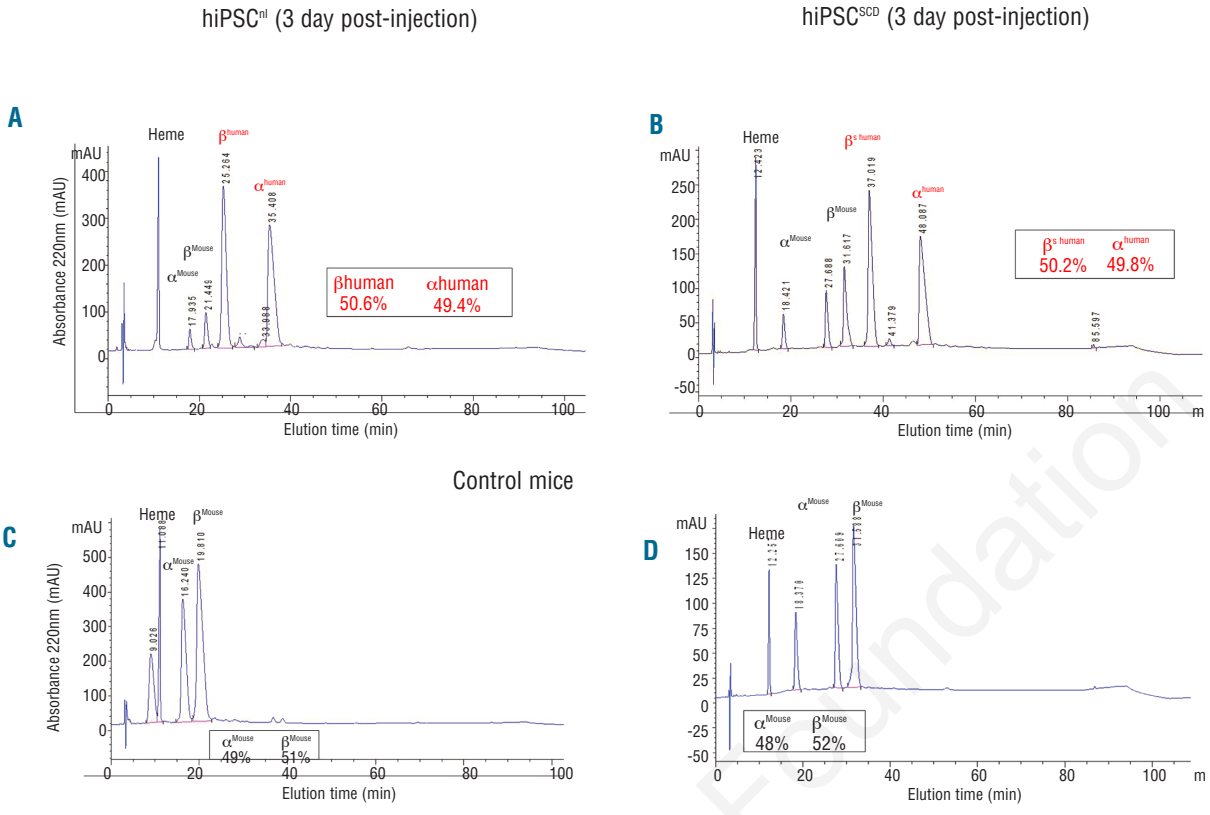

Figure 6. Characterization of human erythroid cells injected into NOD/SCID mice. Representative RP-LC and CE-HPLC profiles of the human goblin chains identified by mass spectrometry on: 3 days post-injection (A) hiPSC^{nl}, (B) hiPSC^{SCD}, (C and D) control mouse blood.

Sorted CD235a$^+$ human cells represented 2-10% of the total population.

At three days post injection, the modulation of the human globin chains was complete with: i) for hiPSC^{nl}: 50.6% beta and 49.4% alpha chains (Figure 6A); and ii) for hiPSC^{SCD} 50.2% S beta and 49.8% alpha chains (Figure 6B). As control, we analyzed in parallel the murine globin chains (Figure 6C and D). It should be noted that beyond four days no CFSE$^+$ cells were observed in the peripheral blood of the mice. Furthermore, analysis of globin chains by RHPLC at 7-day post injection reveals the absence of human chains which, as expected, correlates the absence of CFSE$^+$ cells.[25,30,31]

Discussion

To exploit the potential of hiPSC for the generation of RBC, we had previously designed a cell culture protocol which directly commits normal hiPSC to definitive erythropoiesis,[5] i.e. the production of enucleated RBCs. However the synthesis of hemoglobin was blocked at the stage of fetal hemoglobin (HbF). The almost exclusive synthesis of HbF was not related to the origin of the reprogrammed cells since, *in vitro*, its production predominates whether one starts from hiPSC lines obtained from human fetal (IMR90) or adult fibroblast (FD-136) cells or from an hESC line (H1).[5] We now report that the synthesis of HbF is not the consequence of an intrinsic defect of terminal maturation of hiPSC, since a complete switch from HbF to adult Hb could be obtained after *in vivo* injection of progenitors into NOD/SCID mice. We, therefore, show for the first time that hiPSC can reach complete maturation, i.e. nucleus expulsion and adult Hb synthesis, in the model of erythropoiesis. This suggests that hiPSC-derived erythropoiesis mimics that of human development and recapitulates accordingly the globin gene switch. It also indicates that complete maturation of immature erythroid cells can take place under the influence of an adult hematopoietic microenvironment, as we have already described for cord blood cells,[30] and in accordance with observations of Hayakawa *et al.*[32] that the combination of the murine marrow microenvironment and cytokines is sufficiently potent to mimic the normal post-natal human hemoglobin switching process. We assume that the delay required for the cells to achieve complete hemoglobin switching is linked to their stage of maturation.[33]

Several studies have shown that the *in vitro* erythroid differentiation of pluripotent stem cells results in the expression of fetal and embryonic globins rather than adult beta globin.[5,34-36] In demonstrating an *in vivo* assay that the erythroid cells derived from hiPSC can adopt the characteristics of adult cells, we show that the embryonic and/or fetal expression pattern displayed by pluripotent stem cell derivatives is the consequence of an incomplete recapitulation of ontogenic hematopoietic differentiation

during *in vitro* culture.

Further studies will be needed to eliminate this defect of the *in vitro* differentiation method and better identify the factors necessary to obtain terminally differentiated adult cells *in vitro*. Although based on two iPSC lines, the results validate the proof of concept that iPSC are capable of full terminal maturation, at least in the erythroid pathway.

Using a model of hereditary hemoglobin disease, sickle cell disease, we show that hiPSC also constitutes a model for cell therapy which consists of taking advantage of the culture conditions to modify the cell characteristics in a therapeutic setting, without the use of gene therapy or drugs. Indeed, the presence of HbF in re-programmed cells can be used beneficially to functionally replace the deficient adult globin by normal HbF. As regards the possible use for autologous transfusion, it is clear that RBC produced *in vitro* are no longer capable of changing the type of hemoglobin in the absence of nuclei. The almost exclusive presence of functional tetrameric HbF confers on these hiPSC^{SCD}-RBC the characteristics of cord blood RBC[25] in terms of oxygen transport capacity, deformability, sickling and adherence, despite the persistence of the homozygous mutation in erythroid progenitors.

The present therapeutic arsenal for SCD relies on transfusion and on the *in vivo* induction of HbF in SCD-RBC that, through insertion into the HbS polymer during its formation, reduces the cell sickling. The medicinal approach using hydroxyurea is that most widely employed. However, its mechanism is subject to debate since, if it is thought to intervene by inducing the synthesis of HbF,[20] it could nonetheless act through other mechanisms, notably by modulating the adhesion of sickle cell RBC to the endothelium.[37] In the light of the work of Hanna *et al.*[16] in animals, hiPSC could represent a future tool for gene therapy based on targeted genomic modification. By injecting into homozygous SCD mice differentiated cells derived from iPSC that were repaired *in vitro* by homologous recombination of the hemoglobin gene, these authors were, in fact, able to reconstitute a heterozygous hematopoietic system.

Our results suggest that generating patient-specific blood cells from their own pluripotent stem cells could benefit these patients and increase the possibilities of treatment.[15] We recently established the proof of principle for transfusion of in *vitro* generated enucleated RBC in humans.[31] The approach we now report provides an alternative to functional normalization of sickle cell RBC for auto transfusion purposes in the hiPSC model.[5,11,15]

In conclusion, using the model of hematopoiesis, we show for the first time that hiPSC can achieve terminal maturation, *in vitro* in terms of enucleation and *in vivo* in terms of synthesis of adult Hb. Furthermore, the data for SCD suggest that hiPSC could represent a very valuable model system to study genetic disorders of erythropoiesis. Since iPSC cells can proliferate indefinitely and can be selected for a phenotype of interest,[5,11,15] they are potential candidates to organize complementary sources of RBCs for transfusion. However, the procedures need to be optimized before their use in clinical practice can be considered. Several crucial points remain to be resolved. Notably, these include the optimization of erythrocyte proliferation and differentiation, and the definition of good manufacturing practice (GMP) conditions for industrial production.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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