Tough decoy targeting of predominant let-7 miRNA species in adult human hematopoietic cells

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Abstract

Background: In humans, the heterochronic cascade composed of the RNA-binding protein LIN28 and its major target, the let-7 family of microRNAs (miRNAs), is highly regulated during human erythroid ontogeny. Additionally, down-regulation of the let-7 miRNAs in cultured adult CD34(+) cells or the over-expression of LIN28 in cultured erythrocytes from pediatric patients with HbSS genotype causes increased levels of fetal hemoglobin (HbF) in the range of 19–40% of the total. Therefore, we hypothesized that focused targeting of individual let-7 miRNA family members would exhibit regulatory effect on HbF expression in human adult erythroblasts.

Methods: The expression levels of mature let-7 family members were measured by RT-qPCR in purified cell populations sorted from peripheral blood. To study the effects of let-7 miRNAs upon globin expression, a lentiviral construct that incorporated the tough decoy (TuD) design to target let-7a or let-7b was compared with empty vector controls. Transductions were performed in CD34(+) cells from adult healthy volunteers cultivated ex vivo in erythropoietin-supplemented serum-free media for 21 days. Downstream analyses included RT-qPCR, Western blot and HPLC for the characterization of adult and fetal hemoglobins.

Results: The expression of individual let-7 miRNA family members in adult peripheral blood cell populations demonstrated that let-7a and let-7b miRNAs are expressed at much higher levels than the other let-7 family members in purified adult human blood cell subsets with expression being predominantly in reticulocytes. Therefore, we focused this study upon the targeted inhibition of let-7a and let-7b with the TuD design to explore its effects upon developmentally-timed erythroid genes. Let-7a-TuD transductions significantly increased gamma-globin mRNA expression and HbF to an average of 38%. Let-7a-TuD also significantly decreased the mRNA expression of some ontogeny-regulated erythroid genes, namely CA1 and GCNT2. In addition, the erythroid-related transcription factors BCL11A and HMGA2 were down- and up-regulated, respectively, by let-7a-TuD, while ZBTB7A, KLF1 and SOX6 remained unchanged.

Conclusions: Overall, our data demonstrate that let-7 miRNAs are differentially expressed in human hematopoietic cells, and that targeted inhibition of the highly-expressed species of this family is sufficient for developmentally-specific changes in gamma-globin expression and HbF levels.

Keywords: let-7, miRNAs, let-7a, let-7b, Gamma-globin, Fetal hemoglobin, HbF
Background
MicroRNAs (miRNAs) are small (mostly 18–21 nucleotides long), non-coding RNAs, highly conserved across evolution and involved in the regulation of messenger RNAs (mRNAs). Intracellular miRNAs cause post-transcriptional repression of multiple mRNAs to which they bind [1]. The let-7 family of miRNAs in humans consists of twelve genes that encode nine mature miRNAs (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98). The expression of mature let-7 miRNAs can be regulated at the transcriptional and post-transcriptional levels, where post-transcriptional repression of let-7 (at both pri-let-7 and pre-let-7 stages) is mainly mediated by the RNA-binding protein LIN28 [2]. Two human homologs of the C. elegans lin28 gene were identified and named LIN28A and LIN28B [3]. Interestingly, LIN28 proteins and their let-7 miRNA targets have several reported functions including regulation of developmental timing [4–7], stem cell pluripotency, and differentiation of skeletal muscle [8, 9].

In humans, reticulocyte levels of let-7 miRNAs increase with the fetal-to-adult developmental transition [10]. LIN28B expression is silenced during the same developmental switch. Transgenic increases of LIN28 proteins in adult erythroblasts, which as a consequence down-regulate the let-7 miRNAs, cause the cells to manifest fetal-like features [11, 12]. Augmented expression of LIN28A/B also precipitated a rise in fetal hemoglobin (HbF) levels and amelioration of the sickling morphologies of nucleated erythrocytes cultured in vitro from pediatric patients with sickle cell disease (HbSS genotype) [11, 12]. Earlier efforts aimed toward the reduced expression of let-7 by "sponge" targeting of the miRNA family seed region [11] resulted in mild HbF increases compared with LIN28 over-expression in the same cells [11]. Therefore, it remained inconclusive whether suppression of the let-7 family, or targeting of individual let-7 species are sufficient to cause the robust developmentally-specific changes in cellular phenotype that were manifested by LIN28 [11].

Here we investigate the expression levels of the individual let-7 miRNAs in human blood cells, and further explore the role of let-7 miRNAs upon ontogeny-related gene expression in the erythroid lineage. Expression of individual let-7 miRNA family members was quantitated in human peripheral blood cell populations, allowing a more focused strategy for reducing let-7 levels in the adult erythroblasts. Finally, lentiviruses designed specifically for let-7a and let-7b miRNA targeting were transduced in erythroblasts and explored for their regulation of HbF and other developmentally-regulated genes.

Methods
Ethics statement
Written informed consent was obtained from all research subjects prior to participation in this study. Approval for the research protocol and consent documents using primary erythroblasts and peripheral blood samples was granted by the Intramural National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board.

miRBase
Mature let-7 miRNAs sequences were obtained from the miRBase database release 21 (http://mirbase.org). Details of the miRBase database have been previously described [13–17].

Peripheral blood samples
Peripheral blood cells were isolated using Ficoll-Paque Premium (GE Healthcare, Pittsburgh, PA) following manufacturer’s instructions. Fresh post-ficoll peripheral blood cells were used for cell sorting based on forward and side scatter using the BD FACSAria I flow cytometer (BD Biosciences, San Jose, CA). Lymphocytes and monocytes were sorted from the post-ficoll interface. Neutrophils were obtained from the post-ficoll packed red cells, after lysis with ACK lysing buffer following manufacturer’s protocol (Life Technologies, Grand Island, NY) and sorted based on forward and side scatter. Reticulocytes were obtained after filtration through a Purecell Neonatal High Efficiency Leukocyte Reduction Filter (PALL, Germantown, MD) and RNA from reticulocytes was extracted using Trizol LS following manufacturer’s instructions (Thermo Fisher Scientific, Grand Island, NY).

Cell culture
Cryopreserved healthy adult human CD34(+) cells were cultured ex vivo in a 3-week serum-free system consisting of three phases (phase I: days 0–7, phase II: days 7–14 and phase III: days 14–21) as previously described [11, 18].

Recombinant viral transduction
Lentiviral particles with tough decoy (TuD) design [19], constructed to inhibit human let-7a or let-7b miRNAs (catalog numbers: HLTUD0001 and HLTUD0007, respectively) and negative vector control (HLTUD001C) were purchased from Sigma Aldrich (St. Louis, MO). A lentivirus shRNA vector to knockdown
BCL11A (clone TRCN0000033449) and the respective lentiviral control (SHC002V) were also acquired from Sigma Aldrich. On culture day 3 of phase I, CD34(+) cells were transduced with the following lentiviral particles: let-7a-TuD, let-7b-TuD, BCL11A knockdown and each respective negative vector control (MOI of 6). After 24 h, puromycin (Sigma Aldrich) was added to the culture. On culture day 7, cells were transferred to phase II medium containing EPO and cultivated at the conditions previously described without puromycin [18].

Cell counts and cell morphology analyses
Cell counts were performed throughout the culture period in a Z1 Coulter Particle Counter (Beckman Coulter, Indianapolis, IN) following manufacturer’s instructions. Cell morphology was analyzed with the preparation of cytospins followed by Wright–Giemsa staining. Briefly, cytospins were prepared by centrifugation of the cytoslides using the Shandon Cytospin 4 (Thermo Fisher Scientific) at 1000 rpm for 2 min. Cytoslides were stained with Wright–Giemsa (Sigma Aldrich, St. Louis, MO) for 50 s followed by two 1-min washes in distilled water.

Flow cytometry analyses
Erythroid differentiation was assessed with antibodies directed against CD71 and glycophorin A (Invitrogen, Carlsbad, CA) on culture days 14 and 21 using the BD FACSAria I flow cytometer (BD Biosciences) as previously described [20]. Enucleation was quantitated by thiazole orange (TO) staining (Sigma) on culture day 21. Fetal hemoglobin distribution was assessed with antibody directed against fetal hemoglobin (Life Technologies) at culture day 21 as previously described [21].

Quantitative PCR for mRNAs
Total RNA was isolated using miRNeasy mini kit with QIAzol (Qiagen) following manufacturer’s instructions and complementary DNA (cDNA) was synthesized using SuperScript III reverse transcriptase (Thermo Fisher) following manufacturer’s instructions as previously described [22, 23]. RT-qPCR assays and conditions were performed as previously described [11, 22–25]. Assay-on-Demand Gene Expression Product (Thermo Fisher Scientific/Applied Biosystems) were used as follows: CA1 (Hs01100176_m1), GCNT2 (Hs00377334_m1), BCL11A (Hs00256254_m1), HMGA2 (Hs00971724_m1), ZBTB7A (Hs00792219_m1), KLF1 (Hs00610592_m1), SOX6 (Hs00264525_m1), LIN28A (Hs04189307_g1), and LIN28B (Hs01013729_m1). Absolute quantification for each target mRNA was determined by comparison with a standard curve that was run in parallel with biological samples as previously described [23]. Reactions were performed in triplicate.

Quantitative PCR analysis for the let-7 family of miRNAs
Complementary DNA and real-time PCR reaction using Taqman microRNA assay (Applied Biosystems, Grand Island, NY) were performed as previously described [10, 22] for let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98. Absolute quantification for each target miRNA was determined by comparison with a standard curve that was run in parallel with biological samples as previously described [22]. Standard curves were prepared on the basis of the synthetic targeted mature miRNA oligonucleotide of known concentration (at least five 1:10 serial dilutions) as previously described [22]. Reactions were performed in triplicate. A representative standard curve and its correspondent amplification plot for each let-7 miRNA family member is shown in Additional file 1.

Western blot analyses
Nuclear and cytoplasmic extracts from culture day 14 erythroblasts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL) as previously described [11]. Western blot protocols and conditions were performed as previously described [11]. Blots were probed with antibodies against CA1 (Abcam, Cambridge, MA), GCNT2 (Santa Cruz Biotechnology, Dallas, TX), BCL11A (Abcam), HMGA2 (GenTex, Irvine, CA), ZBTB7A (Abcam), KLF1 (Abcam) and SOX6 (Santa Cruz Biotechnology). Histone H3, Lamin B1 or Beta-Actin (all from Abcam) were used as loading controls.

Colonization formation assay
CD34(+) cells from three independent donors were transduced with let-7a tough decoy vector (catalog number: HLTUD0001, Sigma) or negative vector control (catalog number: HLTUD001C, Sigma) overnight and then mixed in MethoCult H4034 Optimum media (Stem Cell Technologies, Vancouver, Canada) supplemented with puromycin for colony formation assay with duplicate wells following manufacturer’s protocol as previously described [18]. Colonies of erythroid progenitors (BFU-E and CFU-E), granulocyte–macrophage progenitors (CFU-GM, CFU-G and CFU-M) and multipotential granulocyte, erythroid, macrophage, megakaryocyte progenitors (CFU-GEMM) were counted for each donor and condition on culture day 14.

HPLC for adult and fetal hemoglobins
Samples for HPLC analysis were prepared and analyzed as previously described [23, 26].

Statistical analysis
Replicates are expressed as mean ± SD values and significance was calculated by two-tailed Student’s t-test.
Results
Reticulocytes contain higher total levels of mature let-7 miRNAs than other peripheral blood cell populations

The mature sequences of let-7 miRNA family members are defined and well-conserved across multiple species. As shown in Additional file 2, let-7a is the most well-conserved family member across evolution. The alignment and nucleotide differences of the human mature let-7 family members in comparison to the human mature let-7a miRNA is shown in Fig. 1 (sequences were obtained on miRBase, http://mirbase.org/, Release 21). Following the in silico comparison of the let-7 miRNAs sequences, we explored the expression levels of mature let-7a and the related miRNA family members in purified mononuclear cell populations and reticulocytes from peripheral blood. Interestingly, reticulocytes have higher levels of total let-7 miRNAs compared to monocytes, lymphocytes, and neutrophils: 2.0E ± 0.6E copies/ng; lymphocytes: 1.1E ± 0.6E copies/ng; neutrophils: 2.0E ± 0.1E copies/ng and reticulocytes: 1.7E ± 0.8E copies/ng. Unexpectedly, among the individual family members, let-7a and let-7b were identified as the predominant members of the let-7 family in peripheral blood cell populations. Hence, we became more interested in the effects of focused reductions of these highly-expressed species, let-7a and let-7b, in erythroblasts.

Studies of cell proliferation and differentiation showed decreased enucleation upon let-7 miRNAs focused inhibition in adult erythroblasts cultivated ex vivo

To investigate the inhibition of let-7a and let-7b miRNAs, lentiviral constructs that incorporated the Tough Decoy (TuD) design targeting let-7a or let-7b were compared with control vector. Transductions were performed in CD34(+) cells from adult healthy volunteers cultivated ex vivo in erythropoietin-supplemented serum-free media for 21 days. The effects of miRNA inhibition by TuD vectors has been shown to be accompanied by a decrease of target miRNA levels [27], as a result of target-miRNA sequestration [27, 28] and degradation, the latter via the ‘tailing and trimming’ pathway [28]. As shown in Fig. 3a, b, inhibition of let-7 miRNAs by TuD caused reduction in the levels of let-7 species as assessed by RT-qPCR at culture day 14 (let-7a RT-qPCR: control: 1.4E ± 0.7E ± 2.4E ± 0.6E copies/ng, let-7a-TuD: 1.6E ± 0.6E ± 4.6E ± 0.5E copies/ng, p = 0.0003; let-7b RT-qPCR: control: 1.0E ± 0.7E ± 4.9E ± 0.5E, let-7b-TuD: 1.6E ± 0.5E ± 5.1E ± 0.5E, p = 0.00002). Importantly, both let-7a-TuD and let-7b-TuD reduced the expression of both let-7a and let-7b miRNAs, demonstrating a more focused, but not exclusive targeting of miRNA by this technology. When the total levels of let-7 miRNAs were compared between let-7a-TuD and let-7b-TuD designs, the let-7a-TuD vector was more efficient in reducing the total levels of let-7 miRNAs compared to let-7b-TuD (Fig. 3c; 88% versus 75% suppression of total let-7 miRNA levels after let-7a-TuD and let-7b-TuD transductions, respectively). Since the data suggested that let-7a-TuD resulted in a greater reduction in total levels of let-7 miRNAs compared to let-7b-TuD, the let-7a-TuD vector was utilized for subsequent studies.

Cell proliferation and erythroblast differentiation were compared between control transductions and let-7a-TuD. Similar cell counts were observed between let-7a-TuD versus control transductions at culture days 14 and 21. As shown in Fig. 5a and Additional file 3A–D, and glycophorin A (GPA) were performed at culture days 14 and 21. As shown in Fig. 5a and Additional file 3A–D, erythroblast differentiation (CD71 and GPA) was not affected by let-7a-TuD. Thiazole orange staining on culture day 21 showed that both let-7a-TuD and control transductions achieved enucleation, however, there was a significant decrease in the percent of enucleated cells in let-7a-TuD compared to control transductions (control: 45.4 ± 7.0%, let-7a-TuD: 30.7 ± 3.9%, p = 0.01; Fig. 5b and Additional file 3E). Finally, HbF distribution was

![Fig. 1](image-url)  
*Fig. 1* The human let-7 family of miRNAs. The nucleotide differences of the human mature let-7 family members in comparison to the human mature let-7a miRNA are marked in red font. Sequences were obtained using miRBase (http://mirbase.org/), Release 21
assessed by HbF staining at culture day 21. As shown in Fig. 5c, pancellular expression of HbF was observed upon let-7a-TuD compared to control transductions (control: 53.3 ± 6.7, let-7a-TuD: 85.4 ± 4.7, p = 0.02, Additional file 3F).

**Let-7a-TuD increases gamma-globin mRNA and fetal hemoglobin levels in cultured adult erythroblasts**

To further characterize the effects of cultured adult erythroblasts, RT-qPCR analysis of the globin genes was performed at culture day 14. No major differences were observed in alpha-, mu-, theta-, zeta-, beta-, delta- and epsilon-globin mRNA levels among let-7a-TuD samples compared to control transductions (Fig. 6a, b). However, the gamma-globin mRNA expression level was significantly increased in let-7a-TuD (control: 1.2E+06 ± 6.8E+05 copies/ng; let-7a-TuD: 1.1E+07 ± 4.5E+06 copies/ng, p = 0.004; Fig. 6b). In addition, hemoglobin profiles (HPLC) were generated at culture day 21, showing a robust increase in HbF levels upon treatment with the TuD lentiviral vector (Fig. 6c, d; control: 4.7 ± 0.6%; let-7a-TuD: 38.2 ± 3.8%, p = 0.00003). For comparison purposes, HPLC analysis of let-7b-TuD was performed and demonstrated that let-7b-TuD caused less pronounced changes in the HbF levels, reaching 29.7 ± 4.5% compared to control transductions at 4.1 ± 0.9% in matched cultures.

**Let-7 miRNAs inhibition by TuD demonstrates additional developmentally-specific gene regulation in adult erythroblasts**

The fetal-to-adult transition in humans is accompanied by increased expression in the levels of carbonic anhydrase I (CA1), which after globin is the second most abundant protein in adult erythroid cells [29]. In addition, increased expression in the levels of glucosaminyl (N-acetyl) transferase 2 (GCNT2) during the fetal-to-adult transition catalyzes the expression of adult blood group I antigen in erythrocytes [30, 31]. To investigate whether let-7a-TuD transduced cells would have effects on these erythroid-relevant developmentally regulated genes, let-7a-TuD cells and control transductions were investigated for the expression of CA1 and GCNT2 by RT-qPCR and Western blot. Interestingly, both CA1 and GCNT2 were significantly down-regulated at the mRNA level in let-7a-TuD samples compared to controls (CA1: control: 3.3.E+04 ± 1.3.E+04 copies/ng; let-7a-TuD: 7.5.E+03 ± 6.9.E+03 copies/ng; p = 0.005; GCNT2: control: 3.8.E+03 ± 7.7.E+02 copies/ng; let-7a-TuD: 2.2.E+02 ± 9.4.E+01; p = 0.002; Fig. 6e, f). However, at
the protein level, only CA1 was down-regulated in let-7a-TuD samples compared to controls, while GCNT2 remained unchanged (Fig. 6g, h). Importantly, LIN28A and LIN28B mRNA transcripts remained at background levels and below the detection limits, respectively, in both let-7a-TuD and control transductions.

Additionally, the erythroid-related genes (BCL11A, HMGA2, ZBTB7A, KLF1 and SOX6) [22, 32–37] were studied for comparison in let-7a-TuD and control transductions. Interestingly, while BCL11A mRNA expression levels were significantly reduced in let-7a-TuD samples compared to control transductions (control: 1.7E+03 ± 4.5E+02 copies/ng; let-7a-TuD: 4.3E+02 ± 1.8E+02 copies/ng; p = 0.003), no significant differences were observed in HMGA2, ZBTB7A, KLF1 and SOX6 transcripts (Fig. 7a–e). However, marked reduction in the protein level of BCL11A was observed after let-7a-TuD (Fig. 7f), while HMGA2 showed a double-band pattern previously reported [38] with a marked increase in the intensity of the upper band after let-7a-TuD (Fig. 7f). Of note, ZBTB7A, KLF1, and SOX6 protein levels demonstrated no consistent change with let-7a-TuD.

Direct knockdown of BCL11A does not significantly affect the let-7 family of miRNAs

Finally, to determine whether direct down-regulation of BCL11A would affect the let-7 family of miRNAs, primary CD34(+) cells were transduced in this experimental system with a lentivirus shRNA vector to knockdown BCL11A as well as the lentiviral vector matched control for comparison. BCL11A knockdown (BCL11A-KD) was confirmed by RT-qPCR (Fig. 8a). Interestingly, BCL11A-KD did not significantly affect the let-7 family of miRNAs (Fig. 8b), which suggests that the let-7 miRNAs are upstream regulators of BCL11A.

Discussion

In this study, we show that the let-7 family of miRNAs is differentially expressed in purified adult human blood, and that let-7a and let-7b are the predominantly expressed family members in the analyzed peripheral blood cell populations, including reticulocytes. Focused suppression of let-7a and let-7b miRNAs with a miRNA Tough Decoy approach in erythroblasts was sufficient to cause robust changes in several developmentally-specific erythroblast genes including increases in gamma-globin mRNA expression and HbF to reach mean levels around 35–40% of the total hemoglobin produced. As such, these data confirm a functional role for erythroblast let-7 miRNAs in globin gene regulation and suggest that targeted reductions of the predominant let-7s should be further explored for application in patients with sickle cell disease and beta-hemoglobin disorders.

The first description of small-RNAs as regulators of developmental timing events was observed in studies of C. elegans, when lin-4 and, subsequently let-7 (from the initial denomination lethal-7) [39–41] were identified.
The mature let-7 miRNAs sequence and its function as a heterochronic regulator are highly conserved across evolution [42], and let-7a is the most well-conserved let-7 family member. Mature let-7a originated from three different genomic loci (let-7a-1, let-7a-2 and let-7a-3) and mature let-7f originated from miRNA precursors of two distinct genomic locations (let-7f-1 and let-7f-2), while all other family members are originated from one precursor miRNA sequence.

Also of interest, we observed that let-7a and let-7b are the major species detected by RT-qPCR in all peripheral blood cell populations analyzed. As reported previously, array-based analyses of the let-7 miRNAs demonstrated similarly high levels of each let-7 family member in human adult reticulocytes rather than the predominance of let-7a and let-7b [10]. The differences between these microarrays versus RT-qPCR results may be due to a higher level of cross-reactivity in let-7 miRNAs array-based detection [43].

Here we aimed our gene transduction studies to suppress the two most prevalent members of the let-7 miRNA family, let-7a and let-7b. Importantly, the high similarity among the mature let-7 miRNA sequences prevented the exclusive targeting by tough decoy (TuD) lentiviral designs. While TuD inhibitors are able to provide a more focused inhibition of miRNAs than other strategies [27, 28], similar non-specificity of TuD constructs for targeted species in the same miRNA family was previously described [19]. These results support the notion that targeted let-7 inhibition is a robust approach towards the manipulation of HbF levels in adult erythroblasts. Genomic targeting of individual let-7 species, perhaps with short palindromic repeat technologies, may be useful for determination of individual let-7 family member effects upon HbF.

Interestingly, the magnitude of total let-7 suppression was proportional to the increase in gamma-globin mRNA and HbF in studies reported to date. It is known that LIN28 proteins regulate let-7 biogenesis and that let-7 miRNAs regulate LIN28 levels by binding to its 3′ untranslated region in a double negative feedback loop [44]. The absence of increased LIN28A or LIN28B
mRNA transcripts after let-7 suppression suggests that the LIN28 genes are transcriptionally silent rather than post-transcriptionally degraded by let-7 in the adult cells. Our study also provides the first evidence that the let-7 effects extend beyond globin gene regulation to include other markers of the fetal-to-adult switch in the erythroid lineage, namely CA1 and GCNT2. Interestingly, reduced levels were observed only at the protein levels of CA1, while GCNT2 levels remained unchanged.

The biological significance of this finding will require further investigation.

The erythroid-related genes BCL11A, HMGA2, ZBTB7A, KLF1 and SOX6 were investigated upon let-7 suppression. The B-cell CLL/lymphoma 11A (BCL11A) is a zinc-finger transcription factor known to regulate gamma-globin and HbF levels in human erythroid cells [32] as well as to rescue the sickle cell disease phenotype in a murine model through the activation of HbF [33]. The High Mobility Group AT-hook 2 (HMGA2) is known as an architectural transcription factor and a target of the let-7 miRNAs [34] that has been recently reported to regulate gamma-globin mRNA and moderately increase HbF levels in human adult erythroblasts in vitro [22]. The Leukemia/Lymphoma-Related Factor (LRF) encoded by the Zinc Finger and BTB Domain Containing 7A (ZBTB7A) gene is also a zinc-finger transcription factor shown to cause robust increases in the HbF levels in human cultured erythroblasts [35]. The Kruppel like factor 1 (KLF1) is an erythroid-specific transcription factor known to regulate the expression of several erythroid genes including BCL11A [36]. Finally, the SRY-box 6 (SOX6) is a transcription factor that contains a conserved DNA-binding domain and was demonstrated to physically interact and co-occupy the human beta-globin cluster with BCL11A and other transcription factors such as GATA1 [37]. Interestingly, marked modulations were observed only at BCL11A (down-regulation at both the mRNA and protein levels) and HMGA2 (up-regulation at the protein level).

Overall, we interpret our data as demonstrating that reduction of let-7, in the absence of other potential LIN28 effects, is a main driver of these developmentally-regulated genes in erythroblasts. While LIN28 effects upon the expression of BCL11A have been inconsistent in prior studies [11, 12], this study shows that robust let-7 reduction is sufficient to reduce BCL11A as well as to increase HMGA2 for increased gamma-globin transcription. Future studies should be aimed toward understanding how this well-conserved miRNA family is able to regulate erythroid gene activity associated with the fetal-to-adult transition in humans. Since let-7 has a more generic role in timing worm development, such studies may ultimately demonstrate how the let-7 developmental clock circuit functionally evolved in human tissues.

**Conclusions**

The let-7 family of miRNAs is differentially expressed among monocytes, lymphocytes, neutrophils, and reticulocytes from adult human blood and higher expression
Fig. 6  Focused inhibition of let-7 miRNAs regulates gamma-globin and HbF levels in human erythroblasts ex vivo. Let-7a-TuD and control transductions were investigated for α, μ-, θa-, and ζa-globins, and β, δ-, γ-, and ε-globins. RT-qPCR analyses were performed at culture day 14.  

- **a**: Representative HPLC tracings. HbF and HbA peaks are labeled on each graph (y-axis, mVolts; x-axis, elution time in minutes).  
- **b**: HPLC analysis of hemoglobin from let-7a-TuD compared to control transductions. HPLC samples were collected at culture day 21. Let-7a-TuD effects in CA1 and GCNT2 mRNA levels. RT-qPCR analyses were performed at culture day 14. Open bars represent control and black bars represent let-7a-TuD. Mean value ± SD of five independent donors for each condition. p values were calculated using two-tailed Student’s t-test. Western blot analyses of CA1 and GCNT2 in the cytoplasmic extracts of three independent donors at culture day 14 upon let-7a-TuD compared to control transductions. Blots were probed with anti-CA1 or anti-GCNT2 antibodies as indicated. Beta-Actin was used as loading control. C, control (negative control vector) transduction, 7a-TuD or 7a, let-7a tough decoy design. *p < 0.05.
Fig. 7 Let-7 miRNAs focused inhibition modulates the expression levels of BCL11A and HMGA2. Let-7a-TuD effects in a BCL11A, b HMGA2, c ZBTB7A, d KLF1 and e SOX6 mRNA levels. RT-qPCR analyses were performed at culture day 14. Open bars represent control and black bars represent let-7a-TuD. Mean value ± SD of at least four independent donors for each condition. p values were calculated using two-tailed Student’s t-test. f Western blot analyses of BCL11A, HMGA2, ZBTB7A, KLF1 and SOX6 in the nuclear extracts of three independent donors at culture day 14 upon let-7a-TuD compared to control transductions. Blots were probed with anti-BCL11A, anti-HMGA2, anti-ZBTB7A, anti-KLF1 or anti-SOX6 antibodies as indicated. Lamin B1 and Histone H3 were used as loading controls. C, control (negative control vector) transduction; 7a-TuD or 7a, let-7a tough decoy design. *p < 0.05
levels of the let-7 miRNAs were observed in reticulocytes. Remarkably, let-7a and let-7b are predominantly expressed species in all peripheral blood cell populations analyzed. Also of interest, targeted reduction of let-7a was more efficient than let-7b, and focused targeting of let-7a in erythroblasts is sufficient to cause robust increases in gamma-globin mRNA expression and HbF to mean levels around 38% of the total hemoglobin produced. Targeting of individual let-7 genes or additional RNA transcripts from the let-7 cascade may be useful for therapeutic induction of HbF levels in patients with sickle cell disease or other beta-hemoglobinopathies.
Consent for publication
Not applicable.

Ethics approval and consent to participate
Written informed consent was obtained from all research subjects prior to participation in this study. Approval for the research protocol and consent documents using primary erythroblasts and peripheral blood samples was granted by the Intramural National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board.

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