The mRNA-Binding Protein IGF2BP1 Restores Fetal Hemoglobin in Cultured Erythroid Cells from Patients with β-Hemoglobin Disorders

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INTRODUCTION

Sickle cell disease (SCD) and β-thalassemia are caused by structural abnormality or inadequate production of adult hemoglobin (HbA, αβ2), respectively. Individuals with either disorder are asymptomatic before birth because fetal hemoglobin (HbF, αγ2) is unaffected. Thus, reversal of the switch from HbF to HbA could reduce or even prevent symptoms these disorders. In this study, we show that insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) is one factor that could accomplish this goal. IGF2BP1 is a fetal factor that undergoes a transcriptional switch consistent with the transition from HbF to HbA. Lentivirus delivery of IGF2BP1 to CD34+ cells of healthy adult donors reversed hemoglobin production toward the fetal type in culture-differentiated erythroid cells. Analogous studies using patient-derived CD34+ cells revealed that IGF2BP1-dependent HbF induction could ameliorate the chain imbalance in β-thalassemia or potently suppress expression of sickle β-globin in SCD. In all cases, fetal γ-globin mRNA increased and adult β-globin decreased due, in part, to formation of contacts between the locus control region (LCR) and γ-globin genes. We conclude that expression of IGF2BP1 in adult erythroid cells has the potential to maximize HbF expression in patients with severe β-hemoglobin disorders by reversing the developmental γ- to β-globin switch.

The β-thalassemias result from various alterations in the β-globin gene or its promoter that impact levels of HbA. Individuals with some level of β-globin protein are classified as β+, while those that lack expression have a β0 genotype. The severity of β-globin deficiency determines how much α-globin remains unpaired to form insoluble aggregates that cause RBC destruction and severe anemia, which is treated by frequent blood transfusions.2

Rare individuals with SCD or β-thalassemia also inherit genetic changes that cause HbF to be produced into adult life.3,4 These people have less severe disease or are asymptomatic depending on levels and distribution of HbF in RBCs.4 Medications that enhance HbF are being used and continue to be developed, but they are not a cure.5 Bone marrow (BM) transplantation from human leukocyte antigen (HLA)-matched siblings is a cure, but insufficient donors limit widespread use of this treatment.6,7 Alternative donor sources such as HLA-matched unrelated persons,8 HLA-mismatched family members,9 and umbilical cord blood units10 are being explored, but evidence is still needed to draw conclusive recommendations about safety and efficacy of these options. Thus, gene therapy using the patient’s own hematopoietic stem/progenitor cells (HSPCs, CD34+) creates an opportunity to increase availability of curative therapy while eliminating risks associated with heterologous transplant.11,12

Gene addition or modification strategies are being explored as treatment for β-hemoglobin disorders. Lentiviral vectors that encode for high-level, erythroid-specific expression of human γ-globin genomic sequences have been used by our group and others to improve or

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correct mouse models of β-thalassemia or SCD, as well as erythroid progeny of CD34+ cells obtained from patients with these disorders. Other groups have demonstrated therapeutic benefit in similar mouse models and cultured patient cells using lentiviral vectors to express wild-type or modified versions of human β-globin. Clinical trials are ongoing for many of these approaches, with the most data available for lentiviruses expressing the human β-globin (βT87Q) with a beneficial γ-globin substitution. Interim results from these trials indicate that lentivirus delivery of βT87Q-globin gene is safe, well tolerated, and sufficient to achieve consistent clinical benefit in patients with less severe forms of transfusion-dependent β-thalassemia.

An alternative to globin gene addition is modulation of factors that regulate the endogenous fetal γ-globin (γ4 and Aγ) genes. The transcription factors B cell CLL/lymphoma 11A (BCL11A) and leukemia/lymphoma-related factor (LRF) encoded by the ZBTB7A gene independently suppress γ-globin expression in adult erythroid cells, and inhibition of either factor favors HbF production. Recently, insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) was identified as a fetal-specific factor and positive regulator of HbF. IGF2BP1 is highly expressed during early development but suppressed in adult tissues; this timing pattern corresponds with the transition from HbF to HbA. Indeed, expression of IGF2BP1 in healthy adult erythroblasts caused a transcriptional switch from β- to γ-globin and robust increase in HbF. In this study, we confirm the ability of IGF2BP1 to potently induce HbF in adult erythroblasts and extend this function to cultured cells from patients with β-thalassemia major or SCD. In patient cells, IGF2BP1-dependent induction of HbF ameliorated the chain imbalance that occurs in β-thalassemia major or potently suppressed expression of sickle β-globin in SCD. Reversal of globin gene expression was achieved at the transcriptional level by increased γ-globin combined with decreased β-globin mRNA due, in part, to renewed interaction between the locus control region (LCR) and γ-globin genes. Based on these results, we propose that erythroid-specific expression of IGF2BP1 could provide dominant and curative levels of HbF in RBCs derived from transduced HSPCs of patients with SCD or severe β-thalassemia.

RESULTS

IGF2BP1 Is Highly Expressed in Human Fetal Erythroblasts but Silenced in Adult Erythroblasts

The beneficial effects of HbF on the severity of β-thalassemia and SCD have fueled long-standing efforts to identify factors controlling expression of the endogenous fetal γ-globin genes. These studies have identified numerous gene products that can be modulated to induce HbF expression, including IGF2BP1. Association between IGF2BP1 and developmental hemoglobin switching was initially revealed by comparing RNA and protein expression for reticulocytes and culture-differentiated erythroid cells that predominantly produced HbF versus those that did not. To confirm fetal-specific expression of IGF2BP1, we used our two-phase culture model of human erythropoiesis. In this system, CD34+ cells are first proliferated and then differentiated for 7 days each. Terminal stage cultures consist of mostly orthochromatic erythroblasts, and hemoglobin production mirrors the developmental state of the starting cell population. Specifically, CD34+ cells isolated from fetal liver (FL) produce predominantly HbF while those from adult BM produce mostly HbA. Erythroblasts were generated from FL CD34+ cells purchased from Lonza (Walkersville, MD, USA) or adult BM CD34+ cells obtained from either Lonza or the Yale Cooperative Center of Excellence in Hematology (Yale School of Medicine, New Haven, CT, USA). qRT-PCR was used to quantify transcript levels of IGF2BP1
as a percentage of the internal control RNaseP (Figure 1A). This analysis also included primer-probe sets capable of detecting all BCL11A splice variants or ZBTB7A, two well-known transcriptional repressors of the fetal $\gamma$-globin genes.34-37 IGF2BP1 was highly expressed in fetal erythroblasts (42 ± 19% of RNaseP), but at background levels in adult counterparts (0.01% ± 0.01%), a 4,200-fold change. Alternatively, BCL11A transcripts were modestly increased in adult cells (0.2% ± 0.02% fetal versus 1.6% ± 0.3% adult), and LRF/ZBTB7A was expressed at similar levels in both groups (1.0% ± 0.4% fetal versus 1.7% ± 0.8% adult).

A prior study used culture-differentiated human fetal and adult erythroblasts and the chromatin immunoprecipitation (ChIP)-sequencing method to develop a genome-wide map of histone modifications and make comparisons across the $\beta$-globin locus.43 Consistent with differential globin gene expression for these developmental stages, epigenetic modifications typical of active chromatin regions (H3K27ac, H3K4me3, and H3K36me3) were enriched at the $\gamma$- and $\alpha$-globin genes in fetal cells and the $\beta$-globin gene in adult cells. Conversely, repressive chromatin markers (H3K9me3 and H3K27me3) were detected at the $\gamma$-globin genes in adult cells and $\beta$-globin in fetal cells. We re-analyzed these data to define the chromatin pattern for IGF2BP1, BCL11A, and LRF/ZBTB7A for fetal and adult erythroblasts. For IGF2BP1, active chromatin modifications were observed in fetal cells and the repressive marks limited to adult cells (Figure 1B). Consistent with these marks, flow cytometry analysis of culture-differentiated erythroblasts confirmed that IGF2BP1 was expressed in fetal cells, which produce HbF, versus adult cells, which do not (Figure S1). Conversely, BCL11A and LRF/ZBTB7A exhibited active chromatin marks in both cell types, and these signals were enriched in adult cells (Figures S2A and S2B). From these data, we conclude that IGF2BP1 expression is specific to fetal erythroid development and undergoes a transcriptional switch that correlates with the transition from HbF to HbA production.

**IGF2BP1 Reverses Hemoglobin Expression in Healthy Adult Erythroblasts**

Previously, it was shown that lentivirus-mediated expression of IGF2BP1 in culture-differentiated erythroblasts of healthy adult donors could reverse hemoglobin production to the fetal type.39 This was accomplished with a lentiviral vector that utilized a relatively weak human spectrin alpha gene (SPTA1) promoter to drive erythroid-specific expression of IGF2BP1 and a puromycin resistance gene coupled by an internal ribosomal entry site (IRES) element (SIiP, Figure S3A). We replaced the IRES with the 2A self-cleaving peptide from porcine teschovirus-1 (P2A) to reduce the size of the lentiviral vector cargo as well as provide efficient cleavage between IGF2BP1 and downstream marker genes.44 The 2A sequence was linked to a puromycin resistance gene or ZsGreen fluorescent reporter35 to permit drug selection or visual confirmation and enrichment of transduced cells by fluorescence-activated cell sorting (FACS), respectively (Figure S3A). To determine the impact of these modifications on IGF2BP1 function, we transduced CD34+ cells from four healthy adult donors with the parental SIiP virus or versions that included the 2A-puromycin (SI2AP) or 2A-ZsGreen (SI2AZG) cassette at equivalent multiplicities of infection (MOIs) (Figure S3B). Control cells were transduced using mock conditions or with an MSCV-regulated GFP virus, which we have shown to provide high levels of gene transfer in human CD34+ cells and subsequent expression in erythroid progeny.17,42 Transduced cells were grown for 2 days before the IGF2BP1 fraction was enriched by FACS for ZsGreen or selected with puromycin. After 5 days of additional growth, cells were collected to obtain total RNA and protein, perform flow cytometry analysis, or for plating into differentiation medium (Figure S3B).

**Table 1. Increased Levels of $\gamma$-Globin and HbF in Erythroblasts Derived from Adult CD34+ Cells Transduced with IGF2BP1 Lentiviral Vectors**

| Transduction       | $\gamma$-Globin mRNA% | % HbF± SEM | % HbA± SEM | % HbFb/total (HbF + HbA) % | % HbAb/total (HbF + HbA) % |
|---------------------|-----------------------|------------|------------|-----------------------------|-----------------------------|
| Mock                | 0.06 ± 0.02           | 4.5 ± 2.4  | 4.5 ± 2.4  | 4.5 ± 2.4                   | 4.5 ± 2.4                   |
| GFP                 | 0.10 ± 0.04           | 6.3 ± 1.8  | 6.3 ± 1.8  | 6.3 ± 1.8                   | 6.3 ± 1.8                   |
| SIiP                | 0.57 ± 0.20           | 46.5 ± 0.10| 46.5 ± 0.10| 46.5 ± 0.10                 | 46.5 ± 0.10                 |
| SI2AP               | 0.52 ± 0.10           | 33.1 ± 5.5 | 33.1 ± 5.5 | 33.1 ± 5.5                  | 33.1 ± 5.5                  |
| SI2AZG              | 0.50 ± 0.11           | 36.1 ± 2.5 | 36.1 ± 2.5 | 36.1 ± 2.5                  | 36.1 ± 2.5                  |

Data are mean ± SEM.

aRatio of $\gamma$-globin to total ($\gamma$-globin + $\beta$-globin) mRNA was determined by qRT-PCR.
bPercentage of HbF to total (HbF + HbA) was determined by HPLC.

As previously observed,39 SIiP vector-transduced cells overexpressing IGF2BP1 favored production of HbF. Molecular analysis confirmed that SIiP-mediated HbF production resulted from a significant shift in the ratio of $\gamma$-globin/total ($\gamma$- + $\beta$-globin) mRNA compared to mock and GFP controls (Table 1; Figure 2A). Importantly, our modified SPTA1-IGF2BP1-2A vectors (SI2AP and SI2AZG) yielded similar increases in $\gamma$-globin over total $\beta$-like globin mRNA (Table 1; Figure 2A). High-performance liquid chromatography (HPLC) analysis of lysates from differentiated cells confirmed that IGF2BP1-mediated effects on globin transcription coincided with high levels of HbF (HbF/[HbF + HbA]) protein (Table 1; Figure 2B). To verify that HbF induction was coordinated with vector-mediated expression of IGF2BP1, we performed flow cytometry on erythroblasts that had been permeabilized and incubated with antibodies to IGF2BP1 and HbF. Minor cell populations with low levels of HbF or IGF2BP1 were identified for mock and GFP control cells, and only a few percent co-expressed both proteins (Figures 2C and 2D; % IGF2BP1+/HbF±, mean ± SEM: mock, 1.1 ± 0.7; GFP, 1.5 ± 0.4). Alternatively, IGF2BP1-transduced cells exhibited high levels of both IGF2BP1 and HbF that was predominantly coordinated (Figures 2C and 2D; % IGF2BP1+/HbF±, mean ± SEM: IGF2BP1, 45.6 ± 8.3). Thus, expression of IGF2BP1 in adult erythroblasts can potently reverse hemoglobin production toward a fetal-like phenotype, and this function is independent of the marker gene used to identify and/or enrich the transduced cell populations.

**IGF2BP1 Augments HbF in $\beta$-Thalassemia Major Erythroblasts**

Having established function of the newly constructed 2A vectors in healthy erythroblasts, we wanted to determine whether IGF2BP1
could induce HbF levels in cultured cells from patients with hemoglobin disorders. This was first tested using CD34+ cells isolated from BM of two patients with b-thalassemia, which we have shown to have suboptimal levels of HbA when differentiated in culture.17 Cells were mock treated or transduced with a lentivirus encoding for the GFP control or the IGF2BP1-2A-puromycin cassette under control of the erythroid-specific SPTA1 promoter (SI2AP) or the constitutive SFFV promoter (FI2AP). Transduced cells were expanded for 2 days and then cultured for 5 more days in a low concentration of puromycin to enrich the IGF2BP1 fraction. At this time point, cells were collected for isolation of total RNA, protein, or flow cytometry analysis and the remainder were plated into differentiation medium.

Control b-thalassemia cells (mock and GFP) had very low levels of IGF2BP1 mRNA, which were greatly elevated for IGF2BP1-transduced cells (Table 2). Flow cytometry for IGF2BP1 protein correlated with qRT-PCR results, as only a small percentage of positive cells was detected in control populations, which was greatly increased in IGF2BP1 transduced populations (Table 2; Figure 3A). These levels of IGF2BP1 protein were only visible by western blot for the SI2AP and FI2AP samples (Figure 4B), which mirrors results for b-thalassemia cells. The basal level of 3γ-globin mRNA in controls was very low to undetectable in control samples and substantially increased in cells transduced with SI2AP and FI2AP vectors (Table 2). IGF2BP1 protein was detected in mock and GFP control cells by flow cytometry, but percentages were greatly increased for transduced populations (Table 2; Figure 4A). These levels of IGF2BP1 protein were only visible by western blot for the SI2AP and FI2AP samples (Figure 4B), which mirrors results for b-thalassemia cells. The basal level of 3γ-globin mRNA in controls was lower than that seen in b-thalassemia cells, likely due to the fact that SCD
cells express wild-type levels of $\beta^S$-globin. The level of $\gamma$-globin rose in response to IGF2BP1 whereas $\beta$-globin was reduced, a shift that almost exclusively favored $\gamma$-globin (Table 2; Figure 4D). Cellulose acetate electrophoresis (Figure 4D) and HPLC (Figure S4) confirmed the transcriptional switch with IGF2BP1-transduced cells predominantly producing HbF and mock and GFP controls producing mainly HbS (Table 2; Figure 4D). Thus, IGF2BP1-mediated effects may be sufficient to provide dominant and potentially curative levels of HbF in SCD patients, but this will require additional patient samples to confirm.

**IGF2BP1 Induces $\gamma$-Globin/LCR Looping**

The LCR is an enhancer that physically interacts with the globin gene promoters to drive high-level, erythroid-specific expression during development. In fetal erythroblasts, the LCR binds upstream of the $\beta$- and $\gamma$-globin genes, leading to HbF production, whereas in adult cells, the LCR localizes with the $\beta$-globin gene promoter to confer HbA expression. To determine whether IGF2BP1 expression would cause the LCR to re-establish connections with the $\gamma$-globin genes, a chromosome conformation capture (3C) experiment was performed to assess the relative interaction of the LCR with the $\gamma$- or $\beta$-globin genes in adult erythroblasts. CD34$^+$ cells from a healthy adult donor were transduced in triplicate with either GFP control or SI2AP lentivirus particles. Transduced cells were grown for 2 days and then selected with puromycin to enrich the IGF2BP1 population. After 5 days of expansion, samples were equally divided and fixed with formaldehyde to stabilize DNA/protein interactions or transferred to differentiation medium. Hemoglobin electrophoresis showed uniform induction of HbF (mean of 42% by HPLC) for samples expressing IGF2BP1 (Figure 5A). Consistent with other studies, control cells demonstrated interaction between the LCR with $\beta$-globin gene (Figure 5B). IGF2BP1 significantly increased the interaction frequency of the LCR with the $\gamma$-globin gene (Figure 5B). The experiment was repeated using the FI2AP lentivirus and GFP control. Mean levels of HbF (66% by HPLC) surpassed those achieved using the SI2AP lentivirus (Figure S5A). Relative interaction of the LCR with the $\gamma$-globin genes was, however, not further augmented (Figure S5B). These data demonstrate that IGF2BP1 acts to partially re-localize the LCR to allow expression of the $\gamma$-globin genes in adult erythroblasts, which was confirmed by increased expression of both $\delta$- and $\gamma$-globin proteins compared with GFP control when lysates from differentiated cells were analyzed by reverse-phase HPLC (Figure 5C).

**DISCUSSION**

Our data confirm previous findings that IGF2BP1 is expressed at high levels in fetal erythroblasts and at very low to undetectable levels in adult erythroblasts. This is not unexpected, as multicellular organisms have networks of temporally regulated genes responsible for controlling major developmental events, including those that occur during the transition from fetal to adult life. The IGF2BP family consists of three proteins (IGF2BP1, 2, and 3). All three encode two RNA recognition motifs and four heterogeneous nuclear ribonucleoprotein (hnRNPs K homology domains. IGF2BP2 is the only member that continues to be expressed in adult life, while the other two IGF2BP genes are downregulated as tissues mature. IGF2BP1 has well-defined roles in controlling the stability, localization, and translation of RNA targets, such as IGF2, $\beta$-actin, tau, CD44, and $\beta$-catenin. These functions are essential for normal growth and development, as mice deficient in IGF2bp1 have dwarfism and impaired intestinal development. The presence of IGF2BP1 in some tumors has led to the suggestion that it is oncogenic. Activation of IGF2BP1 in cancer could result from reduced expression of let-7 microRNAs, which regulate thousands of transcripts, including the IGF2BPs. Nevertheless, oncogenic potential is cell- and tissue type-dependent, as IGF2BP1 can act to suppress proliferation and invasiveness of metastatic cells. This diversity of effects may be due to the large predicted number of potential IGF2BP1 target RNAs in cell lines and primary cells. Genome-wide association studies (GWASs) have not identified any potential IGF2BP1 single-nucleotide polymorphisms (SNPs) linked to human disease whereas SNPs in the second intron of IGF2BP2 are associated with type 2 diabetes.

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**Table 2. Increased Levels of $\gamma$-Globin and HbF in Erythroblasts Derived from Bone Marrow CD34$^+$ Cells of $\beta$-Hemoglobinopathy Patients Transduced with IGF2BP1 Lentiviral Vectors**

|                      | $\beta$-Thalassemia Patient 1 | $\beta$-Thalassemia Patient 2 | SCD Patient |
|----------------------|--------------------------------|--------------------------------|-------------|
|                      | Mock | GFP | SI2AP | FII2AP | Mock | GFP | SI2AP | FII2AP | Mock | GFP | SI2AP | FII2AP |
| IGF2BP1 mRNA$^a$     | 1.4  | 0.7 | 8.6   | 101.8  | 0.03 | 0.03 | 17.3 | 65.8   | 0.18 | 0.06 | 46    | 141   |
| % IGF2BP1$^b$        | 8    | 9   | 59    | 79     | 8    | 13   | 52   | 60     | 32   | 37   | 70    | 74    |
| $\gamma$-Globin mRNA | 61.1 | 73.1 | 92.3 | 99.2  | 76.5 | 86.6 | 97.6 | 98.8   | 33.0 | 56.8 | 91.2  | 96.5  |
| % HbA or HbS$^c$     | 27   | 24  | 19    | 9      | 26   | 27   | 9    | 12     | 81   | 74   | 33    | 23    |
| % HbF$^d$            | 23   | 23  | 8     | 0      | 15   | 12   | 4    | 0      | 4    | 5    | 0     | 0     |
| % HbF$^d$            | 50   | 53  | 73    | 91     | 59   | 61   | 87   | 88     | 15   | 21   | 45    | 66    |

$^a$IGF2BP1 mRNA as percentage of RNaseP determined by qRT-PCR.

$^b$Percentage of IGF2BP1-positive cells determined by flow cytometry.

$^c$Ratio of $\gamma$-globin to total $\beta$-like globin ($\gamma$-globin + $\beta$-globin) mRNA determined by qRT-PCR.

$^d$Percentage to total hemoglobin (HbF + HbA/S + HbA$s$) determined by HPLC.
The best characterized of the γ-globin regulators is the zinc-finger transcription factor BCL11A. We found that BCL11A is expressed in fetal erythroblasts and upregulated in adult counterparts, which is consistent with its role as a suppressor of the γ-globin genes. Analysis of published ChIP-sequencing data for fetal and adult erythroblasts complement this finding, as the BCL11A locus exhibited active chromatin marks in both cell populations that were enhanced in adult cells. Conversely, IGF2BP1 was marked by histone modifications typical of repressed chromatin in adult cells. Indeed, IGF2BP1 protein was significantly lower for differentiated erythroblasts from BM CD34⁺ cells compared with FL counterparts.

Lentivirus-mediated overexpression of IGF2BP1 in differentiated erythroblasts of healthy adult donors and patients with β-thalassemia disorders reversed the pattern of globin gene expression to favor the fetal type. Reverse-phase HPLC analysis of globin chains revealed a preferential increase in \( \gamma \)-versus \( \alpha \)-globin for β-thalassemia cells (Figure 3F) that was not apparent for healthy donors (Figure 3C). This deferential effect on globin gene expression is unexpected and will require further experimentation to explain.

A previous study established that IGF2BP1 overexpression in adult erythroblasts reduced levels of BCL11A protein. This effect was...
considered to occur post-transcriptionally, as IGF2BP1 overexpression had no effect on BCL11A mRNA level or stability. Sequence analysis of RNA that immunoprecipitated with IGF2BP1 identified BCL11A. Quantification of BCL11A transcripts in polysome fractions revealed no difference for control and IGF2BP1-expressing cells. This result could be explained by findings that IGF2BP1-containing RNP granules lack factors (eIF4E, eIF4G, and 60S ribosomal subunits) needed for translation of associated mRNAs, which could include BCL11A. Molecular characterization of IGF2BP1-containing RNPs and embodied mRNAs will be required to address this possibility.

Data from previous studies and those provided herein demonstrate that IGF2BP1-mediated reprogramming of hemoglobin expression in erythroblasts is achieved by a simultaneous increase in γ-globin and decrease in β-globin mRNA. Our 3C analysis revealed that IGF2BP1 restores long-range interactions between the LCR and γ-globin genes. There was, however, no appreciable effect on proximity of the LCR to the β-globin gene even in the situation where γ-globin mRNA made up two-thirds of total γ+β mRNA (Figure S5). A change in the LCR-β-globin interaction frequency has been varyingly observed upon induction of γ-globin transcripts. Repositioning of the LCR may be explained by reduction in BCL11A protein. However, it is intriguing to speculate that IGF2BP1 may interact with components of the LDB1/GATA-1/TAL1/FOG1/LMO2-containing complex or other multi-protein complexes involved in β-globin chromatin looping. This possibility can be addressed by targeted identification of proteins that immunoprecipitate with IGF2BP1 or by characterizing global changes in protein expression in adult erythroblasts reprogrammed to express HbF.

The benefit of HbF on severity of β-thalassemia and SCD is well documented. As a result, decades of research have been devoted to understanding the factors and mechanisms controlling the switch from HbF to HbA during development. Identification of proteins and non-coding RNAs involved in this process offers an opportunity to develop novel approaches to reactivate HbF. This can be accomplished by downregulating repressors or expressing activators of the endogenous γ-globin genes, respectively, using gene delivery and/or
gene editing techniques. We have shown that lentivirus-mediated expression of IGF2BP1 is sufficient to cause potent reversal of adult hemoglobin production to the fetal type in erythroblasts derived from transduced CD34+ cells of patients with β-thalassemia or SCD. Reprogramming of high levels of hemoglobin expression is achieved at the transcriptional level by increased γ-globin combined with decreased β-globin mRNA, which shifts the total β-like globin mRNA profile to favor γ-globin. IGF2BP1 expression was predominantly coordinated with pancellular expression of HbF. That said, we consistently detected a small population of IGF2BP1-positive cells that lacked HbF (Figure 2C). The reason for this is currently unknown. IGF2BP1-dependent induction of HbF ameliorated the chain imbalance that occurs in β-thalassemia or potently suppressed expression of β-like globin in SCD. This is a critical point for SCD where γ-globin must compete with β-like globin for α-globin to yield beneficial HbF tetramers. Based on these data, it is possible that erythroid-specific expression of IGF2BP1 could provide dominant and curative levels of HbF in children or adults with β-thalassemia or SCD. Transplantation studies performed using healthy mice and models of β-thalassemia or SCD will be required to rigorously test the utility of IGF2BP1 as a new therapeutic avenue for patients with severe β-hemoglobin disorders.

MATERIALS AND METHODS

Lentiviral Vector Construction and Production

**SPTA1-IGF2BP1-IRES-Puromycin (SI2AP)**

Details regarding construction of the self-inactivating (SIN) lentiviral vector (pLVX; Clontech Laboratories, Mountain View, CA, USA) encoding for bicistronic expression of the human IGF2BP1 cDNA (1,724 bp) and a puromycin resistance gene up to the 5’-end of this gene. The fragment was provided in a cloning vector, excised with BstXI and XmaI, and inserted into the same sites of SFFV to replace the IRES.

**SPTA1-IGF2BP1-2A-ZsGreen (SI2AZG)**

An 892-bp gBlock fragment was synthesized by IDT that again included IGF2BP1 sequences from the BstXI contiguous with the 2A peptide and puromycin gene sequences up to the XmaI site on the 5’-end of this gene. The fragment was provided in a cloning vector, excised with BstXI and XmaI, and inserted between the same sites of SPTiP to replace the IRES.

**SFFV-IGF2BP1-2A-Puromycin (FI2AP)**

The erythroid-specific SPTA1 promoter was replaced with the constitutive spleen focus-forming virus (SEFV) promoter. The SEFV promoter was PCR amplified from plasmid CL20-SFFV42 (a gift from Brian Sorrentino) with the addition of 5’-ClaI and 3’-XhoI restriction sites, and inserted into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA, USA). A 404-bp fragment was excised from a sequence-verified clone with ClaI and XhoI and inserted into SPTiP cut with the same enzymes to remove the SPTA1 regulatory element.

Lentiviral vector particles pseudotyped with vesicular stomatitis virus glycoprotein (VSV-g) were prepared using human embryonic kidney 293T/17 cells (ATCC, Manassas, VA, USA; CRL-11268) and a four-plasmid transient transfection system.63 Viral supernatants were concentrated 10- to 20-fold by centrifugation at 20,000 rpm for 90 min at 4°C under vacuum and stored as aliquots at −80°C. Viral titer was determined by overnight transduction of 1 × 10^7 K562 human erythroleukemia cells (ATCC, CCL-243) with serial dilutions of thawed virus in the presence of 8 μg/mL Polybrene. Flow cytometry was used to determine the percentage of cells that expressed ZsGreen.
on day 7 post-transduction or survived puromycin selection (propa-
dium iodide negative fraction), which began 2 days after transduction
and continued for 5 days, to calculate transducing units (TU) per mL
of lentivirus for CD34+ cell transductions.

**CD34+ Cell Transduction and Erythroid Culture**

Cytokine-mobilized peripheral blood or BM cells from healthy adult
donors or patients with β-thalassemia major or SCD were collected
according to protocols approved by the Institutional Review Board
of St. Jude Children’s Research Hospital. CD34+ cells were recovered
by positive selection using immunomagnetic beads, and cryopre-
served cell products were obtained without personal identifiers. Purity
and viability of processed cells was ≥90% for healthy donors and
≥80% for β-thalassemia or SCD patients. Purified CD34+ cells
were also purchased from Lonza (Walkersville, MD, USA) or the
Yale Cooperative Center of Excellence in Hematology (Yale School
of Medicine, New Haven, CT, USA) to make comparisons between
fetal and adult sources, respectively.

CD34+ cells were first cultured for 48 h in expansion medium consist-
ing of StemSpan SFEM II (STEMCELL Technologies, Vancouver, BC,
Canada) containing 2 U/mL human recombinant erythropoietin
(EPO), 10 ng/mL stem cell factor (SCF), 1 ng/mL human recombi-
nant interleukin-3 (IL-3), and 1 μM each dexamethasone and β-estradiol.
Cells were transferred to RetroNectin-coated plates (Clontech
Laboratories, Mountain View, CA, USA) and transduced with lentivi-
ral particles (MOI of 10–20). After 48 h of exposure, transduced
cells were enriched by FACs or selected for drug resistance by adding
a low concentration of puromycin (0.5 μg/mL) to the culture me-
dium. Enriched cell populations were cultured for 7 days at a concen-
tration of 1–2 × 10^6 cells/mL before they were collected for isolation
of RNA (5 × 10^2 cells) or protein (2 × 10^6 cells). The remaining cells
(ranging from 10 to 30 million) were transferred to differentiation
medium (StemSpan SFEM II, 4 U/mL human recombinant EPO,
and 0.5 mg/mL holo-transferrin) and cultured for 4–7 days at a con-
centration of 5 million cells/mL. Differientiated cells were lysed and
hemoglobin tetramers evaluated by cellulose acetate hemoglobin elec-
trophoresis and/or HPLC.

**qPCR**

RNA was extracted (PureLink RNA mini; Life Technologies,
Carlsbad, CA, USA) from cells 7 days after transduction, quantitated
by NanoDrop, and 400 ng was used for first-strand cDNA synthesis
(VILO with DNsase; Invitrogen, Carlsbad, CA, USA). cDNAs
were diluted to 100 ng/μl and 200 ng/reaction was used to detect
IGF2BP1 (Hs00198023_m1), γ-globin (Hs00361131_g1), β-globin
(Hs00747223_g1), BCL11A (Hs00256254_m1), LRF/ZBTB7A
(Hs00792219_m1), and RNaseP (internal control, 4403328). TaqMan
primer-probe sets were from Applied Biosystems. Data are expressed
as percentage of RNaseP levels.

**Western Immunoblot**

Whole-cell lysates, prepared with M-PER (mammalian protein
extraction reagent) supplemented with 1 × HALT protease inhibitors
(Thermo Fisher Scientific, Waltham, MA, USA), were separated by
SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) mem-
branes (Immobilon-P, Millipore). Signals were detected with Super-
Signal West Pico (Pierce, Thermo Fisher Scientific, Waltham, MA,
USA) and captured with a CCD (charge-coupled device) camera
(Syngene, Frederick, MA, USA). Primary antibodies used were as fol-
lows: IGF2BP1 (Cell Signaling Technology, Danvers, MA, USA; clone
D33A2), BCL11A (Abcam, Eugene, OR, USA; clone 14B5), GAPDH
(Sigma-Aldrich, St. Louis, MO, USA; clone GAPDH-71.1) and α-
tubulin (Active Motif, Carlsbad, CA, USA; clone 5-8-1-2). Horse-
radish peroxidase (HRP)-conjugated secondary antibodies (goat
anti-mouse and goat anti-rabbit) were from Thermo Fisher Scientific.

**Flow Cytometry**

Erythroblasts (1 × 10^7 per condition) were treated with a Fix & Perm
cell permeabilization kit (Invitrogen) according to the manufacturer’s
instructions. Staining was performed during the permeabilization
step. IGF2BP1 was detected with a monoclonal antibody (Cell
Signaling Technology; clone D33A2, 1:100 dilution) and then Alexa
Fluor 667-conjugated goat anti-rabbit secondary antibody (Life Tech-
nologies; 1:2,000 dilution). Cells treated with secondary antibody
alone were used as a control. HbF was detected using phycoerythrin
(PE)-conjugated mouse anti-human fetal hemoglobin monoclonal
antibody (BD Pharmingen, San Jose, CA, USA; clone 2D12, 1:500
dilution); PE-conjugated mouse immunoglobulin G (IgG) (BD Phar-
mingen; 1:500 dilution) served as a control. Data were collected using
the FACSARia II flow cytometer (BD Biosciences, San Jose, CA, USA)
and analyzed with FlowJo v10.0 software. The percentage positive
cells and geometric mean fluorescence intensity (MFI) was deter-
mained for each condition. In selected experiments, cells were simul-
taneously stained with antibodies to both IGF2BP1 and HbF and
the percentage of double-labeled cells was determined.

**Hemoglobin Analysis**

Differentiated erythroblasts (10–20 million) were lysed in hemolysate
reagent (Helena Laboratories, Beaumont, TX, USA; 20 μL per 10
million cells) and refrigerated overnight. Supernatants were cleared
of debris by centrifugation (14,000 rpm at 4°C for 15 min), and hem-
globin tetramers were identified by cellulose acetate electrophoresis as
described[17] and HPLC using a G7 analyzer (Tosoh Bioscience, San
Francisco, CA, USA) and β-thalassemia settings. Globin chain
expression was performed for selected samples on a TSP Specta
HPLC system using a LiChristopher 100 RP-8 column and a gradient
of acetonitrile-methanol-sodium chloride.[66] Controls included a
standard with known amounts of adult (HbA and HbA2), fetal
(Hbf), and sickle (Hbs) hemoglobin (AFSA2 Hemo Control, Helena
Laboratories), as well as hemolysates prepared from adult peripheral
blood or umbilical cord blood samples.

**3C Assay**

The 3C assay was performed as described[68] with minor modifications.
CD34+ cells from a healthy adult donor were transduced with control
or IGF2BP1 lentivirus in triplicate. Culture-differentiated erythro-
blasts (8–9 million per condition) were collected 8 days after
transduction and fixed with 1% formaldehyde. Isolated nuclei were digested overnight with EcoRI and ligated for 4 h. Interaction frequency between the anchor fragment and regions of interest in the β-globin locus were determined by SYBR Green quantitative real-time PCR using published primers. Interaction frequency between two fragments within the α-tubulin gene was used for the internal normalization control. Interaction frequency for the fragment containing the Gγ-globin gene is representative of both the Aγ- and Gγ-globin genes, as these sequences cannot be effectively discriminated due to high sequence homology.

Statistical Analysis
Microsoft Excel or GraphPad Prism 5 was used to determine descriptive statistics (mean ± SD or SEM), and significant differences between mean values were determined by an unpaired Student’s t test (two-tailed) or one-way ANOVA with a Newman-Keuls post hoc test. Values of p are indicated by asterisks in the figures with level of significance reported.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
C.B.C. performed the research, analyzed data, and wrote the manuscript. J.G., K.P., C.B., and Y.T.L. contributed to performing the research. D.L. performed reverse-phase HPLC and edited the manuscript. X.G. and A.D. performed 3C experiments and edited the manuscript. J.L.M. provided critical reagents, technical advice, and edited the manuscript. A.W. designed the research, analyzed data, wrote the manuscript, and was responsible for organization of the research effort. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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