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An Enhancer LncRNA Regulates NFE2 Expression and Proliferation in Human Leukemic K562 Cells*

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Abstract **Objective** Transcription factor NFE2 was observed abnormal expression in myeloproliferative neoplasm (MPN) patients. However, how *NFE2* is transcriptionally regulated remains ambiguous. This study aims to explore the elements and molecular mechanisms involved in the transcriptional regulation of *NFE2*. **Methods** Active enhancers were predicted by public NGS data and conformed experimentally *via* dual luciferase reporter assay. After that, PRO-seq and GRO-seq data was used to detect enhancer RNAs transcribed from these enhancers. RACE was utilized to clone the full length enhancer RNA (eRNA) transcripts, and RT-qPCR was used to measure their expression in different leukemia cell lines as well as the transcript levels during induced differentiation. Finally, to investigate the molecular function of the eRNA, overexpression and knockdown of the eRNA *via* lentivirus system was performed in K562 cells. **Results** We identified three enhancers regulating *NFE2* transcription, which located at −3.6k, −6.2k and +6.3k from *NFE2* transcription start site (TSS) respectively. At the −3.6k enhancer, we cloned an eRNA transcript and characterized that as a lncRNA which was expressed and located in the nucleus in three types of leukemia cell lines. When this lncRNA was overexpressed, expression of *NFE2* was upregulated and decreases of K562 cell proliferation and migration ability were observed. While knocking down of this lncRNA, the level of NFE2 decreases correspondingly and the proliferation ability of K562 cells increases accordingly. **Conclusion** We identified an enhancer lncRNA that regulates *NFE2* transcription positively and suppresses K562 cell proliferation.

Key words NFE2, enhancer, lncRNA, cellular proliferation
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NFE2 is a transcription factor expressed almost exclusively in the hematopoietic system[1-2], and was first identified in extracts of erythroid cells because of its DNA binding activities[3]. The most clearly articulated role of NFE2 is its regulation of megakaryocyte biogenesis and function. Complete deficiency of NFE2 leads to defective platelet synthesis resulting in over 90% of mouse pups dying from hemorrhage[4-5]. In addition, NFE2 has been shown to play a role in the maturation of the erythroid lineage[6]. Clinically, overexpression of *NFE2* has been found in patients with polycythemia vera (PV), a disease characterized by the overproduction of red blood cells and in some cases megakaryocytes and platelets[7]. Recently, the relevance of aberrant *NFE2* expression to tumors has been noted. The *NFE2* gene represents a common integration site for the Friend erythroleukemia virus in mice. This suggests that loss of NFE2 function in this model system may contribute to leukemogenesis[8]. More direct evidence of this is that the reintroduction of NFE2 into a non-producer

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erythroleukemic cell line resulted in significantly reduced cell growth rates *in vitro* and *in vivo*[9]. Surprisingly, in some recent reports, overexpression of NFE2 has been significantly correlated with the development of myeloproliferative neoplasms. In patients with myeloproliferative neoplasms (MPN), increased expression of NFE2 has been observed[10]. In addition, insertional and deletional mutations in *NFE2* have been identified in MPN patients. The truncated NFE2 protein, despite the loss of DNA binding activity, enhances the activity of wild-type NFE2 and led to erythrocytosis, thrombocytosis and neutrophilia[11].

Early studies identified two alternative promoters of *NFE2,* with the 1F (fetal) promoter used more frequently in the fetal liver and the 1A (adult) promoter used with greater frequency in the adult bone marrow[12]. Thus, all promoters mentioned in this paper refer to the 1A promoter. Expression of *NFE2* is regulated by cytokines or growth factors. In megakaryocytes, interleukin-1β and platelet-derived growth factor (PDGF) were found to facilitate *NFE2* expression[13-15] while interleukin-4 had the contrary effect[16]. *NFE2* expression is also controlled by other transcription factors. GATA1[17] as well as RUNX1[18] are the major regulators of *NFE2* transcriptional activity and function in maturation of megakaryocytes and platelet generation. NF-κB, as a negative regulator of *NFE2*, may be responsible for maintaining low levels of *NFE2* in early erythroid progenitors[19]. In MPN patients with *NFE2* overexpression, AML1 has been shown to upregulate NFE2[20]. A typical positive feedback mechanism controls *NFE2* in MPN patients, where overexpressed NFE2 leads to an increase of epigenetic enzyme JMJD1C, and more JMJD1C in turn enhances *NFE2* transcription[21].

Here, we identified *cis*-regulatory elements associated with the *NFE2* gene, and we cloned an enhancer RNA (eRNA) transcript of one of the enhancers. We identified this eRNA as a lncRNA and demonstrated its regulatory function on *NFE2*. In addition, we found this lncRNA suppressed the proliferation of K562 cells.

1 Materials and methods

1.1 Cell culture and treatment

K562 (CCL-243, ATCC), HL-60 (CCL-240, ATCC), and U937 (CRL-1593.2, ATCC) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (10099141, Gibco) and 1% penicillin-streptomycin-glutamine solution (SV30082.01, Hyclone). All cell lines were cultured at 37℃ in a humidified atmosphere containing 5% CO_2. For treatment, K562, HL-60, or U937 cells were seeded at a density of 1×10^8/L, the final concentrations of 30 μmol/L hemin (51280, Sigma-Aldrich), 0.16 μmol/L all-trans-retinoic acid (ATRA) (R2625, Sigma-Aldrich) or 2 μmol/L 12-O-tetradecanoylphorbol 13-acetate (TPA) (S1819, Beyotime) were added to the media to induce erythroid, monocytic, and granulocytic differentiation, respectively, and then cultured for 48 h.

1.2 Chromatin immunoprecipitation （ChIP）

ChIP experiments were performed as previously described[22]. In brief, 1×10^7 cells were fixed in 1% formaldehyde for 10 min at room temperature and sonicated to shear the chromatin. Immunoprecipitation of crosslinked chromatin was performed overnight at 4℃ with H3K27ac and H3K4me1 antibodies (ab4729 and ab8895, Abcam). An equal amount of isotype immunoglobulin G (IgG) was used as background control. Primers for ChIP-qPCR are shown in Table S1.

1.3 Dual−luciferase reporter assay

The *NFE2* promoter (chr12: 54 300 825−54 301 741, reverse complement, hg38) was amplified, digested with *Xho*I and *Bgl*II, and cloned into the pGL4 luciferase reporter vector (E6651, Promega). The upstream regions −3.6k (chr12: 54 303 806−54 305 005, reverse complement, hg38), −6.2k (chr12: 54 306 477−54 307 773, reverse complement, hg38) and downstream +6.3k (chr12: 54 294 275−54 295 160, reverse complement, hg38) were amplified and inserted into pGL4-*NFE2*-promoter mentioned above *via Xho*I and *Sac*I digestion. Then these constructs were cotransfected into HEK-293T cells with pRL-TK vector (E2241, Promega) using TurboFect Transfection Reagent (R0531, ThermoFisher) according to the manufacturer’s instructions. Dual-Luciferase Reporter Assay System (E1910, Promega) was used to measured luciferase activity on a FlexStation 3 multimode microplate reader. All assays were performed in triplicate and repeated at least 3 times.

1.4 Rapid−amplification of cDNA ends （RACE）

Total RNA was isolated using TRIzol reagent (15596018, Invitrogen). Then 1 μg of total RNA was reverse-transcribed and amplified with SMARTer RACE 5'/3' Kit (634858, Takara). Amplified cDNA fragments were inserted into pEASY-Blunt Zero Cloning Vector (CB501, TransGen, China). After that, the constructs were sequenced and blasted to reference genome sequence to determine if it is the required sequence. Primers for RACE are shown in Table S1, and spliced −3.6k-lncRNA sequence was shown in Document S1.

1.5 Quantitative real−time PCR analysis

Extracted total RNA was reverse-transcribed to cDNA using PrimeScript™ RT reagent kit with gDNA eraser (RR047A, TaKaRa) and then diluted to 1 mg/L. To measure the levels of candidate RNAs, the iTaq™ Universal SYBR Green Supermix (1725124, Bio-Rad) and a Light Cycler 480II real-time PCR machine were used. Data was normalized to human GAPDH transcripts. Relative quantitation was carried out by the comparative threshold cycle (CT) method. The primer sequences are listed in Table S1.

1.6 Nucleus extraction and isolation

About 1×10^7 cells were collected by centrifugation, washed twice with 10 ml of pre-cooled PBS, centrifuged at 1 000 *g* for 5 min. The supernatant was discarded, the cells were resuspended in 800 μl of hypotonic buffer and incubated on ice for 10 min to swell the cells. The cells were adequately broken up using a Dounce homogenizer after which 10% triton-100 was added and thoroughly mixed. After centrifugation at 1 500 *g* for 5 min at 4℃, the supernatant and the precipitate were collected separately.

1.7 Lentivirus production and cell transduction

For creation of the overexpression vectors, −3.6k-lncRNA sequence was cloned and ligated into the pLVX-IRES-Neo vector (632181, Clontech) which had been digested by *Eco*RI and *Bam*HI. For knockdown vectors, oligonucleotides for sh−3.6k-lncRNA were annealed and ligated into the pLKO.1-puro vector (8453, Addgene) digested with *Eco*RI and *Age*I. After that, packaging plasmids pCMV-VSVG (8454, Addgene), pCMV-DR8.91 (PVT2323, Life Science Market) and the relevant lentiviral transfer vectors were cotransfected into HEK-293T cells in a mass ratio of 1∶9∶10. After 48 h, the media containing lentivirus particles were collected. To infect K562 cells, hexadimethrine bromide (H9268, Sigma-Aldrich) was added to a final concentration of 8 mg/L. An appropriate number of cells were cultured in this lentivirus-containing medium and collected after 72 h.

1.8 Cell proliferation assay

Collected cells were diluted to 2×10^7/L with complete culture media. One hundred μl of cell suspension was added to each well of the 96-well plate and three replicates of each sample were set up. The wells without the addition of cells were used as blank controls and transferred to the cell culture incubator for incubation. Three h before each test time point, 10 μl of CCK-8 (C0042, Beyotime) solution was added to each well and continued incubation in the cell incubator. The absorbance was measured at 450 nm and the mean value calculated.

1.9 Cell migration assay

Six hundred μl of complete media was added to the wells of a 24-well plate. Cells were resuspended in serum-free medium to 5×10^8/L and 100 μl of cell suspension was seeded into the transwell upper chamber, then the chambers were placed into the 24-well plate. After 16 h of incubation, the medium was removed from the chambers and the chambers were removed with forceps and washed twice in a beaker containing PBS. The chambers were placed in a new 24-well plate, 1 ml of 3.7% paraformaldehyde was added and incubated for 20 min at room temperature. Residual formaldehyde was removed from the chambers, after that, the chambers were washed twice with PBS and placed in a new 24-well plate. The plates were incubated for 15 min at room temperature and protected from light with 1 ml of Giemsa staining solution. Wash the chambers with PBS and place in a new 24-well plate, gently wiping away any cells that have not migrated from the upper chamber with a cotton swab. Photographs were taken under an inverted microscope and the number of cells in different fields of view was counted and averaged.

1.10 Bioinformatics and statistical analysis

The ChIP-seq datasets downloaded from the ENCODE project were visualized with the IGV. PRO-seq and GRO-seq raw sequencing data were downloaded and aligned to hg38 reference genome. The resulting bam files were converted to bigwig format and then visualized. Data was obtained from at

least three independent experiments and are expressed as the *means*±standard deviation (*SD*). Statistical significance (P<0.05) for all the above experiments was assessed by Student's two-tailed *t*-test.

2 Results

2.1 Identification of 3 enhancers for the *NFE2* promoter

The tissue specificity of *NFE2* expression almost exclusively in hematopoietic progenitors and differentiated cells of the erythroid, megakaryocytic, granulocytic and mast cell lineages means that its transcription is tightly regulated[1-2]. To investigate its transcriptional regulation mechanism, we predicted enhancers that might be involved in regulating NFE2 expression. Combining two histone modifications, H3K27ac and H3K4me1, with chromatin accessibility analysis such as DNase I hypersensitive sites is commonly used to predict enhancers[23-24]. Besides, transcriptional co-activator CBP and p300 are identified to bind at enhancers[25]. Therefore, we downloaded ChIP-seq data of H3K27ac (ENCSR000 AKP), H3K4me1 (ENCSR000AKS), CBP (ENCSR00 0ATT) and p300 (ENCSR000EGE) as well as DNase-seq (ENCSR000EKS) and ATAC-seq (ENCSR868 FGK) data of K562 cell line which displays a high level of *NFE2* (Figure 1a). These signals indicate three potential enhancers located at upstream 3.6k, 6.2k and downstream 6.3k basepairs from the *NFE2* transcription start site (TSS). ChIP-qPCR was further performed to confirm the H3K27ac (Figure 1b) and H3K4me1 (Figure 1c) enrichment at these regions.

Dual luciferase reporter experiment was performed to further verify the enhancer activity of these three regions. DNA fragments containing the corresponding H3K27ac and H3k4me1 peaks, named −3.6k, −6.2k and +6.3k, respectively, were cloned and inserted upstream of the *NFE2* promoter controlling a firefly luciferase reporter gene (Figure 1d) and then the constructs were transfected into HEK-293T cells. Compared with the non enhancer group, the −3.6k, −6.2k and +6.3k fragments all showed significantly increased luciferase activity (Figure 1e). Among these, +6.3k fragment displayed the highest enhancer activity. Taken together, these data suggest that these fragments contain enhancer sequences for the *NFE2* promoter.

2.2 Two enhancers show bidirectional transcription signals

Enhancer transcription was found to be a widespread occurrence in the genome[26-27]. Bidirectional transcription is the main form of enhancer transcription[27-28]. eRNAs have been proved to be involved in the regulation of target genes in a variety of ways, for examples, enhancing the formation or stability of the enhancer-promoter loops[29-31], assisting in the recruitment of transcription factors and co-regulators and regulating their activity[32-34], facilitating RNA Pol II pause-release to promote transcription elongation[35]. Thus, we explored whether these three enhancer regions have transcriptional signals. PRO-seq (GSM1480327) and GRO-seq (GSM1480325) data were downloaded and used to evaluate the likelihood of transcripts being present in these regions (Figure 2a). These data demonstrate that both −3.6k and −6.2k enhancers have a bidirectional transcriptional signal. Based on these signals, we successfully cloned the eRNA in one direction of the −3.6k region, referred to below as −3.6k− , by the RACE technique (Figure 2b). A negative sign denotes that the sequence of this eRNA is consistent with the minus strand sequence of the human reference genome. In brief, this eRNA is 1 859 nt long and extends from 3 779 bp to 1 920 bp upstream of *NFE2* TSS. Considering that we used oligo T to enrich the eRNA and that there is a typical polyA signal 16 bp away from the 3' end, this suggests that the eRNA molecule has a polyA structure (Document S1). Subsequently, using the protein coding potential prediction tool CPC2 (coding potential calculator 2) to predict its coding potential, we found that this eRNA do not have coding potential.

We then explored the expression of −3.6k-lncRNA in different types of leukemic cells (Figure 2c). In K562, HL-60, and U937 cell lines expressing NFE2, −3.6k-lncRNA was present in lower abundance of transcript level. To further clarify the regulatory function of the lncRNA on NFE2, we used hemin, TPA and ATRA to induce differentiation of K562, U937 and HL-60 cells for 48 h, respectively. After induction of erythroid differentiation in K562 cells using hemin, *NFE2* was significantly increased and −3.6k-lncRNA level had a corresponding variation (Figure 3a). However, after induction of U937 and HL-60 cells, the transcript levels of both *NFE2* and

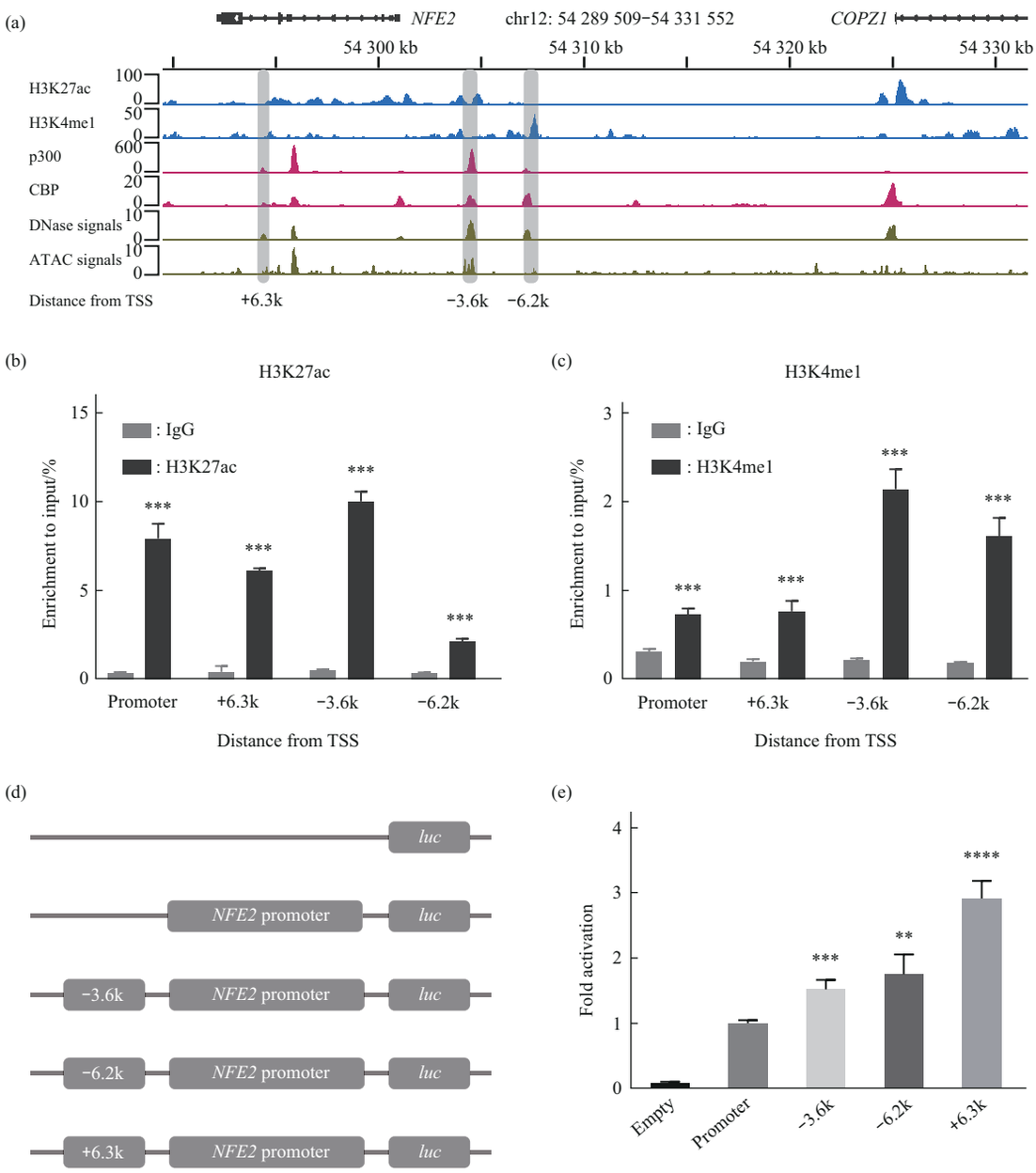

Fig. 1　The −3.6k, −6.2k and +6.3k regions of *NFE2* are active enhancers

(a) ChIP-seq data display that 3 regions are enriched for active histone modification, transcription co-activators and chromatin accessible signals located at −3.6k, −6.2k and +6.3k from *NFE2* TSS. Data were downloaded from the ENCODE consortium and visualized using the IGV genome browser. (b, c) ChIP-qPCR was performed to detect the enrichment of H3K27ac and H3K4me1 at indicated regions in K562 cells. Values are represented as a percentage of input normalized by IgG control. (d) A schematic view displays constructs for enhancer activity assay. The pGL4.10-*NFE2*-promoter was used as control. (e) Dual-luciferase reporter assay was performed to show the enhancer activity of indicated regions in HEK-293T cells. Firefly luciferase signals were normalized to renilla luciferase signals. Data for graphing were obtained from the *means±SD* of three individual experiments, and *P* values were calculated using Student's *t*-test (***P*<0.01; ****P*<0.001; *****P*<0.000 1) in (b), (c) and (e).

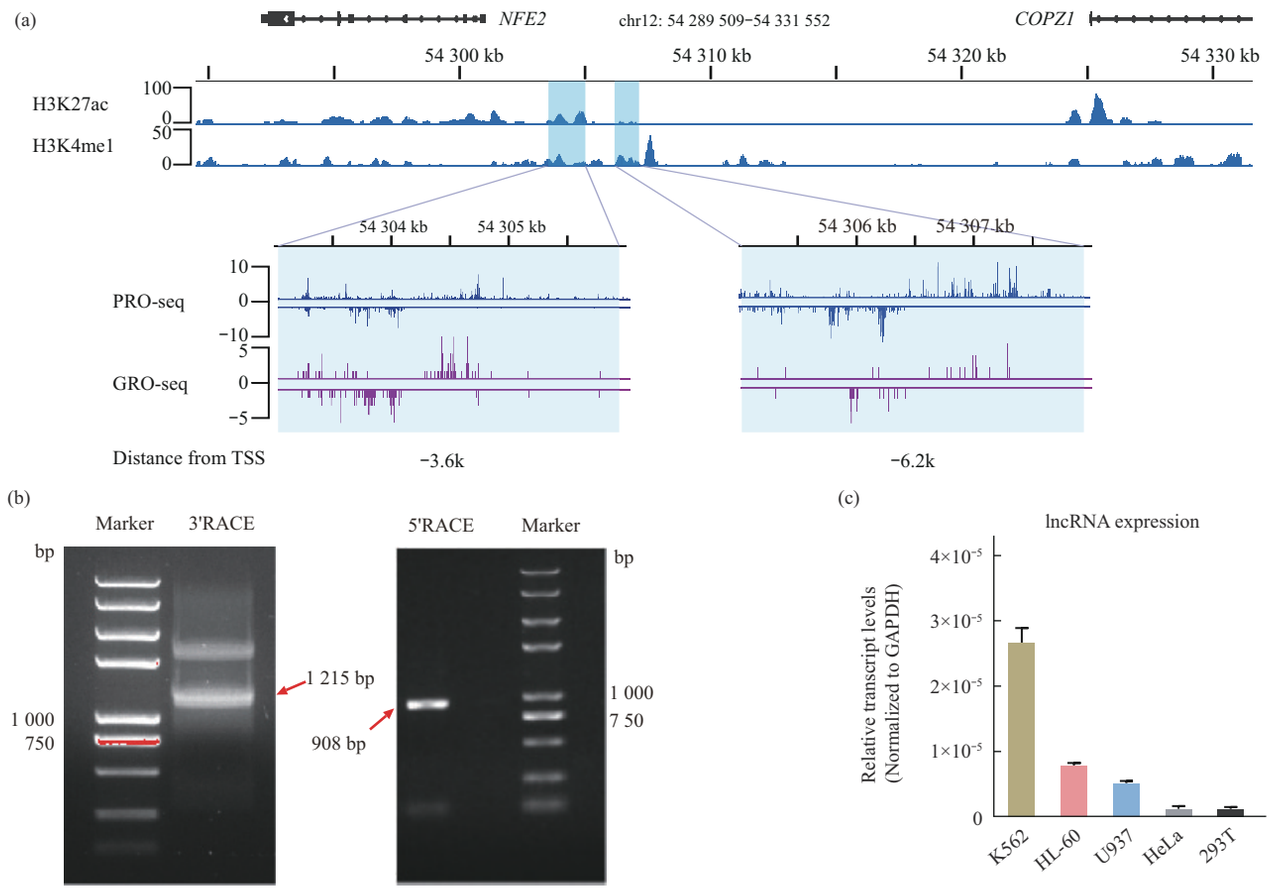

Fig. 2　−3.6k and −6.2k enhancers transcribe bidirectionally producing enhancer RNAs

(a) Nascent RNA-seq data show that −3.6k and −6.2k enhancers undergo bidirectional transcription. Raw data was download from GEO database[36] and then aligned to hg38 human genome after trimming. (b) The 5' and 3' end sequences of the −3.6k-eRNA were identified by 5' RACE and 3' RACE with an overlap, respectively. A full-length eRNA sequence of 1 859 nt was obtained after splicing. (c) The expression of −3.6k-lncRNA in indicated cells. RT-qPCR showing the relative eRNA transcript levels (normalized to GAPDH) in indicated cell lines. Data for graphing were obtained from the *means*±*SD* of three individual experiments. −3.6k-lncRNA expresses in different cell lines.

−3.6k-lncRNA decreased (Fig 3b, c). These results are consistent with the reported levels of NFE2 at different stages of hematopoietic cell differentiation, where NFE2 is highly expressed in erythrocytes but lower in monocytes and granulocytes. Compared to the adjacent gene *COPZ1*, −3.6k-lncRNA maintained a consistent pattern of change with *NFE2* after induction in different cell lines, providing clues that this lncRNA regulates *NFE2* gene transcription.

Depending on the localization and specific interactions with DNA, RNA and proteins, lncRNAs exhibit different functions that ultimately affect gene expression in a variety of biological and physiological environments[37]. Therefore, we then isolated cytoplasmic and nuclear fractions of the above 3 cell lines and determined the content of −3.6k-lncRNA in the different fractions by RT-qPCR as a judgment of the subcellular localization of this lncRNA. GAPDH and 18S rRNA were used as cytoplasmic marker, U1 and MALAT1 were used as cytosolic marker. Our results suggest that in all 3 cell lines −3.6k-lncRNA is localized in the nucleus (Figure 3d−f). Compared to approximately 90% −3.6k-lncRNA localization in the nucleus in K562 cells, this value is slightly lower in HL-60 and U937 cells, which may be due to lower accuracy as a result of lower abundance of this lncRNA in these two cell lines.

2.3　−3.6k−lncRNA suppresses cell proliferation and migration of K562 cells

To further understand the role of −3.6k-lncRNA in leukemia cells, we separately overexpressed and

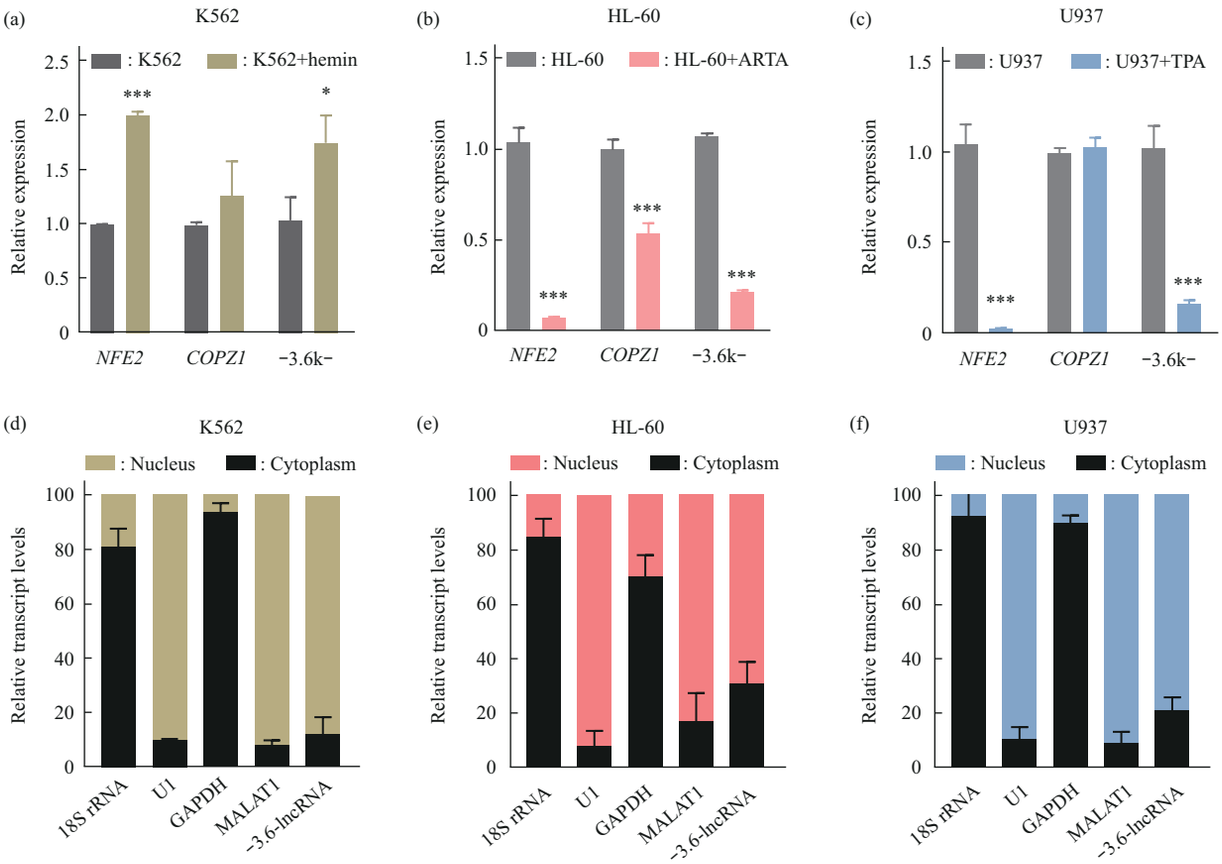

Fig. 3　Changes of −3.6k−lncRNA expression after induced differentiation of leukemia cell lines and subcellular localization of this lncRNA

(a−c) Changes of −3.6k-lncRNA, *NFE2* and *COPZ1* expression during differentiation of leukemia cells. K562, HL-60, and U937 cells were treated with hemin (30 μmol/L), ATRA (0.16 μmol/L) or TPA (2 μmol/L) for erythroid, granulocytic differentiation, and monocytic for 48h, respectively. RT-qPCR was performed to quantify indicated transcripts abundance. GAPDH was used as an internal control. (d−f) Cellular localization of −3.6k-lncRNA. Cytoplasmic and nuclear fractions were extracted from K562, U937 and HL-60 cells, and RT-qPCR results indicating the distribution of −3.6k-lncRNA, U1 (a nuclear marker), MALAT1 (a nuclear marker), 18S rRNA (a cytoplasmic marker) and GAPDH (a cytoplasmic marker) in the nuclear and cytoplasmic fractions. Data for graphing were obtained from the *means*±*SD* of three individual experiments, and *P* values were calculated using Student's *t*-test (**P*<0.05; ****P*<0.001).

knocked down the lncRNA in K562 cells by a lentiviral system. RT-qPCR of total RNA shows successful overexpression and knockdown of −3.6k-lncRNA (Figure 4a, d). Not surprisingly, overexpression of −3.6k-lncRNA increased the transcript level of *NFE2* (Figure 4b) and a significant decline of *NFE2* was found after knockdown (Figure 4e) providing direct evidence that this lncRNA regulates *NFE2* transcription. We then examined the role of −3.6k-lncRNA in the proliferation and migration of K562 cells. The optical density of infected cells, which responds to the number of cells, was measured by the CCK-8 assay every 24 h after seeding. Our results show that overexpression of −3.6k-lncRNA significantly reduces the proliferative capacity of K562 cells (Figure 4c), while the rate of cellular proliferation is accelerated in cells with decreased lncRNA levels (Figure 4f). In addition, migratory capacity of −3.6k-lncRNA overexpression K562 cells, which was detected *via* transwell migration assay, was observed having an apparent decrease (Figure 4g, h). However, no significant variation was observed in −3.6k-lncRNA knockdown cells (Figure 4i).

Taken together, these results suggest that −3.6k-lncRNA is involved in the regulation of NFE2 expression and plays an important role in the proliferation and migration of K562 cell.

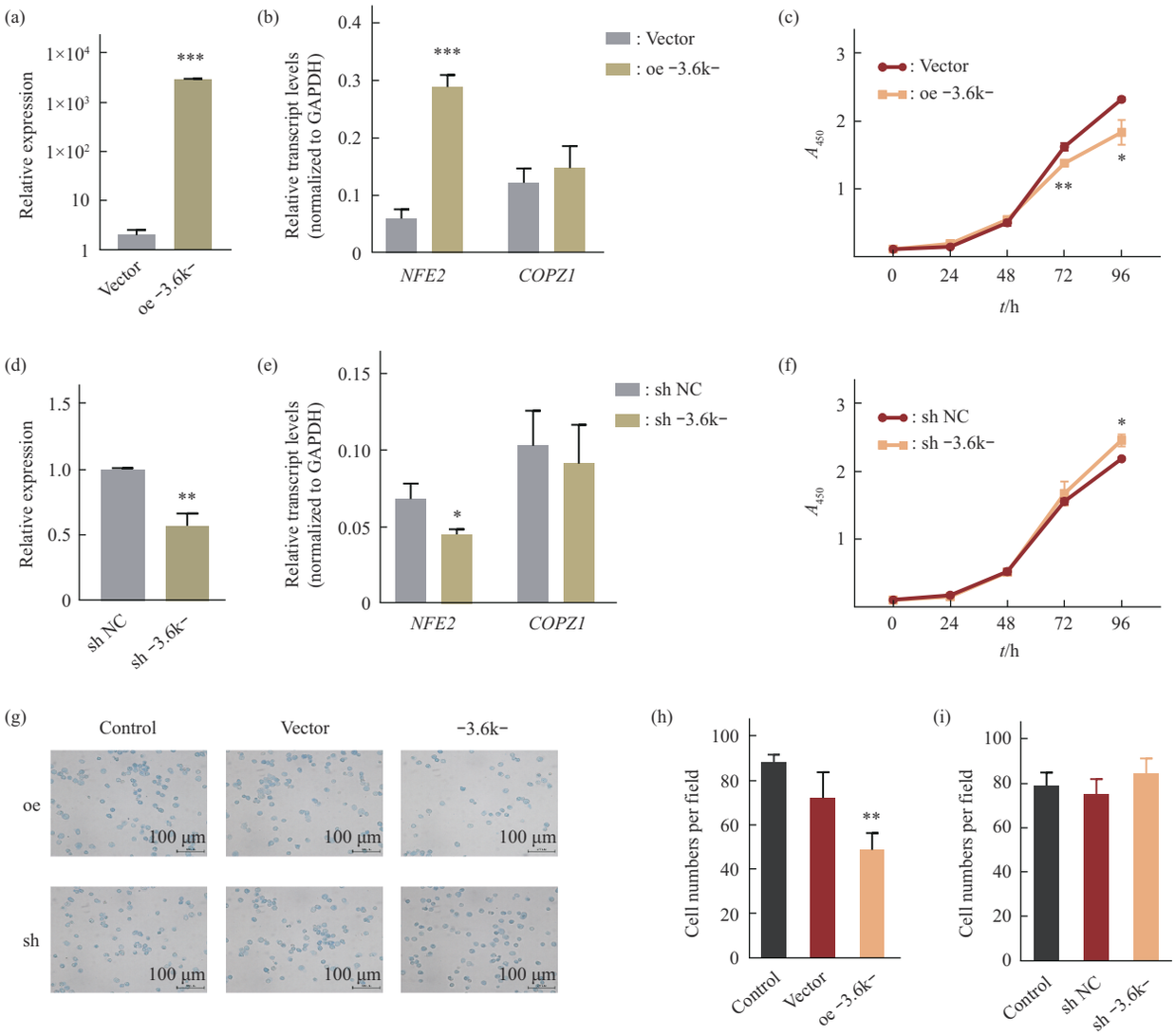

Fig. 4　−3.6K−lncRNA affects *NFE2* transcription and K562 cell proliferation

(a, b) RT-qPCR results show the levels of −3.6k-lncRNAs, *NFE2* and *COPZ1* in K562 cells after infected with pLVX-neo (vector) or pLVX-3.6k-lncRNA overexpression (oe −3.6k−) lentivirus. GAPDH was used as an internal control. (c) The proliferation ability of K562 cells was detected by CCK8 assays after infected with relevant lncRNA overexpressing lentivirus. (d, e) Corresponding expression in K562 cells after infected with pLKO-NC (sh NC) or pLKO−3.6k-lncRNA (sh −3.6k−) lentivirus. (f) The proliferation ability determined by CCK8 assays. (g−i) Transwell migration assay indicating the migration capability of K562 cells after overexpression (upper panel) or knockdown (lower panel) of −3.6k-lncRNA and corresponding quantitative statistics ((h) for overexpression and (i) for knockdown). Data for graphing were obtained from the *means*±*SD* of three individual experiments, and *P* values were calculated using Student's *t*-test (**P*<0.05; ***P*<0.01; ****P*<0.001).

3　Discussion

Enhancers are a class of *cis*-DNA regulatory elements that are capable of transcription and contain transcription factor recognition sequences. Enhancers are located proximal or distal to the promoter of a target gene and establish physical contact with the promoter of target gene through chromatin interactions, thereby regulating gene expression[38]. Enhancers in cancer cells promote the transcriptional expression of oncogenes by interacting with promoters, which in turn leads to tumorigenesis. Therefore, enhancers can be regarded as potential targets for tumor therapy. For instance, PD-L1, a cell surface receptor on tumor cells playing an significant role in immune escape[39], when a distal enhancer of

PD-L1 was knocked out *via* CRISPR-cas9, *PD-L1* expression was significantly reduced at both mRNA and protein levels, and immune escape of cancer cells was effectively inhibited[40]. Current studies observe abnormal expression of eRNAs in tumor samples compared to normal tissue[41]. Enhancer overactivation is a unique phenomenon in tumorigenesis, and targeting eRNAs would be a potential new anti-cancer therapeutic strategy[41]. The clinical relationship between *NFE2* and tumors has been reported in many papers[42-44]. However, the mechanism that leads to the overexpression of *NFE2* in patients with myeloproliferative neoplasms has only been sparsely reported. *JMJD1C*, encoding a histone demethylase, is one of the target genes of NFE2. Its protein level is elevated in patients with MPN due to the overexpression of *NFE2*[21]. Furthermore, JMJD1C, in turn, reduces the level of histone methylation in the promoter and upstream regions of *NFE2*, thereby enhancing the expression of *NFE2*. In this study, we identified 3 enhancer regions in the vicinity of the *NFE2* promoter. The −3.6k region has been shown to significantly enhance the transcription of *NFE2* in the presence of AML1 transcription factor binding[20]. Among enhancers measured by dual-luciferase reporter assay, +6.3k region displays the highest enhancer activity. It is worth noting that the +6.3k region is adjacent to the *NFE2* 1F promoter, which is almost not used in adult bone marrow cells. Therefore, it is worthwhile to further investigate the role of the +6.3k enhancer in the transcription of adult and fetal types of NFE2. Besides, we found a new transcript of −3.6k enhancer and its regulatory activity for *NFE2*. The levels of this lncRNA during leukemic cells differentiations vary consistently with *NFE2*. Thus, in addition to the enhancer we identified, the lncRNA itself could also serve as a novel target for the treatment of MPN.

In our K562 cell model overexpressing −3.6k-lncRNA, our data show that overexpression of this lncRNA has a significant inhibitory effect on the proliferation of tumor cells. K562 cells with −3.6k-lncRNA knock-down have an enhanced proliferative capacity. These data are consistent with the studies that in the Friend virus-induced mouse model of erythroleukemia, the absence of NFE2 promotes tumor growth by accelerating the rate of cellular proliferation[9], but differ from studies suggested that overexpression of NFE2 promotes the conversion of MPN to AML[20, 45]. One possible reason for this is that −3.6k-lncRNA has an additional way of regulating cell proliferation as a lncRNA compared to the NFE2 protein. In addition, it is also possible that *NFE2* is differentially expressed in different cells at different stages of development, just as NFE2 overexpression causes different phenotypes in early and late erythrocyte maturation. Gene ontology analysis of target genes of NFE2 predicted by *NFE2* ChIP-seq data (ENCSR552YGL) in K562 cells reveals that the functions of many target genes are involved in intercellular communication and cell motility. In our study, a significant enhancement in the migratory capacity of K562 cells was observed only upon overexpression of −3.6k-lncRNA, while there appeared to be no effect upon knockdown. Hence, not only the correlation between NFE2 and migration ability needs to be further verified, but the mechanism by which this lncRNA is associated with it also needs to be investigated in greater depth. In addition, the failure to clone the remaining transcripts in the two enhancers of upstream with bidirectional transcription suggests that they may not have typical polyA structures. This leads to their rapid degradation after transcription, making them difficult to be captured[38]. Further research is needed to confirm their sequences and their role in the transcriptional regulation of *NFE2*. In conclusion, how −3.6k-lncRNA regulates *NFE2* gene expression and whether there are effects on cell proliferation that bypass *NFE2* demand further investigation.

4 Conclusion

In this study, we first identified 3 enhancers that regulate *NFE2* transcriptional activity, located at −3.6k, −6.2k, and +6.3k relative to the *NFE2* TSS. Subsequently, we cloned the −3.6k-eRNA and identified it as a lncRNA. We found that this lncRNA mainly localizes in the nucleus and is expressed in three leukemia cell lines (K562, U937, and HL-60). Finally, through overexpression and knockdown experiments, we demonstrated that this lncRNA participates in the regulation of the target gene *NFE2*, and plays a role in inhibiting the proliferation and migration of K562 cells.

Supplementary Available online (http://www.pibb.ac.cn or http://www.cnki.net):

PIBB_20230105_Table_S1.pdf
PIBB_20230105_Doc_S1.pdf

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人*NFE2*基因上游增强子lncRNA调控*NFE2*基因转录和K562细胞增殖*

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摘要　目的　转录因子NFE2的异常表达在许多骨髓增殖性肿瘤患者中被观察到，然而造成这种异常的转录调控机制尚不明确，本研究旨在探究参与*NFE2*转录调控的元件和分子机制。**方法**　首先通过公共数据库中ChIP-seq数据和ATAC-seq数据预测*NFE2*基因的潜在增强子元件，并通过双荧光素酶报告实验进行体外验证。随后，通过PRO-seq和GRO-seq数据结合RACE技术克隆这些增强子RNA转录本，经在线编码潜能预测工具分析认为其为lncRNA，通过RT-qPCR检测该lncRNA在不同白血病细胞系中和这些细胞诱导分化前后的表达变化及其亚细胞定位。最后，通过慢病毒系统在K562细胞中过表达和敲降该lncRNA以探究其功能。**结果**　鉴定出调控*NFE2*转录的3个增强子元件，分别位于*NFE2*转录起始位点−3.6k，−6.2k和+6.3k区域，这些元件插入*NFE2*启动子上游均能增强下游萤火虫荧光素酶的表达。克隆出−3.6k增强子负链方向的转录本，将其鉴定为−3.6k-lncRNA。本研究发现，该lncRNA在K562、U937和HL-60这3种白血病细胞系中均有一定程度的表达，且均定位于细胞核内。当该lncRNA在K562细胞中过表达，*NFE2*水平随之提高，细胞增殖和细胞迁移能力受到抑制；当其被敲降时，*NFE2*水平相应降低而K562细胞增殖能力随之升高。**结论**　本文鉴定了调控人*NFE2*基因转录的3个增强子元件和一条增强子lncRNA转录本，并验证了该lncRNA对*NFE2*转录的正调控作用以及对K562细胞增殖能力具有抑制作用。

关键词　*NFE2*基因，增强子，长链非编码RNA，细胞增殖

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