Induction of adult levels of β-globin in human erythroid cells that intrinsically express embryonic or fetal globin by transduction with KLF1 and BCL11A-XL

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ABSTRACT

A major barrier to the clinical use of erythrocytes generated *in vitro* from pluripotent stem cells or cord blood progenitors is failure of these erythrocytes to express adult hemoglobin. The key regulators of globin switching KLF1 and BCL11A are absent or at a lower level than in adult cells in K562 and erythroid cells differentiated *in vitro* from induced pluripotent stem cells and cord blood progenitors. Transfection or transduction of K562 and cord blood erythroid cells with either KLF1 or BCL11A-XL had little effect on β-globin expression. In contrast, transduction with both transcription factors stimulated β-globin expression. Similarly, increasing the level of BCL11A-XL in the induced pluripotent stem cell-derived erythroid cell line HiDEP-1, which has levels of endogenous KLF1 similar to adult cells but lacks BCL11A, resulted in levels of β-globin equivalent to that of adult erythroid cells. Interestingly, this increase in β-globin was coincident with a decrease in ε– and ζ–, but not γ-globin, implicating BCL11A in repression of embryonic globin expression. The data show that KLF1 and BCL11A-XL together are required, but sufficient to induce adult levels of β-globin in induced pluripotent stem cell and cord blood-derived erythroid cells that intrinsically express embryonic or fetal globin.

Introduction

The generation of human red blood cells *in vitro* for transfusion therapy is a major goal of health services globally. In recent years, development of systems for the generation of erythrocytes *in vitro* have progressed rapidly using progenitor cells isolated from adult peripheral blood (PB), umbilical cord blood and human pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells (iPSC)). Progenitors isolated from cord blood have the distinct advantage of a greater proliferative capacity than those isolated from PB[1] whereas iPSC and immortalized erythroid progenitor cell lines derived from cord blood and iPSC[2,3] have the potential to provide an inexhaustible source of progenitors for the generation of large numbers of red blood cells (reviewed in Anstee *et al.*,[4] Kaufman[5] and Peyrard *et al.*[6]). However, these progenitor cell sources express predominantly the β-like globins γ, or ε and γ, subunits of fetal (HbF) and embryonic (HbE) globin, in contrast to adult erythroid cells, which express β-globin. Although re-activation of fetal globin is considered a potential therapy for patients with β-hemoglobinopathies, it is unlikely that regulatory authorities will allow the use of a transfusion product containing such globins, not least because it would be inferior to the existing product derived from blood donations; HbE and HbF have very different biochemical and physical properties to adult globin (HbA). Inducing the expression of β-globin in these cells is, therefore, desirable before they can be taken forward for therapeutics. BCL11A and KLF1 are transcription factors critically involved in the switch to and expression of β–globin. BCL11A is a zinc finger (ZF) transcription factor identified from genetic association studies of HbF levels[7-10] and shown to be a repressor of γ–globin expression in humans.[10] Multiple variants of BCL11A are expressed although the three main forms reported are BCL11A-XL, BCL11A-L and BCL11A-S.[11,12] Developmental analysis of human erythroblasts shows full-length forms of BCL11A robustly expressed in adult cells, at substantially lower levels in fetal cells, and absent in primitive erythroblasts,[10] an inverse relationship to the expression of HbF in these cells. In adult erythroid cells, full-length BCL11A occupies several discrete regions within the human β-globin cluster including HS3 of the LCR, the ε-globin gene and an intergenic region near the δ-globin gene.[12,13] BCL11A also associates with erythroid transcription factors, transcriptional co-repressors, and chromatin-modifying enzymes in these cells.[14] Knockdown of BCL11A in human definitive erythroblasts results in increased expression of HbF[12] and reconfiguration of the 3D chromatin loop formation at the β-globin locus such that the γ genes are preferentially associated with the LCR.[15] In addition, introduction of a human β-locus transgene into BCL11A knock-out mice results in impaired silencing of the γ-globin genes in the definitive erythroid lineage.[16] BCL11A is, therefore, a potential target for reactivation of HbF in patients with β-hemoglobinopathies. Indeed, such an effect has recently been demonstrated in sickle cell disease transgenic mice, whereby inactivation of BCL11A corrects the hematologic and pathological

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defects associated with sickle cell disease through HbF induction.[17]

KLF1 (EKLF) is an erythroid-specific transcription factor essential for β-globin expression, definitive erythropoiesis, and also the switch from HbF to HbA.[18-20] The role of KLF1 in β-globin expression has been extensively studied (reviewed by Siatecka and Bieker[21]). KLF1 null mice die in utero around embryonic Day 14-15 due to failure of β-globin gene expression during fetal liver erythropoiesis.[18] β-globin expression is also absent in KLF1 null mice containing a human β locus transgene, whereas γ-globin is increased.[20] Similarly, knockdown of KLF1 in adult erythroblasts results in an increase in the γ- to β–globin ratio, and notably reduces expression of BCL11A.[22] There are increasing data to show that KLF1 also regulates many other erythroid genes and hence plays a critical and central role in erythropoiesis.[18,23-26]

KLF1 has been shown to interact *in vivo* with the locus control region as well as with the β–globin proximal promoter.[27] Although the exact mechanism by which KLF1 regulates β-globin expression is not yet fully elucidated, available data indicate that KLF1 plays a central role in promoting interaction of the locus control region with the proximal β-globin promoter, resulting in β-globin expression in adult erythroid cells.[28] As such, targeted knockdown of KLF1 has also been proposed as a strategy for activating HbF in individuals with sickle cell disease and β-thalassemia.

With such compelling data demonstrating a significant role for KLF1 and BCL11A in the expression of β-globin, we surmised that *in vitro*-generated erythroid cells intrinsically expressing ε- or γ-globin likely have absent or reduced expression of one or both these transcription factors. If so, we reasoned that induced or increased expression of KLF1 and/or BCL11A in these cells would induce expression of β-globin, a concept not previously investigated.

In this paper, we confirm both these hypotheses in K562, CB, iPSC and the immortalized iPSC-derived erythroid progenitor cell line HiDEP-1. Furthermore, we show that KLF1 and BCL11A are required above a threshold level, but together are sufficient to induce a level of β-globin expression equivalent to that of adult erythroid cells. Interestingly, our iPSC and HiDEP-1 cells expressed predominately embryonic (ε and ζ) globin, the levels of which decreased coincident with the increase in β-globin on transduction of HiDEP-1 cells with BCL11A-XL, implicating BCL11A in repression of embryonic globin expression. Overall, our data show the feasibility of using forward programming approaches to induce adult globin expression in erythroid cells generated *in vitro* from cord blood and pluripotent stem cells.

Methods

Plasmid construction

To prepare pBabe-puro HAII WT KLF-1, wild-type KLF1 was amplified by PCR, cloned into pCR®2.1-TOPO vector, then subcloned into the EcoRI site of pBabe-puro (pBp) HAII (plasmid 14738, Addgene Inc., Cambridge, MA, US). KLF1 was also inserted into pXLG3, and BCL11A-XL was amplified by PCR and inserted into pXLG3-eGFP, both using In-Fusion cloning system (Clontech).

Cell isolation and culture

K562 cells (European Collection of Cell Cultures, Salisbury, UK) were incubated in Iscove's modified Dulbecco's medium with L-glutamine supplemented with 10% fetal calf serum. Leukocyte reduction system cones and cord blood units were obtained from healthy donors who gave their written informed consent for research use in accordance with the Declaration of Helsinki and after approval by the local research ethics committees (Southmead Research Ethics Committee reference 08/H0102/26 and Bristol Research Ethics Committee reference 12/SW/0199). $CD34^+$ cells were isolated and incubated for eight days in a 3-stage erythropoietic culture system.[29] The human C19 iPSC line was expanded and differentiated as described by Trakarnsanga *et al.*[30] HiDEP-1 cells are a human iPSC line derived from amniotic fibroblast-like cells, differentiated into erythoid cells and immortalized at the pro-erythroblast stage by transduction with inducible HPV-type 16-E6/E7.[3] HiDEP-1 cells were maintained in Stemspan SFEM (Stem Cell Technology) with $3U.mL^{-1}$ erythropoietin (EPO) (Neorecormon), 10^{-6} M dexamethasone (Sigma) and $1\mu g.mL^{-1}$ doxycycline (Takara Bio) and differentiated in tertiary erythroid culture medium.[29]

Nucleofection

Cord blood cells and K562 cells were transfected using the Amaxa Nucleofection® system (Lonza Cologne AG, Cologne, Germany) with the Amaxa® Human $CD34^+$ Cell Nucleofector® Kit and Amaxa® Cell Line Nucleofector® Kit V, respectively, following the manufacturer's protocols.

Lentiviral preparation and transduction

HEK 293T cells were transduced with constructs pMDG2 (viral coat), pCMVR8.91 (packaging protein) and pXLG3-eGFP-BCL11A-XL, pXLG3-KLF1 or pCMV-VSV-G-RSV-Rev, pCAG-HIVgp (packaging plasmids) and CSII-EF-BCL11A-XL-IRES-Puro using Polyethylenimine (PEI). PEI/DNA solutions were incubated with the cells for 4 h, after which the media was replaced. After 48 h, media containing virus was filtered and concentrated using Lenti-X concentrator (Takara Bio Europe SAS/Clontech, France). Cells were incubated with 1 mL of selected virus(es) with the addition of polybrene at $8\ \mu g.mL^{-1}$.

Standard and quantitative polymerase chain reaction analysis

RNA (400 ng) was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Paisley, UK). BCL11A and KLF1 expression was analyzed by PCR. β-globin expression was analyzed by PCR and quantitative (q)PCR. All methods have been described previously.[26] Sequences of the primers used are shown in *Online Supplementary Table S1.*

Antibodies

KLF1 (H-210), β–globin (37-8), γ-globin (51-7), α-globin (D-16) were all from Santa Cruz Biotechnology, Santa Cruz, CA, USA, Ctip1/BCL11A (14B5), ε-globin (ab156041) and CHD4/Mi2β (ab54603) were from Abcam, Cambridge, MA, USA.

Tandem mass tag labeling and mass spectrometry

Cell lysates were digested with trypsin and labeled with tandem mass tag (TMT) reagents according to the manufacturer's protocol (Thermo Fisher Scientific). After labeling, samples were combined in equal amounts and fractionated by strong cation exchange using an Ettan LC system (GE Healthcare) prior to analysis by nanoLC-MS/MS. Data acquisition and processing was performed as described in Steinberg *et al.*[31]

Results

Transfection of K562 cells with KLF1 and BCL11A-XL induces β-globin expression

K562 is an erythroid cell line that expresses ε- and γ-globin, but not β-globin. Both KLF1 and BCL11A protein were absent in the K562 cells used in our study (Figure 1A, control lane), consistent with previous data.[32-34] However, different clones of K562 cells have been described which express just the shorter[12] or all variants of BCL11A.[35] When we looked for BCL11A-XL, BCL11A-L and BCL11A-S transcripts in our K562 cells using primers specific for each BCL11A variant, no transcripts were detected (*Online Supplementary Figure S1*). In contrast, transcripts for all three variants were detected in erythroid cells differentiated from adult PB progenitors.

To determine whether induced expression of KLF1 and/or BCL11A could induce β-globin expression in K562, cells were transiently transfected with pCDNA3-3Flag-BCL11A-XL (BCL11A-XL; 10 μg), pBabe-puro HAII-KLF1 (HA-KLF1; 10 μg) or co-transfected with both plasmids (5 or 10 μg of each). Full-length BCL11A-XL was used, as this is the most abundant variant in adult erythroid cells.[12] Following transfection (20 h), cells transfected with BCL11A-XL or HA-KLF1 expressed the respective proteins, co-transfected cells expressed both proteins (Figure 1A). Transfection with BCL11A-XL or HA-KLF1 alone increased the level of β-globin transcript by 5.9±1 and 7.5±1.3 fold, respectively, compared to non-transfected K562 cells (Figure 1B). However, co-transfection with 5 μg or 10 μg of BCL11A-XL and HA-KLF1 increased expression of β-globin by 305.2±90.6 and 887±82.7 fold, respectively (Figure 1B). A correlation between the levels of KLF1 and BCL11A-XL and β-globin expression was also seen when the level of KLF1 and BCL11A-XL decreased between 24 and 48 h post transfection, with a concomitant decrease in β-globin expression (Figure 1C-E). We also co-transfected K562 cells with pCDNA3-3Flag-BCL11A-L or pCDNA3-3Flag-BCL11A-S along with HA-KLF1 (10 μg of each). However, in contrast to BCL11A-XL,

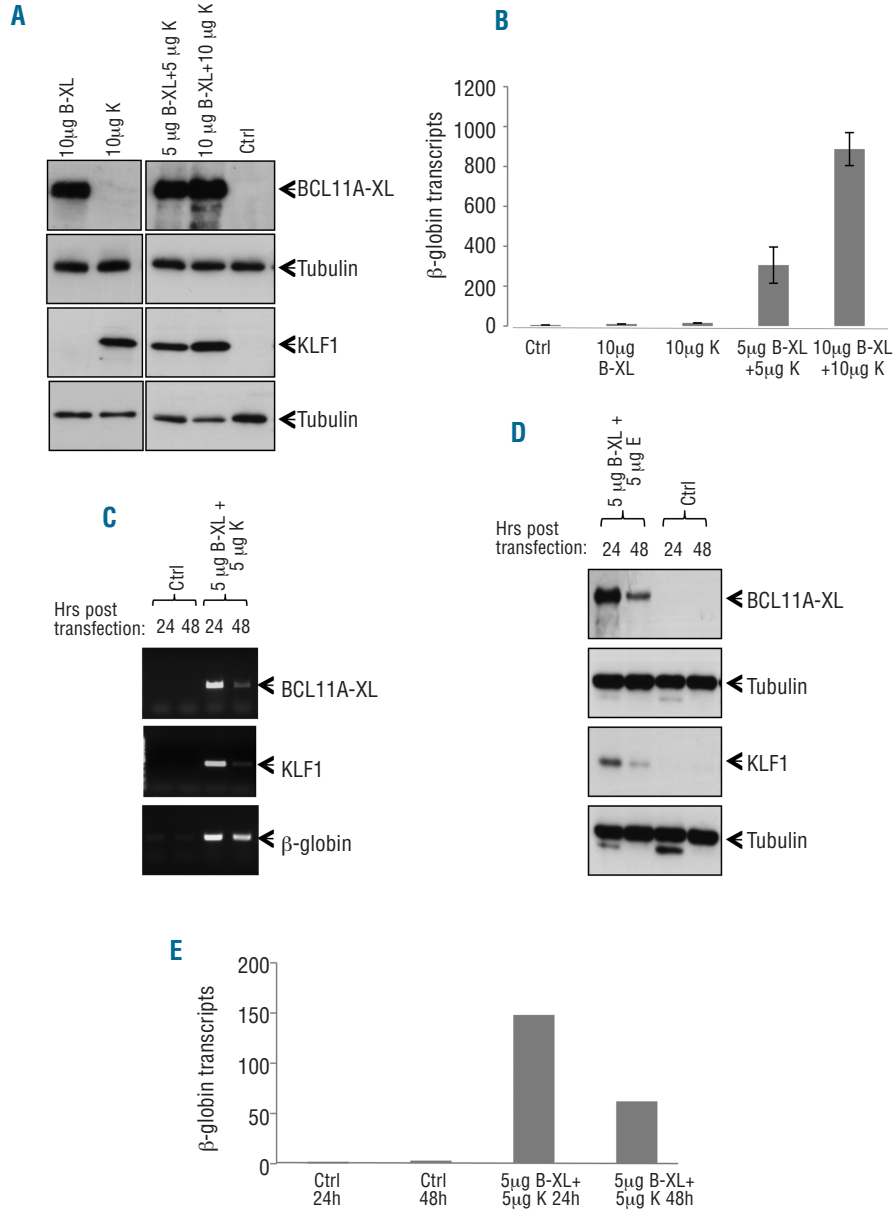

Figure 1. Co-transfection of K562 cells with KLF1 and BCL11A-XL induces β-globin expression. K562 cells were transfected with 10 μg of pCDNA3-3Flag-BCL11A-XL (B-XL), 10 μg of pBp HA-KLF1 (K) or co-transfected with 5 or 10 μg of each plasmid. Control (Ctrl) K562 cells were mock transfected. (A) Western blot of total protein from transfected and control cells 20 h post transfection probed with BCL11A and KLF1 antisera. (B) Relative β-globin transcript levels in transfected and control cells analyzed by qPCR, normalized to transcripts for PABPC1 and calibrated to control cells. Mean±SEM; n=3. (C) BCL11A-XL, KLF1 and β-globin transcripts 24 and 48 h post transfection analyzed by PCR. (D) Western blot of total protein from K562 cells 24 and 48 h post transfection probed with antisera to BCL11A and KLF1. (E) β-globin transcripts in co-transfected and K562 control cells at 24 and 48 h post transfection analyzed by qPCR, normalized to PABPC1 expression and calibrated to K562 control. Western blots were stripped and re-probed with antisera to Tubulin as a protein loading control.

co-transfection with neither BCL11A-L nor BCL11A-S increased the expression of β-globin (*Online Supplementary Figure S2*).

As the duration of expression of KLF1 and BCL11A-XL was short following transient transfection, we transduced K562 cells with pXLG3-eGFP-BCL11A-XL and/or pXLG3-KLF1 lentiviral constructs. Approximately 80% efficiency was achieved for the single and 30% for dual transductions. The level β-globin protein was increased 48 h post transduction with both BCL11A-XL and KLF1, similar to the increase observed following co-transfection, relative to control cells (*Online Supplementary Figure S3*). There was no notable increase in levels of β-globin protein following transduction with KLF1 or BCL11A-XL alone.

Expression of KLF1 and BCL11A in erythroid cells generated in vitro *from adult, cord blood and iPSC progenitors*

Erythroid cells generated *in vitro* from cord blood and iPSC CD34$^+$ progenitors also express predominantly HbF,

Table 1. Comparison of the level of globin subunits in HiDEP-1 cells before and after transduction with BCL11A-XL and adult erythroid cells.

Globin subunit	Peptides	Unique peptides	HiDEP-1/PB	HiDEP-1-BCL11A/PB
Hemoglobin alpha 1	5	4	1.44	2.66
Hemoglobin beta	6	2	0.06	0.86
Hemoglobin gamma-A	10	2	3.54	4.58
Hemoglobin gamma-G	10	1	7.99	17.97
Hemoglobin epsilon	8	6	58.30	8.47
Hemoglobin zeta	6	5	79.91	9.67
Hemoglobin delta	6	4	0.40	3.73
Hemoglobin mu	2	2	1.69	2.03
Hemoglobin theta	1	1	2.68	2.62

Cell lysates of HiDEP-1 cells, HiDEP-1 cells transduced with BCL11A-XL (HiDEP-1-BCL11A) and erythroid cells differentiated from adult peripheral blood progenitors (PB) at the same developmental stage were digested with trypsin and labeled with tandem mass tag (TMT) reagents prior to analysis by nanoLC-MS/MS. The raw data files were processed and quantified using Proteome Discoverer software v.1.4 (Thermo Scientific) and searched against the UniProt/SwissProt Human database release v.57.3 (20326 entries) using the SEQUEST (v.28, rev. 13) algorithm. Peptides and unique peptides: the total number of peptide sequences and number of unique peptides identified for that protein. Comparative levels were calculated using unique peptides only.

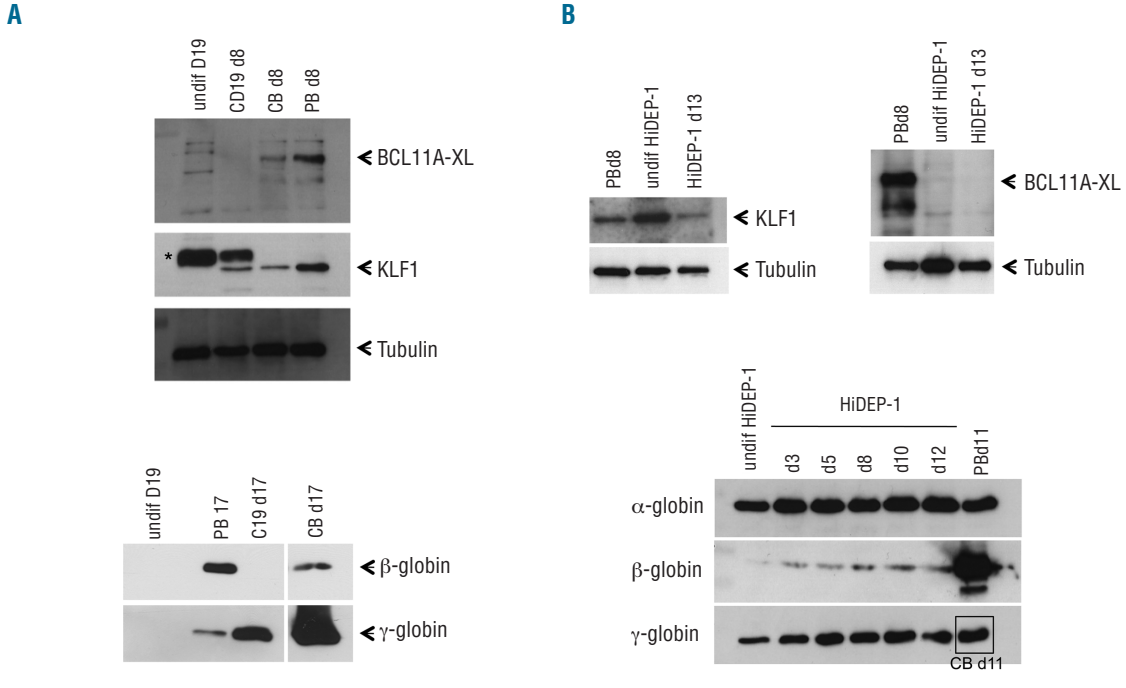

Figure 2. Expression of KLF1, BCL11A and globin subunits by erythroid cells differentiated *in vitro* from adult, cord blood and C19 iPSC progenitors and HiDEP-1 cells. Western blots of (A) undifferentiated (undif) C19 iPSCs, and C19, adult (PB) and cord blood (CB) CD34$^+$ cells differentiated for 8 and 17 days in erythroid culture media. *Non-specific band detected by antibody (B) undifferentiated HiDEP-1 (undif), HiDEP-1 differentiated for 3, 5, 8, 10 and 12 days in erythroid culture medium and adult (PB) and cord blood (CB) *in vitro* derived erythroid cells at Day 8 and/or 11 in culture. Blots were probed with antibodies to KLF1, BCL11A, α, β and γ globin, stripped and re-probed with an antibody to tubulin as a protein loading control. For analysis of β-globin in C19 and HiDEP-1 cells, 20 μg protein was resolved by SDS-PAGE. For all other samples and analysis 3 μg protein was used. Box indicates cord blood (CB) erythroid sample. Top image in panel A from Trakarnsanga *et al.*[30]

or HbE and HbF. We, therefore, compared the level of KLF1 and BCL11A in these cells with that of adult erythroid cells, and correlated levels with their globin expression profiles.

Progenitors from cord blood, adult PB, the iPSC line C19, and the iPSC-derived erythroid progenitor cell line HiDEP-1 were differentiated in erythroid culture media, and comparisons were made between erythroid cells at similar stages of differentiation, the stage determined using morphological analysis. Erythroid cells from cord blood progenitors had lower levels of both KLF1 and BCL11A-XL when compared with adult cells, and expressed predominantly γ, with a low level of β-globin (Figure 2A). C19-derived erythroid cells expressed a similar level of KLF1 to cord blood cells following differentiation, but BCL11A was absent (Figure 2A). These cells expressed γ-globin indicating erythroid differentiation, but no β-globin. In contrast, the level of KLF1 in HiDEP-1 cells was at least as high as that in adult erythroid cells; however, BCL11A was absent. The level of KLF1 decreased in these cells during differentiation, as in adult erythroid cells. These cells also expressed predominantly γ-globin, although a low level of β-globin was also present (Figure 2B), both globins increasing during differentiation. These data suggest a correlation between the levels of both KLF1 and BCL11A and globin expression profiles.

Differentiated HiDEP-1 cells exhibit an erythroid phenotype

Erythroid cells differentiated from HiDEP-1 cells have previously been shown to express transcripts for a number of erythroid genes[3] and, as shown in Figure 2B, they synthesize α, γ and low-level β-globin protein, indicating erythroid differentiation. We further confirmed erythroid differentiation of HiDEP-1 cells morphologically (*Online Supplementary Figure S4A*), by their synthesis and localisation of hallmark erythroid proteins GPA, GPC, Band 3, Rh, RhAG and CD71 (*Online Supplementary Figure S4B and C*), and detected up to 16% enucleation, similar to that of other iPSC-derived erythroid cells.[2,36]

As shown above, HiDEP-1 cells express a similar level of endogenous KLF1 to adult erythroid cells on differentiation in erythroid culture. However, in mouse erythroid cells, KLF1 is detected in both the cytoplasm and nucleus[37,38] with developmental stage-specific nuclear import potentially involved in the switch to adult globin expression.[38,39] We, therefore, compared the sub-cellular localization of KLF1 in HiDEP-1 cells with adult PB CD34$^+$ derived erythroid cells at the same developmental stage (Figure 3A). In both, KLF1 was detected in the cytoplasmic and nuclear fractions (Figure 3B), with at least as much KLF1 detected in the nuclear fraction of HiDEP-1 as in adult cells. The sequence of KLF1 in the HiDEP-1 cells was also confirmed as wild type (*data not shown*).

Transduction of HiDEP-1 cells with BCL11A-XL induces adult level β-globin expression

Next, we explored whether induced expression of BCL11A-XL in HiDEP-1 cells could increase the expression of β-globin. Initially, HiDEP-1 cells were transduced with CSII-EF-BCL11A-XL-IRES-Puro and differentiated in erythroid culture medium. BCL11A-XL was detected in the nuclear fraction of transduced HiDEP-1, but not non-transduced cells (Figure 3B), and showed a similar sub-cellular localization to endogenous BCL11A in adult erythroid cells. Following transduction, a robust increase in the level of β-globin protein was induced in the HiDEP-1 cells (Figure 3C). There was no observed increase in the nuclear level of KLF1 coincident with the increase in β-globin expression (Figure 3B), consistent with data from Quadrini *et al.*,[37] but in contrast to that of Shyu *et al.*[38,39]

Cell transduction routinely results in a heterogeneous population of cells expressing different levels of induced protein. To determine whether cells expressing a higher level of BCL11A-XL express a higher level of β-globin, we transduced HiDEP-1 cells with the pXLG3-eGFP-BCL11A-

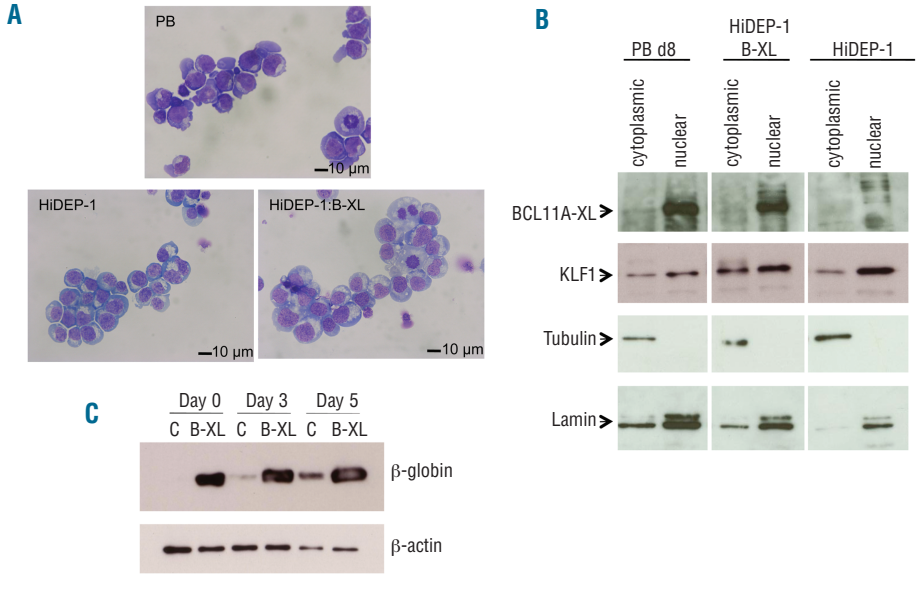

Figure 3. Induced expression of β-globin in HiDEP-1 cells transduced with BCL11A-XL. (A) HiDEP-1 cells before and 9 days after transduction with BCL11A-XL (HiDEP-1:B-XL) and erythroid cells differentiated from adult PB progenitors at Day 8 in erythroid culture (PB) stained with May-Grunwald Giemsa reagent and analyzed by light microscopy. (B) Western blots of cytoplasmic and nuclear fraction of cells in (A) probed with antibodies to KLF1 and BCL11A. Antibodies to tubulin and lamin were used to determine purity of the subcellular fractions, (C) whole cell lysate of HiDEP-1 cells (C) and HiDEP-1 cells transduced with BCL11A-XL (B-XL) before, 3, and 5 days after differentiation in erythroid culture medium, probed with antibody to β-globin. Antibody to β-actin was used as a protein loading control. For all blots, 20 μg protein was resolved by SDS-PAGE.

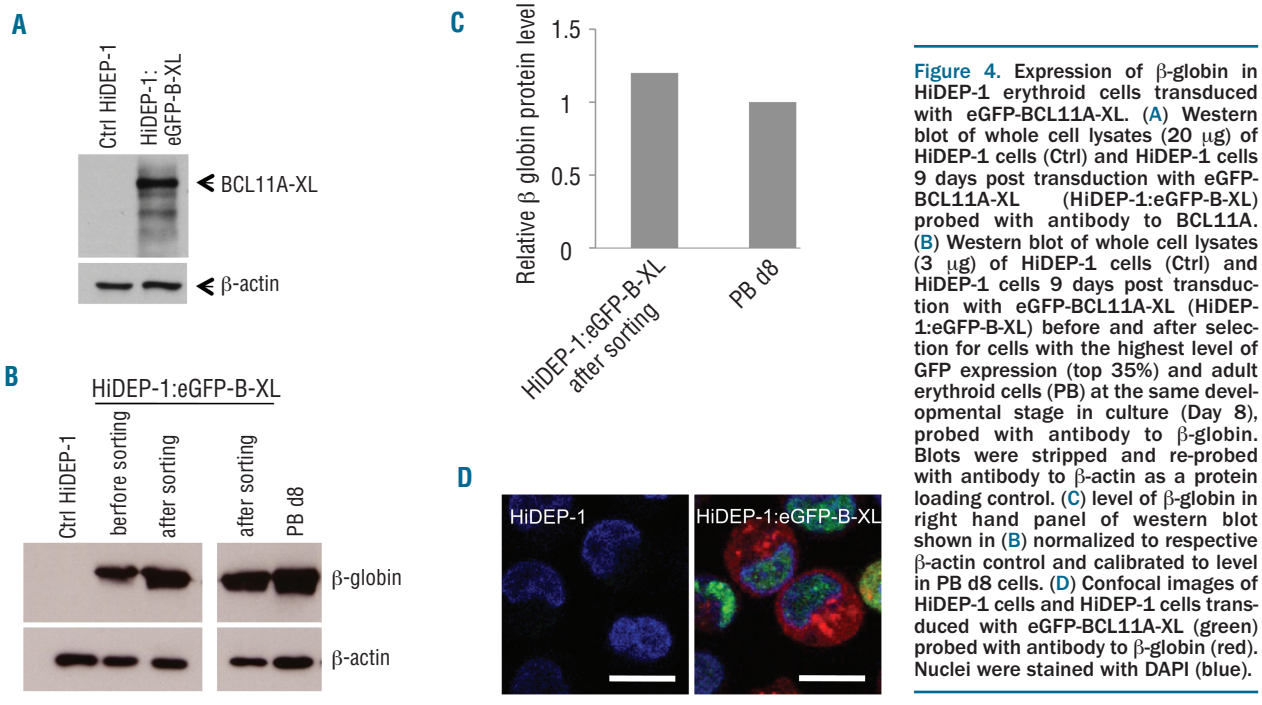

Figure 4. Expression of β-globin in HiDEP-1 erythroid cells transduced with eGFP-BCL11A-XL. (A) Western blot of whole cell lysates (20 μg) of HiDEP-1 cells (Ctrl) and HiDEP-1 cells 9 days post transduction with eGFP-BCL11A-XL (HiDEP-1:eGFP-B-XL) probed with antibody to BCL11A. (B) Western blot of whole cell lysates (3 μg) of HiDEP-1 cells (Ctrl) and HiDEP-1 cells 9 days post transduction with eGFP-BCL11A-XL (HiDEP-1:eGFP-B-XL) before and after selection for cells with the highest level of GFP expression (top 35%) and adult erythroid cells (PB) at the same developmental stage in culture (Day 8), probed with antibody to β-globin. Blots were stripped and re-probed with antibody to β-actin as a protein loading control. (C) level of β-globin in right hand panel of western blot shown in (B) normalized to respective β-actin control and calibrated to level in PB d8 cells. (D) Confocal images of HiDEP-1 cells and HiDEP-1 cells transduced with eGFP-BCL11A-XL (green) probed with antibody to β-globin (red). Nuclei were stained with DAPI (blue).

XL construct, enabling us to monitor protein expression and select those cells with the highest level of BCL11A-XL. Transduction efficiency was routinely 60-70%. Transduced cells expressed eGFP-BCL11A-XL (Figure 4A), which was again localized to the nucleus (Figure 4D and *Online Supplementary Figure S5*). Cells with the highest levels of GFP expression (35% of expressing cells) were collected and the level of β-globin compared to that of cells prior to sorting. The level of β-globin was 1.5-fold higher in those cells expressing the highest levels of eGFP-BCL11A-XL, compared to the level of the entire population before sorting (Figure 4B, left panel). Moreover, the level of β-globin in these cells was equivalent to that of adult erythroid cells (Figure 4B, right panel, and 4C). Transduced HiDEP-1 cells proliferated at the same rate as non-transduced cells and differentiated normally, as determined by morphological analysis and GPA expression at Day 12 in tertiary erythroid culture medium (*Online Supplementary Figure S6*).

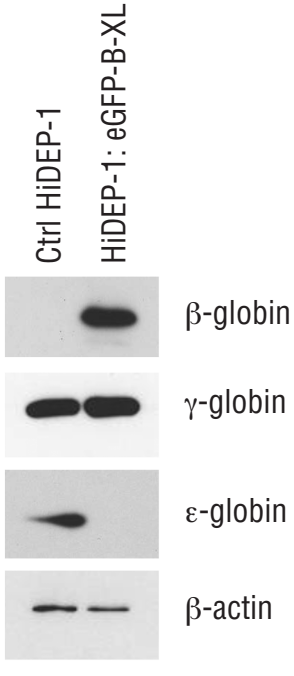

Figure 5. Level of β-, γ- and ε-globin in HiDEP-1 cells before and after transduction with eGFP-BCL11A-XL. Western blot of whole cell lysates (10 μg) of HiDEP-1 cells (Ctrl) and HiDEP-1 cells 9 days post transduction with eGFP-BCL11A-XL (HiDEP-1:eGFP-B-XL) probed with antibodies to β-, γ- and ε-globin and β-actin.

To obtain more detailed information on the expression of globin subunits, we used TMT labelling and mass spectrometry to quantify and compare protein levels in HiDEP-1 cells before and after BCL11A-XL transduction with that of erythroid cells differentiated from adult PB progenitors at the same developmental stage (Figure 3A). As can be seen in Table 1, the level of β-globin in HiDEP-1 cells before transduction was 17-fold less than that in adult cells. Gamma globin A and G were both at a slightly higher level in HiDEP-1 compared to adult cells; however, the embryonic globin subunits (ε and ζ) were at a strikingly higher level (58- and 80-fold higher than adult cells, respectively), indicating that the cells express predominantly embryonic globin. We have used the same methodology to analyze the total globin expression profile of erythroid cells differentiated from iPSC lines C19, OCE1 and OPM2 with that of adult cells, and found the level of β-globin was 25-fold lower in these compared to adult cells and that they also expressed predominately embryonic globin subunits.[30] Following transduction of HiDEP-1 cells with BCL11A-XL, the level of β-globin increased 14-fold;

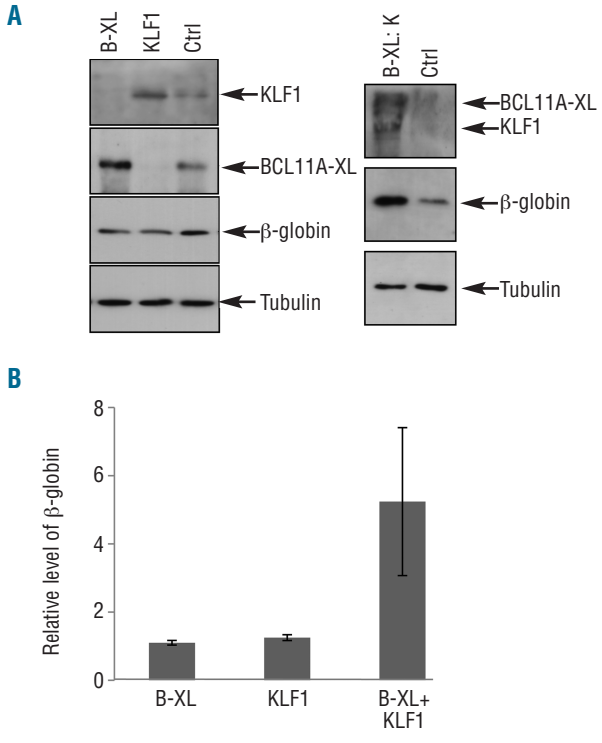

Figure 6. Co-transfection of erythroid cells differentiated *in vitro* from cord blood progenitors with KLF1 and BCL11A-XL increases β-globin expression. Erythroid cells differentiated in vitro from cord blood (CB) CD34+ cells were transfected with 5 μg of pCDNA3-3Flag-BCL11A-XL (B-XL), 5 μg of pBp HA-KLF1 (K) or co-transfected with 5 μg of each plasmid. Control cells were mock transfected. Cells were collected 17 h post transfection. (A) Western blot of total protein (3 μg) from transfected cells probed with BCL11A and KLF1 antisera, then sequentially stripped and re-probed with β-globin and tubulin antisera. (B) Relative β-globin protein levels in single and co-transfected cells normalized to tubulin expression and calibrated to level in control cells (mean±SEM; n=3).

δ-globin also increased 9-fold. Interestingly, the level of γ-globin did not decrease following transduction; instead, the level of both ε- and ζ-globin decreased by 7- and 8-fold, respectively. The level of β-, γ- and ε-globin in HiDEP-1 cells before and after transduction with BCL11A-XL was confirmed by western blot (Figure 5).

Increased expression of both BCL11A-XL and KLF1 is required to increase the expression of β-globin by erythroid cells differentiated from cord blood progenitors

Cord blood red blood cells contain predominantly HbF (~65%) in contrast to adult RBCs that contain predominantly HbA (~94%). As shown in Figure 2A, cord blood-derived erythroid cells have lower levels of both KLF1 and BCL11A compared to adult cells (consistently 5-10 fold lower). To increase the level of KLF1 and/or BCL11A-XL in these cells, we initially used transfection with pCDNA3-3Flag-BCL11A-XL (5 μg) and/or pBabe-puro HAII-KLF1 (5 μg). Following transfection (17 h), the level of both KLF1 and BCL11A-XL was increased (Figure 6A). No detectable increase in the level of β-globin was detected in cells transfected with KLF1 or BCL11A-XL alone; however, a 5.2+2.2 fold increase was observed following transfection with both KLF1 and BCL11A-XL compared to non-transfected cells (n=3) (Figure 6A and B; western blots shown are representative of all 3 experiments). A similar effect was obtained following transduction of cord cells with CSII-EF-BCL11A-IRES-Puro and pXLG3-KLF1 (*Online Supplementary Figure S7*); however, co-transduction efficiency was low (<10%).

Expression of Mi2β in K562 cells and erythroid cells generated in vitro from adult, cord blood and iPSC progenitors

During the preparation of this manuscript, Amaya *et al.*[40] showed that Mi2β binds and positively regulates both KLF1 and BCL11A genes, with knockdown of Mi2β resulting in decreased expression of both, with a corresponding increase in γ-globin expression. We thus hypothesized that the level of Mi2β would be lower in erythroid cells expressing predominantly fetal globins than in adult cells. If so, Mi2β could be an attractive target for manipulation, increasing the level of both KLF1 and BCL11A, and thus β-globin directly. We, therefore, examined our comparative proteomic data obtained from tandem mass tag (TMT) labeling of cord blood, iPSC and adult erythroid cell peptides (J Frayne, unpublished data, 2013) but found no variation in the level of Mi2β between the different erythroid cells. This was confirmed by western blot analysis of adult, CB and HiDEP-1 erythroid, and K562 cells (*Online Supplementary Figure S8*).

Discussion

Cord blood and iPSC are attractive sources of progenitor cells for the production of erythroid cells *in vitro*. However, one hurdle that remains unresolved is the expression of predominantly HbE and HbF, rather than HbA, by the resultant erythrocytes.

KLF1 and BCL11A are transcription factors essential for inducing β-globin and repressing γ-globin expression, respectively. We have shown that K562 cells and erythroid cells derived from C19 iPSC and cord blood progenitors have absent or lower levels of BCL11A and KLF1 when compared with adult erythroid cells, which correlates with their low level of β-globin expression. HiDEP-1 cells have levels of KLF1 similar to adult erythroid cells, but lack BCL11A and also have low levels of β-globin.

Increasing the level of KLF1 or BCL11A-XL in K562 or cord blood erythroid cells by transfection or transduction resulted in, at best, a very modest increase in β-globin expression. In contrast, increasing the level of both transcription factors in K562 cells induced a prominent increase in β-globin levels. Similarly, increasing the level of both transcription factors in cord blood progenitors, and BCL11A-XL in HiDEP-1 cells, had a robust effect on β-globin expression, with the resultant level equivalent to that of adult cells. In cord blood cells, this increase may appear low (average 5.2-fold; n=3), but as can be seen in the example western blot in Figure 2, the level of endogenous β-globin is approximately 4-8 fold lower in cord blood than adult erythroid cells; hence, the increase achieved is physiologically significant.

We believe these are the first data demonstrating that β-globin can be induced *in vitro* in cells normally restricted to, or predominantly expressing HbE or HbF. The data also demonstrate that both KLF1 and BCL11A-XL are required and must be above a threshold level, but together are suf-

ficient to induce adult levels of β-globin. These results are consistent with data showing knockdown of BCL11A in adult erythroid cells with normal levels of KLF1 increases the γ to β globin ratio.[12] Knockdown of KLF1 also increases the γ to β globin ratio, but simultaneously decreases the expression of BCL11A relieving repression of the γ-globin gene.[41] A KLF1-BCL11A axis has also been described for compound KLF1::BCL11A mutant mice carrying a human β-globin locus transgene, with repression of γ-globin expression.[42] However, although KLF1 positively regulates the expression of BCL11A in adult erythroid cells, increasing the level of KLF1 in K562 and cord blood cells did not result in an increase in BCL11A. Similarly, HiDEP-1 cells, which have levels of KLF1 similar to adult cells, do not express BCL11A. Therefore, other factors that differ between these cells are likely involved in the regulation of BCL11A.

The iPSC and HiDEP-1 cells used in our study expressed predominately embryonic ε- and ζ-globin subunits. Interestingly, transduction of HiDEP-1 cells with BCL11A-XL resulted in a decrease in the level of these subunits, and not γ-globin, coincident with the increase in β-globin. Our data, therefore, suggest that BCL11A is involved in repressing embryonic globin expression. The lack of effect of BCL11A on γ-globin expression in our cells is likely due to their predominant expression of the embryonic subunits. Such a role for BCL11A in repressing embryonic globin is supported by ChIP-chip data showing binding of BCL11A, along with interacting partners SOX6 and GATA1, to the epsilon globin gene.[15]

A number of BCL11A isoforms have been identified, the most commonly detected being BCL11A-XL, -L and –S,[43] the latter two being spliced within exon 4 to an additional fifth exon not present in BCL11A-XL. The function of these different isoforms is not clear. In our study, transcripts for all three isoforms were detected in erythroblasts derived from peripheral blood CD34$^+$ cells; however, only BCL11A-XL induced β-globin expression when co-transfected with KLF1 in K562 cells.

In conclusion our data are proof of principle that adult levels of β-globin can be obtained in erythroid cells generated *in vitro* from cord blood progenitors and iPSC using forward programming approaches.

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