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A Long Noncoding RNA from the *HBS1L-MYB* Intergenic Region on Chr6q23 Regulates Human Fetal Hemoglobin Expression

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Abstract

The *HBS1L-MYB* intergenic region (chr6q23) regulates erythroid cell proliferation, maturation, and fetal hemoglobin (HbF) expression. An enhancer element within this locus, highlighted by a 3-bp deletion polymorphism (rs66650371), is known to interact with the promoter of the neighboring gene, *MYB*, to increase its expression, thereby regulating HbF production. RNA polymerase II binding and a 50-bp transcript from this enhancer region reported in ENCODE datasets suggested the presence of a long noncoding RNA (lncRNA). We characterized a novel 1283 bp transcript (*HMI-LNCRNA;* chr6:135,096,362–135,097,644; hg38) that was transcribed from the enhancer region of *MYB*. Within erythroid cells, *HMI-LNCRNA* was almost exclusively present in nucleus, and was much less abundant than the mRNA for *MYB*. *HMI-LNCRNA* expression was significantly higher in erythroblasts derived from cultured adult peripheral blood $CD34^+$ cells which expressed more *HBB*, compared to erythroblasts from cultured cord blood $CD34^+$ cells which expressed much more *HBG*. Down-regulation of *HMI-LNCRNA* in HUDEP-2

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cells, which expressed mostly *HBB*, significantly upregulated *HBG* expression both at the mRNA (200-fold) and protein levels, and promoted erythroid maturation. No change was found in the expression of *BCL11A* and other key transcription factors known to modulate *HBG* expression. *HMI-LNCRNA* plays an important role in regulating *HBG* expression, and its downregulation can result in a significant increase in HbF. *HMI-LNCRNA* might be a potential therapeutic target for HbF induction treatment in sickle cell disease and β-thalassemia.

Keywords

Long noncoding RNA; HbF quantitative trait loci; Regulation of HbF expression

INTRODUCTION

Sickle cell disease (SCD) and β-thalassemia major are the most prevalent hemoglobinopathies worldwide. Once found primarily in regions of the world where malaria was and may still be endemic, these diseases are now widespread due to human migration and are increasingly important to global health. Fetal hemoglobin (HbF; $\alpha_2\gamma_2$) can inhibit polymerization of deoxy-sickle hemoglobin, and can also compensate for the lack of adult hemoglobin (HbA; $\alpha_2\beta_2$) in β-thalassemia major. Therefore, HbF is the major modifier of disease severity for both SCD and β-thalassemia major [1,2].

Genome-wide association studies (GWAS) have found multiple single nucleotide polymorphisms (SNPs) marking three major quantitative trait loci (QTL) associated with HbF levels — chr11p15 (*HBB* gene cluster), chr2p16 (*BCL11A*) and chr6q23 (*HBS1L-MYB* intergenic polymorphisms or HMIP) [3]. Together they account for 20–45% of HbF variance in different populations. In addition, other cis-acting elements such as the *HBD-HBBP1* intergenic region and transcription factors including MYB, KLF1, BCL11A, ZBTB7A, CHD4, NR2C1/NR2C2 and KDM1α, play important roles in regulating *HBG* expression [4–7]. Nevertheless, significant gaps of knowledge on the regulation of *HBG* still remain.

The 126 kb *HBS1L-MYB* intergenic region on chr6q23 is between the genes *HBS1L* which is a member of the GTP-binding elongation factor family with no known association with erythroid-specific traits, and *MYB* which encodes for the transcription factor c-MYB. c-MYB regulates proliferation and maturation of erythroid cells, and modulates gene expression within the *HBB* gene cluster [8,9]. A distal enhancer located at ~84 kb upstream of *MYB* has been shown by GWAS, insertional mutagenesis, long-range interaction demonstrable by chromosome conformation capture (3C) analysis, and gene editing with Cas9 nucleases [10–13]. This enhancer encompasses a 3-bp deletion polymorphism (rs66650371), which is surrounded by binding sites for erythroid-specific transcription factors TAL1/E47, GATA, RUNX1, LDB1 and KLF1, and is likely the functional motif to account for most of the effect upon HbF level by this QTL [10,12,13]. Alteration of this enhancer by polymorphisms such as rs66650371 reduced its interaction with the *MYB* promoter, which led to downregulation of *MYB* and upregulation of *HBG* expression. Furthermore, ENCODE datasets annotated RNA polymerase II occupancy and a 50-bp RNA

transcript adjacent to rs66650371. This led us to hypothesize that this transcript is part of a long noncoding RNA (lncRNA) [10]. LncRNAs are usually greater than 200 nucleotides long, are transcribed throughout the genome, and have broad functionality.

We now report the characterization of a novel 1283 bp lncRNA, herein named the *HBS1L-MYB* intergenic long noncoding RNA (*HMI-LNCRNA*). *HMI-LNCRNA* is transcribed from the enhancer for *MYB*, and its downregulation significantly increased *HBG* expression at both the mRNA and protein levels in human adult-like erythroid cells. These observations suggest that *HMI-LNCRNA* has an important role in silencing *HBG* expression in adults, and could become a therapeutic target for increasing HbF in patients with SCD and β-thalassemia major.

MATERIALS AND METHODS

K562 cells

K562 cells were cultured at 37°C in RPMI medium containing 10% FBS and 2% penicillin/streptomycin.

RNA extraction

Total RNA was extracted using RNeasy Mini Kit (Qiagen), treated with DNase (RNase-Free DNase Set, Qiagen), followed by RNA cleanup using RNeasy Mini Kit. For tissue-specificity experiment, multiple human organ RNA panels (Invitrogen and Clontech) were also treated with DNase, followed by RNA cleanup.

Reverse transcription polymerase chain reaction RT-PCR

cDNA was synthesized from total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR reactions were done using the Multiplex PCR kit (Qiagen). The following primers were used to amplify the 1180 bp product: 5′-ATCGCTCATGAGAAATGTGG-3′ (forward) and 5′-GGAACCGCCCTGATAACATT-3′ (reverse).

Rapid amplification of cDNA ends (RACE)

5′- and 3′-RACE were done using the FirstChoice RLM-RACE Kit (Ambion), following the manufacturer's instructions, using SuperTaq Plus Polymerase (Life Technologies) for PCR reactions. The following gene-specific primers were used: 5′-GTCTAATGGTGTGGCTCACAAA-3′ (5′-outer), 5′-CCCCAGCTTCCTTATCTGTAAA-3′ (5′-inner), 5′-TTCACTCTGGACAGCAGATGTT-3′ (3′-outer) and 5′-CGGTTCCCTCAGAAGACACTTA-3′ (3′-inner). RACE PCR products were ligated to pCRII vector using TA Cloning Dual Promoter Kit (Invitrogen), transformed into One Shot INVαF chemically competent *E. coli* (Invitrogen), and grown on LB plates containing ampicillin and X-Gal. Insert-positive white colonies were picked and grown for DNA extraction. PCR to amplify insert (Forward: 5′-TGTGGAATTGTGAGCGGA TA-3′ and Reverse: 5′-GTTTTCCCAGTCACGACGTT-3′), and DNA sequencing were done to determine the 5′- and 3′-ends.

DNA sequencing

PCR products were purified using AccuPrep PCR Purification Kit, and prepared for sequencing using ABI Big Dye Terminator v3.1 Cycle Sequencing Kit. Sequence data was analyzed on FinchTV version 1.5.0. NCBI BLAST was used to determine length and location of sequence.

Human Umbilical Cord Blood-Derived Erythroid Progenitor (HUDEP) cells

HUDEP cells are an immortalized erythroid cell line derived from cord blood $CD34^+$ mononuclear cells [14]. HUDEP-1 and HUDEP-2 cells were maintained in expansion medium—StemSpan SFEM medium (StemCell Technologies) supplemented with SCF (50 ng/ml, Invitrogen), EPO (3 U/ml, Invitrogen), dexamethasone (1 μM, Sigma), doxycycline (1 μg/ml, Clontech), L-glutamine (1%, Life Technologies) and penicillin/streptomycin (2%, Life Technologies). For erythroid maturation, cells were cultured in differentiation medium —IMDM medium (Invitrogen) supplemented with heat inactivated human serum from human male AB plasma (5%, Sigma), EPO (3 U/ml, Invitrogen), insulin (10 μg/ml, Sigma), doxycycline (1 μg/ml, Clontech), holo-transferrin (500 μg/ml, Sigma), heparin (3 U/ml, Sigma), SCF (100 ng/ml, Invitrogen), L-glutamine (1%, Life Technologies) and penicillin/ streptomycin (2%, Life Technologies)—for 5 days. For further erythroid maturation, doxycycline was removed and cells were cultured for 2 more days.

Primary $CD34^+$ mononuclear cells

Primary $CD34^+$ mononuclear cells derived from cord blood and peripheral blood (StemCell Technologies) were expanded for up to 7 days in StemSpan SFEM II medium (StemCell Technologies) supplemented with StemSpan CC100 (1×, StemCell Technologies) and penicillin/streptomycin (2%, Life Technologies). To induce erythroid differentiation, cells were cultured for up to 10 days in StemSpan SFEM II medium (StemCell Technologies) supplemented with SCF (10 ng/ml, Invitrogen), EPO (5 U/ml, Invitrogen), IL-6 (10 ng/ml, Sigma) and penicillin/streptomycin (2%, Life Technologies).

Nuclear and cytoplasmic fractionation

Nuclear/Cytosol Fractionation Kit (BioVision) was used following manufacturer's instructions.

Quantitative PCR (qPCR)

RNA was used for qPCR using TaqMan RNA-to-C_T 1-Step Kit (Applied Biosystems) and the following TaqMan gene expression assays (Applied Biosystems): *HBG1/2* (Hs00361131_g1), *HBB* (Hs00758889_s1), *MYB* (Hs00920556_m1), *HBS1L* (Hs04188641_g1), *HMI-LNCRNA* (custom TaqMan assay designed by Applied Biosystems to target genome position chr6: 135,096,354–135,097,644, hg38; assay ID number AJI1MTQ), *BCL11A* (Hs01093197_m1), *CHD4* (Hs00172349_m1), *KLF1* (Hs00610592_m1), *ZBTB7A* (Hs00252415_s1), *NR2C1* (Hs00915957_m1), *NR2C2* (Hs00991824_m1), *KDM1a* (Hs01002741_m1) and *ACTB* (Hs01060665_g1). QPCR reactions were run on a StepOne Plus qPCR machine (Applied Biosystems). *ACTB* was used as the endogenous control.

Western blot analysis

Cell pellets were suspended in Roche lysis buffer (protease inhibitor, 0.3% NP40, 10% glycerine, 2 mM EDTA, 246 mM NaCl, 10% phosphatase inhibitor, PBS and water), placed on ice for 1 hour and centrifuged at 14,500 rpm for 15 minutes at 4°C to extract protein. Standard methodology was used for western blot analysis. The following antibodies were used: c-MYB (ab109127, Abcam), HBB (16216-1-AP, Proteintech), hemoglobin γ (sc-21756, Santa Cruz Biotechnology) and GAPDH (sc-47724, Santa Cruz Biotechnology).

Plasmids

The following top and bottoms strands were synthetically made and annealed to make the shRNA template for *HMI-LNCRNA*: top strand 5′- GATCCGCTAG TATGTGAAGCACTTAGCTTCCTGTCAGACTAAGTGCTTCACATACTAGCTTTTTG-3′ , bottom strand 5′- AATTCAAAAAGCTAGTATGTGAAGCACTTAGTCTGACAGGAAGCT AAGTGCTTCACATACTAGCG-3′) (135,096,650-135,096,670; hg38). Next, the shRNA template for *HMI-LNCRNA* was ligated to the lentivirus vector pGreenPuro (System Biosciences), upstream the EF1 promoter, and labeled HMI-lncRNA shRNA. pGreenPuro scrambled shRNA (System Biosciences) was used as the negative control. Both lentivectors co-express green fluorescent protein (GFP).

Lentivirus transduction

293T cells were transfected with scrambled shRNA and HMI-lncRNA shRNA using the LentiStarter 2.0 kit (System Biosciences), following manufacturer's instructions, to generate lentiviral particles. HUDEP-2 cells were transduced at an MOI 50 with 5 ug/mL Polybrene, and centrifuged at room temperature for 30 min at 1250 × g. After 48 hours, transduction was repeated. 48 hours post-transduction, cells were cultured in expansion medium with 1 ug/mL Puromycin for up to 2 weeks.

Cell surface staining for FACS

Cells were harvested and washed in FACS buffer, and were resuspended in 100 μl of FACS buffer with antibody, and incubated on ice for 30 minutes. Next, cells were washed twice in FACS buffer by centrifugation at 300g for 5 minutes. Finally, cells were fixed in IC Fixation buffer (eBioscience) for 30 minutes before analyzing the cells using the BD FACScan. The following antibodies were used: CD71-PE (334105, BioLegend) and CD235-PerCP/Cy5.5 (306613, BioLegend).

Immunofluorescence (IF)

Fixed cells were washed in PBS, blocked in 5% BSA (in PBS) and incubated in primary antibody for 1 hour. Next, cells were washed, blocked in 5% normal goat serum and incubated in secondary fluorescent-labeled antibody for 30 minutes. Finally, cells were washed and covered with mounting medium containing DAPI. Images were taken with the Eclipse Ci (Nikon) microscope with a DS-Qi1Mc-03 mono-type (Nikon) camera, using NIS-Elements Br Imaging software (Nikon). The following antibodies were used: Hemoglobin γ (sc-21756, Santa Cruz), Monoclonal Mouse Anti-human β-globin (a gift from IsoLab), Anti-

human alpha globin (Wallac Inc.), and Alexa Fluor-594-conjugated AffiniPure F(ab)$_2$ Fragment Goat Anti-mouse IgG (H+L) (115-586-062, Jackson ImmunoResearch).

RESULTS

Determining the full length of *HBS1L-MYB* intergenic long noncoding RNA

To determine if the 50-bp transcript reported by ENCODE is part of a putative lncRNA, we used the sequence of this transcript as a guide to develop primers consecutively upstream and downstream of this region. cDNA from K562 cells, an immortalized human erythroid cell line, was used for PCR reactions until amplification failed. The longest PCR product amplified was 1180 bp (chr6:135,096,355–135,097,534; hg38) (Fig. 1A).

To obtain the full-length sequence of this RNA transcript, rapid amplification of cDNA ends (RACE) was employed to ascertain its 5′- and 3′-termini (Fig. 1B; Supplemental Figs. 1 and 2). RNA from K562 cells was used for the 3′-RACE, and RNA extracted from the thymus, which was more abundant for this lncRNA (Fig. 2), was used for the 5′-RACE. Moreover, both 3′- and 5′-RACE was repeated using RNA extracted from HUDEP-2 cells, and yielded identical results (Supplemental Fig. 3).

Figure 1C illustrates the coordinates of the full-length lncRNA (chr6:135,096,362–135,097,644; hg38), and its nucleotide sequences are shown in Supplementary Table 1. It was 1283 bp in length, transcribed from a genomic site ~84.9 kb upstream from *MYB* and ~41.5 kb upstream from *HBS1L*, and herein called the *HBS1L-MYB* intergenic long noncoding RNA (*HMI-LNCRNA*). *HMI-LNCRNA* was transcribed from the enhancer for *MYB* and encompassed binding sites for erythroid transcription factors TAL1/E47, GATA, RUNX1, and within the transcript was the site of the HbF-associated 3-bp deletion polymorphism (rs66650371). *HMI-LNCRNA* was intron-less, had no 3′-end polyadenylation signal, and could be presumed to have a 5′-cap as 5′-RACE selected for only RNAs with a 5′-cap. It also contained no open reading frames longer than 300 nucleotides (data not shown); their presumed peptides were not catalogued in protein databases; and therefore they were unlikely to be translated [15].

Tissue-Specificity of *HMI-LNCRNA*

LncRNAs often are expressed in either one or a few cell types and are considered more tissue-specific than most mRNA [16]. The expression pattern of *HMI-LNCRNA* was analyzed by qPCR in 25 different human tissues and cells (Fig. 2). The thymus and testis had by far the highest expression. Erythroblasts derived from cord blood CD34$^+$ mononuclear cells had high expression of *HMI-LNCRNA*, followed by other hematopoietic tissues and cells, such as the fetal liver, bone marrow, spleen and K562 cells. Non-hematopoietic tissues, except for testis where lncRNAs are especially common [17], had mostly minimal expression. *HMI-LNCRNA* exhibited a distinct association with cells of hematopoietic origin. Similar to *HMI-LNCRNA*, *MYB* was also expressed in the thymus and the other hematopoietic tissues and cells (Supplemental Fig. 4).

Expression pattern of *HMI-LNCRNA* and *MYB* in erythroid cells

Within erythroid cells, *HMI-LNCRNA* was expressed almost entirely in the nucleus (Supplemental Fig. 5), suggesting that it may function as a gene-regulating lncRNA. *HMI-LNCRNA* was much less abundant than *MYB* (Supplemental Fig. 6), which is a common characteristic of many lncRNAs compared to their protein-coding counterparts [18]. *MYB* is critical to erythropoiesis; high levels of expression favor proliferation of erythroid progenitor cells, while reduced levels promote erythroid maturation [19]. Additionally, downregulation of *MYB* in erythroid precursors was shown to increase HbF [8,12,13,20]. Therefore, it was important to determine if *HMI-LNCRNA* and *MYB* had similar expression patterns in erythroid cells and if this similarity was associated with differences in *HBG* and *HBB* expression.

Both *HMI-LNCRNA* and *MYB* transcript levels were compared in erythroblasts derived from culture of cord blood $CD34^+$ cells, which expressed more *HBG*, to erythroblasts derived from culture of adult peripheral blood $CD34^+$ cells, which expressed more *HBB* (Fig. 3A). Both *HMI-LNCRNA* and *MYB* were significantly higher in erythroblasts derived from adult peripheral blood $CD34^+$ cells than in erythroblasts derived from cord blood $CD34^+$ cells (Fig. 3A). With erythroid differentiation of erythroblasts derived from peripheral blood $CD34^+$ cells, there was a decline in *HMI-LNCRNA* and in *MYB* expression (Fig. 3B; Supplemental Fig. 7). Next, the same comparison was made in HUDEP-1 cells, which mostly express *HBG*, and HUDEP-2 cells, which exclusively express *HBB* (Fig. 3C). HUDEP-2 cells had significantly higher expression of *HMI-LNCRNA* than HUDEP-1 cells; and there was no difference in *MYB* expression (Fig. 3C). During erythroid maturation of HUDEP-2 cells, both *HMI-LNCRNA* and *MYB* expression decreased similar to erythroblasts derived from peripheral blood $CD34^+$ cells (Fig. 3D). These data show that *HMI-LNCRNA* expression is higher in erythroid cells which express more *HBB*, when compared to erythroid cells which express more *HBG*. With erythroid cell differentiation, the expression of both *HMI-LNCRNA* and *MYB* decreases.

HMI-LNCRNA regulates hemoglobin expression in HUDEP-2 cells

To examine the possible role of *HMI-LNCRNA* in regulating *MYB* and hemoglobin expression, a lentiviral vector co-expressing GFP and a shRNA template targeting *HMI-LNCRNA* (HMI-lncRNA shRNA) was employed to downregulate the *HMI-LNCRNA* in HUDEP-2 cells. Figure 4A displays the timeline of the shRNA transduction experiments. After transduction, cells were cultured in expansion medium containing puromycin to select for lentivector-positive cells. Transduction of HUDEP-2 cells with HMI-lncRNA shRNA led to a 50% knockdown of *HMI-LNCRNA* when compared to naïve (non-transduced) cells and cells expressing scrambled shRNA (Fig. 4B). Reduction in *HMI-LNCRNA* led to a 200-fold increase in *HBG* mRNA, a 30% reduction in *MYB* mRNA, and no change in *HBB* or *HBS1L* mRNA when compared to naïve cells and cells transduced with scrambled shRNA (Fig. 4C). Percent *HBG* increased from less than 1% in naïve and scrambled shRNA cells to more than 20% of total *HBG* and *HBB* expression in cells with HMI-lncRNA shRNA (Fig. 4D). Furthermore, changes in *HBG* and *MYB* mRNA levels were associated with corresponding changes in HBG and c-MYB protein levels (Fig. 4E) shown by Western blot analyses. The up-regulation of HBG production was also confirmed by immunofluorescence

staining using anti-HBG antibody (Fig. 4F). This immunocytological technique showed that high HBG expression were found in approximately 10% of more mature HUDEP-2 cells with knockdown of *HMI-LNCRNA*. There was no change in HBB or HBA protein expression (Supplemental Figs. 8 and 9). Down-regulation of *MYB* expression in HUDEP-2 cells transduced with HMI-lncRNA shRNA was inconsistent (Supplemental Fig. 10).

The expression of key hemoglobin-regulating transcription factor genes (*KLF1, BCL11A, ZBTB7A, CHD4, NR2C1, NR2C2 and KDM1α*), which are all known to downregulate *HBG*, were either unchanged or slightly upregulated in HUDEP-2 cells transduced with HMI-lncRNA shRNA compared to controls (Fig. 5).

Hemoglobin expression during erythroid differentiation of HUDEP-2 cells with knockdown of *HMI-LNCRNA*

In addition to analyzing the effect of downregulating *HMI-LNCRNA* in non-differentiated HUDEP-2 cells, after the two-week selection for transduced cells with puromycin treatment, the cells underwent erythroid maturation for up to seven days as shown in Figure 6A. *HBG* was significantly higher at days 0, 5 and 7 in HUDEP-2 cells with HMI-lncRNA shRNA (Fig. 6B), and *HBG* expression remained at about 20% of total *HBG* and *HBB* transcripts at each time point (Fig. 6C). The increased HBG expression was also corroborated at the protein level (Supplementary Figs. 10 and 11).

Role of *HMI-LNCRNA* in modulating erythroid maturation in HUDEP-2 cells

HUDEP-2 cells resemble basophilic erythroblasts expressing both transferrin receptor (CD71), a cell surface marker for immature erythroblasts, and glycophorin A (CD235), a cell surface marker for more mature erythroblasts [6]. The percentage of cells positive for these markers were examined by flow cytometry in naïve cells, cells expressing scrambled shRNA and cells expressing HMI-lncRNA shRNA. Within both control groups, approximately 55–65% of the cells were positive for both CD71 and CD235 (Fig. 7). In contrast, about 85% of cells transduced with HMI-lncRNA shRNA were positive for both markers. These observations suggest that downregulation of *HMI-LNCRNA* promotes maturation of HUDEP-2 cells.

DISCUSSION

A novel 1283 bp lncRNA in erythroid cells is transcribed from the distal enhancer of *MYB* on chr6q23 highlighted by the 3-bp deletion polymorphism (rs66650371). Knockdown of *HMI-LNCRNA* to half of its normal level increased *HBG* expression by 200-fold and this is reflected by a marked increase in HBG production.

Long non-coding RNAs are important regulators of multiple cellular processes, biomarkers for disease prognosis and possible therapeutic targets [21–24]. Many tissue specific lncRNAs are expressed during hematopoiesis and erythropoiesis [25–29]. To date, long non-coding RNAs that can modulate globin gene expression have not been described.

HUDEP-2 cells, which do not have the 3-bp deletion at rs66650371 (data not shown) and synthesize almost exclusively HBB have been used to study the regulation of *HBG*

expression [6,13,14]. It will be necessary to replicate the present experiments in cultured erythroblasts derived from primary $CD34^+$ progenitor cells from healthy individuals and SCD patients. To determine if upregulation of *HMI-LNCRNA* could reduce *HBG* expression, we overexpressed this lncRNA in HUDEP-1 cells that express primarily *HBG* but saw no change (supplemental Fig. 12).

The expression of HBG in HUDEP-2 cells was heterocellular. With erythroid maturation, the proportion of these HUDEP-2 cells that expressed HBG increased from approximately 10% to 25% (Fig. 4F; Supplementary Fig. 11). It remains to be determined if additional down-regulation of *HMI-LNCRNA* or allowing these HUDEP-2 cells to become fully mature erythroblasts might lead to more cells expressing HBG. It is possible that transduction of HUDEP-2 cells with lentiviral particles was not homogeneous, thus accounting for the heterocellular expression of HBG. The genomic sites for the lentiviral particle integration might also be another factor that could modulate HBG expression [30]. Furthermore, HBG expression in cultured erythroid cells derived from single adult erythroid progenitors is stochastic [31].

The mechanism whereby *HMI-LNCRNA* augments *HBG* expression requires further study. LncRNAs may regulate gene expression by providing the necessary scaffold which transcription factor and other protein complexes can be associated with and be guided to cogent promoters[21,22]. They may recruit chromatin modification enzymes for epigenetic remodeling processes [22,32]. They may also act by promoting intra-chromosomal looping to foster interaction between enhancers and promoters [33,34]. Since *HMI-LNCRNA* is transcribed from the distal enhancer for *MYB*, it is highly probable that *HMI-LNCRNA* is an enhancer RNA (eRNA), and functions by facilitating the interaction between the distal enhancer and the *MYB* promoter. We hypothesized that downregulation of *HMI-LNCRNA* would reduce interaction of the distal enhancer with the promoter of *MYB* and reduce its transcription, which would ultimately lead to increase in *HBG* expression. However, decreased expression of *MYB* was not consistently observed in HUDEP-2 cells transduced with HMI-LncRNA shRNA to downregulate *HMI-LNCRNA* to 50% of normal level (Fig. 4E; Supplementary Fig. 10). Conceivably, additional down-regulation of *HMI-LNCRNA* may result in consistently decreased *MYB* expression. Whether or not interaction between distal enhancer and promoter of *MYB* is attenuated with *HMI-LNCRNA* down-regulation should be informative. Alternatively, *HMI-LNCRNA* might function through a yet undescribed molecular mechanism to modulate *HBG* expression. An important future study is to determine genomic or protein binding sites for *HMI-LNCRNA* [35].

Increasing HbF levels in SCD and β-thalassemia major is a proven approach to treatment. Hydroxyurea, the first FDA-approved drug for SCD, can induce high HbF level. Unfortunately, not all treated patients responded to this agent with sufficient increase in HbF to effect substantial clinical improvement. There has been remarkable progress on gene therapy by different approaches [36–38], and many different agents are also being investigated for their efficacy to induce HbF expression [39–44]. Down-regulating *HMI-LNCRNA* can possibly be an additional therapeutic target for HbF induction. If the increment in HbF increase found *in vitro* can be recapitulated *in vivo* in SCD and β-thalassemia major, it should be clinically beneficial [45].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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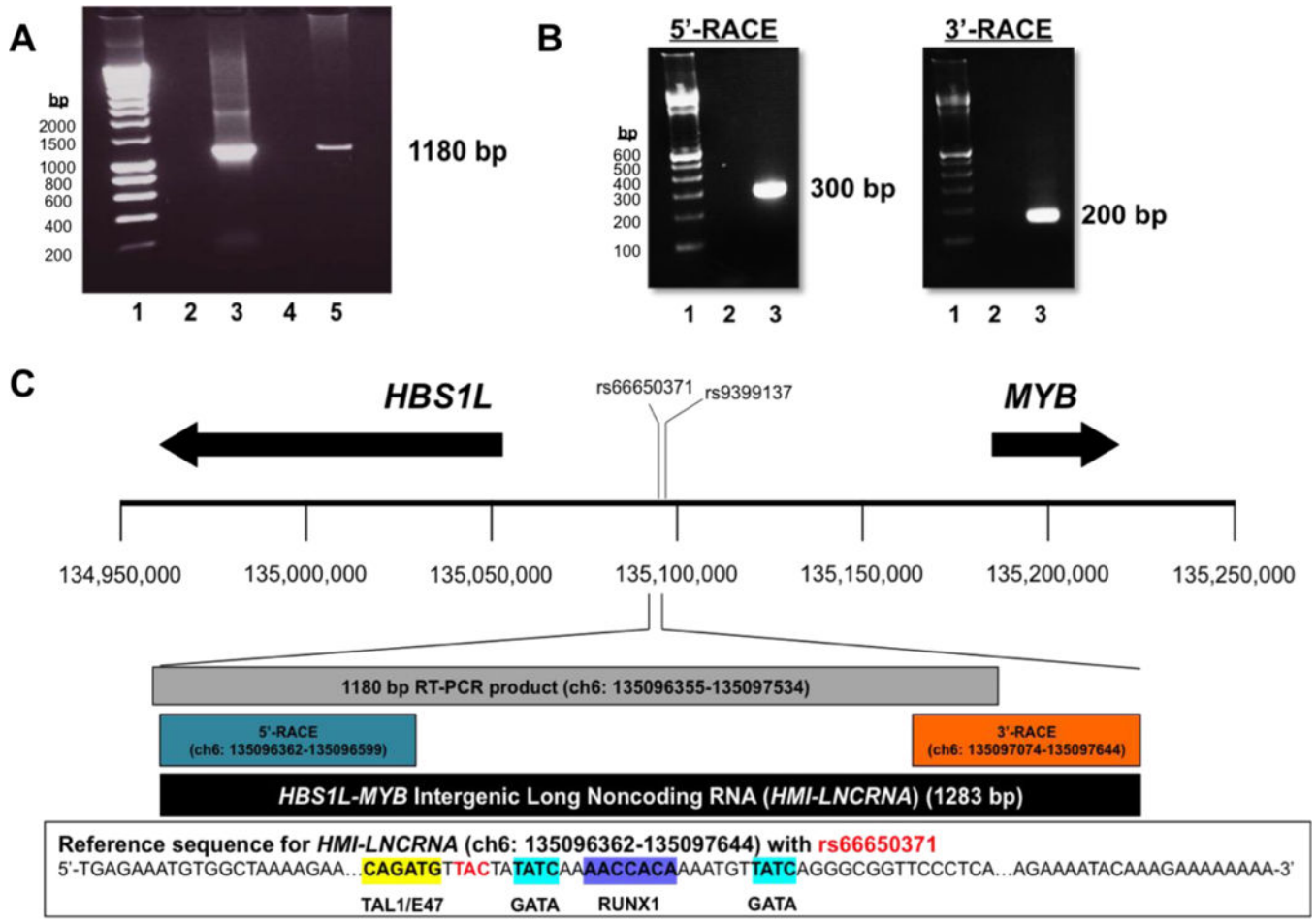

Figure 1. Determining the full length of the transcript found within the *HBS1L-MYB* intergenic region

(**A**) PCR was done to amplify a 1180 bp region within the *HBS1L-MYB* intergenic region using cDNA from K652 cells. Samples were run on agarose gels. Lane 1: 100-bp DNA ladder; lane 2: negative control, PCR mix without DNA input; lane 3: positive control, PCR mix with genomic DNA; lane 4: PCR mix with cDNA generated without reverse transcriptase; and Lane 5: PCR mix with cDNA generated with reverse transcriptase. (**B**) Agarose gels show PCR products from the 5′-RACE reactions (using RNA from thymus) and 3′-RACE reactions (using RNA from K562 cells). For 5′-RACE, lane 1: 100-bp DNA ladder; lane 2: PCR mix with cDNA generated without Tobacco Acid Pyrophosphatase (TAP) treatment; and lane 3: PCR mix with cDNA generated with TAP treatment. For 3′-RACE, lane 1: 100-bp DNA ladder; lane 2: PCR mix with cDNA generated without reverse transcriptase; and lane 3: PCR mix with cDNA generated with reverse transcriptase. (**C**) Illustration of genomic region between chr6: 134,950,000–135,250,000 (hg38), showing approximate locations of the 3-bp deletion polymorphism rs66650371 and rs9399137 a SNP in LD with rs66650371, and *HBS1L* and *MYB* (arrows represent transcription direction and approximate length of genes). Based on DNA sequencing of RACE products, the 5′- and 3′-ends of the transcript were revealed to determine the full length of the transcript, which is 1283 bp in length and named the *HBS1L-MYB* Intergenic Long Noncoding RNA (*HMI-*

LNCRNA). Located in the genomic sequence for *HMI-LNCRNA* are binding sites for erythroid-specific transcription factors TAL1/E47, GATA and RUNX1, and rs66650371. *HMI-LNCRNA* does not include rs9399137.

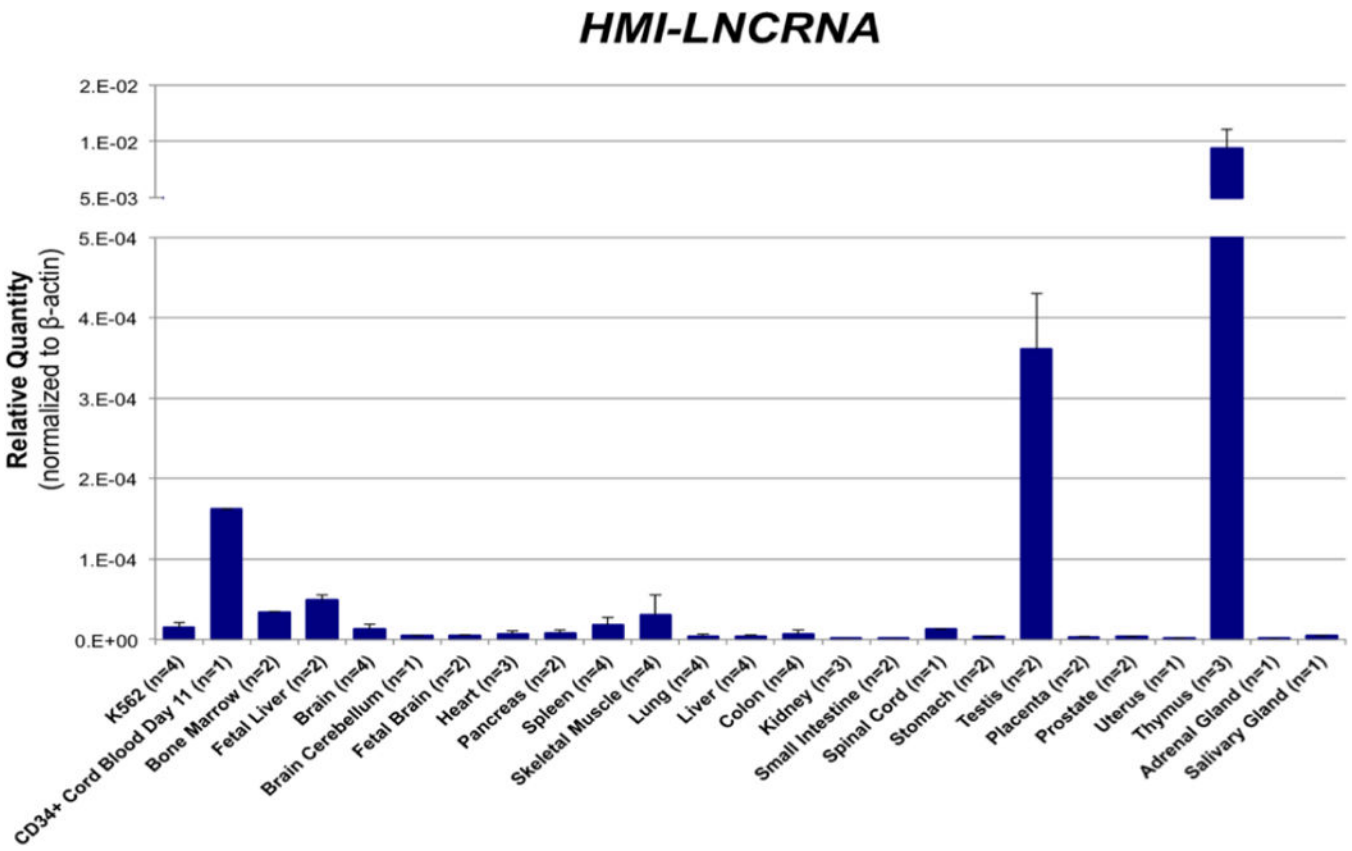

Figure 2. Expression pattern of *HMI-LNCRNA* among various human cells and tissue

Relative quantity of *HMI-LNCRNA* was measured by qPCR in 25 different samples. $CD34^+$ cord blood day 11 is erythroblasts harvested at Day 11 of two-phase expansion and differentiation culture of $CD34^+$ mononuclear cells derived from cord blood (see Supplemental Fig. 6). Means are shown for samples with two or more independent samples. *ACTB* was used as the endogenous control.

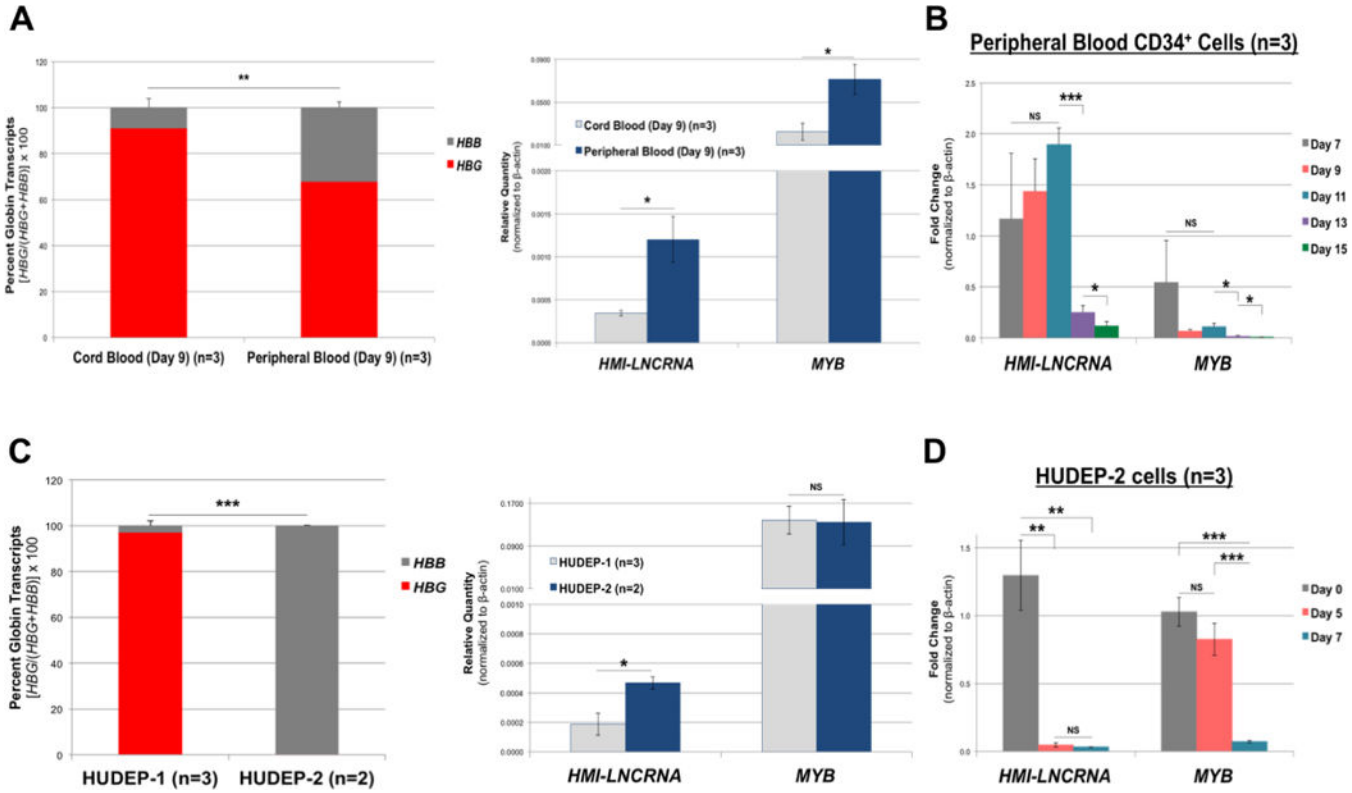

Figure 3. *HMI-LNCRNA* and *MYB* expression pattern in erythroid cells derived from cord blood and peripheral blood CD34$^+$ mononuclear cells, and in HUDEP-1 and HUDEP-2 cells

(**A**) Primary erythroid cells derived from cord blood (n=3) and adult peripheral blood (n=3) CD34$^+$ mononuclear cells (both expanded for 7 days and differentiated for 2 days) were analyzed by qPCR analysis to determine percent *HBG* to *HBB*, and the relative quantity of *MYB* and *HMI-LNCRNA*. (**B**) Fold change of *HMI-LNCRNA* and *MYB* were determined during differentiation of CD34$^+$ mononuclear cells derived from adult peripheral blood (n=3) at Days 7, 9, 11, 13 and 15. (**C**) Percent *HBG* to *HBB*, and the relative quantity of *HMI-LNCRNA* and *MYB* were determined by qPCR analysis in HUDEP-1 (n=3) and HUDEP-2 (n=2) cells (both maintained in expansion medium). (**D**) Fold change of *HMI-LNCRNA* and *MYB* were determined in HUDEP-2 cells at Days 0, 5 and 7 in cultures with differentiation medium. *ACTB* was used as the endogenous control. p-values: *< 0.05; **< 0.005; ***<0.0005; NS (not significant). p-values were obtained by Student T-test.

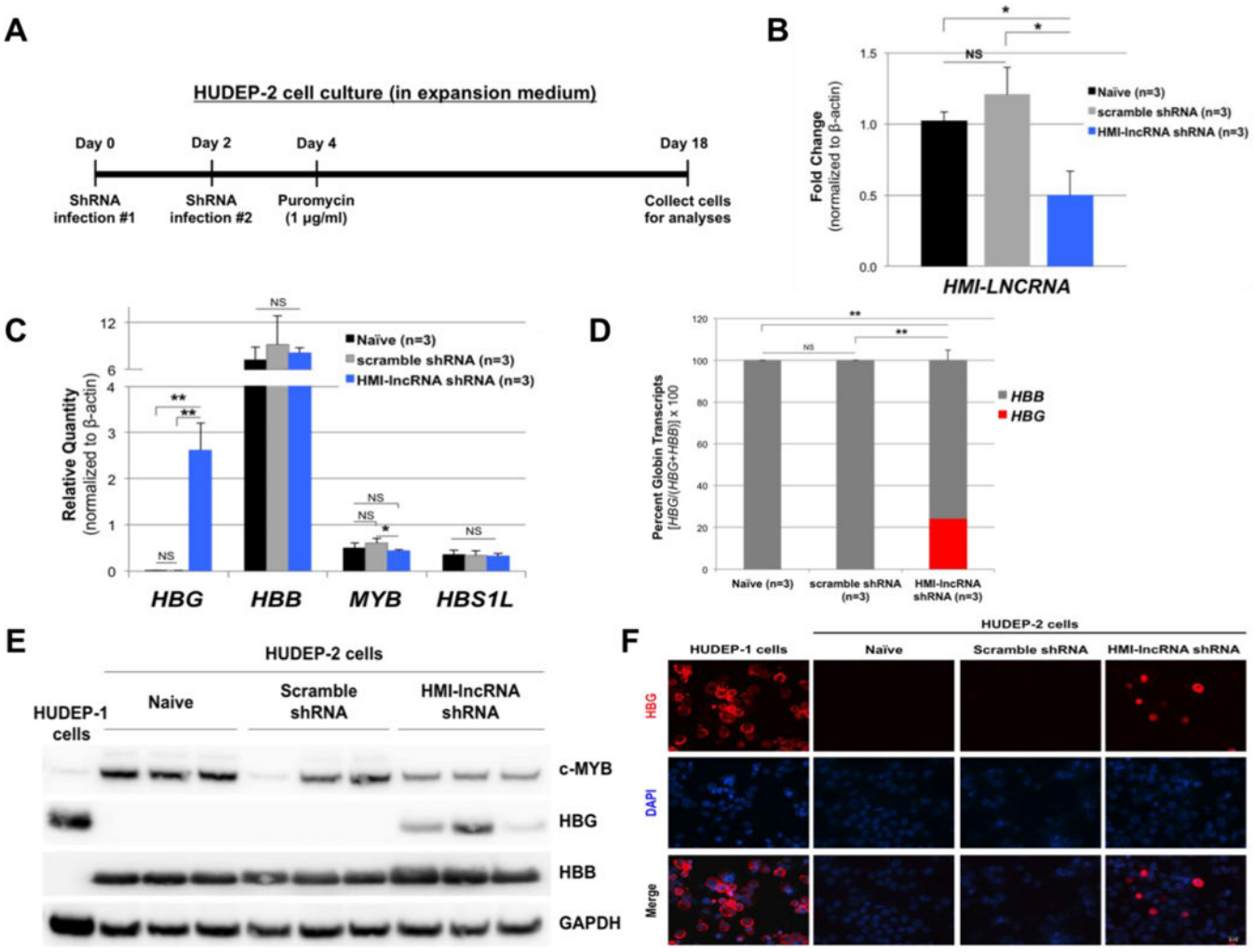

Figure 4. The effects of knocking down *HMI-LNCRNA* on hemoglobin, *MYB* and *HBS1L* in HUDEP-2 cells

(**A**) Illustration of timeline for transduction and culture of HUDEP-2 cells. Cells were maintained in expansion medium. (**B**) Expression level of *HMI-LNCRNA* was determined by qPCR in HUDEP-2 cells that were not transduced (naïve), and transduced with either scrambled shRNA or HMI-lncRNA shRNA lentiviruses by qPCR. (**C**) *HBG, HBB, MYB* and *HBS1L* transcript levels, and (**D**) percent *HBG* and *HBB* out of the total of both transcripts were measured in these same samples. (**E**) Protein expression of c-MYB, HBG and HBB were analyzed by Western blot in naïve HUDEP-2 cells, and cells transduced with scrambled shRNA and HMI-lncRNA shRNA (HUDEP-1 cells were used as control). GAPDH was used as loading control. (**F**) Naïve HUDEP-2 cells, and cells transduced with scrambled and HMI-lncRNA shRNAs were stained with anti-HBG antibody, followed by secondary antibody labeled with Alexa Fluor-594 (in red) and DAPI to stain nuclei (in blue), and imaged with a fluorescent microscope at 40X magnification. For all qPCR analyses, *ACTB* was used as the endogenous control. p-values: *< 0.05; **< 0.005; ***<0.0005; NS (not significant). p-values were obtained by Student T-test.

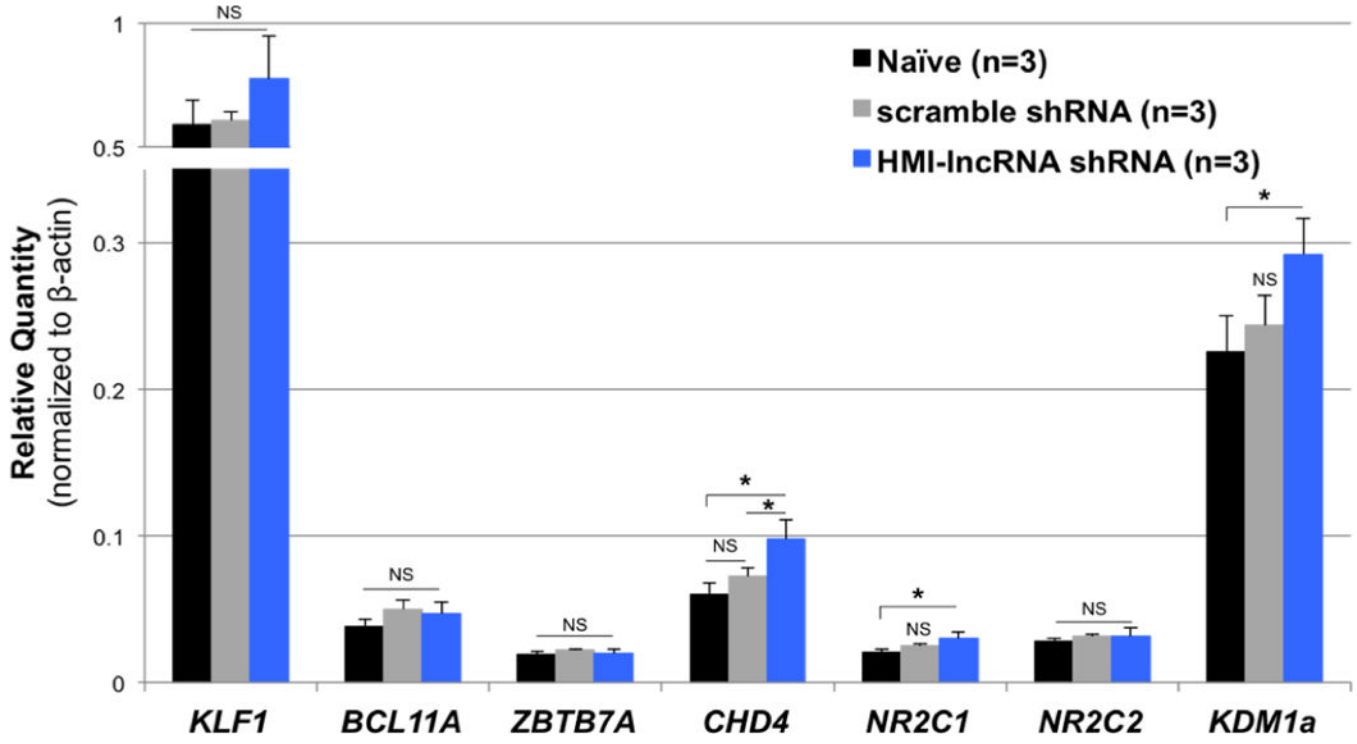

Figure 5. The effects of knocking down *HMI-LNCRNA* on erythroid-regulating transcription factors in HUDEP-2 cells

Relative expression of *KLF1, BCL11A, ZBTB7A, CHD4, NR2C1, NR2C2* and *KDM1a* transcripts were analyzed by qPCR in naïve HUDEP-2 cells, and cells infected with scrambled shRNA and HMI-lncRNA shRNA. Beta actin was used as the endogenous control. p-values: *< 0.05; **< 0.005; ***<0.0005; NS (not significant). p-values were obtained by Student T-test.

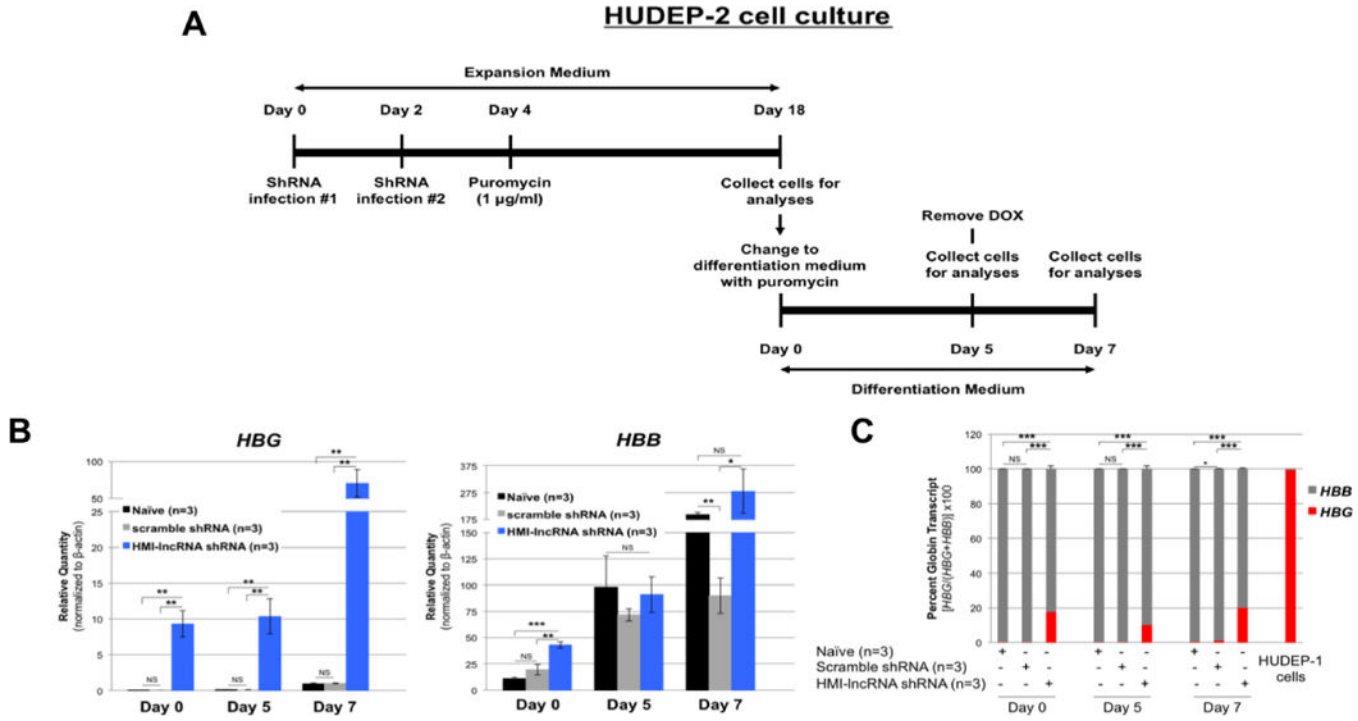

Figure 6. Hemoglobin expression during erythroid differentiation of HUDEP-2 cells with knockdown of *HMI-LNCRNA*

(**A**) Illustration of timeline for infection and culture of HUDEP-2 cells. Cells were maintained in expansion medium for 2 weeks after transduction, and then placed in differentiation medium for up to 7 days. Doxycycline (DOX) was removed at Day 5 to promote erythroid maturation. (**B**) Relative quantity for *HBG* and *HBB* transcripts were analyzed by qPCR in naïve HUDEP-2 cells (n=3), and cells transduced with scrambled shRNA (n=3) and HMI-lncRNA shRNA (n=3) at Day 0, 5 and 7 of differentiation. (**C**) Percent hemoglobin of *HBG* to *HBB* was determined by qPCR. For all qPCR analyses, *ACTB* was used as the endogenous control. p-values: *< 0.05; **< 0.005; ***<0.0005; NS (not significant). p-values were obtained by Student T-test.

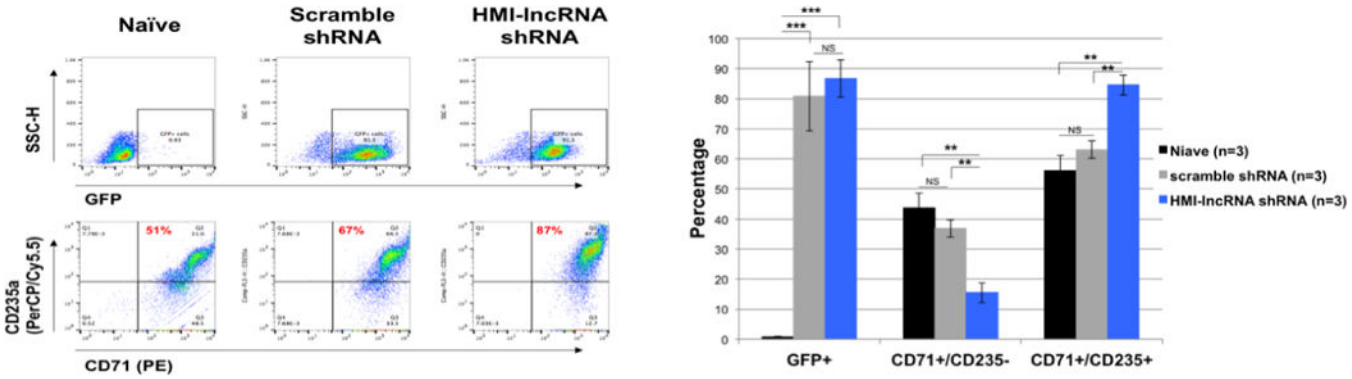

Figure 7. The effects of knocking down *HMI-LNCRNA* on erythroid maturation of HUDEP-2 cells

Naïve HUDEP-2 cells, and cells transduced with scrambled shRNA and HMI-lncRNA shRNA (cultured in expansion medium) were stained with PE-labeled transferrin receptor (CD71) and PerCP/Cy5.5-labeled glycophorin-A (CD235) antibodies, and analyzed by flow cytometry to discriminate between cells that are positive and negative for GFP, CD71 and CD235. Data was analyzed with FlowJo. Average percent GFP+, CD71+/CD235- and CD71+/CD235+ for each group was plotted on a bar graph. p-values: *< 0.05; **< 0.005; ***<0.0005; NS (not significant). p-values were obtained by Student T-test.