Original Article

Negative regulation of *HBG1/2* expression through S6K by long noncoding RNA NR_120526

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Background: High levels of fetal hemoglobin (HbF) may alleviate clinical symptoms in patients with β-thalassemia. A previous study showed that the long noncoding RNA NR_120526 (lncRNA NR_120526) might be involved in regulating HbF levels (*HBG1/2* gene expression). However, the function and mechanism by which NR_120526 regulates HbF expression remains unknown. Here, we investigated the effect of NR_120526 on HbF and its mechanism so as to provide an experimental basis for treating patients with β-thalassemia.

Methods: Chromatin isolation by RNA purification-mass spectrometry (ChIRP-MS) assay, database query, and bioinformatics analysis were performed to explore the proteins that specifically bind to NR_120526 and their interactions. Chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq) were used to determine whether NR_120526 directly regulates the expression of *HBG1/2*. The NR_120526 gene was knocked out (KO) using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology in K562 cells. Finally, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were used to detect the messenger RNA (mRNA) and protein expressions of *HBG1/2*, ribosomal protein S6 kinase B1 (*RPS6KB1*, *S6K*), and Ras homologous family member A (*RhoA*), respectively.

Results: We found that NR_120526 interacts with ILF2, ILF3, and S6K. However, ILF2/ILF3 bound to NR_120526 did not interact with *HBG1/2*, suggesting that NR_120526 may regulate *HBG1/2* expression indirectly. The qRT-PCR results showed no statistical difference in the mRNA expression levels of *HBG1/2*, *S6K*, and *RhoA* between the NR_120526-KO group and negative control (NC) group ($P>0.05$). However, Western blot results showed a significant increase in the protein levels of *HBG1/2*, *S6K*, and *RhoA* in the KO group ($P<0.05$). It was found that NR_120526 inhibited S6K, thereby downregulating RhoA and leading to decreased *HBG1/2* expression.

Conclusions: LncRNA NR_120526 negatively regulates the expression of *HBG1/2* through S6K. These new findings provide mechanistic insights into the regulation of HbF and offer potential therapeutic targets for precision medicine in patients with β-thalassemia.

Keywords: NR_120526; fetal hemoglobin (HbF); RPS6KB1; RhoA; HBG1/2

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Introduction

β-thalassemia is one of the most common monogenic diseases worldwide, with 1.5% of the global population being carriers of mutations in the β-thalassemia gene (1). β-thalassemia is highly prevalent in tropical and subtropical regions; however, it has become more prevalent globally due to recent migration, making it a growing concern for healthcare systems (2). β-thalassemia is caused by a spectrum of mutations that results in a quantitative reduction of β-globin chains that are structurally normal and is an autosomal recessive hereditary anemia characterized by decreased (β^+-thalassemia) or no (β^0-thalassemia) synthesis of β-globin chains (2,3). However, the clinical phenotype of such patients is not entirely dependent on the type of β-thalassemia mutation. Studies have shown that fetal hemoglobin (HbF, α2γ2) levels in patients with β-thalassemia are associated with severe clinical symptoms. Elevated HbF levels are associated with milder symptoms of β-thalassemia, and elevated HbF levels compensate for HbA deficiency in β-thalassemia (4,5). Identification of genetic variants that alter HbF production in combination with the α-globin genotype predicts disease severity in β-thalassemia, but the generation of personalized genetic risk scores to inform prognosis and guide management requires that a larger set of genetic modifications be identified. Various quantitative trait loci (QTLs) controlling HbF levels have been reported, including the β-like globin gene cluster (11p locus), *HBS1L/MYB* (6q locus), and *BCL11A* (2p locus) (6-8). Polymorphisms in Kruppel-like factor 1 (*KLF1*) (8,9) and Gγ-globin *XmnI* restriction loci (8,10) have also been associated with HbF levels. Epigenetic modifications, such as DNA methyltransferase 1 (*DNMT1*) mutation also play a role in the regulation of HbF expression (11). However, HbF synthesis after birth in patients with β-thalassemia is an extremely complex biological process of which the molecular pathogenesis mechanisms remains understudied.

Recently, there has also been a research interest in long noncoding RNA (lncRNA) (12,13). LncRNAs constitute most of the noncoding RNA transcripts and are longer than 200 nucleotides (14). LncRNAs can regulate the expression of target genes through chromatin modification and remodeling, histone modification, and altered ribosomal localization (15), and can also indirectly regulate at the posttranscriptional level by binding to microRNAs acting as competing endogenous RNAs (ceRNAs) (16). In addition, some lncRNAs can also cleave microRNAs from sequences in intronic or exonic regions during maturation (17). Given these key roles in biological processes, lncRNAs are thought to be involved in the development and progression of many diseases, such as malignancies (18), hematopoietic stem cells and other blood disorders (19), and neurological disorders (20). However, the important regulatory role of lncRNAs in β-thalassemia and other hemoglobinopathies still needs to be studied more intensively.

In our previous studies (12,21), it has been confirmed that multiple lncRNAs were differentially expressed in individuals with high HbF levels, and the expression level of NR_120526 was significantly correlated with HbF. That is, NR_120526 was related to the synthesis of HbF but the specific regulatory mechanisms have not been clarified. Therefore, the differential expression of NR_120526, located on chromosome 11, and its mechanism of action in HbF regulation (*HBG1/2* expression) are the focus of our study. We found that NR_120526 might regulate *HBG1/2* protein expression through the ribosomal protein S6 kinase B1 (RPS6KB1, S6K in *Homo sapiens*), thus providing a potential therapeutic target for enhancing HbF in patients with β-thalassemia. We present the following article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-23-174/rc).

Highlight box

Key findings

- This study demonstrated that long noncoding RNA (lncRNA) NR_120526 negatively regulates the expression of *HBG1/2* through S6K.

What is known and what is new?

- High levels of fetal hemoglobin (HbF) may alleviate clinical symptoms in patients with β-thalassemia. Our previous study has confirmed that lncRNA NR_120526 is differentially expressed in individuals with high HbF levels, showing that lncRNA NR_120526 might be involved in regulating HbF levels (*HBG1/2* gene expression). However, the specific regulatory mechanisms have not been clarified.
- This study revealed the role of lncRNA NR_120526 in suppressing *HBG1/2*.

What is the implication, and what should change now?

- This study provides mechanistic insights into the regulation of HbF by lncRNA NR_120526 and offers new potential therapeutic targets for precision medicine in patients with β-thalassemia.

Table 1 Sequences of RNA antisense probes for ChIRP-MS

Probe number	Probe sequence	Probe locations (start)
NR_120526-P1	CTCATGTGTGTAACCCTTAA	240
NR_120526-P2	GGACATAATGGGCAGAGTGA	321
NR_120526-P3	TGGAGAAAGCAGAGACTAGA	424
NR_120526-P4	GATCCTTTCCAAAATTACCT	575
NR_120526-P5	GGAGCTCCTAATGATCACAA	659
NR_120526-P6	CTGGGAGCAACTTCCAGAAA	749

ChIRP-MS, Chromatin isolation by RNA purification-mass spectrometry.

Methods

Cell culture

Human chronic myelogenous leukemia cell lines (K562) were purchased from the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 100 IU/mL penicillin-streptomycin (Solarbio, Beijing, China) in a 5% CO_2 humidified incubator at 37 ℃.

Chromatin isolation by RNA purification-mass spectrometry (ChIRP-MS) assay and bioinformatics analysis for NR_120526–protein interaction

ChIRP-MS assays were performed to explore the proteins that specifically bind to NR_120526 and the interactions between them. Briefly, K562 cells were washed with phosphate-buffered saline (PBS) and cross-linked with formaldehyde. Next, cells were hybridized with RNA antisense probes labeled with biotin and bound to magnetic beads. Sequences of RNA antisense probes designed for NR_120526 are shown in *Table 1*. The control group was treated with nonspecific RNA antisense probes. After a wash to remove unbound material, nuclease was added to prevent cross-linking. After trypsin hydrolysis, peptide desalting, and other strong denaturing processes, the nonspecific binding proteins were removed and the RNA binding proteins (RBPs) were obtained. Thereafter, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to analyze the obtained RBPs. The raw LC-MS/MS data were searched and quantified using MaxQuant (version 1.5.6.0). The protein database used UniProt HUMAN_2019_10, and the quantitative method used MS1 method. Only unique peptides were used for quantification, not variable modified peptides. The false discovery rate (FDR) at the peptide and protein level was controlled at 0.01. Intensity-based absolute protein quantification (iBAQ) was also performed.

Statistical analysis of the quantitative results of the samples was conducted to obtain the corresponding enriched proteins. Gene Ontology (GO) analysis (http://www.geneontology.org) was applied to analyze the enriched proteins, with the cutoff for P values set at 0.05. For protein-protein interaction (PPI) networks, the online software Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 11.0 (https://string-db.org) was used, while Cytoscape 3.7.2 (https://cytoscape.org/) was used to analyze the interactions of enriched proteins at the gene level.

Chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq) assay to confirm the direct regulation of HBG1/2 by NR_120526

K562 cells immobilized with formaldehyde were sonicated. Next, anti-interleukin enhancer-binding factors 2 (ILF2) and anti-interleukin enhancer-binding factors 3 (ILF3) antibodies (Abcam, Cambridge, UK) were added to obtain enriched ILF2/ILF3–DNA complexes. The enriched DNA fragments were purified and polymerase chain reaction (PCR) was performed using primers designed for the *HBG1/2* gene promoters (*Table 2*). If the corresponding PCR product was amplified, ILF2 and ILF3 bound to

Table 2 List of primers for qRT-PCR

Gene information	Primer sequence	Product length(bp)
RHOA (NM_001664)	Forward: AAGCATTTCTGTCCCAACG	273
	Reverse: TCACAAGACAAGGCACCC	
RPS6KB1 (NM_003952)	Forward: TATGGCAAGGTGTTCCAGGTG	294
	Reverse: CAGGTAGAAGCAGGCCGTATC	
HBG1 (NM_000559)	Forward: GACCCAGAGGTTCTTTGACAGC	137
	Reverse: CCTTGAGATCATCCAGGTGCTT	
HBG2 (NM_000184)	Forward: TGGACCCAGAGGTTCTTTGAC	125
	Reverse: AGGTGCTTTATGGCATCTCCC	
LOC100506675 (NR_120526)	Forward: CCATTATGTCCCACTGAAC	121
	Reverse: GGATATTTGGAGAAAGCAG	
GAPDH (NC_000012)	Forward: TGACTTCAACAGCGACACCCA	121
	Reverse: CACCCTGTTGCTGTAGCCAAA	

qRT-PCR, quantitative real-time polymerase chain reaction.

NR_120526 were considered to directly bind to the γ globin gene promoter to regulate its transcription.

NR_120526 gene knockout studies in K562 cells

Four RNA antisense probes [small guide RNA (sgRNA)] were used to knock out the NR_120526 gene (Table S1). The primers used to detect sgRNAs and the Cruiser digestion information used to screen for active sgRNAs are listed in Table S2. Active sgRNAs were then transfected by lentiviral vector (LV-LOC100506675-sgRNA (04859-1), Shanghai Genechem Co., Ltd., Shanghai China) into K562 cells with normal K562 cells (CON) and K562 cells infected with negative control (NC) virus being used as controls. These experiments were performed according to the protocol of the manufacturer of the Knockout and Mutation Detection Kit (Shanghai Genesci Co., Ltd., Shanghai China). Table S3 lists the validation primers for NR_120526 knockout. Figure S1 displays a schematic diagram of NR_120526 knockout. The details of plasmid information of lentivirus-mediated vectors are shown in Figure S2.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to detect messenger RNA (mRNA) expressions of *HBG1/2*, *RPS6KB1(S6K)*, and Ras homolog family member A (*RhoA*) genes. Total RNA was extracted from K562 cells using TRIzol reagent (Invitrogen Life Technologies, Thermo Fisher Scientific) and then reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocols. qRT-PCR was then conducted using a SYBR Master Mixture kit (Takara Bio, Kusatsu, Japan). *GAPDH* was used as the endogenous control. The primers for qRT-PCR are listed in *Table 2*. All experiments were performed in triplicate.

Western blotting

Western blotting was performed to detect the expression of *HBG1/2*, *S6K*, *RhoA*, phosphorylated *S6K* (p-S6K), and phosphorylated *RhoA* (p-RhoA). After lysis of K562 cells, cellular proteins were extracted and quantified using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins of different molecular weights. The electrophoresed proteins were then transferred onto polyvinylidene fluoride membranes (PVDF; MilliporeSigma, Burlington, MA, USA). The transferred proteins were hybridized with primary and secondary antibodies. The Pierce ECL Western Blotting Substrate Kit

(Thermo Fisher Scientific) was used to analyze the relative amounts of proteins hybridized to the antibodies according to the manufacturer's protocol. Gray-level differences were analyzed to quantify protein expression. The primary antibodies of hemoglobin γ (SC-21756) and GAPDH (sc-32233) were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). The primary antibody of S6K1 (#2708) was purchased from Cell Signaling Technology (CST; Danvers, MA, USA). The primary antibodies of p-S6K1 (ab59208), RhoA (ab54835), and p-RhoA (ab41435) were purchased from Abcam. The secondary antibodies [rabbit immunoglobin G (IgG; sc-2004) and mouse IgG (sc-2005)] were purchased from Santa Cruz Biotechnology, Inc. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA). Data are shown as the mean ± standard deviation from 3 biological independent replicates. Student *t*-test or Mann-Whitney test was used to compare the means between the 2 groups, and $P<0.05$ was considered statistically significant.

Results

Bioinformatics analysis and ChIRP-MS found NR_120526 interacted with ILF2/3

To explore the role of NR_120526 in thalassemia, ChIRP-MS assays were performed to explore the proteins that specifically bind to NR_120526; compared to a control group, a total of 29 proteins were identified (Table S4). GO analysis was subsequently performed to explore the function of these proteins' interactions with NR_120526. GO analysis revealed that in terms of biological processes, these proteins bound to NR_120526 were involved in protein localization to the endoplasmic reticulum and cotranslational protein targeting to the membrane; in terms of cellular components, they were shown to be involved in endoplasmic reticulum chaperone complexes and cell-substrate junctions; and in terms of molecular functions, they were found to be involved in structural components of the ribosome (*Figure 1A*). For PPI analysis, a PPI network was constructed of these 29 proteins using the STRING database (http://string-db.org). The results showed that ILF2 and ILF3 are proteins with strong interactions (*Figure 1B*). To further investigate the interactions at the gene level of the enriched proteins bound to NR_120526, we used Cytoscape 3.7.2 software (GeneMANIA tool) for gene interaction analysis. The results, depicted in *Figure 1C*, show close interactions between all selected genes under the weighted approach of automatic selection, with different lines and colors indicating different types of interactions. *ILF2*, *ILF3*, and *S6K* were found in the network, as well as many other genes. Based on the bioinformatics analyses and ChIRP-MS, ILF2 and ILF3 were found to bind specially to NR_120526, and they also interacted with S6K, which might play a function in regulating the expression of *HBG1/2*. However, the underlying mechanisms still need to be clarified by further research.

ChIP-seq revealed that NR_120526 may regulate the expression of HBG1/2 indirectly

The aforementioned findings suggested that NR_120526 could bind directly to ILF2/3. However, whether ILF2/3 binds directly to the *HBG1/2* gene promoter to regulate its expression remained unclear. We performed ChIP-seq (Appendix 1) and found that ILF2/3 did not interact with the amplified fragments of the *HBG1/2* genes. These results showed that ILF2/ILF3 binding to NR_120526 did not directly interact with *HBG1/2*, indicating that the NR_120526 may regulate the expression of *HBG1/2* indirectly. Moreover, based on the above bioinformatics analysis, both ILF2 and ILF3 interacted with S6K, suggesting that S6K may play a role in NR_120526/ILF2/3-mediated *HBG1/2* expression, but the related mechanism was not clarified. The literature indicates that RhoA is regulated by the *ARHGAP18* (22), which in turn is involved in the regulation of *HBG1/2* expression (23). Therefore, we speculate that S6K may regulate *HBG1/2* expression by affecting RhoA. Furthermore, as the ILF2–ILF3 complex has been recently found to be a target or regulatory factor of S6K protein family (24), we hypothesized that NR_120526 may regulate *HBG1/2* expression through S6K.

Knockout of NR_120526 increased the protein expression of HBG1/2 via S6K

To confirm our hypothesis, we performed a knockout

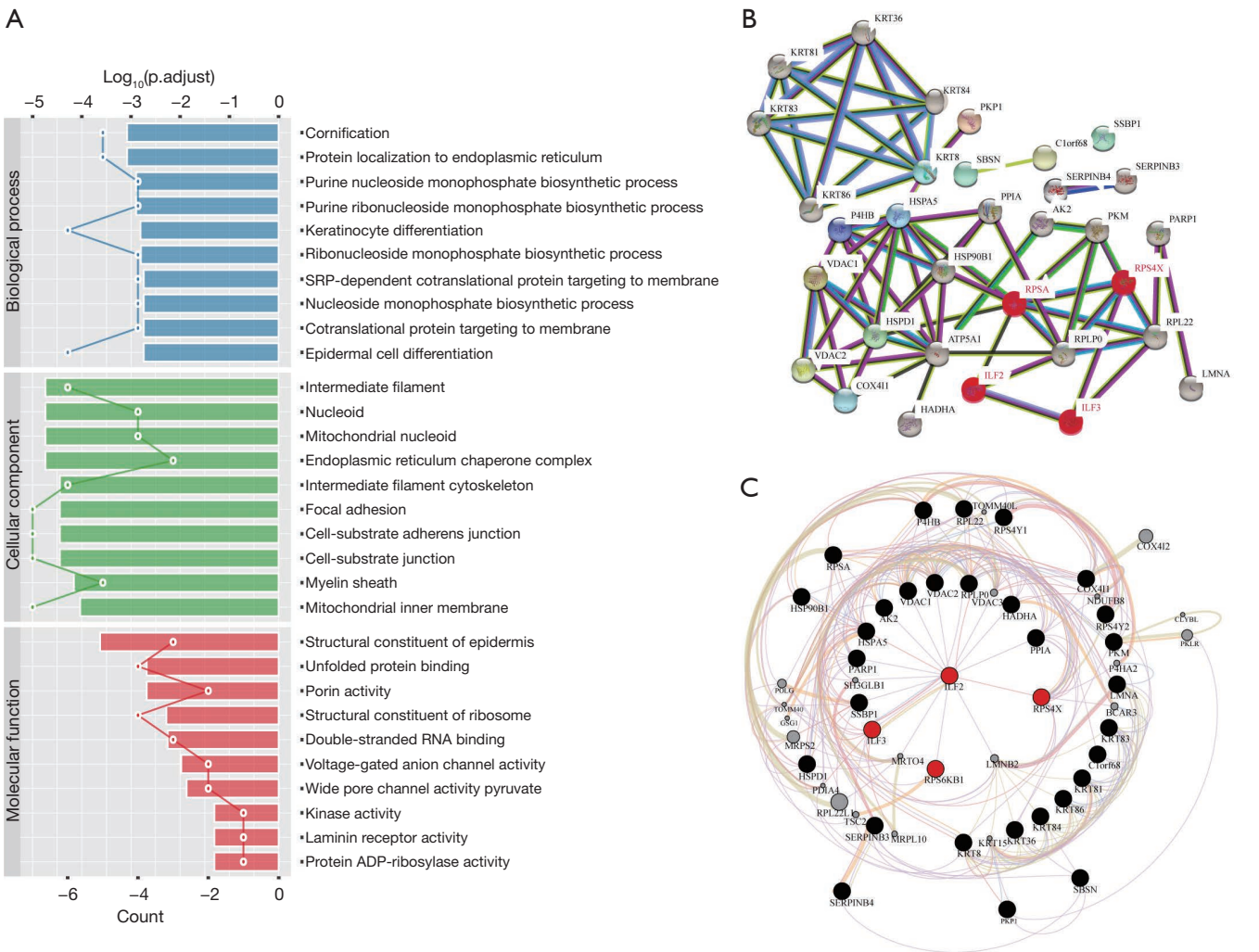

Figure 1 ChIRP-MS and bioinformatics analysis for NR_120526-protein interaction. (A) GO analysis of a total of 29 proteins identified in ChIRP-MS assays that specifically bound to NR_120526 in KO group cells. (B) Interaction network of proteins that specifically bound to NR_120526 in NR_120526-KO group cells. ILF2 and ILF3 were the proteins with significant interactions. (C) Interaction of enriched proteins in K562 cells at the gene level; *ILF2*, *ILF3*, and *RPS6KB1* were the genes with significant interactions. ChIRP-MS, chromatin isolation by RNA purification-mass spectrometry; GO, gene ontology; KO, knockout.

study of NR_120526 gene in K562 cells. The results of the screening of active sgRNAs are shown in Figure S3. The results of the confirmation of the knockout effect of NR_120526 gene are shown in *Figure 2A*. The PCR amplification primers were designed on both ends of the sequence transing the knockout sequence, and a positive amplified band indicated a successful knockout. The PCR results showed that a positive fragment of the target gene was detected in the amplification products of the NR_120526-knockout (KO) group, and no positive fragment was detected in the NC group (*Figure 2A*), indicating that the NR_120526 gene was successfully knocked out. A qRT-PCR experiment was then performed to detect the mRNA expression of *HBG1/2*, *S6K*, and *RhoA* genes, and no statistical difference was observed between the NR_120526-KO group and the NC group (*Figure 2B*).

The total protein expression levels of the target genes, including *HBG1/2*, *S6K*, and *RhoA*, were then analyzed

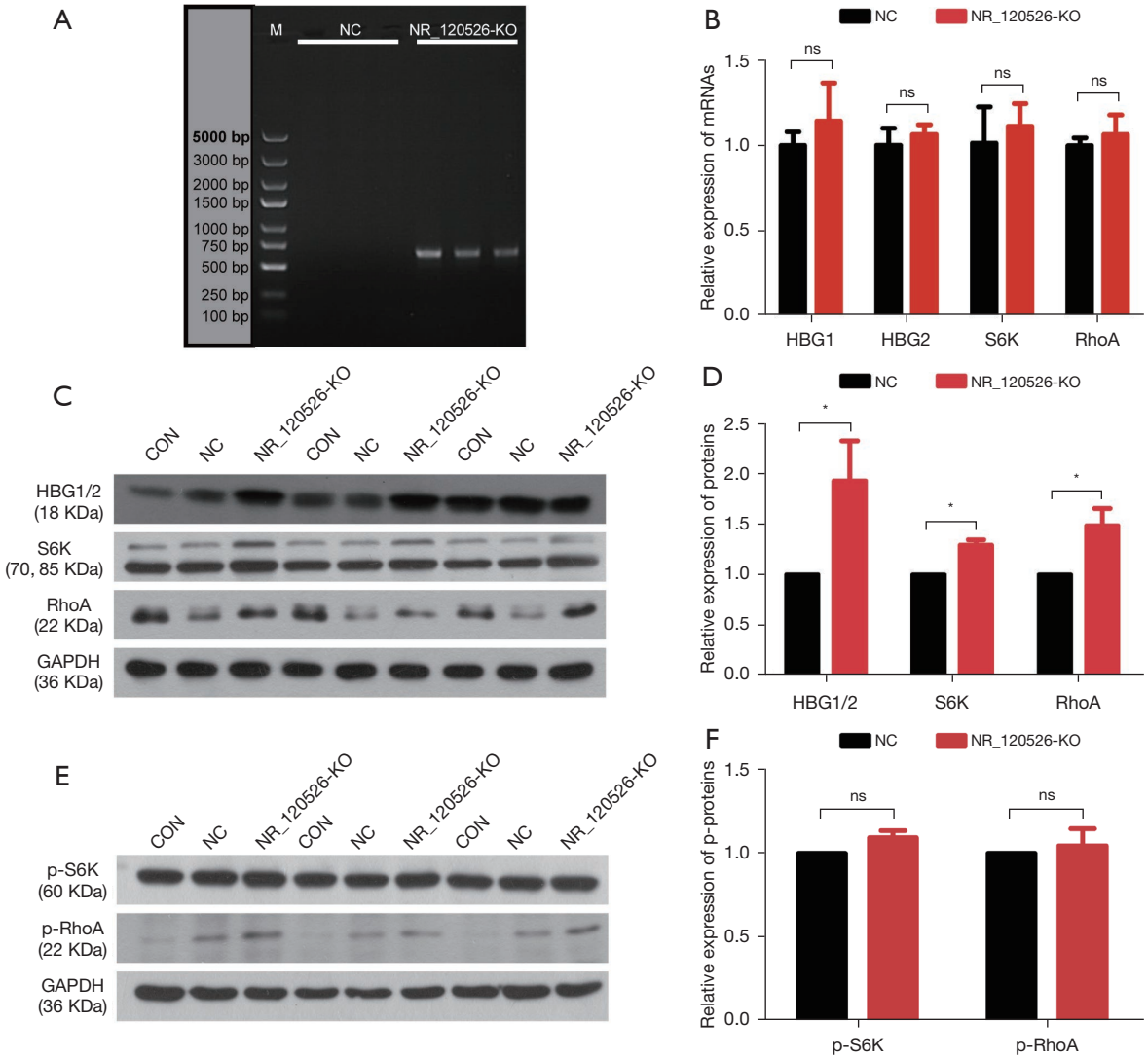

Figure 2 NR_120526 knockout increased *HBG1/2* expression via S6K. (A) Confirming PCR results of successful knockout, an amplified fragment with a length of 653 bp was found while a theoretical negative fragment with a length of 5964 bp was not found in the NR_120526-KO group. (B) mRNA expressions of *HBG1/2* and *RPS6KB1* determined by qRT-PCR. (C,D) Total protein expressions of *HBG1/2* measured using Western blot analysis. (E,F) Phosphorylated protein levels measured using Western blot analysis. *, P<0.05; ns, P>0.05. NC, negative control group; KO, knockout; CON, blank control group; qRT-PCR, quantitative real-time polymerase chain reaction.

by Western blot (*Figure 2C,2D*). As expected, knockout of NR_120526 gene resulted in a significant increase in *HBG1/2* expression levels. Interestingly, S6K and RhoA protein levels were also upregulated compared to the NC group. The phosphorylation levels of the above proteins were also analyzed to identify the functional genes affecting *HBG1/2* expression. There was no statistical difference in p-S6K and p-RhoA between the 2 groups (*Figure 2E,2F*).

Discussion

An important reason for the phenotypic diversity of patients

with β-thalassemia is the marked individual differences in HbF levels, and high levels of HbF have been shown to alleviate clinical manifestations in β-thalassemia patients (25). Reactivating the γ-globin gene (*HBG1/2*) expression to induce HbF is a feasible strategy for the treatment of β-thalassemia. Recent studies have demonstrated that some lncRNAs can influence HbF (*HBG1/2* expression), and play an important role in the progression of β-thalassemia disease (12,26-29). Morrison *et al.* (28) reported that *HMI-LNCRNA* was transcribed from the enhancer region of *HBS1L-MYB* and plays an important role in regulating *HBG* expression, and its downregulation can result in a significant increase in HbF. Ma *et al.* (29) reported that lncRNA *HBBP1* enhances *HBG1/2* expression through the erythroblast transformation-specific (ETS) transcription factor ELK1. Currently, there are few studies on lncRNA NR_120526. The role of lncRNA NR_120526 in the occurrence and development of β-thalassemia has not been reported. This study is the first to demonstrate that NR_120526 negatively regulates the expression of *HBG1/2* through S6K and reduces HbF levels, and is linked to clinical manifestations in patients with β-thalassemia.

The NR_120526 gene was discovered two decades ago (30). It was shown that NR_120526 has low expression in the mammalian testes (31), brain, kidneys (32), and reticulocytes. NR_120526 was also found to be expressed in the stomach tissue of human embryos at the 20th week of gestational age of development (33). In the present study, evidence at the protein level after knockout confirmed that NR_120526 negatively regulated *HBG1/2* expression, but no significant changes in *HBG1/2* transcript levels were observed, suggesting that the regulation of *HBG1/2* by NR_120526 occurs at the protein level. Combining these findings with the results of CHIP-seq, we concluded that NR_120526 may not negatively regulate *HBG1/2* directly but rather indirectly through several complexes or regulatory factors.

Using ChIRP-MS assay, we found that ILF2 and ILF3 specifically bind to NR_120526. ILF2 and ILF3 are constitutively expressed chromatin-interacting proteins (34). Previous studies have shown that ILF2/3 associates with chromatin enrichment at active promoters and strong enhancers (35) and that knockout of ILF2 leads to a significant reduction in mRNAs in adult β-rat and β-mouse individuals (36). More importantly, the ILF2/3 complex is known to be an important member of the protein family that regulates S6K (24). Combining these observations with the results of gene interaction analysis, we hypothesized that NR_120526 may also be involved in the regulation of S6K, which in turn causes changes in the subsequent pathway and affects the expression of *HBG1/2*.

In addition, previous studies have confirmed that knockdown *ARHGAP18* results in increased *HBG1/2* expression (23), while knockout of *ARHGAP18* enhances RhoA activity (22,37). These results suggest that *ARHGAP18*/RhoA is also involved in the regulation of *HBG1/2* expression. Based on the upregulation of S6K after the knockout of NR_120526, we confirmed that NR_120526 inhibited the expression of *S6K*. Our study suggests that NR_120526 may inhibit S6K, resulting in the downregulation of RhoA and the decrease of *HBG1/2* expression in turn. Double knockout/knockdown (i.e., knocking out/down S6K or RhoA along with lncRNA NR-120526) was expected to perform to confirm the results in the future if available, and the mechanisms by which NR_120526 inhibits S6K and by which RhoA activation induces the expression of *HBG* still remain to be investigated further also.

The present study enriches the regulatory network of *HBG1/2* gene expression and provides new clinical insights into the phenotypic diversity of β-thalassemia. In the future, we can try to up-regulate the expression of *HBG1/2* expression by knockout NR_120526, so as to improve the level HbF and ultimately alleviate the clinical phenotype of thalassemia patients. Notably, although the application of lncRNAs in β-thalassemia is promising, it also faces many problems and challenges. For example, does this lncRNA play the same functions in cells of different origins, especially those showing low basal HbF levels? In addition, current studies on the effect of lncRNA on HbF are mostly limited to *in vitro* experiments and lack *in vivo* experiments. In the future, other cell lines such as primary human erythroid cells derived from hematopoietic stem cells or even animal models will be needed to verify its function and observe the results. Furthermore, clinical transformation of lncRNAs therapy also relies on interdisciplinary collaboration to improve tolerability, specificity and delivery. We believe that these challenges will eventually be

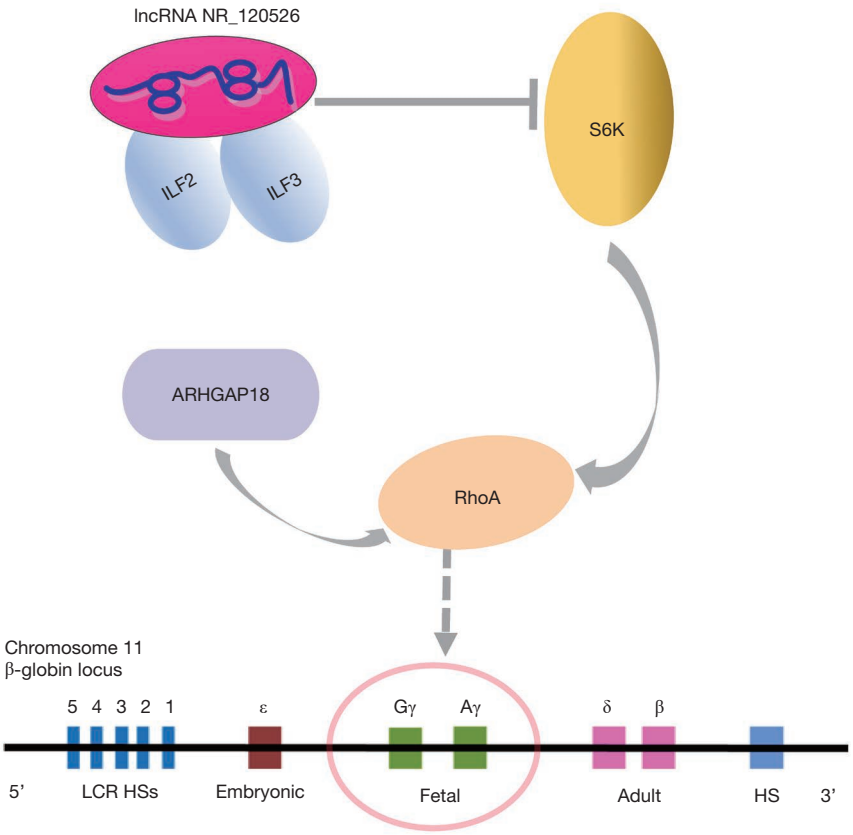

Figure 3 Mechanism diagram of NR_120526 negatively regulating *HBG1/2* expression.

overcome as science advances.

Conclusions

In summary, this study revealed the role of lncRNA NR_120526 in suppressing *HBG1/2* (*Figure 3*) and potentially provides new targets for the treatment of β-thalassemia.

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Footnote

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Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-23-174/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.

com/article/view/10.21037/tp-23-174/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Kattamis A, Kwiatkowski JL, Aydinok Y. Thalassaemia. Lancet 2022;399:2310-24.
2. Taher AT, Weatherall DJ, Cappellini MD. Thalassaemia. Lancet 2018;391:155-67.
3. Wu C, Zhang X, Ye X, et al. Co-existence of myeloproliferative neoplasias and β-thalassemia with IVS-2-654 mutation-a case report. Transl Cancer Res 2020;9:2069-73.
4. Forget BG. Molecular basis of hereditary persistence of fetal hemoglobin. Ann N Y Acad Sci 1998;850:38-44.
5. Sankaran VG, Nathan DG. Thalassemia: an overview of 50 years of clinical research. Hematol Oncol Clin North Am 2010;24:1005-20.
6. Uda M, Galanello R, Sanna S, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. Proc Natl Acad Sci U S A 2008;105:1620-5.
7. Lettre G, Sankaran VG, Bezerra MA, et al. DNA polymorphisms at the BCL11A, HBS1L-MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci U S A 2008;105:11869-74.
8. Zakaria NA, Islam MA, Abdullah WZ, et al. Epigenetic Insights and Potential Modifiers as Therapeutic Targets in β-Thalassemia. Biomolecules 2021;11:755.
9. Perkins A, Xu X, Higgs DR, et al. Krüppeling erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. Blood 2016;127:1856-62.
10. Galanello R, Sanna S, Perseu L, et al. Amelioration of Sardinian beta0 thalassemia by genetic modifiers. Blood 2009;114:3935-7.
11. Gong Y, Zhang X, Zhang Q, et al. A natural DNMT1 mutation elevates the fetal hemoglobin level via epigenetic derepression of the γ-globin gene in β-thalassemia. Blood 2021;137:1652-7.
12. Jia W, Jia S, Chen P, et al. Construction and Analysis of a Long Non-Coding RNA (lncRNA)-Associated ceRNA Network in β-Thalassemia and Hereditary Persistence of Fetal Hemoglobin. Med Sci Monit 2019;25:7079-86.
13. Finotti A, Fabbri E, Lampronti I, et al. MicroRNAs and Long Non-coding RNAs in Genetic Diseases. Mol Diagn Ther 2019;23:155-71.
14. Chi Y, Wang D, Wang J, et al. Long Non-Coding RNA in the Pathogenesis of Cancers. Cells 2019;8:1015.
15. Deans C, Maggert KA. What do you mean, "epigenetic"? Genetics 2015;199:887-96.
16. Rahaman M, Mukherjee M, Bhattacharya S, et al. Exploring the crosstalk between long non-coding RNAs and microRNAs to unravel potential prognostic and therapeutic biomarkers in β-thalassemia. Mol Biol Rep 2022;49:7057-68.
17. Shi Y, Wang Y, Luan W, et al. Long non-coding RNA H19 promotes glioma cell invasion by deriving miR-675. PLoS One 2014;9:e86295.
18. Zhao Y, Zhu Z, Shi S, et al. Long non-coding RNA MEG3 regulates migration and invasion of lung cancer stem cells via miR-650/SLC34A2 axis. Biomed Pharmacother 2019;120:109457.
19. Cruz-Miranda GM, Hidalgo-Miranda A, Bárcenas-López DA, et al. Long Non-Coding RNA and Acute Leukemia. Int J Mol Sci 2019;20:735.
20. Li L, Zhuang Y, Zhao X, et al. Long Non-coding RNA in Neuronal Development and Neurological Disorders. Front Genet 2018;9:744.
21. Lai K, Jia S, Yu S, et al. Genome-wide analysis of aberrantly expressed lncRNAs and miRNAs with associated co-expression and ceRNA networks in β-thalassemia and hereditary persistence of fetal hemoglobin. Oncotarget 2017;8:49931-43.
22. Maeda M, Hasegawa H, Hyodo T, et al. ARHGAP18, a GTPase-activating protein for RhoA, controls cell shape, spreading, and motility. Mol Biol Cell 2011;22:3840-52.
23. He Y, Luo J, Chen Y, et al. ARHGAP18 is a novel gene under positive natural selection that influences HbF levels

in β-thalassaemia. Mol Genet Genomics 2018;293:207-16.
24. Pavan IC, Yokoo S, Granato DC, et al. Different interactomes for p70-S6K1 and p54-S6K2 revealed by proteomic analysis. Proteomics 2016;16:2650-66.
25. Thein SL. Molecular basis of β thalassemia and potential therapeutic targets. Blood Cells Mol Dis 2018;70:54-65.
26. Fakhr-Eldeen A, Toraih EA, Fawzy MS. Long non-coding RNAs MALAT1, MIAT and ANRIL gene expression profiles in beta-thalassemia patients: a cross-sectional analysis. Hematology 2019;24:308-17.
27. Ma J, Liu F, Du X, et al. Changes in lncRNAs and related genes in β-thalassemia minor and β-thalassemia major. Front Med 2017;11:74-86.
28. Morrison TA, Wilcox I, Luo HY, et al. A long noncoding RNA from the HBS1L-MYB intergenic region on chr6q23 regulates human fetal hemoglobin expression. Blood Cells Mol Dis 2018;69:1-9.
29. Ma SP, Xi HR, Gao XX, et al. Long noncoding RNA HBBP1 enhances γ-globin expression through the ETS transcription factor ELK1. Biochem Biophys Res Commun 2021;552:157-63.
30. Deloukas P, Schuler GD, Gyapay G, et al. A physical map of 30,000 human genes. Science 1998;282:744-6.
31. Bu D, Luo H, Jiao F, et al. Evolutionary annotation of conserved long non-coding RNAs in major mammalian species. Sci China Life Sci 2015;58:787-98.
32. Duff MO, Olson S, Wei X, et al. Genome-wide identification of zero nucleotide recursive splicing in Drosophila. Nature 2015;521:376-9.
33. Szabo L, Morey R, Palpant NJ, et al. Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development. Genome Biol 2015;16:126.
34. Guan D, Altan-Bonnet N, Parrott AM, et al. Nuclear factor 45 (NF45) is a regulatory subunit of complexes with NF90/110 involved in mitotic control. Mol Cell Biol 2008;28:4629-41.
35. Wu TH, Shi L, Lowe AW, et al. Inducible expression of immediate early genes is regulated through dynamic chromatin association by NF45/ILF2 and NF90/NF110/ILF3. PLoS One 2019;14:e0216042.
36. Karmakar S, Mahajan MC, Schulz V, et al. A multiprotein complex necessary for both transcription and DNA replication at the β-globin locus. EMBO J 2010;29:3260-71.
37. Vautrin-Glabik A, Botia B, Kischel P, et al. IP(3)R3 silencing induced actin cytoskeletal reorganization through ARHGAP18/RhoA/mDia1/FAK pathway in breast cancer cell lines. Biochim Biophys Acta Mol Cell Res 2018;1865:945-58.

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