Disruption of the BCL11A Erythroid Enhancer Reactivates Fetal Hemoglobin in Erythroid Cells of Patients with β-Thalassemia Major

Nikoletta Psatha,1,6 Andreas Reik,2,6 Susan Phelps,3 Yuanyue Zhou,2 Demetri Dalas,3 Evangelia Yannaki,1,4 Dana N. Levasseur,5 Fyodor D. Urnov,2 Michael C. Holmes,2 and Thalia Papayannopoulou3

1Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, WA, USA; 2Sangamo Therapeutics, Richmond, CA, USA; 3Division of Hematology, Department of Medicine, University of Washington, Seattle, WA, USA; 4Hematology Department, BMT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece; 5Bioverativ Therapeutics, Waltham, MA, USA

In the present report, we carried out clinical-scale editing in adult mobilized CD34+ hematopoietic stem and progenitor cells (HSPCs) using zinc-finger nuclease-mediated disruption of BCL11a to upregulate the expression of γ-globin (fetal hemoglobin). In these cells, disruption of the erythroid-specific enhancer of the BCL11A gene increased endogenous γ-globin expression to levels that reached or exceeded those observed following knockout of the BCL11A coding region without negatively affecting survival or in vivo long-term proliferation of edited HSPCs and other lineages. In addition, BCL11A enhancer modification in mobilized CD34+ cells from patients with β-thalassemia major resulted in a readily detectable γ-globin increase with a preferential increase in G-gamma, leading to an improved phenotype and, likely, a survival advantage for maturing erythroid cells after editing. Furthermore, we documented that both normal and β-thalassemia HSPCs not only can be efficiently expanded ex vivo after editing but can also be successfully edited post-expansion, resulting in enhanced early in vivo engraftment compared with unexpanded cells. Overall, this work highlights a novel and effective treatment strategy for correcting the β-thalassemia phenotype by genome editing.

INTRODUCTION

Patients with severe β-globin thalassemias require life-long red blood cell transfusions along with iron chelation treatment. The only curative option is allogeneic hematopoietic cell transplantation, available only to a subset of patients having a suitable donor.1 For patients without an allogeneic hematopoietic cell transplantation option, recent molecular approaches employing gene-modified autologous cells are emerging as therapeutic alternatives. Gene addition protocols through viral vectors, using autologous stem cells, have been studied in vitro for the last 20 years, and since 2006, several small clinical trials have been either completed or initiated.2,3 These trials offer clear promise with significant clinical improvement in a proportion of patients.

Although innovative protocols for capturing mainly highly purified genetically engineered cells transduced with lentiviral vectors have been proposed to improve efficiency4, significant challenges remain, and the long-term safety risks associated with the use of viral vectors are being currently evaluated. Apart from β- or γ-globin gene addition in hematopoietic stem cells by traditional lentiviral vectors, several other protocols aiming to increase or reactivate fetal hemoglobin (HbF) have been explored because this outcome would be applicable for amelioration of all β-globin disorders, irrespective of the genetic mutation. Tested strategies for HbF reactivation involve either forced chromatin looping, mediated by a lentiviral vector expressing the looping factor LDB1 linked to a zinc-finger protein binding the γ-globin promoter,5 the re-creation of γ-globin promoter mutations leading to hereditary persistence of HbF (HPFH) conditions,6,7 or downregulation or inhibition of trans-factors involved in γ-globin silencing8 or disruption of their binding regions.9–11 Attempts to faithfully recreate HPFH γ-globin promoter mutations through gene editing have been associated with low efficiency in normal adult CD34+ cells ex vivo because less efficient homology- or microhomology-mediated repair is required.8,9 By contrast, attempts to induce nuclease-mediated inactivation of HbF “silencers” (i.e. BCL11A,12–14 LRF,8 or KLF115) can be more efficient because they require repair of DSBs with non-homologous end joining (NHEJ). One of several approaches targeting HbF reawakening is suppression of an important developmental silencer of γ-globin, BCL11A. This approach has shown efficiency in all previous studies, and it is favored for several reasons. BCL11A inactivation through NHEJ repair is more efficient than the homology-mediated repair needed to recreate known HPFH sites in the γ-globin promoter, and inactivation of the erythroid-specific BCL11a enhancer6,14,16 alleviates concerns...
about the effect of BCL11a inactivation in non-erythroid cells because BCL11A deficiency in murine models has been associated with impaired B lymphocyte and hematopoietic stem cell (HSC) development or about influencing aspects of erythroid differentiation/maturation raised by targeting other transcription factors involved in silencing (i.e. KLF1 and ZBTB7A). Finally, it is known that patients with BCL11A haploinsufficiency have increased levels of HbF at levels likely to be therapeutic for patients with β-globin disorders.19,20

Lentivirus-mediated erythroid-specific expression of a BCL11A shRNA reflects an additional efficient strategy for HbF upregulation, although this approach may be hampered by cytotoxicity triggered via endogenous microRNA dysregulation and is coupled with theoretical long-term concerns of lentiviral vector genotoxicity and silencing because of genomic position effects.25

In the present study, we explored the functional consequences of zinc-finger nuclease (ZFN)-induced inactivation of the BCL11A-erythroid enhancer in both normal and thalassemic mobilized CD34+ cells in vitro and in vivo, before and after implementation of our expansion protocols, to enhance engraftment of edited HSCs. A similar approach using an updated ZFN architecture is currently being evaluated in a phase 1/2 clinical trial.

RESULTS

Efficient Editing of the BCL11A Enhancer and BCL11A Exon2 in Adult Mobilized CD34+ Cells

In the first set of our experiments, we sought to provide detailed comparisons of the effect of disrupting BCL11A with early-stage ZFN pairs targeting either BCL11A exon 2 or the BCL11A erythroid enhancer using mobilized peripheral blood CD34+ cells under clinical-scale conditions because these cells represent the optimal cell source for gene modification therapies for β-thalassemia.27,28

Peripheral blood CD34+ cells from a plerixafor and granulocyte colony-stimulating factor (G-CSF)-mobilized healthy donor were electroporated at clinical scale with ZFN mRNA targeting either BCL11A exon 2 or the BCL11A erythroid enhancer (Figure S1A). Gene modification levels, as assessed by high-throughput DNA sequencing 2 days after electroporation, were 72% for the BCL11A exon 2-modified cells and 68% for the BCL11A enhancer-modified cells (Figure 1A). To assess the effects of genome editing on erythropoiesis, we used a well-established protocol of in vitro erythroid differentiation.29 First we evaluated possible effects of mRNA-ZFN electroporation and genome editing on growth kinetics and in vitro terminal erythroid maturation. Disruption of the BCL11A erythroid enhancer did not measurably affect erythroid differentiation and/or maturation because cell growth, morphology, and enucleation rate were not obviously different from the untransfected sample at all time points tested (Figures 1B–1D, respectively). Expression of the erythroid-specific surface markers glycophorin A and E-cadherin was almost identical between the edited and unedited samples throughout the maturation process (Figure S2), suggesting concurrent differentiation among the three different samples. However, the BCL11A coding region knockout (KO) had a negative effect on cell growth and survival during maturation, as shown by the decreased total cell yield (Figure 1B). In line with this outcome, the total insertion or deletion (indel) levels tended to decrease in BCL11A exon 2 KO erythroid progenitors (42% versus 72% prior to differentiation) throughout the culture. In contrast to BCL11a KO, in enhancer KO, the frequency of edited cells was retained throughout differentiation (63% versus 68% prior to differentiation) (Figure 1A). The effect on HbF reactivation was evaluated by both fluorescence-activated cell sorting (FACS) and immunocytochemistry, and the γ-globin protein levels were assessed by HPLC. The frequency of HbF+ cells detected by FACS and immunofluorescence was significantly increased in both exon 2- and enhancer-edited samples.
compared with the control (~25% versus <5% HbF+ cells in enucleated red blood cells, respectively) (Figures 2A and 2B). HbF+ mature enucleated red blood cells were present in both exonic and enhancer BCL11A edited samples, as shown by immunocytochemistry (Figure 2C). The differentiated progeny of the transfected cells was sorted based on γ-globin levels and cell size or maturation level, and the sorted fractions were sequenced for evaluation of indel levels (Figure S2). No evident difference was observed in the indel percentage between the smaller, more mature and larger, less differentiated cells of either group (Figure 2D). More than 95% of the enhancer-targeted alleles were edited in the HbF-high cells, suggesting that BCL11A modification was biallelic in almost every cell with very high γ-globin levels (Figure 2D).

As expected, almost no high γ-globin-expressing cells were found in the untransfected sample (data not shown). Reverse-phase HPLC, separating the globin polypeptides, was performed in enucleated and nucleated erythroid cells. Both ex on 2- and enhancer-edited nucleated cells presented a 6-fold increase in γ-globin expression (GγAγ); however, in the enucleated cells, the enhancer ZFN effect was greater than that of the exon ZFN (3.3-fold increase versus 2.3-fold increase, respectively) (Figure 2E; Figure S4). A similar increase in HbF was recently reported for ZFN-edited bone marrow cells.16 Furthermore, qRT-PCR validated this robust effect of BCL11A erythroid enhancer disruption on γ-globin mRNA levels (Figure S5).

Bcl11a Erythroid Enhancer KO Is Sustained Long-Term In Vivo in Both Primary and Secondary Recipients

In addition to in vitro experiments, we evaluated the engraftment potential of edited CD34+ cells from normal donors and the subsequent effect on HbF expression in erythroid cells derived from xenografted progeny. Sublethally irradiated NOD.Cg-PrkdcsidI2Rgtm1Wjl/Szg (NSG) mice were transplanted with 10⁶ CD34+ cells/recipient ( frozen~48 hr after electroporation and thawed on the day of injection); 3 samples were used: non-edited CD34+ cells, CD34+ cells edited with a BCL11A-exon2 ZFN pair (72% modification 2 days post-electroporation) and CD34+ cells edited with BCL11A enhancer-targeted ZFNs (68% modification 2 days post-electroporation). The mice were evaluated 4, 8, 12, and 16 weeks after transplantation for the presence of human CD45+ cells in their peripheral blood and in week 16 for bone marrow engraftment of human cells of different lineages. Human chimerism was comparable among the different groups throughout the experiment, both in the peripheral blood (Figure 3A) and bone marrow (Figure 3B), and multilineage reconstitution was observed in all engrafted mice (Figures 3B and 3C). Colony-forming human progenitor cells were also detected in all recipients at the same frequency (Figure 3D). However, evaluation of the indel percentage within the different human cell subpopulations revealed a higher retention of modification at the enhancer versus the coding region both in peripheral blood (Figure 3E) and in sorted bone marrow-derived cells (Figure 3F), a difference that was more prominent in the lymphoid lineage and the HSC compartment (CD34+/CD38−) and less so in the myeloid lineage, suggesting that KO of the coding region severely impairs B cell and HSC development, in contrast to the influence observed following KO of the BCL11a erythroid enhancer (Figure 3F). Similar results in terms of engraftment and indel levels were obtained 20 weeks after transplantation (Figure S6). To assess HbF reactivation and indel frequencies in the engrafted erythroid cells, we performed ex vivo erythroid differentiation using recipient bone marrow cells because the NSG mouse model used for these experiments does not support human erythropoiesis. After culturing bone marrow cells from the engrafted mice, both BCL11A exon and BCL11A enhancer disruption yielded significantly higher HbF+ cell numbers (Figure 4A) and γ-globin protein levels (Figure 4B) compared with the untransfected control in red blood cells derived from human erythroid progenitors from the bone marrow of the engrafted mice. The in vitro erythroid differentiation from recipient bone marrow-derived human cells was similar under all conditions in terms of cell growth and enucleation (Figures 4C and 4D). Evaluation of the editing levels again revealed that the indel percentage in the BCL11a exon-targeted samples was significantly lower than in the enhancer-targeted samples (Figure 4E) and, furthermore, that the indel percentage in the latter was similar to the total bone marrow levels described above (>30%; Figure 4E), arguing strongly against an erythroid lineage-selective impairment by the enhancer KO. After sacrifice, the human cells were enriched from the primary recipients’ bone marrow and subsequently transplanted into secondary recipients. Similar levels of total human cell engraftment were observed again under all conditions in peripheral blood (Figure 5A) and bone marrow (Figure 5B). The majority of engrafted cells were lymphocytes, as expected (Figure 5C); however, a high frequency of hematopoietic progenitors was found in the recipients’ bone marrow, as shown by FACS for the CD34+/CD38− population (Figure 5B) and by colony assays (Figure 5D). Interestingly, among engrafted cells, the enhancer-edited ones again showed significantly higher indel retention than the coding region-edited cells (Figure 5E). After in vitro erythroid differentiation of the human engrafted cells in secondary recipients (Figure 5F), an increased percentage of HbF+ cells was observed in the enhancer-edited sample (Figure 5G). Cumulative evidence so far suggests that enhancer-edited cells engrafted comparably to untransfected cells and that the enhancer disruption is comparable in HbF reactivation with the coding KO but without impairing the viability, multilineage...
differentiation and reconstitution, and long-term engraftment potential of the genetically modified cells.

BCL11A Editing Efficiently Reactivates HbF in Erythroid Cells of Patients with β0- or β+-Thalassemia

Bcl11A enhancer modification was then tested in mobilized CD34+ cells from thalassemic patients. Specifically, plerixafor- or plerixafor- and G-CSF-mobilized peripheral blood CD34+ cells from two β+/β−, two β/β−, and two β/β−thalasemic patients were electroporated with the BCL11A ZFN mRNA. The genotypes, phenotypes, and mobilization methods of the thalassemic patients are listed in Table 1. The ZFN transfection did not affect the clonogenic capacity of the cells (Figure 6A). The edited cells were cultured 48 hr after transfection, under the same 20-day erythroid culture protocol as the wild-type cells described above. The thalassemic erythroid cells grew and, at least partially, matured in vitro without editing (Figure 6B). Of interest, although deep sequencing initially showed indel levels similar to those observed in wild-type cells, there was an increase in the proportion of thalassemic edited cells during the erythroid differentiation (56.7% ± 4.83% on day 3 versus 65.7% ± 2.1% on day 14). This was interpreted as a potential survival advantage of the edited cells; however, the difference was not statistically significant (Figure 6C). Furthermore, BCL11A enhancer editing increased the percentage of enucleated cells at the end of differentiation (Figure 6D), and it reduced the oxidative stress of both enucleated and nucleated cells by reducing the production of reactive oxygen species (ROS) (Figure 6E). At the end of erythroid differentiation, the frequency of HbF+ cells was increased in the edited thalassemic samples (from 82% prior to transfection to more than 95% after editing) (Figure S7). BCL11A enhancer editing resulted in evident γ-globin increase, comparable with the reactivation observed in wild-type cells, as shown by HPLC (Figure 6F). In a sample from a transfusion-dependent β/β−-thalassemic donor with an IVSII-745/IVSII-745 genotype, β-globin was all but extinguished at the end of differentiation (day 20) (Figure 6F, last panel). Unexpectedly, in all of our thalassemic samples, BCL11A enhancer disruption was found to have a greater effect on γ-globin expression, whereas the Aγ and AγT30,31 chains were less affected (Figures S8A and S8B). A significant preferential γ-globin increase was also observed in wild-type cells from three healthy donors (Figure S8C). Further, in addition to the expected increase in γ-globin protein, comparison of the globin chain peaks shows an increase in the α-globin peak in the enhancer-edited thalassemic cell samples (Figures S8D–S8F). This may indicate that the increase in γ-globin levels is accompanied by stabilization of previously unpaired, and therefore unstable, α-globin protein. On practical grounds, whatever the reasons for the increase (total increase in alpha or decrease in delta), a concomitant increase in stable α-globin protein upon elevation of γ-globin protein would lead to an underestimation of the γ-globin increase when the γ-globin/α-globin ratio is plotted.

Edited HSPCs Expand Well In Vitro under Specific Expansion Protocols

In all gene therapy or genome editing clinical settings, there is a magnified need for large numbers of modified HSCs with the capacity to robustly engraft long-term and to achieve a sustainable therapeutic
effect in vivo. We have previously demonstrated that a 5-day culture with a StemRegenin1 and Ly2228820 (SL) small molecule combination can successfully expand highly engraftable CD34+/CD38−/CD90+ cells, even in comparison with their uncultured counterparts.26 Thus, we decided to test the ability of the edited wild-type and thalassemic cells to expand in vitro under these culture conditions.

After the same expansion protocol, several different HSPC subpopulations (CD34+/CD38−, CD34+/CD38−/CD90+, CD34+/CD38−/CD90−) from the BCL11A enhancer-edited wild-type and thalassemic samples were sorted and sequenced (Figure 7A). Furthermore, in additional experiments, we studied the editing efficiency in expanded cells, an approach that could be applied especially when lower cell numbers are available (Figure 7B). Edited cells, especially CD34+/CD38−/CD90−, were efficiently expanded during the 5-day period without any loss of editing efficiency (Figure 7C). Of interest, day 5 expanded cells of the same phenotype could be also successfully edited (Figure 7C). Overall, either expansion of edited cells or transfection of expanded cells yielded significantly higher numbers of edited CD34+/CD38−/CD90− cells (2.3- and 11.7-fold increase in wild-type cells and 6.3 and 28.3-fold increase in thalassemic cells, respectively) (Figure 7D).

Subsequently, we tested the engraftability of wild-type BCL11A enhancer-edited expanded cells. Sublethally irradiated NSG mice received either unexpanded enhancer-edited cells 2 days after transfection or the 5-day small molecule expanded progeny from the same number of unexpanded cells. At several time points after transplantation, human chimerism was tested. We observed that the expanded cells achieved a more than 2-fold higher engraftment level compared with their unexpanded counterparts (Figure 7E). Sequencing of the engrafted cells showed an almost identical indel frequency between the expanded and unexpanded populations. However, because the overall number of edited cells engrafted was much higher after the ex vivo expansion (Figure 7F), these results could potentially translate into a higher clinical benefit.

DISCUSSION

Editing of autologous cells has emerged as a new promising therapeutic strategy for treatment of patients with β-globin disorders. A leading approach applicable to all types of mutations is the reactivation of HbF. ZFN-based genome editing allows precise modification of the genome to reactivate HbF by targeting a specific 5-bp GATAA motif that is a key regulatory element in the BCL11A erythroid enhancer.13,14 BCL11A is a key regulator of HbF suppression, and by targeting this specific regulatory element, we can selectively knock out BCL11A expression in the erythroid lineage to reactivate HbF expression while retaining the ability of BCL11A to function in support of HSC function and lymphopoiesis. Our current data using mobilized peripheral blood cells build on and expand previous findings comparing BCL11A exonic versus enhancer disruption data using bone marrow CD34+ cell sources.16 In the present study, lower concentrations of ZFNs achieved similar or even higher editing compared with the higher ZFN amounts reported previously, and, in line with their conclusions, we show impaired erythroid cell proliferation only in the BCL11A coding region KO in erythroid cultures. We also present an extensive long-term in vivo comparison between editing of BCL11A exon 2 and the erythroid enhancer. High retention of enhancer modification was observed in primary and secondary transplants in all cell lineages, with HbF increases well maintained in these recipients. In contrast, editing of exon 2 was significantly reduced in vivo, with especially low levels of edited cells detected in secondary recipients.

In addition, by performing clinical-scale editing, we achieved high editing efficiency for both the coding and the enhancer region in contrast to previously reported results.32 Furthermore, the high editing levels observed in our bulk cell population are superior to recently reported editing levels in unsorted bulk in vitro cultures with unclear in vivo performance.10 Current efforts are also being directed toward...
ZFN designs that increase enhancer disruption activity and cleavage selectivity to yield off-target profiles that are below state-of-the-field detection strategies. In addition to clinical-scale editing, for the first time, important information regarding the editing efficiency and phenotypic correction of cells from thalassemic patients is provided. So far, the use of genome editing on thalassemic cells has not been explored, with the exception of gene correction in thalassemic induced pluripotent stem cells (iPSCs). Although presently iPSCs represent tools for screening different approaches, their lack of engraftment potential and inefficiency for terminal maturation are major hurdles to overcome. Here we show a high BCL11A-erythroid enhancer disruption rate in thalassemic cells by an early stage ZFN pair that efficiently increases cellular HbF protein levels. This increase balances the α-globin/β-like globin ratio, resulting in an improved terminal erythroid enucleation rate. Terminal differentiation was improved by decreased red cell oxidative damage because ROS were significantly reduced after editing. Oxidative damage by ROS is generated by hemichrome formation because of an excess of aggregated α-globin chains and is believed to be one of the main contributors to cell death, tissue damage, and hypercoagulability in thalassemic patients. A decrease in ROS levels in a thalassemic mouse model has also been shown to significantly improve their clinical picture. In conclusion, our detailed evaluation of maturation parameters in thalassemic cells after editing uncovered beneficial features of edited cells.

Beyond the above observations, the fact that we have, for the first time, assessed thalassemic samples for reactivation of fetal Hb (HbF) through gene editing provides additional insights. For example, in cells with the β0/β0 genotype, it is very difficult to gauge the effectiveness of the HbF increase when edited cells are compared with control unedited samples because these also produce significant and variable amounts of HbF under in vitro conditions, as documented previously. Assessment of protein levels at terminal maturation stages, including enucleated red blood cells (RBCs), is of critical importance for comparative purposes. Furthermore, the observed increase in α-globin protein levels after editing (Figure 6F; Figure S8E) and the absence of an adult β-globin signal make the two usual readouts of HbF activation, increase in the γ-globin over α-globin and γ-globin over beta-like globin protein signals, much less informative than in wild-type cells. Nevertheless, it was encouraging to document clear increases in γ-globin peaks (Figure 6F; Figure S8F) as well as increases in α-globin peaks for the first time in edited thalassemia CD34+ cells.

In a short in vitro culture system, it is hard to assess the selective survival advantage of edited cells with higher HbF content that is expected to occur in vivo after a longer time period. Nevertheless, because the in vitro editing conditions can be further optimized,
promoters and, possibly, with a differential af
available, ef
are in very low proportions (0.01% or less) in the mobilized samples
suf
it has been widely recognized that a major goal is the modi
For editing autologous samples for transplantation (i.e CD34+ cells),
additional nucleosome remodeling deacetylase (NuRD) complex
on the two
Whether such an increase is only observed with diminished
320 Molecular Therapy: Methods & Clinical Development Vol. 10 September 2018
BCL11A-related
zygotes is generally 3:2.42 Moreover in a subsequent study, it was
documented that the G
Because non-edited thalassemic samples in our current cultures had
compared with the edited ones (Figure S8A), it would
ratio in peripheral red blood cells of β-thalassemia homo-
ygotes is generally 3:2.42 Moreover in a subsequent study, it was
documented that the G
findings in non-thalassemic samples as well. A similar
MATERIALS AND METHODS
Healthy and Thalassemic Donor Collection, Culture, and Editing
Mobilized leukapheresis products were procured from healthy donors
following a mobilization regimen of 10 μG G-CSF/kg/day for 5 days and
240 μg plerixafor/kg/day on the evenings of days 4 and 5. On days 5 and
6 of mobilization, peripheral blood mononuclear cells were collected by
apheresis with a COBE Spectra apheresis system. During apheresis
collection, 150 mL of autologous plasma was collected and then added
to the cell collection to sustain cell viability during transit and storage.
CD34+ cell purification was performed as described by Yannaki et al.46 Platelet
depletion on the leukapheresis product was performed using the Lovo cell processor device (Fresenius Kabi) before enrichment
for CD34+ cells using the CliniMACS Plus instrument (Miltenyi Bio-
tec). The collected cells were cultured in CD34 culture medium at 1 ×
10^8 cells/mL in a VueLife 750-C1 gas-permeable bag (Saint Gobain,
Gaithersburg, MD). For large-scale transfections, CD34+ HSPCs
were washed with MaxCyte electroporation buffer and split into three
experimental conditions: 1 × 10^6 cells were left untransfected, 1.98 ×
10^8 cells were transfected with SB-BCLmR mRNA (targeting exon 2) in
1 × 10^8 cells/mL at a dose of 120 μg/mL, and 1.75 × 10^9 cells were
transfected with 46801-2a-47923 mRNA (targeting the erythroid
enhancer) in 1 × 10^9 cells/mL at a dose of 120 μg/mL. CD34+ cell
cultures were then transiently incubated in a 30°C, 5% CO2 incubator for
18–24 hr. Subsequently, cells were diluted to 1 × 10^6 cells/mL in CD34
culture medium and incubated at 37°C, 5% CO2. The cell viability
was maintained above 70% throughout the process. The conditions for
the collection of the thalassemic cells were described in two previous clinical
trials.46,47 Small-scale transfections of 2 × 10^5 cells were performed
2 days after thawing the cells using a BTX electroporator (ECM 830,
Holliston, MA; voltage, 250 V; pulse length, 5 ms) in 100 μL of BTX
Express electroporation solution with 8 μg of the 46801-2a-47923
mRNA. The thalassemia patient samples had been frozen twice before
thawing and transfection, probably resulting in lower cell survival and
editing efficiencies. The CD34 culture medium consisted of Stem Span
H3000 supplemented with CC110 cytokine cocktail (STEMCELL

| Table 1. Phenotype, Genotype, and Mobilization Scheme of the Thalassemic Patients |
| Phenotype | Genotype | Mobilization |
| β^+β^+ | IVS1-1/IVS1-1 | plerixafor |
| β^+β^- | CD39/CD39 | plerixafor |
| β^-β^- | Gδ39/IVS1-110 | G-CSF+plerixafor |
| β^-β^+ | CD39/IVS1-110 | G-CSF+plerixafor |
| β^-β^- | IVS1-110/IVS1-110 | plerixafor |
| β^-β^- | IVSII-745/IVSII-745 | plerixafor |

our present data encourage further in vitro studies using edited pa-
tient samples to explore whether that may potentially predict in vivo
outcomes.

Despite certain ambiguities in assessing the HbF increase in vitro after
editing, a detailed assessment of protein levels unveiled additional
novel observations using the thalassemic samples. The preferential in-
crease in Gγ compared with Aγ in all homozygous β-thalassemic
samples when BCL11A was reduced is intriguing. This observation is
reminiscent of earlier findings. Previous studies have found that
the Gγ/Aγ ratio in peripheral red blood cells of β-thalassemia homo-
ygotes is generally 3:2.42 Moreover in a subsequent study, it was
documented that the Gγ/Gγ+Aγ ratio in the bone marrow of these
patients is lower than in peripheral blood, leading to the speculation
that cells with mainly Gγ somehow had a preferential survival.43
Because non-edited thalassemic samples in our current cultures had
more Aγ compared with the edited ones (Figure S8A), it would
appear that the increase in HbF in the BCL11A enhancer-edited
samples is accompanied by a coordinate increase in Gγ. In our earlier
studies of in vitro cultures of fetal liver, neonatal, and adult samples,
we concluded that changes in Gγ/Gγ+Aγ ratios in these samples are
regulated independent of the total amount of fetal globin, but the spe-
cific regulatory influences were unclear.44 Of further interest is that, in
our patients with haploinsufficiency of BCL11A, there was also an in-
crease in the Gγ/Aγ ratio in their peripheral blood,19 pointing to a
BCL11A-related finding in non-thalassemic samples as well. A similar
tendency was also observed in normal CD34+ cells (Figure 2E).
Whether such an increase is only observed with diminished
BCL11A but not with other modalities that increase HbF needs to
be pursued in subsequent studies. Nevertheless, the differential effect
on the two γ-globin promoters could be interpreted to suggest direct
binding of the developmental regulator BCL11A on the γ-globin
promoters and, possibly, with a differential affinity dependent on
additional nucleosome remodeling deacetylase (NuRD) complex
members.

For editing autologous samples for transplantation (i.e CD34+ cells),
it has been widely recognized that a major goal is the modification of a
sufficient number of engraftable long-term HSCs. Because the latter
are in very low proportions (0.01% or less) in the mobilized samples
available, efficient expansion of these cells ex vivo is a highly desirable
means of achieving that goal. Toward this end, we used a previously
successful protocol to expand engraftable HSCs.26 Under these condi-
tions, we have now shown, by using both normal and thalassemic
samples, that we can not only significantly expand ex vivo cells with
an engraftable edited HSC phenotype within 5 days but also efficiently
edit HSCs after expansion. Although, by this method, larger-scale
transfections and higher amounts of ZFN mRNA are required, the
expanded, more primitive CD34+ cells, if originally insuffi-
cient in numbers, could be purified and edited efficiently in isolation
without the co-presence of large numbers of the CD34+/CD38+ pop-
ulation. As recently proposed,4 the edited HSCs could then be co-
transplanted with unedited progenitor or precursor cells. The current
studies provide a promising roadmap for efficient autologous editing
and expansion of the HSPC pool in β-thalassemia patients who may
not efficiently mobilize, providing a therapeutic modality for a
broader spectrum of this population. The finding that engraftable
HSC counts can be augmented could serve other patient populations
with hemoglobin disorders, including sickle cell disease.
Technologies). For \(\alpha\) \textit{vivo} expansion, normal and thalassemic cells were cultured under serum-free conditions in the medium described above, supplemented with 1 \(\mu\)M StemRegenin1 (SR1, Cellagen Technology) and 100 nM Ly2288820 (Ly, Selleckchem). For colony formation assays (colony-forming unit [CFU]-granulocyte macrophage [GM] and burst-forming unit-erythroid [BFU-E]), cells were plated at a density of 750–2,000 cells/mL in semi-solid methylcellulose-based medium containing cytokines, ColonyGEL (ReachBio).

Zinc Finger Nucleases

ZFN pairs were expressed from mRNAs containing 2a self-cleaving peptide linkers. mRNAs were generated by \textit{in vitro} transcription at Eufets (Idar-Oberstein, Germany).

The Bcl11a enhancer-targeting ZFN mRNA 46801-2a-47923 contained the following ZFNs:

46801: DQSNLRA RPYTLRL SGYNLEN TSGSLTR DQSNLRA AQCLFH
Binding site: AGCAACGTAGCTGTGCAC (small letters refer to bases skipped by the DNA binding domain)

47923: RSDHTLQ QSGLAR QKGTILG QSSDLRS RRDNLSH
Binding site: CAGGCTCCAGGAAGG.

The Bcl11a exon 2-targeting mRNA SB-BCLmR contained the following ZFNs:

44490: RSDHTLQ DRSAIAL RSDSLSR DRSVRTK RSDHLSA QRSNLKVR
Binding site: CAACGGGCGGTGCTTGGG

43490: DRSNLSR LRQNLIM LQSLNMR RSDHLSR QSGNLR QRNDKRS
Binding site: CCAGAAGGGGATCATGAC.

MiSeq Deep Sequencing

The efficiency of ZFN-mediated gene modification was assessed by deep DNA sequencing. The region of interest (containing the ZFN binding sites within the BCL11A locus) was PCR-amplified, and the level of modification was determined by paired-end deep sequencing on an Illumina MiSeq. To generate libraries compatible with the Illumina MiSeq sequencing platform, adaptors, barcodes, and a flow cell binder (short DNA sequence) were attached to the target-specific amplicons using two sets of fusion primers in sequential PCRs. The primers used for the MiSeq Adaptor PCR are shown in Table 2.

Insertion or Deletion Quantitation

Loci were PCR-amplified from genomic DNA (gDNA), and the levels of modification were determined by paired-end deep sequencing on an Illumina MiSeq. Paired sequences were merged via SeqPrep (https://github.com/jstjohn/SeqPrep). Merged FASTQ reads were filtered on the following criteria: quality score of 15 or higher at all positions; the 5’- and 3’-terminal 23 bp must match the expected amplicon exactly, the read must not map to a different locus in the target genome as determined by Bowtie2 with default settings, and deletions must be less than 70% of the amplicon size or less than 70 bp long. Filtered sequences were then aligned to the expected sequence using the Needleman–Wunsch algorithm, with penalties set to prioritize single insertions or deletions. Contiguous gaps in either the target or query alignment were counted as indels.

Erythroid Differentiation

For erythroid differentiation of both wild-type and thalassemic CD34+ cells, we used a previously described three-step differentiation protocol. Briefly, 2 days after transfection, 2\(–\)5 \(\times 10^4\) CD34+ cells were cultured for 7 days in Step-1 medium consisting of IMDM (Gibco) supplemented with 1 \(\times\) GlutaMAX, 100 U/mL penicillin/streptomycin, 5% human AB+ plasma, 330 mg/mL human holo-transferrin, 20 mg/mL human insulin, 2 U/mL heparin, 1 mM hydrocortisone (all four from Sigma-Aldrich), 3 U/mL recombinant human erythropoietin (EPO) (Janssen Products), 100 ng/mL stem cell factor (SCF; PeproTech), and 5 ng/mL interleukin-3 (IL-3) (PeproTech). On day 7 (step 2), hydrocortisone and IL-3 were removed from the medium, and the cells were cultured for 4 days. On day 11, SCF was also removed, and the cells were cultured for 7–10 more days until enucleation was observed. For erythroid differentiation of human progenitor cells engrafted in NSG recipients, 2–3 \(\times 10^5\) hCD45+ cells from recipient bone marrow were cultured under the same conditions as described above. For the evaluation of maturation, erythroid cells were collected at different time points for cytosin preparation. Cytosides were then stained with PROTOCOL Hema 3 Fixative and Solution (Thermo Fisher Scientific), a method comparable with the Wright-Giemsa stain.

TaqMan/qRT-PCR Analysis of Globin mRNA Ratios

The ratios of \(\gamma\)-globin mRNA to \(\alpha\)-globin mRNA and \(\gamma\)-globin mRNA to \(\beta\)-globin mRNA were assessed to evaluate the effects of ZFN activity on globin mRNA levels. Whole-cell RNA was isolated from...
from *in vitro*-generated erythrocytes using the High Pure RNA Isolation Kit (Roche). The levels of mRNA for the individual globin genes (α-globin, β-globin, and γ-globin) were then measured by real-time qRT-PCR on the CFX96 C1000 Touch Real-Time PCR Detection System (Bio-Rad) using the following commercially available probe sets: α-globin (HBA)-Hs00361191_g1, β-globin (HBB)-Hs00758889_s1,
and γ-globin (HBG)-Hs00361131_g1 (Applied Biosystems). Each sample was measured twice, thus yielding a technical replicate. The data were analyzed using the Bio-Rad CFX Manager software. The data from this approach can be used to accurately quantitate, for example, the change in the γ-globin mRNA/β-globin mRNA ratio that occurs following a given experimental treatment. By its nature, this method does not yield the absolute ratio of the number of γ-globin mRNA molecules to the number of β-globin mRNA molecules.

**HPLC**

Individual globin chain levels were quantified on a Shimadzu Prominenence instrument with an SPD-10AV diode array detector and an LC-10AT binary pump (Shimadzu, Kyoto, Japan). A gradient mixture of 0.1% trifluoroacetic acid in water/acetonitrile was applied at a rate of 1 mL/min using a Vydac C4 reverse-phase column (Grace Discovery Sciences, Albany, OR). The IVSII-745/IVSII-745 *+/− samples (Figure 6F) were analyzed on alternate columns from Hichrom (UK). Adult and fetal globin chains were identified by comparing their mobilities with that of reference samples (adult peripheral blood and cord blood).

**Flow Cytometry**

Erythroid cells were collected at different time points and stained with e-Cadherin (67A4), a marker of early erythropoiesis, and GlyA (JC159) (Dako), a marker of late erythropoiesis. For evaluation of enucleation, the cells were washed and stained with NucRed (Thermo Scientific) according to the manufacturer’s instructions. To evaluate the frequency of HbF+ cells, erythroid cells were collected at different time points and stained with the following fluorochrome-conjugated antibodies: CD34 (8G12) and CD38 (HIT2) (BD Biosciences) and CD90 (5E10) (Miltenyi Biotech) and acquired in BD FACS-Calibur.

**Xenotransplantation**

Eight-week-old NSG mice were sublethally irradiated (300 rad) and transplanted with $1 \times 10^6$ unedited and edited CD34+ cells. Donor chimera was assessed every 4 weeks as the frequency of human cells in total white blood cells by flow cytometry after red blood cell lysis and staining with human CD45 (BD Biosciences). Upon sacrifice, 16 or 20 weeks after transplantation, the chimeric bone marrow was harvested and assayed for level of engraftment and multilineage reconstitution after staining with the following antibodies: CD45 (2D1), CD19 (HIB19), CD33 (P67.6), CD34 (8G12), and CD38 (HIT2) (BD Biosciences) and CD41 (VIPL3) (Invitrogen). For the secondary transplantsations, bone marrow cells were enriched for human CD45+ cells by immunomagnetic separation (hCD45 microbeads, Miltenyi Biotech), and the same number of human cells ($5 \times 10^6$) was injected into each secondary recipient. Secondary recipients were sacrificed 10 weeks after transplantation. All in vivo experiments were conducted with approval from the institutional animal care and use committee.

**Statistical Analysis**

Results are expressed as mean – SE, and statistical significance was determined by ANOVA with Bonferroni correction using Minitab v.16 or with Student’s t test for pairwise comparisons; p < 0.05 was considered statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes eight figures and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.08.003.

**AUTHOR CONTRIBUTIONS**

Writing – Original Draft, N.P.; Methodology, N.P. and A.R.; Investigation, N.P., A.R., S.P., Y.Z., and D.D.; Writing – Review & Editing, A.R., E.Y., D.N.L., M.C.H., and T.P.; Resources, E.Y.; Conceptualization, F.D.U. and T.P.; Supervision, T.P.

**CONFLICTS OF INTEREST**

A.R., F.D.U., and M.C.H. are or were full-time employees and equity owners of Sangamo Therapeutics when these studies were conducted. D.L. was a full-time employee and equity owner of Bioverativ when these studies were conducted.

**ACKNOWLEDGMENTS**

This study was supported by NIH grant R01-DK101328–01A1 (to T.P.). N.P. was supported by the Cooley’s Anemia Foundation.

**REFERENCES**

1. Higgs, D.R., Engel, J.D., and Stamatooyannopoulos, G. (2012). Thalassaemia. Lancet 379, 373–383.
2. Negre, O., Eggimann, A.-V., Beuzard, Y., Ribel, J.A., Bourget, P., Borworpinyno, S., Hongeng, S., Hacein-Bey, S., Cavazzana, M., Lebouche, P., and Payen, E. (2016). Gene
Therapy of the β-Hemoglobinopathies by Lentiviral Transfer of the βAT87Q Globin Gene. Hum. Gene Ther. 27, 148–165.

3. Walters, M.C., Rasko, J., Hongeng, S., Kwiatkowsi, I., Schiller, G.J., Kletzel, M., Ho, P.J., Vichinsky, E., von Kalle, C., Cavazzana, M., et al. (2015). Update of Results from the Northstar Study (HGB-204): A Phase 1/2 Study of Gene Therapy for Beta-Thalassemia Major Via Transplantation of Autologous Hematopoietic Stem Cells Transduced Ex-Vivo with a Lentiviral Beta AT87Q-Globin Vector (LentGlobin BBI055). Blood 126, 201.

4. Zonari, E., Desantis, G., Petrillo, C., Boccalatte, F.E., Lidonnici, M.R., Kastate-Rudnicki, A., Areti, A., Ferrini, G., Naldini, L., and Gentner, B. (2017). Efficient Ex Vivo Engineering and Expansion of Highly Purified Human Hematopoietic Stem and Progenitor Cells Populations for Gene Therapy. Stem Cell Rep 8, 977–990.

5. Breda, L., Motta, I., Lorenso, S., Gemmo, C., Deng, W., Rupow, J.W., Abdulmalik, O.Y., Manwani, D., Blobel, G.A., and Rivella, S. (2016). Forced chromatin looping raises fetal hemoglobin in adult sickle cells to higher levels than pharmacologic inducers. Blood 128, 1139–1143.

6. Traxler, E.A., Yao, Y., Wang, Y.-D., Woodard, K.J., Kurita, R., Nakamura, Y., Hughes, J.R., Hardison, R.C., Blobel, G.A., Li, C., and Weiss, M.J. (2016). A genome-editing strategy to treat β-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition. Nat. Med. 22, 987–990.

7. Wiernert, B., Funnell, A.P.W., Norton, L.J., Pearson, R.C., Wilkinson-White, L.E., Lester, K., Vadalas, J., Porteus, M.H., Matthews, J.M., Quinlan, K.G., and Crossley, M. (2015). Editing the genome to introduce a beneficial naturally occurring mutation associated with increased fetal globin. Nat. Commun. 6, 7085.

8. Masuda, T., Wang, X., Maeda, M., Canver, M.C., Sher, F., Funnell, A.P., Fisher, C., Suciu, M., Martyn, G.E., Norton, L.J., et al. (2016). Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin. Science 351, 285–289.

9. Martyn, G.E., Wiernert, B., Yang, L., Shah, M., Norton, L.J., Durdach, J., Kurita, R., Nakamura, Y., Pearson, R.C.M., Funnell, A.P.W., et al. (2018). Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. Nat. Genet. 50, 498–503.

10. Antoniani, C., Meneghini, V., Lattanzi, A., Félix, T., Romano, O., Magrin, E., Weber, L., Pavan, G., El Hoss, S., Kurita, R., et al. (2018). Induction of fetal hemoglobin synthesis by CRISPR/Cas9-mediated editing of the human β-globin locus. Blood 131, 1960–1973.

11. Liu, N., Hargreaves, V.V., Zhu, Q., Kurland, I.V., Hong, J., Kim, W., Sher, F., Macias-Trevino, C., Rogers, J.M., Kurita, R., et al. (2018). Direct Promoter Repression by BCL11A Controls the Fetal to Adult Hemoglobin Switch. Cell 173, 430–442.e17.

12. Sankaran, V.G., Menne, T.F., Xu, J., Akie, T.E., Schiller, G.J., Ho, P.J., Vichinsky, E., von Kalle, C., Cavazzana, M., et al. (2015). Ex vivo generation of fully mobilized CD34+ cells represent an optimal graft source for thalassemia gene therapy. Blood 126, 1616–1619.

13. Giarratana, M.-C., Kobari, L., Lapillonelle, H., Chalmers, D., Kiger, L., Cynober, T., Marden, M.C., Wajcman, H., and Dosay, L. (2005). Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat. Biotechnol. 23, 69–74.

14. Segal, G., Ricco, G., Mazza, U., Camaschella, C., Pich, P.G., Giannazi, E., Righetti, P.G., Giglioni, B., Comi, P., et al. (1979). Human T gamma globin chain is affected by elevated fetal hemoglobin syndromes. Clin. Chim. Acta 84, 193–202.

15. Marden, M.C., Wajcman, H., and Douay, L. (2005). Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat. Biotechnol. 23, 126–127.
37. Rund, D., and Rachmilewitz, E. (2005). β-thalassemia. N. Engl. J. Med. 353, 1135–1146.

38. Kong, Y., Zhou, S., Kihm, A.J., Katein, A.M., Yu, X., Gell, D.A., Mackay, J.P., Adachi, K., Foster-Brown, L., Louden, C.S., et al. (2004). Loss of α-hemoglobin-stabilizing protein impairs erythropoiesis and exacerbates β-thalassemia. J. Clin. Invest. 114, 1457–1466.

39. Franco, S.S., De Falco, L., Ghaffari, S., Brugnara, C., Sinclair, D.A., Mattei, A., Iolascon, A., Mohandas, N., Bertoldi, M., An, X., et al. (2014). Resveratrol accelerates erythroid maturation by activation of FoxO3 and ameliorates anemia in β-thalassemic mice. Haematologica 99, 267–275.

40. Breda, L., Casu, C., Gardenghi, S., Bianchi, N., Cartegni, L., Narla, M., Yazdanbakhsh, K., Musso, M., Manwani, D., Little, J., et al. (2012). Therapeutic hemoglobin levels after gene transfer in β-thalassemia mice and in hematopoietic cells of β-thalassemia and sickle cells disease patients. PLoS ONE 7, e32345.

41. Holmes, M.C., Reik, A., Rebar, E.J., Miller, J.C., Zhou, Y., Zhang, L., Li, P., and Vaidya, S. (2017). A Potential Therapy for Beta-Thalassemia (ST-400) and Sickle Cell Disease (BIVV003). Blood 130, 2066.

42. Ricco, G., Mazza, U., Turi, R.M., Pich, P.G., Camaschella, C., Saglio, G., and Bernini, L.F. (1976). Significance of a new type of human fetal hemoglobin carrying a replacement isoleucine replaced by threonine at position 75 (E19) of the gamma chain. Hum. Genet. 32, 305–313.

43. Saglio, G., Camaschella, C., Guerrasio, A., Cambrin, G.R., Capaldi, A., Pich, P.G., Trento, M., and Mazza, U. (1982). G gamma and a gamma globin chain synthesis in bone marrow and peripheral blood of beta-thalassaemia homozygotes. Br. J. Haematol. 52, 225–231.

44. Papayannopoulou, T., Kurachi, S., Brice, M., Nakamoto, B., and Stamatoyannopoulos, G. (1981). Asynchronous synthesis of HbF and HbA during erythroblast maturation. II. Studies of G gamma, A gamma, and beta chain synthesis in individual erythroid clones from neonatal and adult BFU-E cultures. Blood 57, 531–536.

45. Baldwin, K., Urbina, F., Romero, Z., Campo-Fernandez, B., Kaufman, M.L., Cooper, A.R., Masiu, K., Hollis, R.P., and Kohn, D.B. (2015). Enrichment of human hematopoietic stem/progenitor cells facilitates transduction for stem cell gene therapy. Stem Cells 33, 1532–1542.

46. Yannaki, E., Papayannopoulou, T., Jonlin, E., Zervou, F., Karponi, G., Xagorari, A., Becker, P., Pasha, N., Ratsis, I., Kaloyannidis, P., et al. (2012). Hematopoietic stem cell mobilization for gene therapy of adult patients with severe β-thalassemia: results of clinical trials using G-CSF or plerixafor in splenectomized and nonsplenectomized subjects. Mol. Ther. 20, 230–238.

47. Yannaki, E., Karponi, G., Zervou, F., Constantinou, V., Bouinta, A., Tachynopoulou, V., Kotta, K., Jonlin, E., Papayannopoulou, T., Anagnostopoulou, A., and Stamatoyannopoulos, G. (2013). Hematopoietic stem cell mobilization for gene therapy: superior mobilization by the combination of granulocyte-colony stimulating factor plus plerixafor in patients with β-thalassemia major. Hum. Gene Ther. 24, 852–860.