RED CELLS, IRON, AND ERYTHROPOIESIS

LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts ex vivo

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Key Points

- LIN28B regulates HbF expression in erythroblasts that are cultured from umbilical cord and adult human blood.
- LIN28B expression manifested a more fetal-like phenotype among adult human erythroblasts.

Reactivation of fetal hemoglobin (HbF) holds therapeutic potential for sickle cell disease and β-thalassemias. In human erythroid cells and hematopoietic organs, *LIN28B* and its targeted *let-7* microRNA family, demonstrate regulated expression during the fetal-to-adult developmental transition. To explore the effects of *LIN28B* in human erythroid cell development, lentiviral transduction was used to knockdown *LIN28B* expression in erythroblasts cultured from human umbilical cord CD34+ cells. The subsequent reduction in *LIN28B* expression caused increased expression of *let-7* and significantly reduced HbF expression. Conversely, *LIN28B* overexpression in cultured adult erythroblasts reduced the expression of *let-7* and significantly increased HbF expression. Cellular maturation was maintained including enucleation. *LIN28B* expression in adult erythroblasts increased the expression of *γ-globin*, and the HbF content of the cells rose to levels >30% of their hemoglobin. Expression of carbonic anhydrase I, glucosaminyl (*N*-acetyl) transferase 2, and *miR-96* (three additional genes marking the transition from fetal-to-adult erythropoiesis) were reduced by *LIN28B* expression. The transcription factor BCL11A, a well-characterized repressor of *γ-globin* expression, was significantly down-regulated. Independent of LIN28B, experimental suppression of *let-7* also reduced BCL11A expression and significantly increased HbF expression. *LIN28B* expression regulates HbF levels and causes adult human erythroblasts to differentiate with a more fetal-like phenotype. (*Blood*. 2013;122(6):1034-1041)

Introduction

In humans and some other mammals, the composition of hemoglobin tetramers in erythrocytes switch from fetal hemoglobin (HbF) ($\alpha 2\gamma 2$) to adult hemoglobin (HbA) ($\alpha 2\beta 2$) during the last stages of fetal development until early infancy.[1] HbF is the most important known modifier of the clinical symptoms for patients with sickle cell disease (SCD) and β-thalassemias, which are among the most common genetic disorders worldwide.[2,3] In patients with SCD, the polymerization of sickle hemoglobin results in erythrocyte deformation and hemolysis.[4] SCD patient's clinical outcomes are largely improved by inhibition of the polymerization by HbF.[5] In β-thalassemias, decreased production of β-globin causes imbalanced globin polypeptide chain synthesis, and leads to severe effects on the erythroid cells' maturation and survival. The loss of β-globin expression may be compensated by an increase in HbF production that leads to improvement of the clinical phenotype.[6]

The molecular mechanisms underlying the switch from HbF to HbA are still largely unknown. Genome-wide association studies (GWAS) in both normal individuals and patients with β-hemoglobinopathies have identified *BCL11A*, *HSB1L-MYB*, and *HBB* clusters as having an association with the persistence of HbF in adults.[7-10] Suppression of the BCL11A transcription factor causes an increase in HbF levels.[11]

Lin28 is a highly conserved gene that is expressed in the early stages of development of multicellular organisms. In the nematode, *Caenorhabditis elegans*, *lin28* regulates developmental timing at the larvae L2 stage.[12] Mammals express 2 homologs of the *C. elegans lin28* gene known as *LIN28A* and *LIN28B*.[13,14] Human LIN28 genetic variation correlates with developmental timing characteristics, such as variation in height,[15] timing of puberty,[16-18] and age at natural menopause.[16] The effects of LIN28 protein depend, in part, on the cell type in which it is expressed, as evidenced by its role in promoting the pluripotency of stem cells,[19] as well as the differentiation of skeletal muscle cells.[20]

In part, LIN28 acts through the 3′ end uridylation of the precursor molecule *pre-let-7*, ultimately leading to the degradation of microRNA (miRNA) molecules.[21-23] Interestingly, expression of the *let-7* family of miRNAs was correlated with the fetal-to-adult developmental transition in circulating human reticulocytes.[24] Those data suggest a potential link between *LIN28B* expression, the regulation of the *let-7* miRNAs, and vertebrate hematopoiesis. In

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addition to binding to the terminal loop of *let-7* miRNAs, LIN28 binds to GGAGA motifs in messenger RNAs (mRNAs).[25] Moreover, a predicted fetal stage-specific enhancer for *LIN28B* was identified using human primary erythroid cells,[26] and in murine lymphocytes, *Lin28b* expression regulates the fetal-like phenotype.[27] Here, we investigated LIN28B as a candidate regulator of the fetal-to-adult transition of hemoglobin expression and the erythroblast phenotype using an experimental approach developed for ex vivo engineering of human erythrocytes.

Methods

Cell culture

All related studies were performed after human subjects review and National Institutes of Health Institutional Review Board approval. These studies were conducted in accordance with the Declaration of Helsinki. Human cord blood CD34+ cells were obtained from All Cells (Emeryville, CA) and ReachBio LLC (Seattle, WA). For the ex vivo culture, a 21 day serum-free system consisting of 3 phases was used. During phase I of the culture (day 0 to day 7): adult or cord blood CD34+ cells were placed in medium containing StemPro-34 complete medium (L-glutamine, pen-strep, and StemPro-34 nutrient supplement) (Invitrogen, Carlsbad, CA) with 50 ng/mL SCF (HumanZyme, Chicago, IL), 50 ng/mL FLT3 Ligand (HumanZyme) and 10 ng/mL IL-3 (HumanZyme). After phase I, the cells were transferred to phase II (day 7 to day 14). During this expansion phase, the cell count was monitored on days 9, 11, and 14 to maintain cell counts less than 2×10^6 cells/mL. Phase II medium was comprised of the following: StemPro-34 complete medium, 4 U/mL erythropoietin (EPO) (Amgen, Thousand Oaks, CA), 10 ng/mL SCF, 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO), 3 U/mL heparin (Hospira, Lake Forest, IL), and 0.8 mg/mL holo Transferrin (Sigma-Aldrich). On culture day 14, cells were counted and transferred to phase III medium at 8×10^5 cells/mL for the remaining 7 days of culture. Phase III medium was comprised of the following: StemPro-34 complete medium, 4 U/mL EPO, 3 μM RU486 (Sigma-Aldrich), 10 μg/mL insulin, 3 U/mL heparin, and 0.8 mg/mL holo Transferrin.

RNA IP

RNA immunoprecipitation (IP) was performed according to the manufacturer's instructions (Magna RIP RNA-Binding Protein Immunoprecipitation Kit, Millipore, Billerica, MA) with equal amounts of lysates from human cord blood cells using antibodies against LIN28B (catalog #4196; Cell Signaling, Danvers, MA) or IgG (catalog #PP64; Millipore). The immunoprecipitated RNA was purified before reverse transcription-polymerase chain reaction with primers to primary *let-7d* miRNA (Assays-on-Demand Gene Expression Product #Hs03302562_pri; Applied Biosystems, Grand Island, NY).

Recombinant viral transduction

Human *LIN28B* was subcloned into an MSCV-IRES-Puro-based vector, as previously described.[27] Human *LIN28A* overexpression lentiviral particles were purchased from Qiagen (Valencia, CA) and used for comparison studies. Human *LIN28B* knockdown clones were purchased from Sigma Aldrich. *BCL11A* knockdown clones were selected as previously described.[11] A miRNA sponge experimental approach was used for suppression of *let-7*.[28] The *let-7* sponge plasmid (MSCV puro *let-7* sponge) was purchased from Addgene (Cambridge, MA; plasmid, catalog #29766). Tissue culture dishes were coated with 20 μg/mL poly-L-lysine (Sigma-Aldrich) for 1 hour at 37°C. One day before transfection, the 293T cells were seeded at 4×10^6 cells per dish. Retroviral supernatants were produced by transient Lipofectamine 2000 (Invitrogen) cotransfection of 293T cells with the RetroMax packaging vector pCL-Eco (Imgenex, San Diego, CA) and the *let-7* sponge plasmid vector (20 μg each) following the manufacturer's instructions. Seventy-two hours post-transfection, cell supernatant was harvested and concentrated with Retro-X concentrator solution (Clontech, Mountain View, CA) according to the manufacturer's instructions. Empty vectors were used as controls. On culture day 3 of phase I, the cells were transduced with either *LIN28*, *BCL11A*, or *let-7* sponge viral particles (estimated multiplicity of infection: 12). After 24 hours, Puromycin (Sigma-Aldrich) was added to the culture. On culture day 7, cells were transferred to phase II medium containing EPO and Puromycin until culture day 9. After culture day 9, cells were cultivated at the conditions previously described without Puromycin.

Flow cytometry analyses

Immunostaining with antibodies directed against CD71 and glycophorin A (Invitrogen) were performed to assay erythroid differentiation on culture day 21, as previously described,[29] using the BD FACSAria I flow cytometer (BD Biosciences, San Jose, CA). Enucleation was quantitated using thiazole orange staining. Enucleated erythroid cell populations were isolated by filtering day 21 cells through Purecell Neo Leukocyte Reduction filters (Pall, Covina, CA).

Confocal microscopy

For confocal microscopy, slides containing culture day 14 cells were probed against human LIN28B (Cell Signaling). Laser scanned confocal images were obtained from LSM 5 Live Duoscan (Carl Zeiss, Oberkochen, Germany) and analyzed with Zen2007 software.

HPLCs for HbA and HbF

Samples for hemoglobin profiles (HPLCs) were prepared and analyzed as previously described.[29]

Quantitative polymerase chain reaction analysis

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays and polymerase chain reaction conditions were performed as previously described.[30] Additional primers and probes used in this study are described in supplemental Table 1. To evaluate miRNA expression, reactions, and data normalization were performed as previously described.[24]

Western blot analysis

Nuclear and cytoplasmic extracts were prepared from culture day 14 erythroblasts using NE-PER Nuclear Protein Extraction Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. Equal amounts of protein (20 to 30 μg) were separated by NuPAGE Novex 4% to 12% Bis-Tris gel electrophoresis in 4-morpholinepropanesulfonic acid buffer and transferred using the iBlot Blotting System onto nitrocellulose membranes (Invitrogen). Blots were probed with antibodies against human CA1 (Abcam, Cambridge, MA), glucosaminyl (*N*-acetyl) transferase 2 (GCNT2) (Santa Cruz Biotechnology, Dallas, TX), BCL11A (Abcam), and LIN28B (Cell Signaling), and the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Immunoreactive proteins were detected and visualized using ECL Plus Western blotting detection reagents (GE Healthcare, Waukesha, WI). All blots were probed with β-actin (Abcam) antibody as a loading control.

Statistical analysis

Replicate data are expressed as mean value ± standard deviation with significance calculated by two-tailed Student *t* test.

Results

LIN28B binds primary *let-7* transcripts and regulates HbF in human cord blood CD34+ cells

We previously reported that decreased HbF expression in adult human reticulocytes compared with those present in cord blood is associated with increased *let-7* levels in adult reticulocytes.[24] Because *let-7* miRNA expression is downregulated by the binding of LIN28

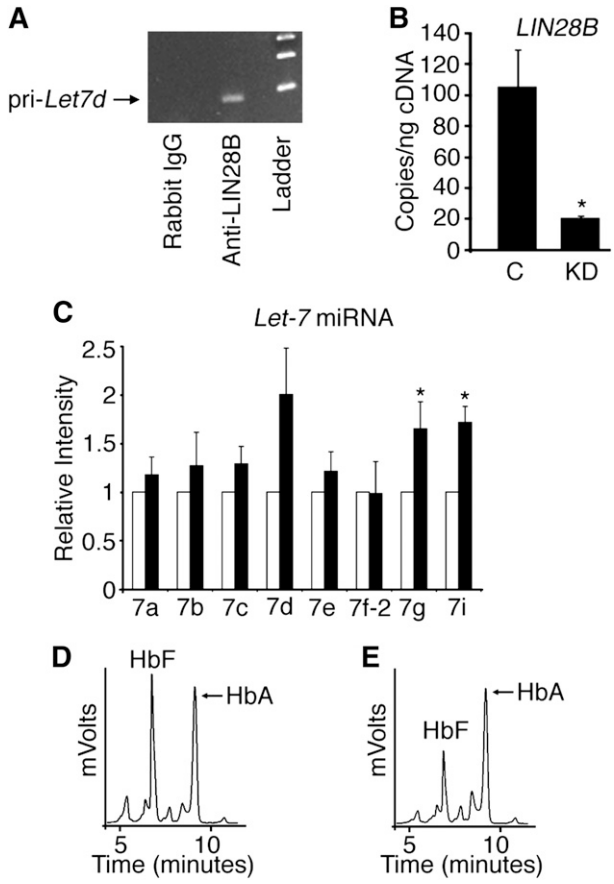

Figure 1. *LIN28B* regulates HbF levels in cultured human cord blood erythroblasts. (A) Detection of primary *let-7d* miRNA in erythroblasts cultured from human CD34+ cord blood cells after RNA IP with antibodies against LIN28B or control (rabbit IgG) with DNA ladder in the right lane shown for comparison. (B) *LIN28B* knockdown (*LIN28B*-KD) confirmation by qRT-PCR quantitation of copy number per nanogram complementary DNA (cDNA) (copies/ng cDNA). *LIN28B*-KD and control samples were evaluated. (C) The relative expression levels of the *let-7* family of miRNAs were determined by qRT-PCR. Open bars represent control samples and black bars represent *LIN28B*-KD. Standard deviation bars are shown. *$P < .05$ in triplicate experiments. Hemoglobin profiles demonstrated by HPLC analysis are shown for (D) control and (E) *LIN28B*-KD cultures. HbF and HbA peaks are labeled on each graph (y-axis: mVolts; x-axis: elution time in minutes). C, empty vector control; KD, *LIN28B* knockdown; Pri-*Let7d*, primary *let-7d* miRNA.

proteins, the absence of *LIN28B* expression in adult human bone marrow compared with its detection in fetal liver[27] suggests that both *LIN28B* and *let-7* are developmentally regulated in erythroid tissues. Initial studies were performed to determine if LIN28B binds endogenous *let-7* primary transcripts in cord blood-derived erythroblasts. RNA IP in CD34+ cord blood lysates was performed and quantitated by reverse transcription-polymerase chain reaction for primary *let-7d*. The *let-7d* was chosen based on its expression level in cord blood cells.[24] Primary *let-7d* was enriched by LIN28B IP compared with the IgG control (Figure 1A).

To investigate a potential role for cord blood *LIN28B* expression in HbF regulation, CD34+ cord blood cells were transduced with lentivirus encoding a short hairpin RNA (shRNA) against *LIN28B* and cultured ex vivo in erythropoietin-supplemented serum-free media for 21 days. Two shRNA clones (TRCN0000122191 and TRCN0000122599) demonstrated equivalent results; the results using clone TRCN0000122191 are shown in Figure 1. *LIN28B* knockdown (*LIN28B*-KD) was confirmed by qRT-PCR (control: 105 ± 24 copies/ng; *LIN28B*-KD: 20.7 ± 0.9 copies/ng; $P = .02$), as demonstrated in Figure 1B. *Let-7* miRNAs were measured by qRT-PCR in *LIN28B*-KD cells. As shown in Figure 1C, *LIN28B*-KD caused increased expression of several *let-7* species (*let-7a*, *let-7b*, *let-7c*, *let-7d*, and *let-7e*), and the increased levels of *let-7g* and *let-7i* reached statistical significance compared with the controls ($P = .05$ and $P = .01$, respectively). Hemoglobin expression profiles for control and *LIN28B*-KD were determined by standard HPLCs (Figures 1D-E). Triplicate experiments demonstrated a significant reduction in HbF expression in *LIN28B*-KD cells compared with the controls (*LIN28B*-KD: 31.0 ± 5.3%; control: 54.2 ± 7.7%; $P = .03$).

LIN28B expression activates HbF during adult human erythropoiesis

In addition to studies of cord blood-derived erythroblasts, *LIN28B* expression in adult erythroblasts was examined. When compared with the cord blood erythroblasts, adult erythroblasts demonstrated a major reduction in *LIN28B* expression (0.1 ± 0.4 copies/ng). To increase *LIN28B* expression, the cells were transduced with a retrovirus encoding *LIN28B*. *LIN28B* overexpression (*LIN28B*-OE) was confirmed by qRT-PCR (*LIN28B*-OE: 1.8E+04 ± 3.5E+02 copies/ng; $P = .01$) and Western blot analyses (Figure 2A-B). In transduced erythroblasts, LIN28B was expressed predominantly in the cytoplasm without distinct nuclear localization as shown by confocal microscopy (Figure 2C-F).

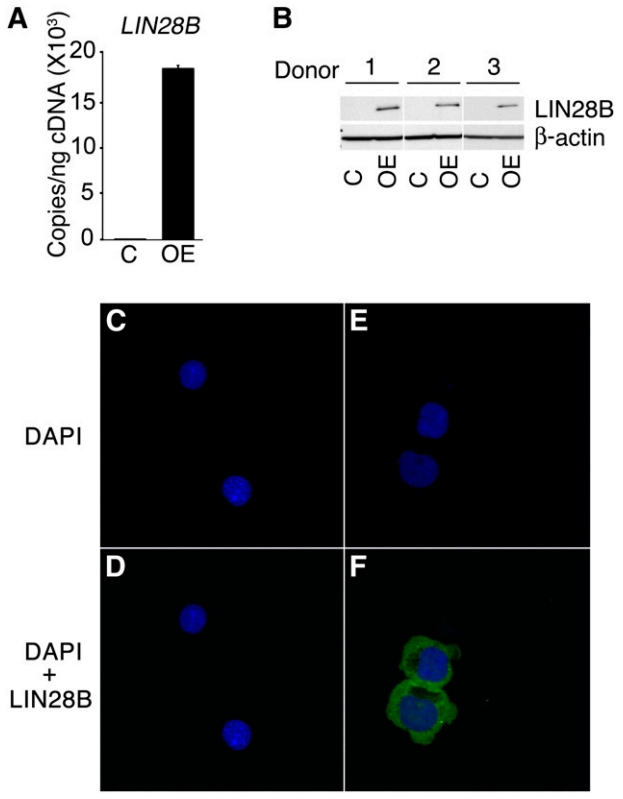

Figure 2. *LIN28B* overexpression in adult human erythroblasts. *LIN28B*-OE was confirmed by (A) qRT-PCR quantitation of copy number per nanogram complementary DNA (cDNA) (copies/ng cDNA); (B) Western blot analysis and (C-F) confocal images of control and *LIN28B*-OE cells were stained with DAPI (4′,6-diamidino-2-phenylindole) (blue) and LIN28B (green). Cells were transfected with empty vector (C-D), and *LIN28B* (E-F). Analyses were performed at culture day 14. Mean value ± standard deviation of 3 independent donors for each condition. C, empty vector control; OE, *LIN28B* overexpression.

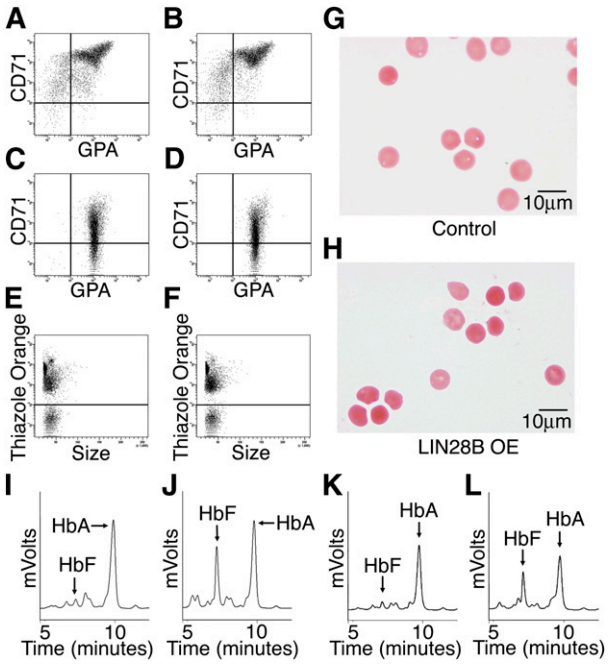

Figure 3. *LIN28B* overexpression enhances HbF levels in adult human erythroblasts. Flow cytometry analyses of (A) control and (B) *LIN28B*-OE at culture day 14, and (C) control, and (D) *LIN28B*-OE at culture day 21 stained with anti-transferrin receptor (CD71) and anti-glycophorin A (GPA) antibodies. Enucleation is represented by staining of culture day 21 cells with thiazole orange for (E) control and (F) *LIN28B*-OE. The enucleated cells were imaged in panels (G) control and (H) *LIN28B*-OE. HPLC analysis of hemoglobin from (I) control and (J) *LIN28B*-OE, and (K) control and (L) *LIN28A*-OE samples performed at culture day 21 shown for comparison with *LIN28B*-OE. HbF and HbA peaks are labeled on each graph (y-axis: mVolts; x-axis: elution time in minutes). Data are representative of more than 3 independent experiments. *LIN28B*-OE, *LIN28B* overexpression; *LIN28A*-OE, *LIN28A* overexpression.

Erythroblast differentiation and the patterns of hemoglobin expression were compared between control and *LIN28B*-OE cells. Flow cytometric analysis of erythroblast plasma membrane markers, transferrin receptor (CD71), and glycophorin A were performed at culture days 14 and 21 for erythroblast differentiation (Figure 3A-D). Surprisingly, *LIN28B*-OE did not overtly affect erythroid cell maturation when compared with empty vector controls. Furthermore, thiazole orange staining of nuclei on culture day 21 in *LIN28B*-OE and control cells showed comparable results (Figure 3E-F) (*LIN28B*-OE enucleation: 38 ± 2.1% compared with control: 25 ± 9.3%), thus indicating the ability of LIN28B-OE erythroblasts to undergo terminal maturation. The culture-generated erythrocytes were purified and further studied. The *LIN28B*-OE erythrocytes were morphologically equivalent to controls for all analyzed samples (Figure 3G-H). HPLCs showed markedly increased HbF levels in the *LIN28B*-OE cells (Figure 3I-J) (*LIN28B*-OE: 33.6 ± 9.4%; control: 5.8 ± 4.5%; $P = .01$). For comparison, a different lentiviral vector encoding the other human *LIN28* gene (*LIN28A*) was used. A robust increase in HbF expression was also detected in *LIN28A* transduced cells (Figure 3K-L). Taken together, these data demonstrate that overexpression of *LIN28A* or *LIN28B* is sufficient for the manifestation of high-level HbF expression in cultured adult human erythroblasts.

Adult erythroid cells expressing *LIN28B* manifest a more fetal-like phenotype

The fetal-to-adult hemoglobin transition in erythrocytes encompasses a reduction in the expression of the fetal ($^{A}\gamma/^{G}\gamma$-globin) genes among erythroblasts, coinciding with an increased expression of the adult (β- and δ-globin) genes. This process begins during the later stages of fetal development and continues through early infancy. Based on the high level of HbF expression in *LIN28B*-OE cells, globin gene expression patterns were quantitated by qRT-PCR. The expression levels of α, μ, θ, ζ, β, δ, γ, and ε globin genes were evaluated for *LIN28B*-OE and compared with controls. As shown in Figure 4A-B, only the fetal γ-globin mRNA expression is significantly heightened with *LIN28B*-OE (control: 5.1E+06 ± 2.6E+06 copies/ng; *LIN28B*-OE: 1.8E+07 ± 5.8E+06 copies/ng; $P = .04$). The increased expression of γ-globin was balanced by reductions in β-globin and δ-globin. No changes were observed in α, μ, and θ globins, and the low level expression patterns of ζ and ε globins indicated only minor increases.

The fetal-to-adult transition in humans is further characterized by an increase in the carbonic anhydrase I (*CA1*) gene expressed in erythrocytes,[31] as well as the carbohydrate modification due to the augmented expression of *GCNT2*.[32-34] Therefore, expression of *CA1* and *GCNT2* were investigated by qRT-PCR with *LIN28B*-OE. As shown in Figure 4C-D, both *CA1* and *GCNT2* were significantly

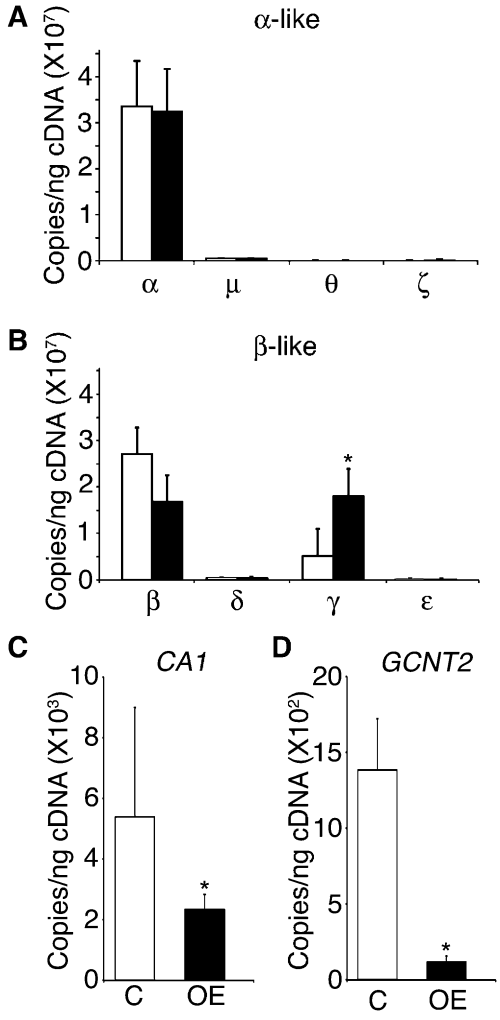

Figure 4. *LIN28B* expression regulates γ-globin and erythroid-related fetal genes in adult erythroblasts. (A) α, μ, θ, and ζ globins, (B) β, δ, γ, and ε globins, (C) *CA1*, and (D) *GCNT2* mRNA expression analysis of *LIN28B*-OE compared with control samples. Open bars represent control and black bars represent *LIN28B*-OE. qRT-PCR analyses were performed at culture day 14. Mean value ± standard deviation of 3 independent donors for each condition. *P* values were calculated using two-tailed Student *t* test. C, empty vector control; OE, *LIN28B* overexpression. *$P < .05$.

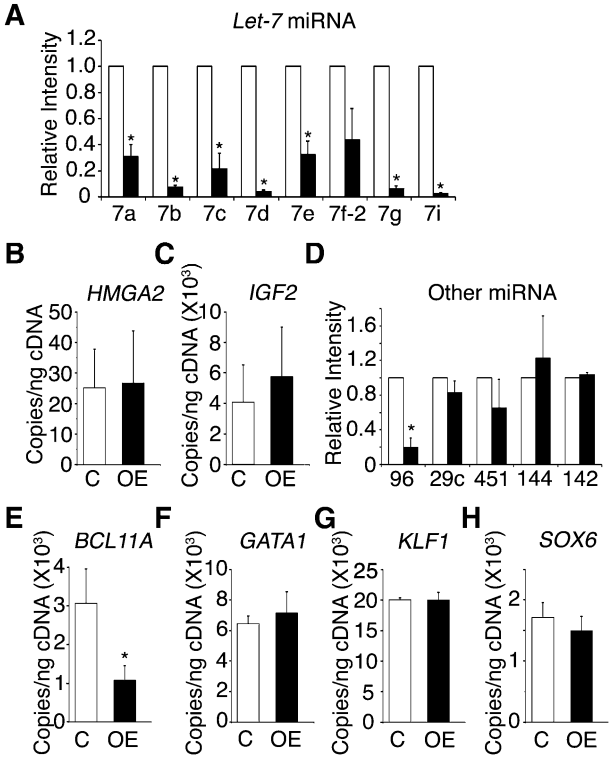

Figure 5. *LIN28B* modulation of adult CD34+ cells toward a fetal-like phenotype involves the *let-7* family of miRNAs, *miR-96*, and *BCL11A*. *LIN28B*-OE compared with control samples in (A) the relative expression levels of the *let-7* family of miRNAs (open bars represent control and black bars represent *LIN28B*-OE), the mRNA expression levels of (B) *HMGA2* and (C) *IGF2*, (D) the relative expression levels of *miR-96*, *miR-29c*, *miR-451*, *miR-144*, and *miR-142*, and the mRNA expression of the transcription factors (E) *BCL11A*, (F) *GATA1*, (G) *KLF1*, and (H) *SOX6*. The qRT-PCR analyses were performed at culture day 14. The miRNAs relative expression levels (y-axis) in the control cells were defined as a level of one for comparison. Error bars denote ± standard deviation of 3 independent donors for each condition. *P* values were calculated using two-tailed Student *t* test. C, empty vector control; OE, *LIN28B* overexpression. $^*P < .05$.

reduced with *LIN28B*-OE (*CA1,* control: 5E+03 ± 4E+03 copies/ng; *LIN28B*-OE: 2E+03 ± 5E+02 copies/ng; $P = .01$; *GCNT2,* control: 1E+03 ± 3E+02 copies/ng; *LIN28B*-OE: 1E+02 ± 4E+01 copies/ng; $P = .01$). Western blot analysis using anti-human CA1 antibody in *LIN28B*-OE was compared with control samples at culture day 21 confirming a reduction in CA1 levels; however, a major change in GCNT2 protein expression was not detected (supplementary Figure 1A-B).

LIN28B suppresses *let-7* and *miR-96* miRNAs

One characteristic effect of LIN28 proteins is the suppressed maturation of the *let-7* family of miRNAs.[21,35] Therefore, the expression patterns for several *let-7* family members (*let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f-2*, *let-7g*, and *let-7i*) were investigated in *LIN28B*-OE erythroblasts. The *let-7* family members demonstrated robust and consistent suppression with a greater than 70% reduction in *LIN28B*-OE compared with the control (Figure 5A). The chromatin modifier *HMGA2* is a validated target of *let-7*, which also modulates growth and differentiation.[36-38] Retroviral integration of globin encoding vectors into the *HMGA2* locus resulted in hematopoietic clonal dominance at the integration site, as well as increased HbF expression.[39] *Lin28* protein also increases the efficiency of insulin-like growth factor 2 (IGF2) protein translation in muscle cells.[20] *IGF2* is expressed at high levels in fetal hepatocytes and supports expansion of hematopoietic stem cells in the fetal liver.[40] Despite the strong suppression of the *let-7* family, *HMGA2* was not significantly up-regulated in *LIN28B*-OE samples (Figure 5B). *IGF2* expression was slightly increased in the *LIN28B*-OE samples (Figure 5C).

In addition to the *let-7* family of miRNAs, *miR-96* and *miR-29c* are preferentially expressed in adult human peripheral blood reticulocytes when compared with cord blood samples.[24] Recently, *miR-96* expression in reticulocytes was demonstrated to increase during human ontogeny,[24] and *miR-96* knockdown resulted in increased γ-globin expression in human erythroblasts.[41] However, the *miR-96* effects on HbF expression in mature erythroid cells were not defined. Three erythroid miRNAs that are not developmentally regulated (*miR-451*, *miR-144*, and *miR-142*) were tested as controls. Among the group, a significant reduction was observed only in *miR-96* (Figure 5D), suggesting possible targeting of this miRNA by LIN28B.

LIN28B regulates BCL11A expression

Additional genetic mechanisms underlying γ-globin expression and HbF production were sought, including the potential for *LIN28B*-OE to regulate erythroid transcription factors. *BCL11A* is an important transcriptional silencer of HbF in model systems, as well as in humans.[11,26,42] *KLF1* knockdown cells were reported to downregulate *BCL11A* and activate γ-globin.[43] In addition, *SOX6* expression has been detected during erythroid development and was implicated in playing a cooperative role with *BCL11A* in the regulation of γ-globin.[26] The expression patterns of the transcription factors *BCL11A*, *KLF1*, and *SOX6* were investigated, along with the erythroid transcription regulator, *GATA1* (Figure 5E-H). Among the group, only *BCL11A* was affected by *LIN28B*-OE, and *LIN28B*-OE caused a 65% reduction in *BCL11A* expression (control: 3.07E+03 ± 1.5 E+02 copies/ng; *LIN28B*-OE: 1.07E+03 ± 18 copies/ng; $P = .02$) (Figure 5E).

Next, we investigated whether LIN28B and BCL11A regulate each other. In accordance with our mRNA expression data, *LIN28B*-OE downregulated BCL11A at the protein level, as shown in Figure 6A-B. To investigate whether BCL11A modulates LIN28B expression, primary CD34+ cells were transduced with the shRNA lentivirus knockdown vector of *BCL11A* and lentiviral controls. *BCL11A* knockdown (Figure 6C) was evaluated and the results showed no effect on LIN28B expression (Figure 6D).

Let-7 miRNAs regulate BCL11A expression and HbF

To investigate if the main targets of LIN28B, the *let-7* family of miRNAs, are responsible for the regulation of HbF, adult CD34+ erythroid cells were transduced with a *let-7* sponge retrovirus.

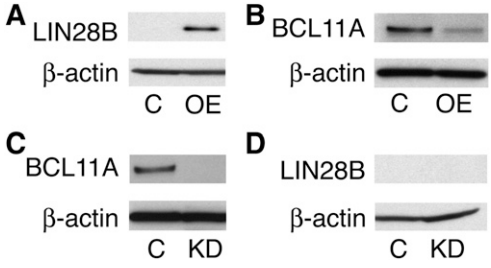

Figure 6. LIN28B is an upstream regulator of BCL11A. Western blot analyses with protein extracts from empty vector control (C) versus *LIN28B*-OE (OE) cells (A-B), or *BCL11A* knockdown (KD) cells (C-D). The blots were probed with anti-LIN28B, and anti-BCL11A as labeled at the left of each band pair. An anti-β-actin probe was used as a loading control.

LIN28A and *LIN28B* expression remained less than the qRT-PCR detection threshold after transduction (data not shown). Transduction with the sponge retrovirus resulted in significant suppression of *let-7* (Figure 7A). However, the level of *let-7* suppression was more robust with *LIN28B-OE* than the *let-7* sponge. Comparison of *LIN28B*-OE and *let-7* sponge experiments also demonstrated greater effects of *LIN28B*-OE on BCL11A (Figure 7B) and HbF than achieved after *let-7* suppression (Figure 7C-E). HPLCs demonstrated significantly increased HbF levels in adult erythroid cells with either *LIN28B*-OE or *let-7* sponge compared with controls (control: 3.5 ± 0.3%; *LIN28B*-OE: 31.1 ± 2.90%; $P = .0029$; *let-7* sponge: 19.1 ± 0.2%; $P = .0003$). These results demonstrate that suppression of *let-7* miRNAs is sufficient to reduce BCL11A and increase HbF in adult human erythroblasts, even in the absence of *LIN28A* or *LIN28B* expression.

Discussion

Increasing levels of HbF in adult erythroblasts represent an important therapeutic target for patients with SCD and β-thalassemias. In this study, we demonstrated that *LIN28B* regulates HbF levels during human erythroid cell development. *LIN28B* knockdown in CD34+ cord blood cells significantly reduced HbF expression, and overexpression of *LIN28B* in adult erythroblasts significantly increased HbF levels. Interestingly, HbF levels were also increased in adult cells transduced with a separate vector encoding LIN28A. *LIN28A* and *LIN28B* are thus functionally similar in this regard. *LIN28B* is a more likely candidate as a physiological regulator of HbF, because *LIN28B* is expressed in the fetal liver[27] and the gene possesses fetal-specific chromatin marks[26] in erythroid cells.

Because each cellular transcriptome helps determine its repertoire of RNA species that may be targeted by LIN28 proteins, the LIN28 effects on cellular fate and phenotype are predicted to be variable. GWAS identified *LIN28* genes as regulators of temporally defined events in humans.[15,16] LIN28 proteins are expressed during normal human development, as well as disease states including cancer. In embryonic stem cells, differentiation is associated with decreased LIN28 protein expression.[44] LIN28 acts with other factors to induce the pluripotency of somatic cells.[19] During mammalian development, expression of LIN28 proteins is reduced after embryogenesis. With the exception of cardiac and skeletal muscles, LIN28 proteins are nearly absent in adult tissues. In contrast, increased expression of LIN28 causes differentiation of myoblasts.[20] Unlike stem cells and myoblasts, erythroblast differentiation was largely unaffected by the overexpression of a LIN28 protein. However, significant changes were noted for a subset of genes that are coordinated with developmental timing in humans. The increased expression of the fetal globin in adult CD34+ cells coincided with reduced expression of the adult globin (HBB). In addition to HBB, decreased expression of *CA1* and *GCNT2* genes were detected. These genes mark the developmental transition from fetal-to-adult erythropoiesis.[32-34] The reductions of *let-7*, *HBB*, *CA1*, and *GCNT2* genes on *LIN28B*-OE suggest a more fetal-like erythroid phenotype beyond the expression of HbF. These data support the notion that LIN28B and its targeted RNAs may be involved and should be explored further in the context of developmental changes in human erythroid biology.[45,46]

Examination of *LIN28B* mRNA in cord blood erythroblasts suggests that low level expression is sufficient to regulate *let-7* and

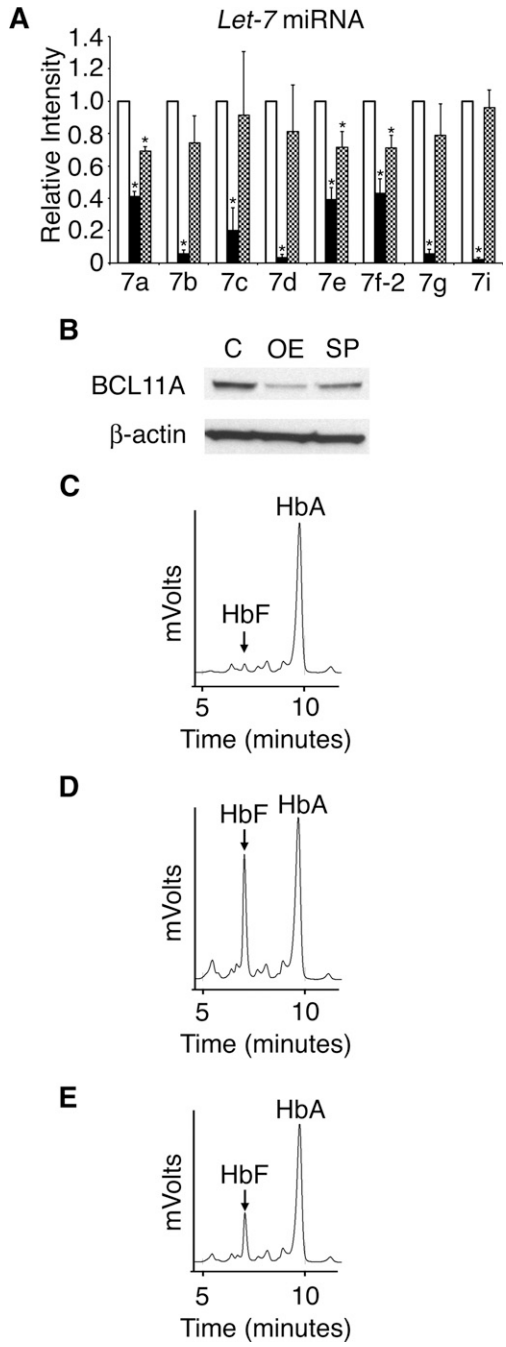

Figure 7. Retroviral suppression of the *let-7* family of miRNAs regulates BCL11A and HbF. (A) Relative expression levels of the *let-7* family of miRNAs (open bars represent control, black bars represent *LIN28B*-OE, and hatch-marked bars represent *let-7* sponge) after transduction of the *let-7* sponge or *LIN28B*-OE encoding retrovirus. The qRT-PCR analyses were performed at culture day 14, and compared with control transductions. The miRNAs relative expression levels (y-axis) in the control cells were defined as a level of one for comparison. Error bars denote ± standard deviation of 3 independent donors for each condition. (B) Western blot analyses with protein extracts from the empty vector control (C) versus *LIN28B*-OE (OE) and *let-7* sponge (SP) cells. The membranes were probed with anti-BCL11A. Anti-β-actin probe was used as a loading control. HPLC analyses of hemoglobin from (C) control, (D) *LIN28B*-OE, and (E) *let-7* sponge samples were performed at culture day 21. HbF and HbA peaks are labeled on each graph (y-axis: mVolts; x-axis: elution time in minutes). Data are representative of 3 independent experiments. *P* values were calculated using two-tailed Student *t* test. C, empty vector control; OE, *LIN28B* overexpression; SP, *let-7* sponge. $^*P < .05$.

HbF in those cells. Significant reduction in HbF expression in cord blood erythroblasts was mediated by reductions in the erythroblast *LIN28B* mRNA levels (105 copies/ng reduced to 21 copies/ng). The effects of reducing *LIN28B* expression are thus amplified to regulate γ-globin RNA levels that are normally detected at 5-log higher levels (10^7 copies/ng) (Figure 4B). In the adult erythroblasts, *LIN28A* or *LIN28B* expression was less than the detection limit in the control cultures. *LIN28B* overexpression in the adult cells reached levels that were approximately 100-fold higher than the cord blood erythroblasts (10^4 copies/ng) (Figure 2A). The viral vector designs did not permit studies of adult-derived erythroblasts that express *LIN28B* in the comparable range of cord blood cells. However, higher level expression of the transduced *LIN28B* in the adult cells caused increases in HbF that approached, but did not exceed, that of cord blood-derived erythroblasts. The lack of increases in HbF to levels greater than detected in cord blood, despite higher *LIN28B* expression, suggests saturation of the globin gene regulating effects in adult cells. These results may be relevant in the context of gene therapy applications,[47] because the requirements for high level γ-globin gene expression may be achieved via lower level expression of a transduced γ-globin gene regulator.

Our studies of *LIN28B* overexpression, as well as *let-7* suppression in the absence of *LIN28A* or *LIN28B,* caused decreased expression of BCL11A as a predominant mechanism for increasing γ-globin mRNA in the adult erythroblasts. Increased BCL11A was also detected after *LIN28B*-KD in the cord blood erythroblasts (data not shown). An initial GWAS suggested that the *BCL11A* locus accounts for approximately 15% of the HbF trait variance in European populations.[7] The role of BCL11A in globin gene regulation was confirmed experimentally using human erythroblasts[11] and genetically-modified mice.[48] Because neither *BCL11A* knockdown nor direct *let-7* suppression had effects on *LIN28A* or *LIN28B* expression, our data suggest that *LIN28B* affects the expression of HbF primarily by targeting *let-7* to act as an upstream regulator of *BCL11A*. We did not observe changes in *KLF1*, *GATA1*, or *SOX6* gene expression in response to *LIN28B*. In addition to binding and inhibiting *let-7*, LIN28 proteins may affect non-*let-7* RNA species[25,49,50]; however, our *let-7* sponge studies suggest that the *let-7* miRNAs are critical targets of LIN28 proteins. Nevertheless, systems biology approaches may be useful to more fully understand how LIN28 proteins and *let-7* regulate the subset of genes described here, as well as the overall erythroid phenotype.

Ultimately, these studies support the original hypothesis that regulated expression of the *let-7* miRNA family is involved in hemoglobin switching during the fetal-to-adult transition of human ontogeny.[24] Because *let-7* and other miRNAs function by reducing the expression of the mRNA to which they bind, it is proposed that suppression of *let-7* causes decreased expression of BCL11A by targeting other RNAs that are expressed in human erythroblasts. Although predicted erythroid mRNA targets of *let-7* were previously reported,[24] the potential role for those genes in regulating BCL11A expression remains vague.

In summary, we demonstrated that *LIN28B* regulates HbF expression in erythroblasts cultured from cord blood and adult CD34+ cells without detected interference with terminal maturation or enucleation. In the adult cells, *LIN28B* acts as an upstream regulator of several genes with an associated fetal erythroid phenotype, including *CA1*, *GCNT2,* and *miR-96*. Overexpression of *LIN28B*, as well as suppression of its major RNA target (the *let-7* family of miRNAs) suppresses *BCL11A,* a well-characterized transcription factor that represses γ-globin gene expression. Our findings have important clinical implications introducing LIN28 proteins and their target RNAs as potential tools to generate therapeutic erythrocytes or aid in the identification of novel candidate molecules for treating SCD and β-thalassemias.

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Authorship

Contribution: Y.T.L., J.F. de V., J.Y., C.B., S.-J.N., E.R.M., K.S.K., A.R., and M.K. performed experiments; Y.T.L., J.F. de V., J.Y., C.B., S.-J.N., E.R.M., K.S.K., A.R., and M.K. analyzed data; Y.T.L., J.F. de V., J.L.M., and S.A.M. wrote the paper; and J.L.M. and S.A.M. conceived, assisted, and directed research.

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