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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Online Supplementary Appendix

Generation and characterization of hiPSC^{SCD}

hAFC were expanded for seven days in Amniochrome II medium (Lonza group Ltd, Switzerland). hAFC (10^5 cells) were infected at the following multiplicities of infection (MOI) for each lentiviral supernatant [Addgene supplier reference number in brackets]: pSin-EF2-Lin28-Pur [16580]: MOI 2; pSin-EF2-Nanog-Pur [16578]: MOI 10; pSin-EF2-Oct4-Pur [16579]: MOI 4; pSin-EF2-Sox2-Pur [16577]: MOI 4. After 24 h, 10^5 infected hAFC were plated on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) and cultured in human ESC medium (DMEM/F12 culture medium supplemented with 20% knockout serum replacement, 10 ng/mL bFGF, 0.1 mM non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1x penicillin/streptomycin, all from Invitrogen). After 15-25 days of culture, single colonies with human ESC-like morphology were manually picked and further expanded and characterized.

The pluripotency of hiPSC^{SCD} was analyzed by FACS analysis and teratoma assay. The expression of pluripotent stem cell markers was detected by Macsquant flow cytometry with MacsQuantify software (Miltenyi Biotech). Briefly, $2.5x10^5$ cells were stained with FITC-conjugated mouse anti-human ESCA-1 and PE-conjugated mouse anti-human SSEA-4 antibodies (Flow Cellect human ESC surface marker characterization kit, Millipore) according to the manufacturer's instructions. To assess Tra1-60 expression, 10^5 cells were stained in 10 μL of PBS with 1 μL of a PE-conjugated mouse anti-human Tra1-60 antibody or 1 μL of a PE-conjugated irrelevant isotype control (BD Biosciences). ESC and iPSC stain positively for Tra1-60, HESCA-1 and SSEA-4. Teratoma formation was induced by injection of two mice, respectively, with $0.5x10^6$ and $3x10^6$ undifferentiated hiPSC^{SCD} cells into the hind leg muscle of NOD/SCID mice. Tumors were removed 6-8 weeks after injection and immediately fixed in 4% paraformaldehyde in PBS before embedding in paraffin. Tumor sections were stained with hematoxylin and eosin and analyzed for the presence of tissues representative of the three germ layers (ectoderm, mesoderm and endoderm).

Gene expression analysis

The expression of exogenous transgenes was measured by Quantitative-PCR on MX3000p instrument (Agilent Technologies). Expression levels were calculated by using the delta-delta-Ct formula using the normalizing gene GAPDH. Primers specific for transgenes or for endogenous transcripts were used (*Online Supplementary Table S1*). For transgene expression, results were calibrated against a single Hela cell line infected with the 4 pSIN-EF2 lentiviruses. For endogenous gene expression, results were calibrated against the original amniotic-derived fluid cell line before cellular reprogramming.

Cells and culture conditions

The hiPSC^{SCD} used in this study were at an average of 25-30 passages and had a normal karyotype. The cells were cultured on mitomycin C (20 μg/mL)-inactivated MEF in DMEM/F12 supplemented with 20% knockout serum replacement, 1 mM L-glutamine, 100 μM β-2-mercaptoethanol, 10 ng/mL bFGF and 0.5% penicillin/streptomycin (Invitrogen).

PCR product purification and sequencing

To confirm the presence of the S mutation in the first exon of the beta-globin gene, DNA was purified from frozen hiPSC^{SCD} cell pellets by phenol/chloroform extraction and PCR analysis of genomic DNA was performed using the following primers:

HBB-F: AACTCCTAAGCCAGTGCCAGAAGA and HBB-R: GCACTTTCTTGCCATGAGCCTTCA.

The expected 549 bp band was purified from a 1.5% agarose gel using a GeneClean kit (Qbiogene). The purified DNA was

sequenced on both strands (MilleGen, France) using a dye-labeled dideoxynucleotide system with the following sequencing primers:

HBBSEQ-F: AACTCCTAAGCCAGTGCCAGAAGA and
HBBSEQ-R: TTCTCTGTCTCCACATGCCCAGTT.

Sequence analysis was performed with APE software (Wayne Davis, Utah University) and results were compared to an original sequence of human beta-globin (UCSC ID: uc001mae.1)

Results

The source of cells for generating hiPSC^{SCD} were human amniotic fluid cells (hAFC) from SCD fetuses. The homozygous HbS mutation in hAFC was confirmed by sequencing.

hiPSC were generated according to the technique of Yu *et al.,*[1] using lentiviral supernatants for the reprogramming genes Lin28, Nanog, Sox2 and Oct4. The hiPSC line from SCD is called PB04 and is recorded in the European registry (*www.hesreg.eu*).

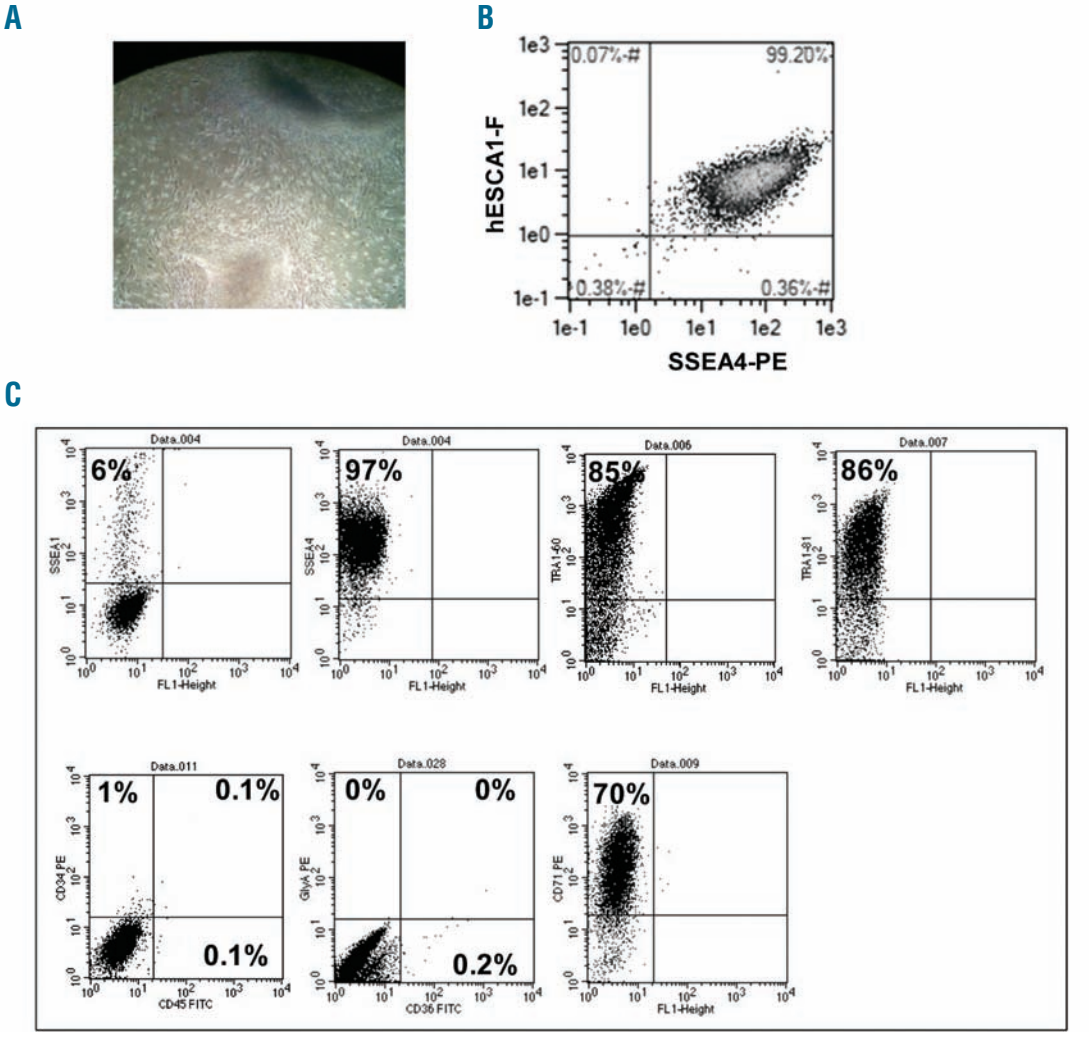
The hiPSC^{SCD} cells showed all the essential characteristics of pluripotent stem cells: (hiPSC^{SCD}) displayed the morphology and characteristics of human embryonic stem cells with expression of Tra1-60, Tra1-81, SSEA-4, hESCA-1 (*Online Supplementary Figure S1*). hiPSC^{SC}D also expressed the endogenous Oct4, Sox2, Nanog, Lin28, KLF4 and CMYC genes at levels comparable to human embryonic stem cells, and down-regulated the transgenic expression after reprogramming (*Online Supplementary Figure S2B*). hiPSC^{SCD} were functionally pluripotent as they formed mature teratomas *in vivo*, containing tissues of all three germ layers (ecto-, meso- and endoderm) (*Online Supplementary Figure S2A*). A homozygous S mutation in the beta-globin gene of sickle cell disease hiPSC^{SCD} cells was confirmed by genomic sequencing (*Online Supplementary Figure S3B*).

References

1. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007;318(5858):1917-20.

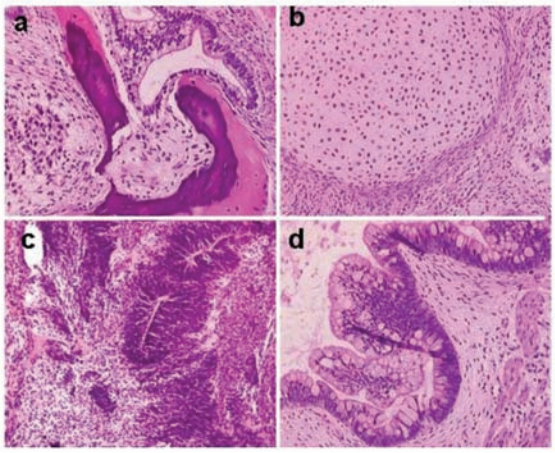
Online Supplementary Table S1.

Primers :	
Transgenic expression : Common reverse primer specific for the pSIN-EF2 lentiviral plasmid : pSINREV : AGATGCATGCGGATCCTTCGAACT Forward primers in the transgenic open reading frame : OCT4-F : CTGGGCTCTCCCATGCATTCAAA SOX2-F : TCACATGTCCCAGCACTACCAGA LIN28-F: AGTGCACAGGGAAAGCCAACCTA NANOG-F: AACATGCAACCTGAAGACGTGTG	Endogenous expression : primers specific of the 5' UTR region OCT4-UTR : AGTTTGTGCCAGGGTTTTTG + ACTTCACCTTCCCTCCAACC SOX2-UTR : AGTCTCCAAGCGACGAAAAA + TTTCACGTTTGCAACTGTCC LIN28 UTR : AGTGGCCTGGATAGGGAAGT + CTTGGCTCCATGAATCTGGT NANOG UTR : TTTGGAAGCTGCTGGGGAAG + GATGGGAGGAGGGGAGAGGA CMYC-endo : TGCCTCAAATTGGACTTTGG + GATTGAAATTCTGTGTAACTGC KLF4-endo : GATGAACTGACCAGGCACTA + GTGGGTCATATCCACTGTCT

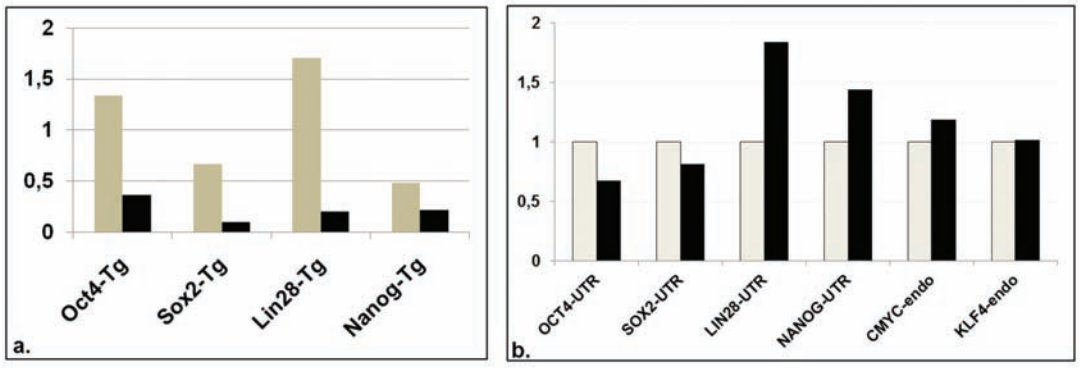


Online Supplementary Figure S1. Morphological and phenotypical characterization of early-passage hiPSCSCD cells (A) hiPSC^{SCD} colony, passage 5. (B) Immunocytometry co-staining of undifferentiated hiPSC^{SCD} cells, with HESCA1 and SSEA4 antibodies. (C) Phenotypic analysis of hiPSC^{SCD}: Representative dot plots from undifferentiated cell markers (SSEA-4, Tra1-60 and Tra1-81), as determined by flow cytometry

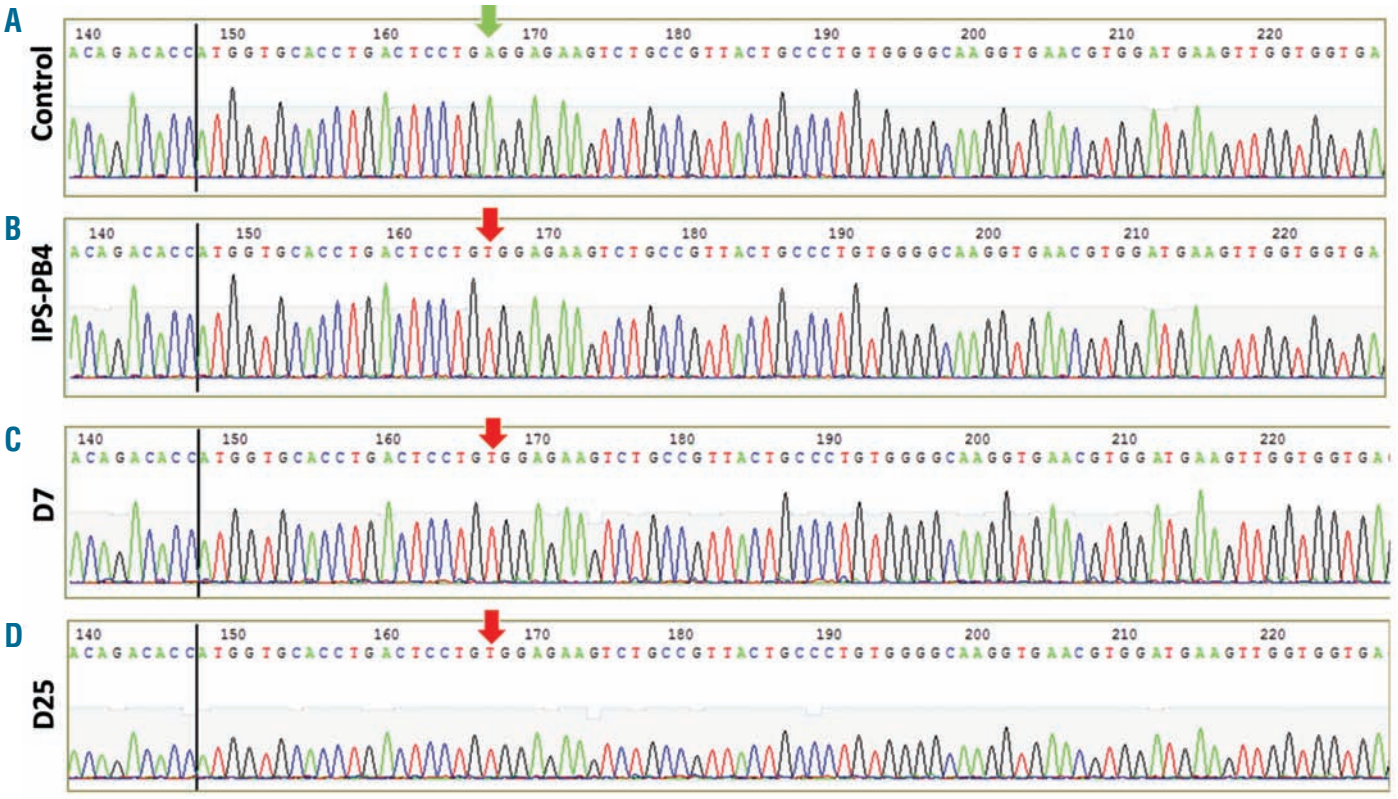
A



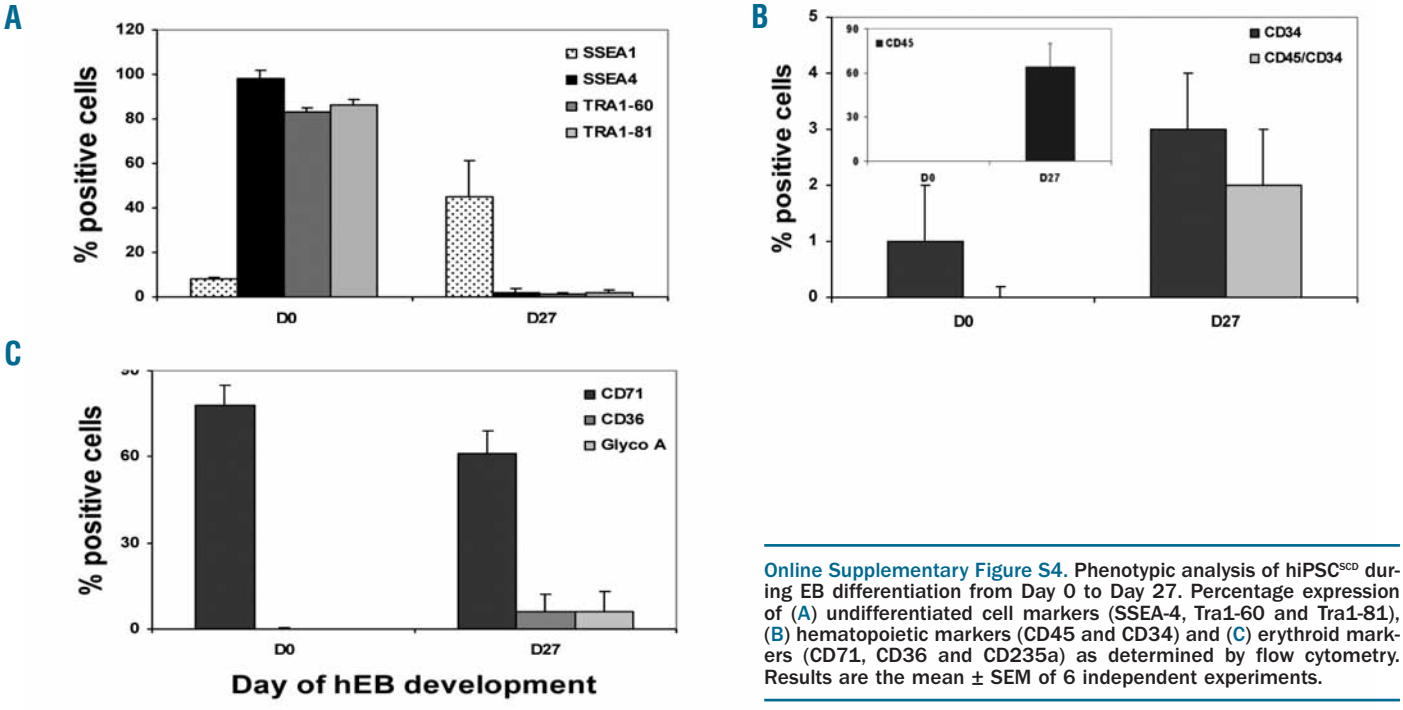
B



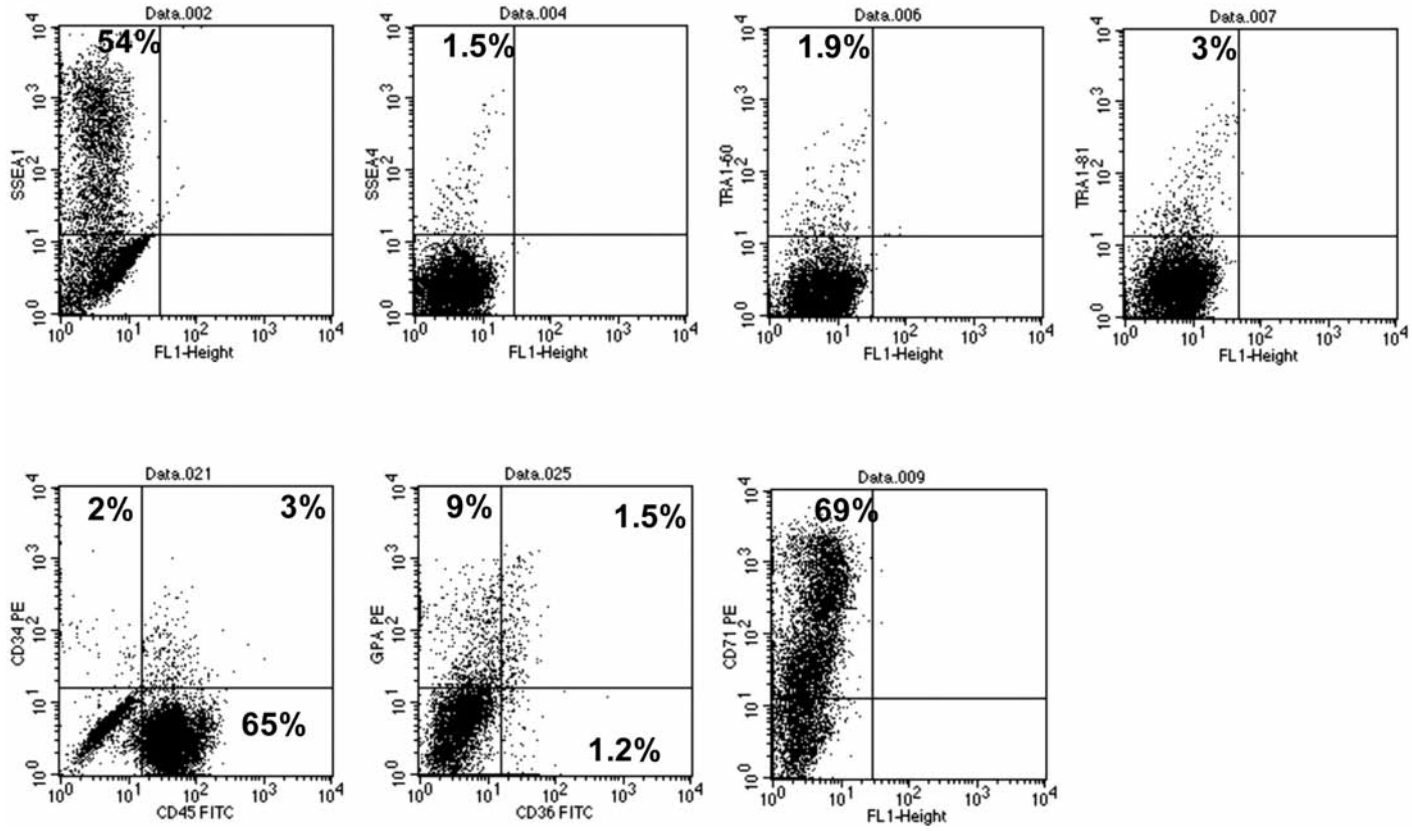
Online Supplementary Figure S2. (A) Histology of teratoma tissues derived from hiPS^{CSCD}. (a) Embryonic-like bone tissue with dark purple calcium deposites (Osteoid substance). (b) Cartilaginous area with embryonic-like chondroblast and safranophilic matrix. (c) Neural crest lining with palisadic pattern suggestive of neuronal tube. (d) Glandular tube with mild dysplasia and mucus droplets reminding of intestinal structures. (B) Gene expression in the hiPSC^{SCD} cell line (Passage 10) (a)Transgene expression: expression of transgenes in hiPSC^{SCD} cell line (black bars) was compared by quantitative PCR with that of infected amniotic-derived cells collected 24 h after infection (gray bars). (b) Endogenous gene expression: expression of the OCT4, SOX2, NANOG, LIN28, CMYC and KLF4 endogenous genes was performed with primers contained or spanning the untranslated regions. Expression in the hiPSC^{SCD} cells (black bars) was compared to the H9 human embryonic stem cells (passage 22) (gray bars).



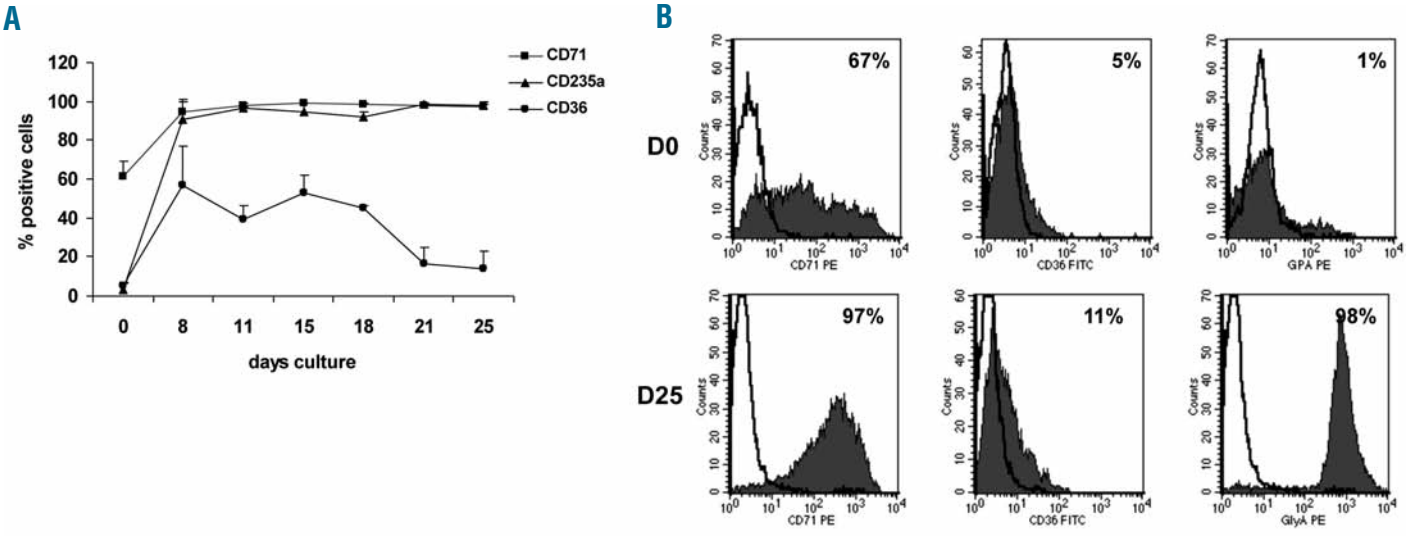
Online Supplementary Figure S3. Confirmation of the presence of the S mutation by genomic sequencing. The first exon of the beta-globin gene was sequenced in (A) the original sequence of human beta-globin used as a control, (B) hiPSC^{SCD} from a patient carrying a homozygous S mutation leading to sickle cell disease. (C) Day 7 erythroblasts differentiated *in vitro* from hiPSC^{SCD} and (D) Day 25 erythroblasts differentiated *in vitro* from hiPSC^{SCD}. The start codon (ATG) is indicated by a black bar. The normal allelic form of beta-globin is indicated by a green arrow and the A>T mutation in patient samples by a red arrow.



Online Supplementary Figure S4. Phenotypic analysis of hiPSC^{SCD} during EB differentiation from Day 0 to Day 27. Percentage expression of (A) undifferentiated cell markers (SSEA-4, Tra1-60 and Tra1-81), (B) hematopoietic markers (CD45 and CD34) and (C) erythroid markers (CD71, CD36 and CD235a) as determined by flow cytometry. Results are the mean ± SEM of 6 independent experiments.



Online Supplementary Figure S5. Phenotypic analysis of D27-hEB. Representative dot plots from undifferentiated cell markers (SSEA-4, Tra1-60 and Tra1-81) hematopoietic markers (CD45 and CD34) and erythroid markers (CD71, CD36 and CD235a) as determined by flow cytometry.



Online Supplementary Figure S6. (A) Analysis of RBC maturation. Kinetics of expression of erythroid markers (CD71, CD36 and CD235a) during erythroid differentiation of hiPSC as determined by flow cytometry. Results are the mean ± SEM of 6 independent experiments. (B) Phenotypic analysis during erythroid differentiation of hiPSC^{SCD}. Representative expression from 6 experiments of erythroid antigens (CD71, CD36 and CD235a) from Day 0 after hEB dissociation to Day 25 as determined by flow cytometry.

Online Supplementary Table S2. Flow cytometric analyses of Day 7 expanded erythroid cells and progenitor cell counts in semi-solid cultures. Results are the mean ± SEM of 3 independent experiments. (A) hiPSC^{nl}. (B) hiPSC^{SCD}.

A

	Positive cells (%)						Clonogeneic progenitors (10^5 cells)		
	CD45	CD34	CD45/CD34	CD71	CD36	CD235a	BFU-E	CFU-E	CFU-GM
Day 0	38±2	4±0.9	1.25±0.4	45±10	2.5±0.2	1±0.5	12±2	2.2±1	<1
Day 7	5±0.5	0.2±0.2	0.3±0.1	97±0.7	66±16	92±7	<1	65±8	<1

B

	Positive cells (%)						Clonogeneic progenitors (10^5 cells)		
	CD45	CD34	CD45/CD34	CD71	CD36	CD235a	BFU-E	CFU-E	CFU-GM
Day 0	64±16	3±1	2±1	49±8	7±6	6±6	15±1	<1	6±1.5
Day 7	10±2	0.3±0.1	1±1	95±4	57±20	89±7	4±1	50±6	<1