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 fetal hemoglobin 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.

Abstract

Reactivation of fetal hemoglobin (HbF) holds therapeutic potential for sickle cell disease and beta-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 utilized 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 over-expression 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 gamma-globin, and the HbF content of the cells rose to levels above 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 gamma-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.
Introduction

In humans and some other mammals, the composition of hemoglobin tetramers in erythrocytes switch from fetal hemoglobin (HbF, α2γ2) to adult hemoglobin (HbA, α2β2) during the last stages of fetal development until early infancy. 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. In patients with SCD, the polymerization of sickle hemoglobin results in erythrocyte deformation and hemolysis. SCD patient’s clinical outcomes are largely improved by inhibition of the polymerization by HbF. In β-thalassemias, decreased production of beta-globin causes imbalanced globin polypeptide chain synthesis, and leads to severe effects on the erythroid cells’ maturation and survival. The loss of beta-globin expression may be compensated by an increase in HbF production that leads to improvement of the clinical phenotype.

The molecular mechanisms underlying the switch from fetal to adult hemoglobin 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. Suppression of the BCL11A transcription factor causes an increase in HbF levels. Lin28 is a highly conserved gene that is expressed in the early stages of development of multicellular organisms. In the nematode, C. elegans, lin28 regulates developmental timing at the larvae L2 stage. Mammals express two homologs of the C. elegans lin28 gene known as LIN28A and LIN28B. Human LIN28 genetic variation correlates with developmental timing characteristics such as variation in height, timing of puberty and age at natural menopause. The effects of LIN28 proteins depend, in part, on the cell type in which it is expressed, as evidenced by its role in promoting the pluripotency of stem cells, as well as the differentiation of skeletal muscle cells.

In part, LIN28 acts through the 3’ end uridylation of the precursor molecule pre-let-7, ultimately leading to the degradation of miRNA molecules. Interestingly, expression of the let-7 family of miRNAs was correlated with the fetal-to-adult developmental transition in circulating human reticulocytes. Those data suggest a potential link between LIN28B
expression, the regulation of the let-7 microRNAs, and vertebrate hematopoiesis. In addition to binding to the terminal loop of let-7 microRNAs, LIN28 binds to GGAGA motifs in mRNAs\textsuperscript{25}. Moreover, a predicted fetal stage-specific enhancer for LIN28B was identified using human primary erythroid cells\textsuperscript{26}, and in murine lymphocytes, Lin28b expression regulates the fetal-like phenotype\textsuperscript{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 \textit{ex vivo} engineering of human erythrocytes.

**Methods**

**Cell culture.** All related studies were performed after human subjects review and NIH IRB 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 \textit{ex vivo} culture, a 21 day serum-free system consisting of three phases was utilized. During phase I of culture (day 0 to day 7): adult or cord blood CD34+ cells were placed in media containing StemPro-34 complete media (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 under 2x10\textsuperscript{6} cells per ml. Phase II medium is comprised of the following: StemPro-34 complete medium, 4 U/ml 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 media at 8x10\textsuperscript{5} cells per ml for the remaining 7 days of culture. Phase III medium is 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 immunoprecipitation (IP). RNA IP was performed following 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 (Cell Signaling, Danvers, MA, catalog #4196) or IgG (Millipore, catalog #PP64). The immunoprecipitated RNA was purified before RT-PCR 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 described27. Human LIN28A over-expression 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 described11. A micro-RNA sponge experimental approach was utilized for suppression of let-728. The let-7 sponge plasmid (MSCV puro let-7 sponge) was purchased from Addgene (Cambridge, MA, plasmid, catalogue #29766), and retroviral supernatants were produced by transient Lipofectamine 2000 (Invitrogen) cotransfection of 293T cells with the RetroMax packaging vector pCL-Eco (Imagenex, San Diego, CA). Seventy-two hours post-transfection, cell supernatant was harvested and concentrated with Retro-X concentrator solution (Clontech, Mountain View, CA) following 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 MOI 12). After 24 hours, puromycin (Sigma) 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 described above 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 described29 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.

HPLC for adult and fetal hemoglobins. Samples for HPLC were prepared and analyzed as previously described.29

Quantitative PCR analysis. Q-RT-PCR assays and PCR conditions were performed as described previously.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 (20-30 µg) were separated by NuPAGE Novex 4–12% Bis-Tris gel electrophoresis in MOPS buffer and transferred using the iBlot Blotting System onto nitrocellulose membranes (Invitrogen). Blots were probed with antibodies against human CA1 (Abcam, Cambridge, MA), 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 ± SD with significance calculated by two-tailed Student’s t test.
Results

LIN28B binds primary let-7 transcripts and regulates fetal hemoglobin in human cord blood CD34+ cells. We previously reported that decreased fetal hemoglobin expression in adult human reticulocytes compared to those present in cord blood is associated with increased let-7 levels in adult reticulocytes\textsuperscript{24}. Since let-7 microRNA expression is down-regulated by LIN28 protein binding, the absence of LIN28B expression in adult human bone marrow compared to its detection in fetal liver\textsuperscript{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 immunoprecipitation in CD34+ cord blood lysates was performed and quantitated by RT-PCR for primary let-7d. Let-7d was chosen based upon its expression level in cord blood cells\textsuperscript{24}. Primary let-7d was enriched by LIN28B immunoprecipitation compared to the IgG control (Figure 1A).

To investigate a potential role for cord blood LIN28B expression in fetal hemoglobin regulation, CD34+ cord blood cells were transduced with lentivirus encoding a short-hairpin RNA (shRNA) against LIN28B and cultured \textit{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 Q-RT-PCR (control: 105 ± 24 copies/ng, LIN28B-KD: 20.7 ± 0.9 copies/ng, p=0.02), as demonstrated in Figure 1B. Let-7 microRNAs were measured by Q-RT-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 to the controls (p=0.05 and p=0.01, respectively). Hemoglobin expression profiles for control and LIN28B-KD were determined by standard HPLC (Figures 1D, 1E). Triplicate experiments demonstrated a significant reduction in HbF expression in LIN28B-KD cells compared to the controls (HbF: LIN28B-KD: 31.0 ± 5.3%; control: 54.2 ± 7.7% p=0.03).

LIN28B expression activates fetal hemoglobin during adult human erythropoiesis. In addition to studies of cord blood derived erythroblasts, LIN28B expression in adult erythroblasts
was examined. When compared to 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 over-expression (LIN28B-OE) was confirmed by Q-RT-PCR (LIN28B-OE: 1.8E+04 ± 3.5E+02 copies/ng, p=0.01) and Western 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).

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 (GPA), 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 to 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 to 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). Hemoglobin profiles (HPLC) 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=0.01). For comparison, a different lentiviral vector encoding the other human LIN28 gene (LIN28A) was utilized. A robust increase in fetal hemoglobin expression was also detected in LIN28A transduced cells (Figure 3K-L). Taken together, these data demonstrate that over-expression of LIN28A or LIN28B is sufficient for the manifestation of high-level fetal hemoglobin 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 (αγ/γγ-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 upon the high-level of fetal hemoglobin expression
in LIN28B-OE cells, globin gene expression patterns were quantitated by Q-RT-PCR. The expression levels of alpha, mu, theta, zeta, beta, delta, gamma and epsilon globin genes were evaluated for LIN28B-OE and compared to controls. As shown in Figure 4A-B, only the fetal gamma-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=0.04). The increased expression of gamma-globin was balanced by reductions in beta-globin and delta-globin. No changes were observed in alpha, mu and theta globins, and the low-level expression patterns of zeta and epsilon 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, as well as the carbohydrate modification due to the augmented expression of glucosaminyl (N-acetyl) transferase 2 (GCNT2). Expression of CA1 and GCNT2 were therefore investigated by Q-RT-PCR with LIN28B-OE. As shown in Figure 4C-D, both CA1 and GCNT2 were significantly reduced upon LIN28B-OE (CA1 - control: 5E+03 ± 4E+03 copies/ng, LIN28B-OE: 2E+03 ± 5E+02 copies/ng; p=0.01; GCNT2 - control: 1E+03 ± 3E+02 copies/ng, LIN28B-OE: 1E+02 ± 4E+01 copies/ng; p=0.01). Western analysis using anti-human CA1 antibody in LIN28B-OE compared to control samples at culture day 21 confirmed 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. 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 to the control (Figure 5A). The chromatin modifier HMGA2 is a validated target of let-7 that also modulates growth and differentiation. Retroviral integration of globin encoding vectors into the HMGA2 locus resulted in hematopoietic clonal dominance at the integration site as well as increased fetal hemoglobin expression. Lin28 protein also increases the efficiency of insulin like growth factor 2 (IGF2) protein translation in muscle cells. IGF2 is expressed at high levels in fetal hepatocytes and supports expansion of hematopoietic stem cells in fetal liver. 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. Recently, miR-96 expression in reticulocytes was demonstrated to increase during human ontogeny, and miR-96 knock down resulted in increased gamma-globin expression in human erythroblasts. However, the miR-96 effects upon fetal hemoglobin 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 gamma-globin expression and fetal hemoglobin (HbF) production were sought including the potential for LIN28B-OE to regulate erythroid transcription factors. BCL11A is an important transcripational silencer of fetal hemoglobin in model systems as well as in humans. KLF1 knockdown cells were reported to down-regulate BCL11A and activate gamma-globin. In addition, SOX6 expression has been detected during erythroid development and implicated in playing a cooperative role with BCL11A in the regulation of gamma-globin. 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. 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=0.02, Figure 5E).

Next, we investigated whether LIN28B and BCL11A regulate each other. In accordance with our mRNA expression data, LIN28B-OE down-regulated 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 fetal hemoglobin.** To investigate if the main targets of LIN28B, the let-7 family of miRNAs, are responsible for the regulation of fetal hemoglobin, adult CD34+ erythroid cells were transduced with a let-7 sponge retrovirus. LIN28A and LIN28B expression remained below the QPCR 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 upon BCL11A (Figure 7B) and fetal hemoglobin than achieved after let-7 suppression (Figures 7D-E). The hemoglobin profiles (HPLC) demonstrated significantly increased HbF levels in adult erythroid cells with either LIN28B-OE or let-7 sponge compared to controls (control: 3.5 ± 0.3%; LIN28B-OE: 31.1 ± 2.90% p=0.0029; let-7 sponge: 19.1 ± 0.2% p=0.0003, Figure 7C-E). These results demonstrate that suppression of let-7 miRNAs is sufficient to reduce BCL11A and increase fetal hemoglobin in adult human erythroblasts, even in the absence of LIN28A or LIN28B expression.

**Discussion**

Increasing levels of fetal hemoglobin (HbF) in adult erythroblasts represents an important therapeutic target for patients with sickle cell disease (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, since LIN28B is expressed in fetal liver^27^ and the gene possesses fetal-specific chromatin marks^26^ in erythroid cells.

Since each cell’s transcriptome helps determine its repertoire of RNA species that may be targeted by LIN28 proteins, the LIN28 effects upon cellular fate and phenotype are predicted to be variable. Genome-wide association studies (GWAS) identified LIN28 genes as regulators of
temporally defined events in humans\textsuperscript{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\textsuperscript{44}. LIN28 acts with other factors to induce the pluripotency of somatic cells\textsuperscript{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\textsuperscript{20}. Unlike stem cells and myoblasts, erythroblast differentiation was largely unaffected by the over-expression 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 the carbonic anhydrase I (\textit{CA1}) and glucosaminyl (N-acetyl) transferase 2 enzyme (\textit{GCNT2}) genes were detected. These genes mark the developmental transition from fetal-to-adult erythropoiesis\textsuperscript{32-34}. The reductions of \textit{let-7}, \textit{HBB}, \textit{CA1} and \textit{GCNT2} genes upon \textit{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\textsuperscript{45,46}.

Examination of \textit{LIN28B} mRNA in cord blood erythroblasts suggests that low-level expression is sufficient to regulate \textit{let-7} and fetal hemoglobin in those cells. Significant reduction in fetal hemoglobin expression in cord blood erythroblasts was mediated by reductions in the erythroblast \textit{LIN28B} mRNA levels (105 copies/ng reduced to 21 copies/ng). The effects of reducing \textit{LIN28B} expression are thus amplified to regulate gamma-globin RNA levels that are normally detected at 5-log higher levels (10\textsuperscript{7} copies/ng, see Figure 4B). In the adult erythroblasts, \textit{LIN28A} or \textit{LIN28B} expression was below the detection limit in the control cultures. \textit{LIN28B} overexpression in the adult cells reached levels that were approximately 100-fold higher than the cord blood erythroblasts (10\textsuperscript{4} copies/ng, see Figure 2A). The viral vector designs did not permit studies of adult derived erythroblasts that express \textit{LIN28B} in the comparable range of cord blood cells. However, higher-level expression of the transduced \textit{LIN28B} in the adult cells caused increases in fetal hemoglobin that approached, but did not
exceed, that of cord blood derived erythroblasts. The lack of increases in fetal hemoglobin above the cord blood levels, 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\textsuperscript{47}, since the requirements for high-level gamma-globin gene expression may be achieved via lower-level expression of a transduced gamma-globin gene regulator.

Our studies of LIN28B over-expression, as well as let-7 suppression in the absence of LIN28A or LIN28B, caused decreased expression of BCL11A as a predominant mechanism for increasing gamma-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\textsuperscript{7}. The role of BCL11A in globin gene regulation was confirmed experimentally using human erythroblasts\textsuperscript{11} and genetically-modified mice\textsuperscript{48}. Since neither BCL11A knockdown nor direct let-7 suppression had effects upon LIN28A or LIN28B expression, our data suggest that LIN28B affects the expression of fetal hemoglobin 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\textsuperscript{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\textsuperscript{24}. Since let-7 and other microRNAs 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 mRNA(s) that are expressed in human erythroblasts. While predicted erythroid mRNA targets of let-7 were reported previously\textsuperscript{24}, the potential role for those genes in regulating BCL11A expression remains vague.
In summary, we demonstrated that LIN28B regulates fetal hemoglobin 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. Over-expression 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 gamma-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 sickle cell disease and beta thalassemias.

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Author Contributions
YTL and JFV performed the experiments, analyzed the data and wrote the paper. JY, CB, SJN, ERM, KSK, AR and MK conducted research and analyzed the data. JLM and SAM conceived, assisted and directed the research and wrote the paper.

Conflict of Interest Statement
No conflict of interests to declare.
References


Figure Legends

Figure 1. *LIN28B* regulates fetal hemoglobin 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 immunoprecipitation 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 Q-RT-PCR quantitation of copy number per nanogram 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 Q-RT-PCR. Open bars represent control samples and black bars represent *LIN28B*-KD. Standard deviation bars are shown, and asterisks denote p<0.05 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). Pri-7d: primary *let-7d* miRNA; C: empty vector control; KD: *LIN28B* knockdown.

Figure 2. *LIN28B* over-expression in adult human erythroblasts was confirmed by (A) Q-RT-PCR quantitation of copy number per nanogram cDNA (Copies/ng cDNA), (B) Western analysis and (C-F) confocal images of control and *LIN28B*-OE cells stained with DAPI (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 ± SD of three independent donors for each condition. C: empty vector control; OE: *LIN28B* over-expression.

Figure 3. *LIN28B* over-expression enhances fetal hemoglobin 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 three
independent experiments. \textit{LIN28B-OE: LIN28B over-expression; LIN28A-OE: LIN28A over-expression.}

\textbf{Figure 4.} \textit{LIN28B} expression regulates gamma-globin and erythroid-related fetal genes in adult erythroblasts. (A) Alpha, mu, theta and zeta globins, (B) beta, delta, gamma and epsilon globins, (C) \textit{CA1} and (D) \textit{GCNT2} mRNA expression analysis of \textit{LIN28B-OE} compared to control samples. Open bars represent control and black bars represent \textit{LIN28B-OE}. Q-RT-PCR analyses were performed at culture day 14. Mean value ± SD of three independent donors for each condition. \textit{P} values were calculated using two-tailed Student’s \textit{t}-test. C: empty vector control; OE: \textit{LIN28B} over-expression. *\textit{p}<0.05.

\textbf{Figure 5.} \textit{LIN28B} modulation of adult CD34+ cells towards a fetal-like phenotype involves the \textit{let-7} family of miRNAs, \textit{miR-96} and \textit{BCL11A}. \textit{LIN28B-OE} compared to control samples in (A) the relative expression levels of the \textit{let-7} family of miRNAs (open bars represent control and black bars represent \textit{LIN28B-OE}), the mRNA expression levels of (B) \textit{HMGA2} and (C) \textit{IGF2}, (D) the relative expression levels of \textit{miR-96, miR-29c, miR-451, miR-144} and \textit{miR-142} and the mRNA expression of the transcription factors (E) \textit{BCL11A}, (F) \textit{GATA1}, (G) \textit{KLF1} and (H) \textit{SOX6}. Q-RT-PCR analyses were performed at culture day 14. miRNAs relative expression levels (y-axis) in the control cells were defined as a level of one for comparison. Error bars denote ± SD of three independent donors for each condition. \textit{P} values were calculated using two-tailed Student’s \textit{t}-test. C: empty vector control; OE: \textit{LIN28B} over-expression. *\textit{p}<0.05.

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

\textbf{Figure 7.} Retroviral suppression of the \textit{let-7} family of miRNAs regulates \textit{BCL11A} and fetal hemoglobin. (A) Relative expression levels of the \textit{let-7} family of miRNAs (open bars represent control, black bars represent \textit{LIN28B-OE}, and hatch-marked bars represent \textit{let-7} sponge) after transduction of the \textit{let-7} sponge or \textit{LIN28B-OE} encoding retrovirus. Q-RT-PCR analyses were performed at culture day 14, and compared with control transductions. miRNAs relative
expression levels (y-axis) in the control cells were defined as a level of one for comparison. Error bars denote ± SD of three independent donors for each condition. (B) Western analyses for protein extracts from 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 analysis of hemoglobin from (C) control, (D) LIN28B-OE and (E) let-7 sponge samples 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 three independent experiments. P values were calculated using two-tailed Student’s t-test. C: empty vector control; OE: LIN28B over-expression; SP: let-7 sponge. *p<0.05.
Lee et al., Figure 2

A

B

Donor 1 2 3

LIN28B

β-actin

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C

D

DAPI

DAPI + LIN28B

E

F
Lee et al., Figure 4

A. α-like

B. β-like

C. CA1

D. GCNT2

Copies/ng cDNA (X10^3)

Copies/ng cDNA (X10^3)

Copies/ng cDNA (X10^3)

Copies/ng cDNA (X10^3)

C  OE  C  OE
Lee et al., Figure 6

A  LIN28B  B  BCL11A
β-actin  β-actin
C    OE  C    OE

C  BCL11A  D  LIN28B
β-actin  β-actin
C    KD  C    KD
Lee et al., Figure 7

A

Let-7 miRNA

Relative Intensity

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

7a 7b 7c 7d 7e 7f-2 7g 7i

B

C O E SP

BCL11A

b-actin

C

HbA

mVolts

Time (minutes)

D

HbF

mVolts

Time (minutes)

E

HbA

mVolts

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

Y. Terry Lee, Jaira F. de Vasconcellos, Joan Yuan, Colleen Byrnes, Seung-Jae Noh, Emily R. Meier, Ki Soon Kim, Antoinette Rabel, Megha Kaushal, Stefan A. Muljo and Jeffery L. Miller