

Large-scale transcriptional profiling and functional assays reveal important roles for Rho-GTPase signalling and SCL during haematopoietic differentiation of human embryonic stem cells

Sun Yung¹, Maria Ledran¹, Inmaculada Moreno-Gimeno², Ana Conesa², David Montaner², Joaquín Dopazo^{2,3,4}, Ian Dimmick¹, Nicholas J. Slater^{1,5}, Lamin Marenah⁵, Pedro J. Real⁶, Iliana Paraskevopoulou¹, Viviana Bisbal², Deborah Burks², Mauro Santibanez-Koref¹, Ruben Moreno², Joanne Mountford⁵, Pablo Menendez⁶, Lyle Armstrong¹ and Majlinda Lako^{1,*}

¹Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK, ²Centro de Investigacion Principe Felipe and ³Functional Genomics Node, INB, CIPF, Valencia, Spain, ⁴CIBER de Enfermedades Raras (CIBERER), Valencia, Spain, ⁵College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK and ⁶Pfizer-Andalusian Government-University of Granada Centre for Genomics and Oncology (GENYO), Spain

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Understanding the transcriptional cues that direct differentiation of human embryonic stem cells (hESCs) and human-induced pluripotent stem cells to defined and functional cell types is essential for future clinical applications. In this study, we have compared transcriptional profiles of haematopoietic progenitors derived from hESCs at various developmental stages of a feeder- and serum-free differentiation method and show that the largest transcriptional changes occur during the first 4 days of differentiation. Data mining on the basis of molecular function revealed Rho-GTPase signalling as a key regulator of differentiation. Inhibition of this pathway resulted in a significant reduction in the numbers of emerging haematopoietic progenitors throughout the differentiation window, thereby uncovering a previously unappreciated role for Rho-GTPase signalling during human haematopoietic development. Our analysis indicated that SCL was the 11th most upregulated transcript during the first 4 days of the hESC differentiation process. Overexpression of SCL in hESCs promoted differentiation to meso-endodermal lineages, the emergence of haematopoietic and erythro-megakaryocytic progenitors and accelerated erythroid differentiation. Importantly, intrasplenic transplantation of SCL-overexpressing hESC-derived haematopoietic cells enhanced recovery from induced acute anaemia without significant cell engraftment, suggesting a paracrine-mediated effect.

INTRODUCTION

Leukaemia and lymphoma are among the most common forms of cancers. A key treatment for both is chemotherapy followed by haematopoietic stem cell (HSC) transplantation. Allogeneic HSCs from cord blood and adult bone marrow (BM) are used for transplantation; however, they are in short supply. For this reason, new stem cell sources such as human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) that can be differentiated at large numbers into blood progenitor cells are very much sought after.

Over the last 12 years, our group and others have developed a number of protocols to direct differentiation of hESCs and hiPSCs to myeloid and lymphoid lineages. These approaches have involved both spontaneous and directed differentiation in the presence of foetal calf serum (FCS)/chemically defined medium and cytokines (1–14), culture with stromal cells derived from haematopoietic niches (15) and overexpression of key haematopoietic factors (16–19). Despite continuous improvements in the efficiency of haematopoietic differentiation, there are still outstanding issues mainly related to the embryonic and foetal nature of hESC/hiPSC-derived cells,

*To whom correspondence should be addressed. Tel: +44 1912418688; Fax: +44 1912418666; Email: majlinda.lako@ncl.ac.uk

scalability of differentiation protocols and low overall engraftment in immunocompromised recipients (20).

To better understand the process of hESC differentiation to haematopoietic lineages and to identify novel regulators that can be used to direct the differentiation to functional blood cells, we undertook a large-scale transcriptional approach of enriched haematopoietic CD31+KDR+ cells produced from feeder- and serum-free differentiation of hESCs (5) at various time points during the differentiation process. This approach is different from other published studies that have focused on transcriptional profiling of mixed cell types produced within embryoid bodies (21–25) and enabled us to identify Rho-GTPase as a key signalling pathway that is involved in the differentiation from hESCs to haematopoietic progenitors. Overexpression of one of the most highly upregulated genes, *SCL*, resulted in enhanced myeloid differentiation and, in particular, accelerated erythroid differentiation of hESCs. Intrasplenic transplantation of *SCL*-overexpressing hESC-derived haematopoietic cells in an acute model of anaemia resulted in faster recovery compared with controls. Importantly, this *in vivo* effect is most likely due to paracrine factors produced by *SCL*-overexpressing hESC-derived haematopoietic cells rather than cell engraftment.

RESULTS

Brief description of the hESC differentiation method

To identify novel haematopoietic regulators that direct hESC differentiation to haematopoietic lineages, we used the serum- and feeder-free differentiation method previously described in Kennedy *et al.* (5) (Supplementary Material, Fig. S1A). We harvested embryoid bodies (EBs) at each day of differentiation and subjected them to quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) analysis (Supplementary Material, Fig. S1B). This indicated a significant downregulation of two key pluripotency markers, *OCT4* and *NANOG* (Supplementary Material, Fig. S1B), and upregulation of meso-endodermal markers, *MIXL1* and *BRACHYURY*, from day 2 of differentiation. Expression of three classical haemangioblast markers (*CD31*, *CD34* and *KDR*) and markers characterizing more mature haematopoietic cells (such as *RUNX1* genes) was upregulated between days 3 and 5 of differentiation (Supplementary Material, Fig. S1B).

We carried out flow cytometry analysis of disassociated EBs at days 4, 6 and 8 of differentiation. In accordance with previously published data (5), double positive CD31+KDR+ (Supplementary Material, Fig. S1C) and CD34+CD31+ (data not shown) populations emerged around day 4 of differentiation, and these increased in percentage in days 6 and 8. Cell sorting of CD31+KDR and CD31–KDR– followed by haematopoietic colony assays revealed that all haematopoietic progenitor activities segregated within the CD31+KDR+ population (Supplementary Material, Fig. S1D), corroborating previously published data (5). Analysis of colony types revealed similar colony types between unsorted and CD31+KDR+ cells at days 4 and 6 of differentiation (Supplementary Material, Fig. S1D); however, at day 8, a significantly higher proportion of multipotent progenitors [colony forming unit granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM)] and

less erythroid progenitors [colony forming unit erythroid (CFU-E) and burst forming unit erythroid (BFU-E)] was observed in the sorted CD31+KDR+ cells compared with the unsorted cell population (Supplementary Material, Fig. S1D).

Largest transcriptional changes occur during the first 4 days of hESC differentiation

The expression of *KDR* and *CD31* has been associated with the emergence of haemangioblasts within differentiating hESC cultures (5,26), and our results suggested that the CD31+KDR+ population contains all the haematopoietic colony forming cells (CFCs) within developing EBs (Supplementary Material, Fig. S1D). For this reason, we purified CD31+KDR+ using flow-activated cell sorting, and RNA from those subpopulations extracted at days 0, 4, 6 and 8 of differentiation was subjected to transcriptional analysis using the Affymetrix gene chip U133 Plus 2. The data from the individual array sets were normalized using the robust multiarray average summarization algorithm. To assess the correlation between biological replicates, principal component analysis (Supplementary Material, Fig. S2A) and visualization of the Pearson correlation coefficients using heatmaps (Supplementary Material, Fig. S2B) were carried out. Both analyses indicated very high correlation between the replicate samples of day 0 (unsorted cells) and a noticeable difference between these samples and those from differentiated cell populations.

As quality controls for gene expression, we carried out qRT–PCR analysis on a relatively large number of genes, including housekeeping genes (*GAPDH*, *RPL13A*, *SDHA* and *TBP*, see Supplementary Material, Fig. S2C), pluripotency markers (*OCT4* and *NANOG*, see Supplementary Material, Fig. S2D), markers of ectoderm (*NESTIN* and *GFAP*, see Supplementary Material, Fig. S2E), endoderm (*FOXA2* and *GATA4*, see Supplementary Material, Fig. S2F), mesoderm (*BRACHYURY* and *MIXL1*, see Supplementary Material, Fig. S2G) and haematopoietic genes (*CD31*, *KDR*, *CD34*, *CD144* and *CD117/c-KIT*, see Supplementary Material, Fig. S2H). This analysis revealed consistent expression patterns between the two methods during the 8-day differentiation window (Supplementary Material, Fig. S2C–H).

To identify major gene expression trends within the data set, we used two complementary strategies: ASCA-genes (27) and ASCAfun (28) (refer to Supplementary Material, Annex for a detailed description of these two methods). The ASCA-genes analysis indicated that 70% of the variability within the data set could be associated with the time factor (and therefore 30% was biological and technical noise). The most important transcriptional change was observed at 4 days after the start of the experiment, followed by a relative maintenance of the expression level at 6 and 8 days (Supplementary Material, Fig. 1A, left panel). This pattern collected 83% of the variability associated with the time. The second most important transcriptional change was associated with a strong regulation of gene expression at 4 days, followed by downregulation at later time points (Fig. 1A, right panel). These results indicate very clear transcriptional patterns in the cell development.

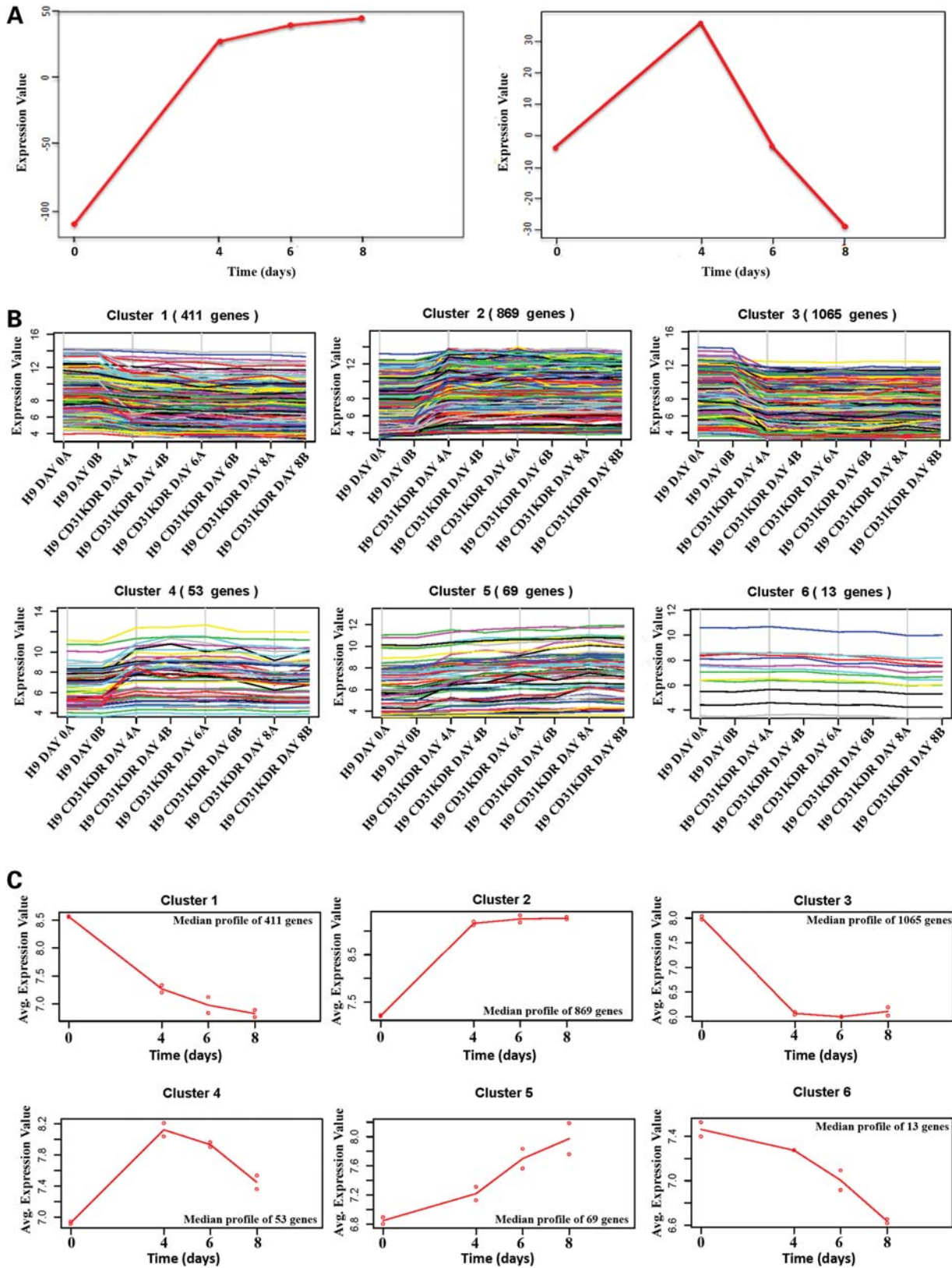


Figure 1. Gene expression changes during hESC differentiation. (A) First (left-hand side panel) and second (right-hand side panel) represent the two major gene expression trends identified by ASCA-genes. The profile shown on the left indicates a strong change at day 4 followed by stabilization of gene expression, whereas the right profile shows a transitory response at day 4. (B and C) Gene clusters of differentially expressed genes detected by maSigPro analysis presented as intensity profiles for all cluster members (B) and mean trajectory plots representative of each gene cluster (C). Note that maSigPro clusters 1, 2 and 3 correspond to ASCA-genes pattern shown in (A) left-hand panel, cluster 4 represents ASCA-genes pattern shown in (A) right-hand panel and clusters 5 and 6 contain minority trends.

A putative role for Rho-GTPase signalling in directing differentiation of hESCs to haematopoietic lineages

To identify differentially expressed genes along the course of the experiment, we employed the R package maSigPro (29) (for more details on this method refer to Supplementary Material, Annex and Table S1), which identified 2480 genes and distributed them among six clusters (Supplementary Material, Fig. 1B and C). Each cluster group shows a unique expression profile, with cluster 1 showing a continuous downregulation pattern from hESCs to haematopoietic progenitors obtained at days 4, 6 and 8 of differentiation and cluster 2 showing a strong upregulation from day 0 to day 4 of hESC differentiation. Clusters 3 and 6 also show a downregulation trend, but the kinetics of these patterns is unique for each group (Fig. 1C). Clusters 4 and 5 showed a strong gene upregulation between days 0 and 4 of differentiation; however, gene expression trends during the rest of differentiation time course were very different, with cluster 4 showing a downregulation trend and cluster 5 showing an upregulation trend. Note that maSigPro clusters 1, 2 and 3 correspond to ASCA-genes pattern 1, cluster 4 represents ASCA-genes pattern 2 and clusters 5 and 6 contain minority trends (refer to Fig. 1A).

We concentrated on further analysis of clusters 2, 4 and 5 since they showed the desired gene expression pattern with a trend of increasing expression between days 0 and 4 of differentiation, a time window during which a clear CD31+KDR+ haematopoietic progenitor subpopulation is observed. Functional enrichment analysis of clusters 4 and 5 revealed that the greatest number of genes clustered within inositol/phosphatidylinositol kinase activity and lipid kinase activity, respectively. Functional enrichment analysis of biological processes of maSigmaPro cluster 2 (Fig. 2A) revealed that the greatest number of genes that was changed during this time point clustered within biological and cellular processes, signal transduction, cellular component organization, cell communication and developmental processes (for more detailed information and node scores, refer to Supplementary Material, Table S2A). When this analysis was carried out for molecular functions and cellular components, the highest scores were obtained for GTPase regulator activity (Ras-Rho axis) and membrane and actin cytoskeleton, respectively (Fig. 2B and C and Supplementary Material, Table S2B and C).

Rho-GTPases are a small family of 22 GTP-binding proteins divided into three main classes: Rho, Rac and Cdc42. They control actin organization and have been shown to play distinct roles in haematopoietic progenitors and mature blood cells throughout development. For example, deletion of Rac1 during the early embryonic development results in impaired intraembryonic haematopoiesis, a decrease in circulating blood progenitors, absence of intra-aortic clusters and foetal liver haematopoietic development (30). In adult haematopoiesis, Rac1 and Rac2 deficiency alters actin assembly in erythrocyte membrane cytoskeleton leading to anaemia (31) and decreased numbers of erythroid progenitors in the BM (32). Deletion of Cdc42 also leads to increased myelopoiesis, decreased erythropoiesis and a blockage in B cell precursor maturation, suggesting an important role for Rho-GTPases in multilineage blood development (33). An important role for Rac1, Cdc42 and Rho A in HSC proliferation, engraftment

and interaction with stroma has been suggested for several Rho-GTPases through induced changes in SDF-1 alpha chemotaxis and chemokines (34–37). In addition to regulating haematopoietic cell fate in normal development, Rho-GTPases have also been involved in the regulation of leukaemic stem cell oncogene-induced proliferation and survival (38).

To gain some further insights into the role of Rho signalling during hESC differentiation processes, we added one of the most well-known inhibitors of this pathway, Y26732 (10 μ m; known to act as a ROCK1/2 inhibitor) to the differentiation media. ROCK1 and 2 are Rho kinases that serve as target proteins for Rho and exert their biological activity by targeting downstream molecules. We noticed a significant downregulation of haematopoietic progenitor cells identified by flow cytometry using single or dual expression of CD34, CD31 and KDR (Supplementary Material, Fig. S3A and B) throughout the 8 days of differentiation. Assessment of total cell numbers indicated a significant increase (1.6-fold day 4, 2.3-fold day 6 and 2.1-fold day 8) in EBs that were differentiated in the absence of ROCK inhibitor, suggesting better survival of other cell types within the EBs. However the reduction in the percentage of haematopoietic progenitors was greater and could not be accounted for by an increase in total cell numbers, pointing to a direct effect of Rho signalling on haematopoietic differentiation of hESCs. We also noticed a smaller degree of downregulation in the expression of pan-haematopoietic marker, CD45 (Supplementary Material, Fig. S3A and B), but throughout the window of differentiation, the percentage of CD45+ expressing cells in both control- and ROCK-treated cultures was relatively small, and longer windows of differentiation with growth factors that promote maturation of haematopoietic progenitors will be needed to assess the role of Rho-GTPase signalling.

An important role for SCL in enhancing *in vitro* myeloid differentiation of hESCs

To identify key regulators of haematopoietic differentiation during the first 4 days of hESC differentiation, we generated a comprehensive gene list that was ranked on the basis of fold change expression (Supplementary Material, Table S3). Confirmatory qRT-PCR analysis was carried out for a number of genes that were previously shown to play a role in haemangioblast development (*CD144* and *CD31*; Supplementary Material, Fig. S2G and H) (26), endothelial cell adhesion and response to phorbol esters (*MMRN2*, *ESAM*, *THBD* and *CD93*; Supplementary Material, Fig. S4A and B) (39–41), proliferation and differentiation of haematopoietic progenitors at the onset of blood specification (*SOX7* and *SOX17*; Supplementary Material, Fig. S4B) (40,42) and leukaemic hematopoiesis (*HOXA9* and *HOP*; Supplementary Material, Fig. S4B) (43,44).

A very interesting and important regulator of HSCs, *SCL* (45), was ranked as the 11th most highly upregulated gene (Supplementary Material, Table S3 and Fig. S4A). Some of the direct targets of *SCL* such as *ERG* and *FLI1* (45–47) were also among upregulated genes during the first 4 days of differentiation (Supplementary Material, Table S3 and Fig. S4A). Given the importance of *Scl* in embryonic and adult HSC specification, we decided to investigate its role in hESC-directed haematopoietic differentiation. We created two human ESC (H1 and H9) *SCL*-overexpressing lines

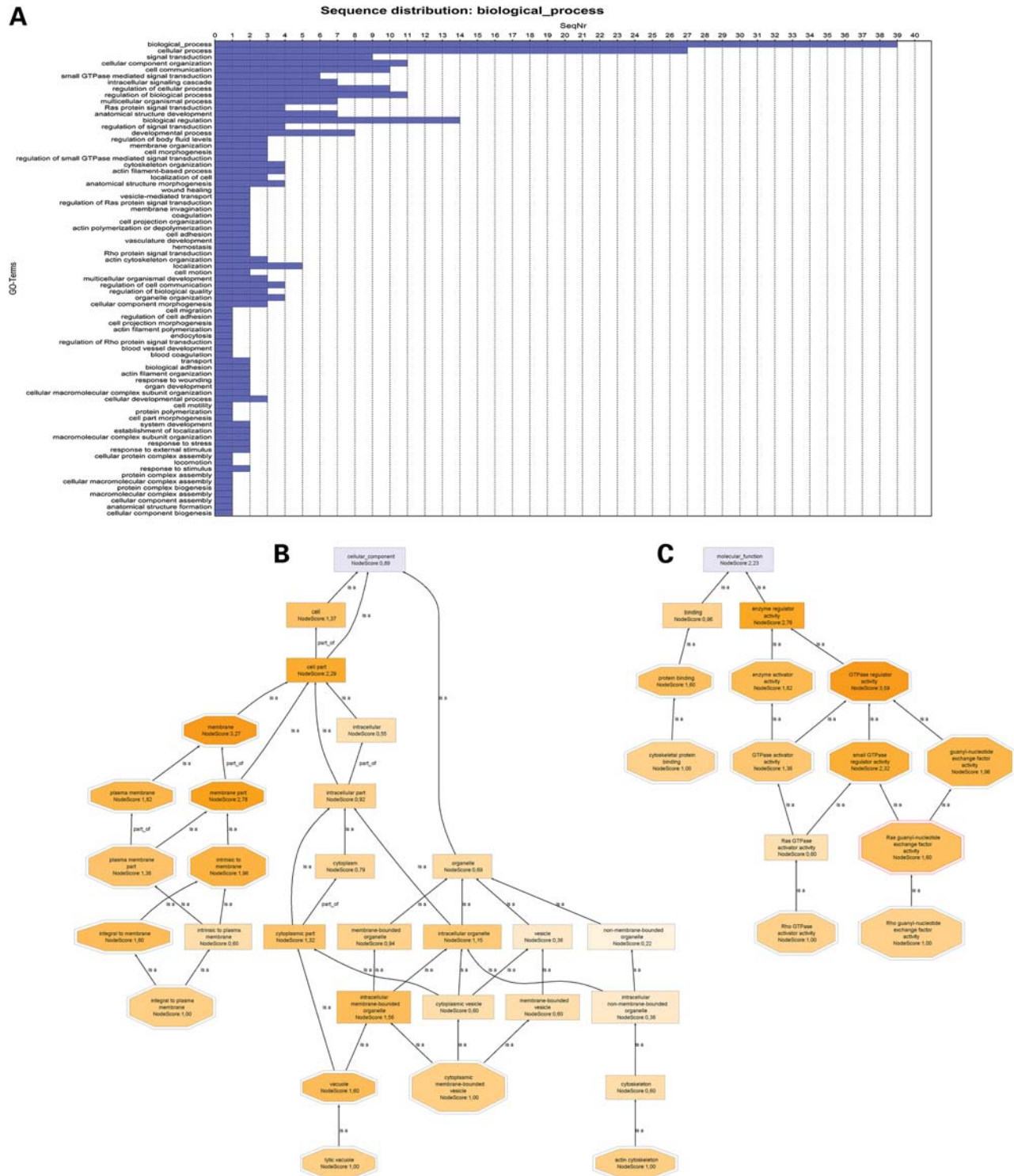


Figure 2. Functional enrichment analysis of hESC differentially expressed genes grouped in cluster 2, as shown in × Figure 1. (A) Bar plot of enriched biological process gene ontology terms. (B and C) Gene ontology graph representation of enriched molecular function and cellular component GO terms. Significant terms are represented as octagonal nodes. Colour intensity of each node reflects the enrichment score.

using the lentiviral vector pFIGW, in which expression of *Scl* and *green fluorescent protein (GFP)* [linked by an internal ribosome entry site (IRES)] was driven by *Ubiquitin C* promoter, as described in Supplementary Material, Annex. Control hESC lines were also created by transducing H1 and

H9 cell lines with the empty pFIGW vector backbone lacking the *Scl* cDNA. Flow cytometry analysis confirmed that the expression of GFP was maintained in both hESCs and EBs in all transduced cell lines (Fig. 3A). qRT-PCR analysis and western blotting were carried out to confirm the

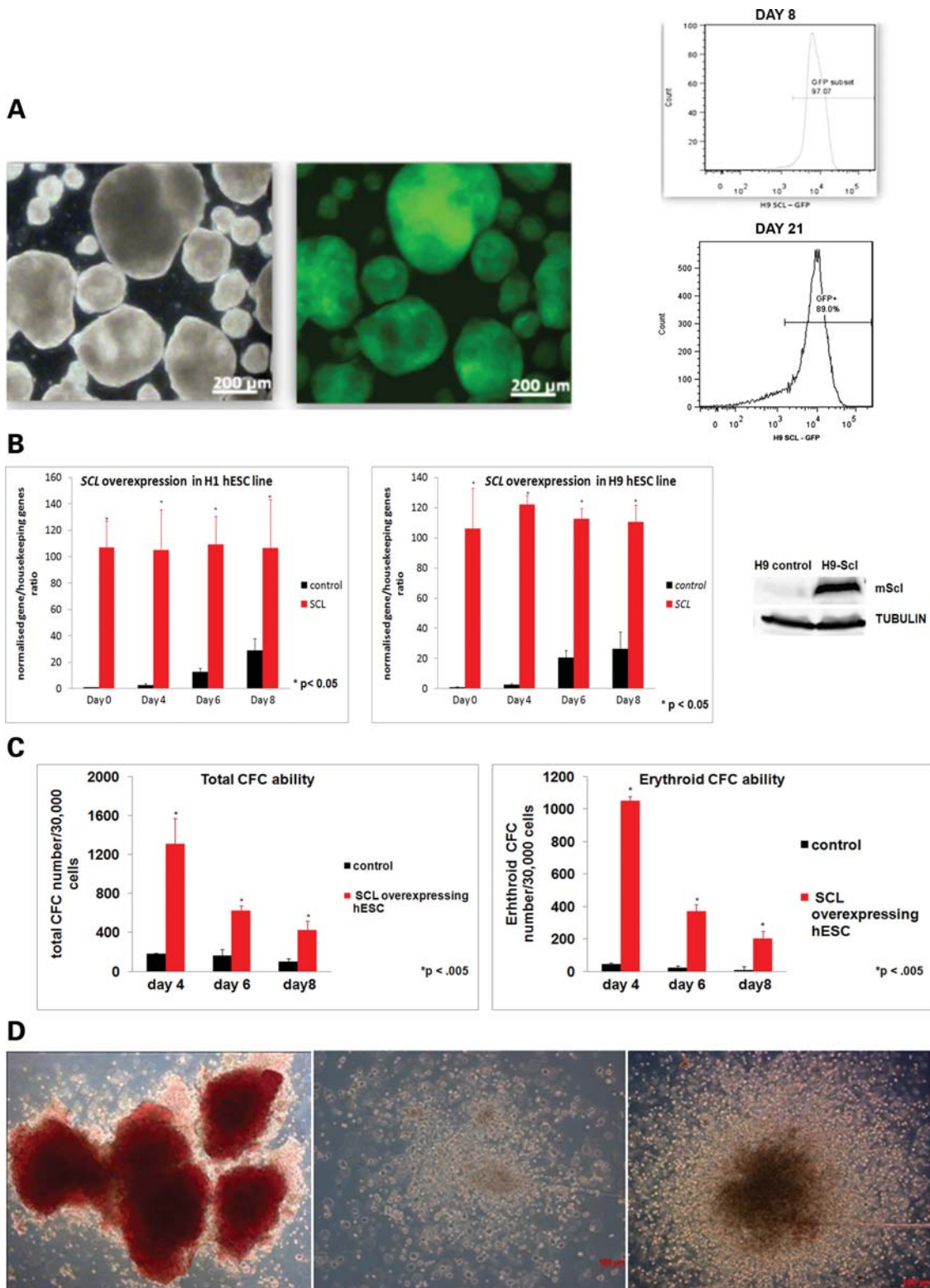


Figure 3. Overexpression of *SCL* enhances differentiation of hESCs to myeloid lineages. (A) Phase (left) and fluorescence (middle) pictures of *SCL* embryoid bodies at day 8 of differentiation. Flow cytometry analysis of EBs derived from *SCL*-overexpressing hESC lines at days 8 and 21 is shown in the right-hand panel. (B) qRT-PCR analysis (left and middle panels) for expression of *SCL* during the 8 days of differentiation in both H9 and H1 ESC lines. Data are presented as mean \pm SEM ($n = 3$). The value for the hESCs was set to 1, and all other values were calculated with respect to that. Right panel represents western blot analysis in H9 control and *SCL*-overexpressing cell lines. (C) Total CFCs and erythroid CFC-forming ability. Data are presented as mean \pm SEM of at least two independent experiments performed in each cell line ($n = 4$). (D) Representative photos of BFU-E, CFU-G and CFU-GEMM obtained from methylcellulose colony assays from differentiation of *SCL*-overexpressing hESC lines.

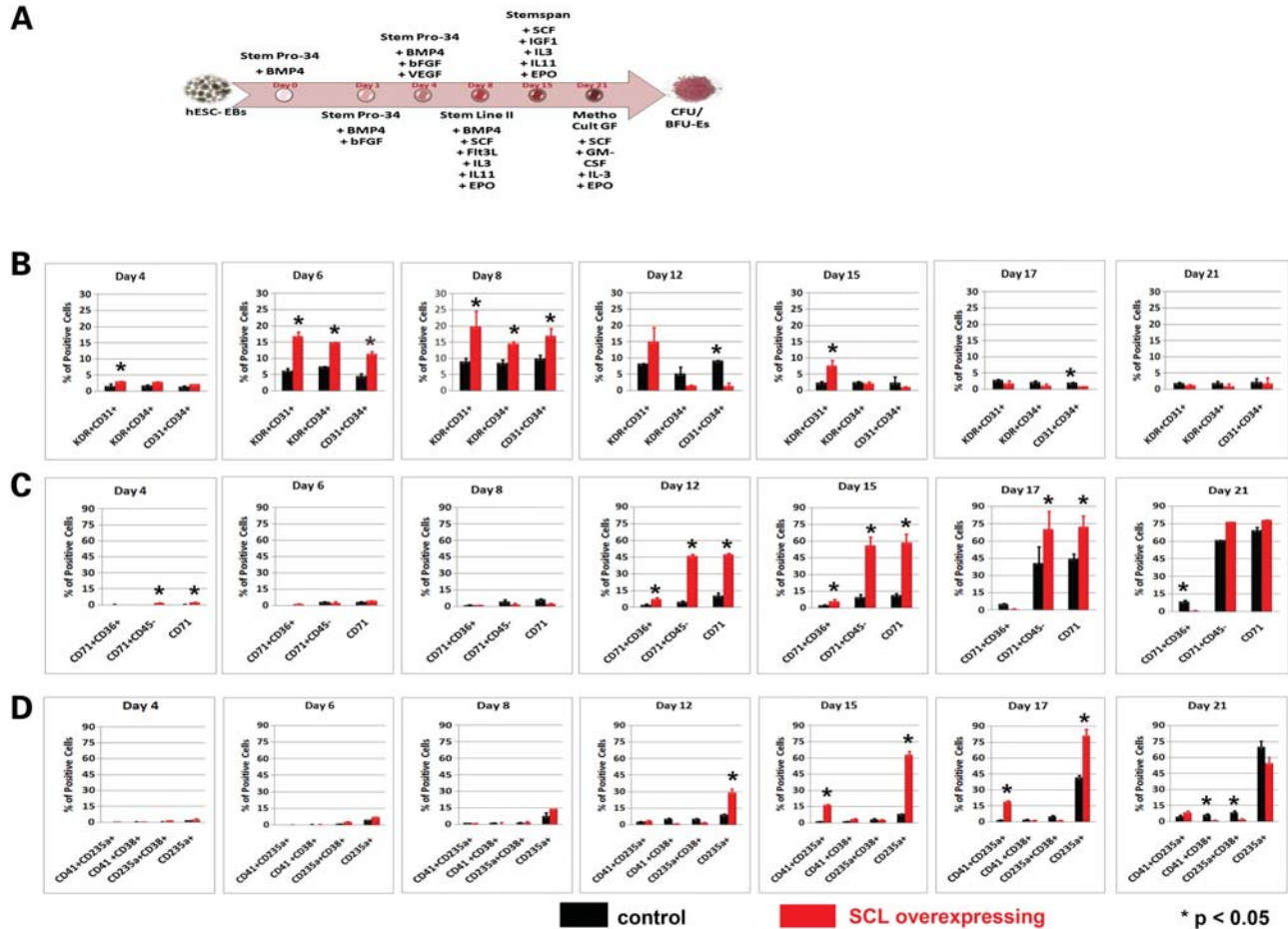


Figure 4. Flow cytometry analysis showing impacts of *SCL* overexpression on the emergence and quantity of haematopoietic, erythroid and megakaryocytic progenitors. (A) Schematic representation of the two-stage differentiation protocol used for erythroid differentiation of *SCL*- and control hESC-overexpressing cell lines. (B) Flow cytometry analysis with haemangioblast markers during the 21-day differentiation time course. (C and D) Flow cytometry analysis with markers of erythro-megakaryocytic cells. (B–D) Data are presented as mean \pm SEM of at least two independent experiments performed in each cell line ($n = 4$).

overexpression of *Scf* throughout the course of differentiation (Fig. 3B). Similar overexpression (around 106-fold) levels were observed in both hESC *SCL*-overexpressing lines, and for this reason, averaged data from both cell lines will be presented for the remainder of this manuscript.

We subjected *SCL*-overexpressing cell lines to the differentiation protocol summarized in Supplementary Material, Figure S1A and carried out haematopoietic CFC assays. At all three points of differentiation, we observed a significant increase in total colony-forming ability (Fig. 3C and D). A close observation of colony-forming data showed that total numbers of CFU-G, CFU-M, CFU-GM and CFU-GEMM were all increased as results of *SCL* overexpression (data not shown), thus suggesting a role in promoting haematopoietic progenitor emergence from hESCs. However, the most significant increase was observed for CFU-E and BFU-E colonies, indicating a role for *SCL* in promoting erythroid differentiation (Fig. 3C).

To investigate in more detail the role of *SCL* in erythroid differentiation, we adapted a two-stage differentiation protocol in which cells are first subjected to haematopoietic conditions that favour emergence and expansion of haemangioblast-like cells (Supplementary Material, Fig. S1A), followed by

a second stage of differentiation which promotes erythroid cell emergence and expansion, as outlined in Figure 4A. Flow cytometry analysis was carried out using the following markers: CD31, KDR, CD34 (expressed in haemangioblast arising during differentiation of hESCs), CD36 (expressed in erythroblasts and declining as cells mature to reticulocytes and mature erythrocytes), CD71 (expressed in erythroid progenitors of foetal liver, cord and peripheral blood (PB) but lost as these cells differentiate to mature erythrocytes), CD41 (bright expression in combination with mature erythroid markers specifies definitive erythroid progenitors) and CD235a (expressed in erythroid precursors and mature erythrocytes). In accordance with the results shown in the previous section, overexpression of *SCL* caused a significant increase in the percentage of haemangioblast-like cells in the first stage of differentiation, up to day 8 (Fig. 4B and Supplementary Material, Fig. S5). However, these were reduced as soon as the cells were subjected to erythroid differentiation conditions (Fig. 4B and Supplementary Material, Fig. S5).

Application of erythroid differentiation conditions resulted in a significant increase in erythroblast progenitor cells marked by CD71 expression as early as day 12 in *SCL*-overexpressing cells

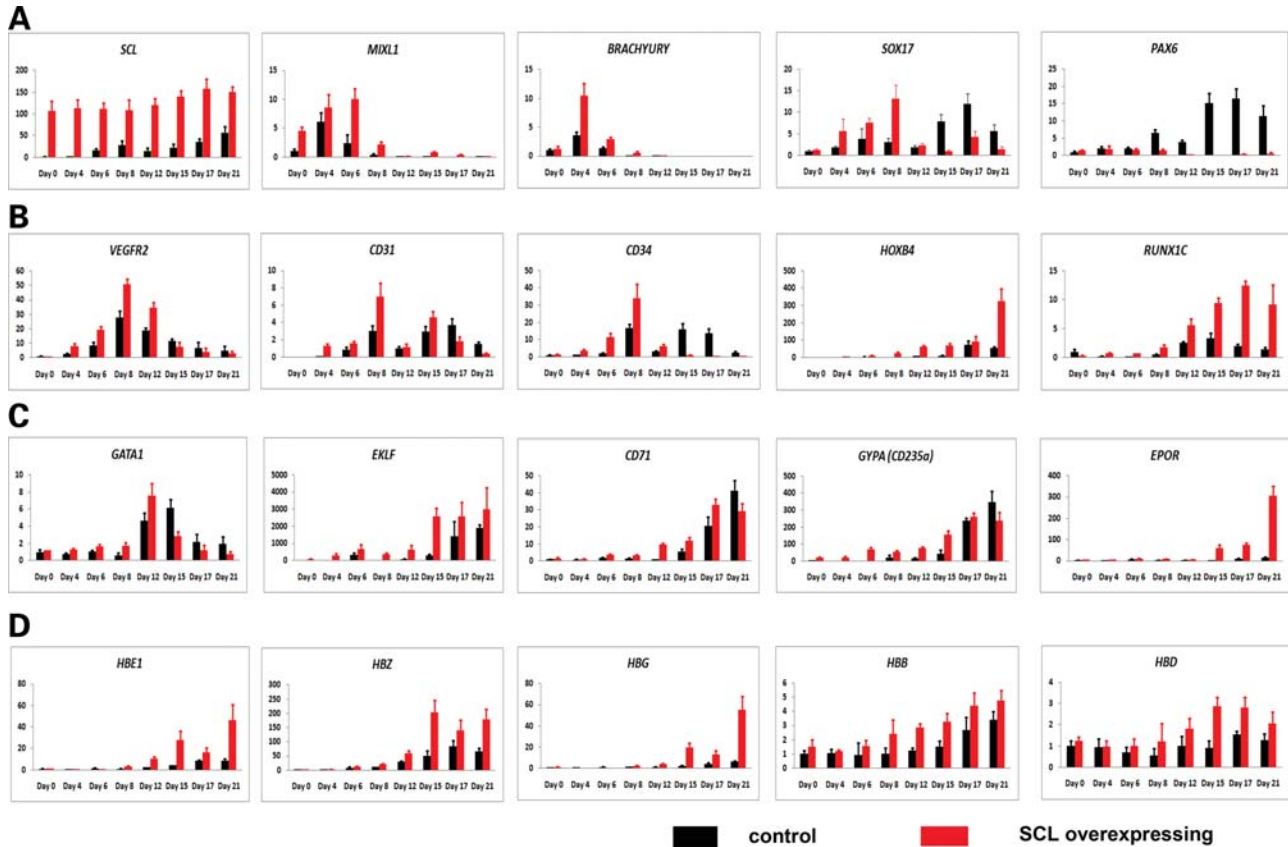


Figure 5. qRT-PCR analysis showing impacts of *SCL* overexpression on hESC germ lineage differentiation, haematopoietic progenitor emergence and erythroid cell differentiation. Y-axis: relative gene expression/housekeeping genes. Data are presented as mean \pm SEM of at least two independent experiments performed in each cell line ($n = 4$).

(Fig. 4C). The majority of CD71-positive cells lacked the expression of not only CD45 but also CD36, perhaps indicating a similarity to foetal liver erythroblasts. The percentage of CD71 increased from day 12 onwards, reaching a plateau of 77% at day 21. Control differentiation cultures lagged behind for most of the differentiation time course; however, by day 21, broadly similar percentages of CD71- and CD235a-positive cells were observed, perhaps due to culture conditions which were designed to promote erythroid differentiation (Fig. 4C and D). qRT-PCR analysis (Fig. 5A) also shows that endogenous *SCL* expression increases in control lines during the course of differentiation, reaching its highest level at day 21. This could also explain increased CD71 and CD235a expression by day 21 in control cultures.

A gradual increase in the expression of CD235a (lagging behind CD71 expression) was observed in *SCL*-overexpressing cells from day 12 to day 17 of differentiation. About 16 and 19% of these CD235a-positive cells also shared the expression of CD41. Dual expression of CD235a and CD41 is indicative of common erythro-megakaryocytic bipotent progenitors arising from hESC differentiation (48). It is worth noting that the percentage of CD235a+CD41+ cells is higher in *SCL*-overexpressing lines compared with control, suggesting an additional role for *SCL* in the emergence of erythro-megakaryocytic progenitors. However, the number of CD235a+CD41- cells is still much higher than that of CD235a+CD41+ cells, arguing in favour of enhanced and

accelerated emergence of erythroid progenitors in *SCL*-overexpressing lines compared with controls (Fig. 4D), which is perhaps due to culture conditions favouring differentiation of these bipotent progenitors to erythroid lineages.

To gain some insights into how *SCL* overexpression might enhance hESC differentiation to haematopoietic progenitor cells and further to erythroid lineages, we carried out qRT-PCR analysis with a large number of markers (Fig. 5A–D). This analysis first showed that *SCL* may have an early effect (first 6 days of differentiation) in promoting differentiation of hESCs to meso-endodermal lineages, as indicated by increased expression of *MIXL1* (marker of meso-endoderm), *BRACHYURY* (marker of primitive streak and mesoderm), *AFP* and *IHH* (data not shown) and *SOX17* (markers of primitive and definitive endoderm; Fig. 5A). This promotion towards meso-endodermal lineages is accompanied by suppression of the ability to differentiate towards ectodermal lineages, indicated by reduced expression of primitive and definitive ectodermal markers *FGF5* (data not shown) and *PAX6* (Fig. 5A). To exclude the possibility that exogenous *SCL* expression is switched off during the second stage of differentiation, upon changing of culture conditions, we performed qRT-PCR analysis (Fig. 5A) and flow cytometry analysis for GFP expression throughout the differentiation time course (Fig. 3A). The expression of exogenous *SCL* and *GFP* (linked by an IRES) was driven by *Ubiquitin C* promoter; therefore, silencing of promoter should be reflected in

both GFP and SCL expression. We observed no significant changes in *SCL* expression by qRT-PCR analysis throughout the differentiation time course (Fig. 5A) and very small changes in GFP expression (98% at day 8 of differentiation to 89% at day 21, Fig. 3A). Together, these data would argue that *SCL* expression is maintained throughout the differentiation, and the very different impacts observed at the early and late stages are due to effects of SCL on the hESC differentiation rather than transgene silencing.

In accordance with flow cytometry results shown earlier, overexpression of *SCL* results in increased expression of markers involved in the specification of haematopoietic progenitors arising during hESC differentiation, such as *KDR* (*VEGFR2*), *CD31*, *CD34* (Fig. 5B) and *LMO2*, *GATA2* and *CDX4* (data not shown) during the first 8 days of differentiation. *SCL* overexpression was accompanied by increased expression of *SOX7* (known to promote the differentiation of early haematopoietic progenitors to both erythroid and myeloid lineages; data not shown) as well as by increased *HOXB4* and *RUNX1C* expression from day 8 to day 21 of differentiation, which may reflect a higher propensity of *SCL*-overexpressing cells to undergo erythro-megakaryocytic differentiation (Fig. 5B).

Changes of culture conditions to enhance erythroid differentiation (after day 8) were associated with an increase in the expression of *GATA1*, a transcription factor known to be essential for terminal maturation of proerythroblasts and shown to be a direct transcriptional target of *SCL*; however, this increase was more prominent in *SCL*-overexpressing cells at day 12 of differentiation (Fig. 5C). In line with enhanced erythroid differentiation, we also noticed a marked increase in the expression of a large number of erythroid markers such as *EKLf*, *CD71*, *EPOR*, *GYPa* (*CD235a*; Fig. 5C), *CD36*, *NACA*, *NFE2L1*, *APOL1*, *APOA1* and *CD47* (data not shown). In addition, the expression of embryonic globins (*HBE1* and *HBZ*), foetal globins (*HbG*) and adult globins (*HbA1*, *HbB* and *HbD*) was significantly increased at the end stages of differentiation (days 15–21; Fig. 5D). This can reflect the role of SCL both in enhancing erythroid differentiation and in directly activating the transcription of globin genes via GATA1/SCL/gfi-1b complex (reviewed in 49).

A paracrine effect for erythroid cells derived from *SCL*-overexpressing hESC lines in enhancing recovery from induced acute haemolytic anaemia

To test whether the erythroid progenitors derived from *SCL*-overexpressing hESC lines would engraft and circulate in the PB of animal models, we created a model of acute haemolytic anaemia in immunocompromised *NOD/LtSz-Scid IL2R γ^{null}* mice by injection with phenylhydrazine (PHZ), as outlined in Materials and Methods. As expected, PHZ injection caused a sudden decrease in red blood cell numbers to 3×10^6 cells/ μ l (Fig. 6A). The numbers of red blood cells rose back to normal levels between days 6 and 11 post PHZ treatment, corroborating previously published data (50). Reticulocytes (immature red blood cells) take about 1–2 days to mature into normocytes in the circulation and under normal conditions that are present at low concentrations (about 1% of total red blood cells). Anaemia causes their proportion to

rise dramatically as production of erythroid progenitors is upregulated in response to Epo production in the kidney. In accordance with this, we observed a sharp rise in the average reticulocyte proportion, reaching 10% of total red blood cell numbers at 4 days post PHZ injection, which declined to normal afterwards (Fig. 6A).

In addition to decreased red blood cell counts, PHZ injections also changed the forward and size scatter of Ter119+ red blood cells observed by flow cytometry (shown in the square gate). One single-cell population (named unaffected) is present before PHZ treatment (Fig. 6B). However, PHZ treatment results in the appearance of a second lower population (named affected), which disappears as red blood cell counts recover (Fig. 6B). We sorted these two cell populations by fluorescence-activated cell sorting (FACS) and assessed their viability using Trypan blue staining (data not shown). This analysis showed that the lower cell population (shown in blue colour in Fig. 6B) had very low viability (97.5% of cells stained with Trypan blue) and small average cell diameter (3.08 μ m). The upper population (shown in red colour in Fig. 6B) had much better cell viability (95% of cells did not stain with Trypan blue) and larger average cell diameter (8.84 μ m). These findings confirm that the upper population of Ter119-stained cells represented the viable red blood cells, whereas the lower one represented the affected and non-viable red blood cells after PHZ treatment. We used the ratio between the unaffected and affected cell population to observe any positive effect that hESC-derived erythroid cells may have in recovery from PHZ-induced acute anaemia and to further assess the role of SCL in this process. To investigate this, we transplanted (intrasplenically) *NOD/LtSz-Scid IL2R γ^{null}* mice with 5×10^6 cells obtained at day 17 of differentiation. We chose day 17 because of the highest CD235a expression and best differences between *SCL*-overexpressing and control lines (refer to Fig. 4D). PB was taken every 2 days, and the ratio between unaffected and affected red blood cells was analysed for each group. As can be seen from Figure 6C, transplanted animals injected with carrier solution only [phosphate-buffered saline (PBS) + 5% FCS] start showing some recovery between days 5 and 8 post PHZ injection, corroborating data shown in Figure 6B. However, animals injected with hESC-differentiated cells from *SCL*-overexpressing lines show an early (from day 3) and significantly better recovery when compared with animals injected with hESC-differentiated cells from control hESC lines or carrier solution (Fig. 6C). Red blood cell counts at day 3 also showed almost double the number of cells in animals injected with *SCL*-hESC-derived cells compared with the controls (data not shown). Animals injected with CD34+ cord blood cells lagged behind *SCL*-differentiated hESC cells, and this could be due to the fact that they are less differentiated compared with hESC-derived cells at day 17; however, by day 8, they also showed a significantly enhanced recovery from PHZ-induced anaemia.

To investigate the engraftment of red blood cells in the PB of PHZ-injected animals, we performed flow cytometry for CD235a expression throughout the 30-day time course (Fig. 6D); however, only very low (maximum 1.5%) circulating cells showed CD235a expression and above all there were no significant differences between control and

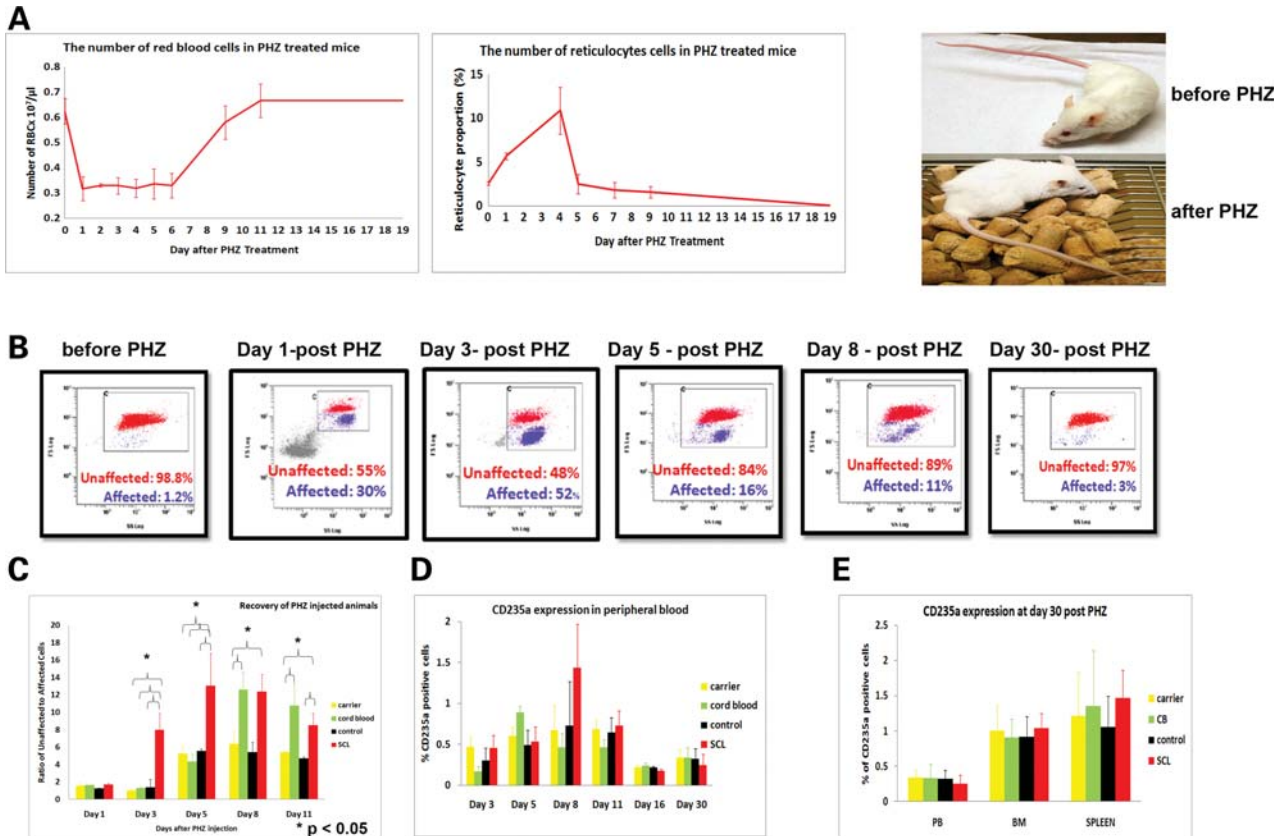


Figure 6. Intrasplenic injections of SCL-hESC-derived cells and recovery from PHZ-induced anaemia. (A) Total numbers of red blood cells and reticulocytes up to 19 days after PHZ treatments in NOD/LtSz-Scid *IL2R γ* ^{null} mice. Data are presented as mean \pm SEM of measurements performed in five mice. Representative pictures of mice prior to and after PHZ injection are shown on the right-hand side. (B) Kinetics of unaffected (shown in red) and affected (shown in blue) gated Ter 199 cell populations up to 30 days post injection with PHZ. Data are presented as mean \pm SEM of measurements performed in five mice. (C–E) Schematic graphs showing recovery from PHZ-induced anaemia (C), percentage of circulating human CD235a cells in PB (D) and percentage of CD235a in PB, BM and spleen at day 30 post PHZ injection (E). Data are presented as mean \pm SEM ($n = 5$ for CD34+ cord blood injections, $n = 6$ for control hESC injections, $n = 7$ for SCL-hESC injections and $n = 6$ for carrier solution injections).

SCL-hESC-derived cells (same for CD34+ cord blood cells). At day 30 post PHZ injection, all animals were sacrificed and flow cytometry analysis was performed on PB, BM and spleen using CD36, CD45, CD235a and CD71 (CD235a data are shown in Fig. 6E). Again, the percentage of engraftment was low and not significantly different between the groups. Consistent with these results, high-performance liquid chromatography (HPLC) analysis of globin chains in samples taken from SCL-overexpressing and control groups at day 17 of differentiation showed no synthesis of haemoglobin chain proteins (Supplementary Material, Fig. S6), despite transcript detection by qRT-PCR (Fig. 5D). This suggests that hESC-derived red blood cells produced with our two-stage differentiation protocol might need a longer time in culture to express the globin chains and to exclude a role for SCL in accelerating or enhancing this final maturation step. In addition, our data suggest a paracrine effect for SCL-hESC-derived cells and CD34+ cord blood cells in either protecting red blood cells from undergoing apoptosis during PHZ-induced anaemia or inducing a proliferative effect on the newly generated red blood cells. This is also corroborated by gene expression studies, which have shown higher expression of erythropoietin, thrombopoietin (Supplementary Material, Fig. S7) and erythropoietic receptor (Fig. 5) in SCL-hESC-derived cells.

DISCUSSION

A full understanding of transcriptional control of embryonic stem cell differentiation is essential for directing this process to yield the desired functional cell types as well as to ensure the scalability and genomic stability in culture. In this study, we undertook a large-scale transcriptional approach and showed that the largest transcriptional changes occur during the first 4 days of differentiation, followed by a relative maintenance at days 6 and 8. Ontology analysis based on molecular function pinpointed Rho-GTPase signalling as key regulator activity. We tested this in our differentiation system using an inhibitor for two downstream Rho targets, ROCK1 and ROCK2, and observed a significant reduction in the number of haematopoietic progenitors emerging at all time points of differentiation. These findings are of great interest since it has been shown that G protein signalling and, in particular, the Ras and Rho-GTPases play an important role in blood cell development (51,52). Rho-GTPases are a small family of 22 GTP-binding proteins divided into three main classes: Rho, Rac and Cdc42. Several members of this family including Rac1, Rac2, Cdc42, Rho-A and Rho-H have already been shown to regulate HSC homing and interaction with the BM microenvironment, proliferation, survival, engraftment and self-renewal of HSCs (53),

proliferation of myeloid cells, enucleation of erythrocytes, balance between erythroid and myeloid fate determination and T cell development and activation (52). Indeed, *RHOJ*, *RHOB* and *RHOH* expression is upregulated during the first 4 days of hESC differentiation, whereas *CDC42* is downregulated (Supplementary Material, Table S3). Although at this stage we do not know which member of this signalling cascade is involved in each stage of differentiation, our study provides for the first time some useful and novel insights into involvement of a key signalling pathway that can be manipulated to achieve the desired differentiation from hESCs.

Various studies in animal models have shown that Scl is a crucial regulator of HSC specification, yolk sac angiogenesis, erythroid and megakaryocytic development, regulation of G₀ to G₁ transit of HSCs and competence of stem cell function upon transplantation (reviewed in 54). Recent ChIP-Seq studies have shown that Scl is one of the 10 major transcriptional regulators in HSCs with the ability to bind to more than 7096 target sequences in haematopoietic progenitor cells, 1015 of which are also co-occupied by LYL1, LMO2, ERG, FLI-1, GATA2 and RUNX1 (45). Despite this, there have been no reports on the role of SCL during haematopoietic differentiation of hESCs, and this formed the second focus of this study. Our *SCL* overexpression studies showed an effect for SCL at multiple levels of hESC differentiation, starting from enhancement of meso-endoderm differentiation (at the expense of ectodermal lineage suppression) as well as enhancement of hESC differentiation to all myeloid lineages *in vitro*. In addition, our data also indicated that *SCL* overexpression promoted the formation of erythro-megakaryocytic progenitors and accelerated their differentiation into erythroid progenitors.

Intrasplenic injection of SCL-overexpressing hESC-derived cells into anaemic immunocompromised recipients resulted in a faster and better recovery when compared with the control group injected with equivalent cells from differentiating control hESC lines. Despite this, we could not detect a significant engraftment of erythroid-derived cells from either of the groups. The faster recovery from anaemia can, however, be explained by growth factors produced by injected cells. The cell mixture derived from differentiation of SCL-overexpressing hESCs at day 17 contains 19% of CD41+CD235a+, which are very likely to be erythro-megakaryocytic progenitors, and 81% of CD41-CD235a+, which are perhaps more mature erythrocytes. This is much higher than the percentage of equivalent cells in the control differentiation group (1.4% of CD41+CD235a+ and 41% of CD41-CD235a+). It can be envisaged that either of these two groups of progenitors can produce growth factors that enhance recovery from anaemia by exerting an effect of proliferation of red blood cells. For example, it has been reported that thrombopoietin and human megakaryocyte growth and development factor (both of which are produced by megakaryocytes) have an effect on red blood cell recovery when administered in irradiated immunocompromised recipients (55). This hypothesis is currently unknown; however, it provides an interesting research avenue for the identification of growth factors/signalling pathways that are produced/enhanced in differentiating *SCL*-overexpressing cells, which can make a difference in recovery from conditions such as anaemia or total body irradiation. As an example of this, it is important to note that

SCL-overexpressing hESC-derived cells also produce higher levels of erythropoietin (and its receptors), whose function is to protect red blood cells from apoptosis.

In summary, our data have shown multiple roles for SCL during differentiation of hESCs, which range from early commitment to meso-endoderm, emergence of haematopoietic progenitors and differentiation to erythrocyte and erythro-megakaryocytic progenitors. Most importantly, *SCL* overexpression accelerates the erythrocytic differentiation programme, thus shortening the time window for the production of hESC-derived erythrocytic progenitors.

MATERIALS AND METHODS

Culture and differentiation of human ESCs

The human ESC lines H9 and H1 (WiCell Inc., Madison, Wisconsin) were routinely passaged and maintained in human ESC media on mitotically inactivated mouse embryonic fibroblast feeder layers, as described in Stojkovic *et al.* (56). One to two passages prior to experiments, human ESCs were transferred to Matrigel-coated plates with feeder-conditioned media as described previously (56).

Haematopoietic differentiation of hESCs in serum- and feeder-free media

The optimized differentiation method described in this section was reported elsewhere (5) and is summarized in Supplementary Material, Figure S1A. For a fuller description, refer to Supplementary Material, Annex.

Erythroid specification of hESC-derived haematopoietic progenitors

The optimized differentiation method is described in Figure 4A. For a fuller description, refer to Supplementary Material, Annex. HPLC of globin chain separation was performed, as described in Supplementary Material, Annex.

Haematopoietic colony assays

All haematopoietic progenitor assays were performed according to manufacturer's instructions using MethoCult GF H4434 (Stem Cell Technologies, Vancouver, Canada). After 12–14 days incubation at 37°C in a 5% CO₂ humidified atmosphere, the plates were scored for CFU-G, CFU-M, CFU-GM, CFU-E, BFU-E and CFU-GEMM colonies under a light microscope.

Flow cytometry analysis

Single cell suspensions of hESC-derived cells were prepared by incubation in Accutase [Chemicon (Millipore), Watford, UK] for 15–30 min at 37°C. The required fluorochrome-conjugated antibodies were added to the cell suspension at the manufacturer's specified concentrations, and the mixture was incubated in the dark at 37°C for 1 h. For details of antibodies, refer to Supplementary Material, Annex. The cells were analysed using the BD FACS LSRII or BD FACS Aria equipped with DIVA software.

Table 1. Sequences of oligonucleotides used for the qRT-PCR analysis

Primer name	Forward sequence	Reverse sequence	Product length (bps)	Annealing temperature (°C)	Melting temperature (°C)
<i>AFP</i>	CTTTGGGCTGCTCGTATGA	ATGGCTTGAAAGTTCGGGTC	176	56	78
<i>BCL2</i>	GTCTGGGAATCGATCTGGAA	AATGCATAAGGCAACGATCC	131	65	79
<i>BRACHYURY</i>	TCAGCAAAGTCAAGCTCACCA	CCCCAACTCTCACTATGTGGATT	102	65	80
<i>CD144</i>	GATCAAGTCAAGCGTGAGTCG	AGCCTCTCAATGGCGAACAC	181	58	81
<i>CD31</i>	GCTGACCCCTTCTGCTCTGTT	TGAGAGGTTGGTGTGACATC	186	55	83
<i>CD34</i>	GAGGCCTCAGTGTCTACTGC	CGATTCATCAGGAAATAGCC	228	65	80
<i>CD71</i>	GTCGCTGGTCAAGTTCGTGATT	AGCAGTTGGCTGTTGTACCTCTC	80	60	80
<i>CD93</i>	CTAGGGCCACCTCACTTTC	GGCAGACAGTTACTCCTGGG	105	60	78
<i>CDX2</i>	CCGAACAGGGACTTGTTAGAG	CTCTGGCTTGGATGTTACACAG	197	65	72
<i>CDX4</i>	GGAAATTCCTTTCCAGCTCC	CTATGCATGGATGCGCAAG	110	60	75
<i>C-KIT (CD117)</i>	GAGTGAAGTGAATGTTGCTGAG	CAAGTGAATTGCAGTCTTCC	169	60	81
<i>E2A (TCF3)</i>	CCCAGGAGCCTGAGCTG	CTGTCACCAACGGGAAGG	93	60	76
<i>EKLF</i>	GGCTGGTCTCAGACTTCAC	CCGGACACACAGATGACTT	110	59	75
<i>EPOR</i>	CAGGCCAGATCTTCTGCTTC	GACGCTCTCCCTCATCCTC	95	60	75
<i>ERG</i>	TCTGTCTTAGCCAGGTGTGG	CGCATTATGGCCAGCACTAT	101	60	81
<i>ESAM</i>	CACTTCCCCTCCCTCCAC	CTGCGGTTTTTGTTCCTGG	108	60	78
<i>FLI1</i>	CGCTGAGTCAAAGAGGGACT	AATGTGTGGAATATTGGGGG	108	60	76
<i>FOXA2</i>	AAGGCATACGAACAGGCACTG	TACACACCTTGGTAGTACGCC	140	65	85
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTTCATGAG	86	53	81
<i>GATA1</i>	CAGGCCAGGGAACCTCCA	ATCACACTGAGCTTGCCACA	96	60	81
<i>GATA2</i>	GGGGACCCTGTCTGCAACGC	GGCAGCTGCACTGAAGGGGG	201	65	85
<i>GATA4</i>	ACACCCCAATCTCGATATGTTG	GTGTCACAGATAGTGACCCCGT	210	58	81
<i>GFAP</i>	AAGAGATCCGCACGCAGTAT	AAGAGATCCGCACGCAGTAT	174	68	86
<i>GYP A (CD235a)</i>	TGCCACACCAGTGGTACTTG	TCATGATCTCAGGATGTATGGAA	91	60	82
<i>HB A1+2</i>	ACTCTTCTGGTCCCACAG	GTGGGGAAGGACAGGAAC	153	57	85
<i>HBB</i>	GCAACCTCAAACAGACACCA	TTAGGGTTGCCATAACAGC	198	65	84
<i>HBD</i>	TCCTGAGGAGAAGACTGCTGTCAATGC	GAGAGGACAGATCCCCAAAGGACTCAA	140	57	83
<i>HBE</i>	ATATCTGCTCCGACACAGC	GCTTGAGGTTGTCCATGTTT	303	65	81
<i>HBZ</i>	ACCAAGGCCAGTCTGAG	CGGGTGGGTGAGGAAGAG	169	53	80
<i>HOP</i>	TCTGTGACGGATCTGCACTC	GCCTTCCGAGGAGAGAC	97	60	77
<i>HOXA9</i>	CCGAGAGGCAGGTCAAGATC	AAATAAGCCCAAATGGCATCA	93	54	79
<i>HOXB4</i>	GTCGTCTACCCTGGATGC	CGTGTGAGGTAGCGGTTGTA	153	59	76
<i>IHH</i>	GCCACGCAAACCTCGTGCCGC	GTCCTTGACGCGTGGGTCATG	208	60	81
<i>LMO2</i>	ATCCCTGCTGACATGCGGCG	CGCAGCTCAGGCAGTCTCG	98	65	86
<i>MIXL1</i>	CAGAGTGGGAAATCCTTCCA	TTCAGAGAGAGGGGAACAGG	207	65	82
<i>MMRN2</i>	CAGCTTGGACATTGGGTAGG	ATCTGCAGAGCTCCAGGACA	104	60	83
<i>NANOG</i>	GATTTGTGGCCCTGAAGAAA	AAGTGGGTTGTTTGGCTTTG	155	65	81
<i>NESTIN</i>	GAGGGAAGTCTTGGAGCCAC	AAGATGTCCCTCAGCCTGG	99	60	84
<i>OCT4-TV1</i>	CGTGAAGCTGGAGAAGGAGA	CTTGGCAAATTGCTCGAGTT	91	65	82
<i>PAX6-TV1</i>	GAGGTCAGGCTTCGCTAATG	TGGTGTGGCTCAAGTGTGT	91	52	85
<i>PU.1</i>	CACAGCGAGTTCGAGAGCTT	GATGGGTACTGGAGGCACAT	194	61	75
<i>RASGRP3</i>	ATTGGGAAATGCATGGAAGA	GGATTCTCTGATTCACTGCC	104	60	79
<i>RPLI3A</i>	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGATTTGTCAA	126	65	80
<i>RUNX1A</i>	AAGACACAGACCCTGGAGA	GCCTTCCATATAACGTGCAT	151	62	80
<i>RUNX1B</i>	CAATGGATCCCAGGTATTGG	CACTGCCTTTAACCTCAGC	107	60	78
<i>RUNX1C</i>	GAAGTCTGAACCCAGCATAGTGGTCAGCAG	GTGGACGTCTTAGAAGGATTCATTCCAAG	231	60	75
<i>SCL</i>	AGCCGATGCCCTCCCTAT	CCGCACAACCTTGGGTGG	104	53	80
<i>SDHA</i>	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	84	65	76
<i>SOX17</i>	TCTGCCTCCTCCACGAAG	CAGAATCCAGACCTGCACAA	101	60	79
<i>SOX7</i>	CTCTTCTGGGACAGCGTCA	GCCAAGGACGAGAGGAAAC	110	60	80
<i>TBP</i>	ATGTTTTTCCCATGAACCA	TCTGGCACAGAAATAACCCC	112	65	73
<i>THBD</i>	CCTCCATGCATCTCATAGCA	CGGGTTGTGTCTGTTCAC	99	60	84
<i>VEGFR2 (KDR)</i>	CACCACTCAAACGCTGACATGTA	GCTCGTTGGCGCACTCTT	95	63	81

RT-PCR and Light Cycler quantitative analysis

Total RNA was extracted from hESCs and differentiating cultures using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription and qRT-PCR analysis were carried out as described in Yang *et al.* (57). All human-specific primers are shown in Table 1.

Microarray analysis

Total RNA was extracted from hESCs and EBs at days 4, 6 and 8 of differentiation using Trizol reagent (Invitrogen), and 10 µg of the RNA sample was used for hybridization to Affymetrix Human U133 Plus 2 array. For further details on hybridization and analysis, refer to Supplementary Material, Annex. These data have been submitted to GEO under the accession number GSE29115.

Construction of *SCL*-overexpressing human ESC lines

The IRES-based polycistronic pFIGW vector in which expression of murine *Scl* and *GFP* was driven by *Ubiquitin C* promoter was kindly provided by Dr M. McCormack and Dr D. Curtis. Lentiviral transductions were performed as described in Supplementary Material, Annex.

Analysis of red blood cell engraftment in NOD/LtSz-*Scid* *IL2Rγ*^{null} mice

NOD/LtSz-*Scid* *IL2Rγ*^{null} mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained under sterile conditions. hESCs were differentiated using the two-stage differentiation protocol described in Figure 4A and collected at day 17. The whole cell mixture was dispersed to single cells using Accutase for 15–30 min, and cells were passed through a filter to remove clumps and finally resuspended in a total volume of 50 µl of PBS with 5% FCS per 5 × 10⁶ cells. To induce anaemia, NOD/LtSz-*Scid* *IL2Rγ*^{null} mice were injected intraperitoneally with PHZ (Sigma-Aldrich, St. Louis, MO; 90 mg/kg) 1 day prior to transplant. Twenty-four hours after the PHZ injection, 5 × 10⁶ hESC-differentiated cells were injected directly into the spleen. Groups of five to seven mice were injected either with cell carrier solution only or hESC-differentiated cells from *SCL*-overexpressing and control hESC lines. Five additional mice were injected with CD34+ cells from cord blood. Blood was drawn from the tail vein at different days using 0.5 mM EDTA as anticoagulant. Total red blood cell and white blood cell counts were performed at each day, in addition to reticulocyte counts by flow cytometry. Staining with Ter119-PE antibody (BD Biosciences) was performed prior to the analysis of dead and live red blood cell population by flow cytometry. Animals were culled at day 30 post PHZ injection, and PB, BM and spleen were collected for flow cytometry analysis.

AUTHORS' CONTRIBUTION

S.Y. performed research, analysed the data, wrote the paper and final approval of manuscript; M.L. performed research, analysed the data and final approval of manuscript; I.M.-G.

performed research, analysed the data and final approval of manuscript; A.C. analysed the data and final approval of manuscript; D.M. analysed the data and final approval of manuscript; J.D. analysed the data and final approval of manuscript; I.D. performed research and final approval of manuscript; N.J.S. performed research and final approval of manuscript; L.M. performed research and final approval of manuscript; P.J.R. performed research and final approval of manuscript; I.P. performed research and final approval of manuscript; V.B. performed research and final approval of manuscript; D.B. analysed the data and final approval of manuscript; M.S.-C. analysed the data and final approval of manuscript; R.M. analysed the data, fundraising and final approval of manuscript; J.M. analysed the data and final approval of manuscript; P.M. analysed the data and final approval of manuscript; L.A. analysed the data, fundraising, and final approval of manuscript and M.L. designed research, analysed the data, wrote the paper, fundraising and final approval of manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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