

A Novel lncRNA, AK130181, Contributes to HIV-1 Latency by Regulating Viral Promoter-Driven Gene Expression in Primary CD4⁺ T Cells

Haiyu Li,¹ Xiangbo Chi,¹ Rong Li,² Jing Ouyang,¹ and Yaokai Chen¹

¹Department of Infectious Disease, Chongqing Public Health Medical Center, Chongqing, China; ²Department of Department of Gastroenterology, Chongqing Public Health Medical Center, Chongqing, China

The functions and mechanisms of long non-coding RNAs (lncRNAs) in latent HIV-1 infection are not yet fully understood and warrant further research. In this study, we identified the newly inhibitory lncRNA AK130181 (also named LOC105747689), which is highly expressed in CD4⁺ T lymphocytes latently infected with HIV, using bioinformatics. We also found that AK130181 is involved in HIV-1 latency by inhibiting long terminal repeat (LTR)-driven HIV-1 gene transcription in a nuclear factor κ B (NF- κ B)-dependent manner. Furthermore, silencing AK130181 significantly reactivates viral production from HIV-1 latently infected Jurkat T cells and primary CD4⁺ T cells. Interestingly, we found that inhibition of AK130181 in resting CD4⁺ T cells from HIV-1-infected individuals treated with highly active antiretroviral therapy significantly increased viral reactivation upon T cell activation *in vivo*. We provide new insights and a better understanding of lncRNAs that play a role in HIV-1 latency, and suggest that silencing AK130181 expression to activate HIV-1 latently infected cells may be a potential therapeutic target for HIV-infected individuals.

INTRODUCTION

Similar to protein-coding RNAs, most long non-coding RNAs (lncRNAs) are also transcribed by RNA polymerase II catalysis, but their sequences are not conserved, and their expression abundance is low, showing strong specificity in tissues and cells. Initially, lncRNAs were regarded as the “garbage” of genome transcription. They were identified as a byproduct of RNA polymerase II transcription and had no biological function. However, recent studies have shown that lncRNAs are involved in many important regulatory processes, such as X chromosome silencing, genomic imprinting, chromatin modification, transcriptional activation, transcriptional interference, and intranuclear transport. These regulatory roles of lncRNAs have also begun to attract wide attention.^{1–3} In total, 4%–9% of transcripts in the mammalian genome sequence are produced by lncRNAs (the corresponding protein-coding RNA ratio is 1%). While the study of lncRNAs has progressed rapidly, the vast majority of lncRNA functions are still not clear.^{4–7} With the advancement of research, a large number of lncRNAs have been found, which has led people to gradually realize that there is “dark matter” in the

genome that we know little about.^{8,9} Studies have shown that lncRNAs play an important role in regulating growth and development, cell differentiation, subcellular structure distribution, evolutionary selection, and the relationships between human diseases.^{10–12}

Human immunodeficiency virus (HIV) is the pathogen of the acquired immunodeficiency syndrome (AIDS). It is the fourth leading cause of human death in the world. From 1981 to 2009, 65 million people worldwide were infected with HIV, resulting in 25 million deaths.¹³ HIV is a lentivirus that causes HIV infection and over time AIDS.^{14–16} At present, there is no effective alternative therapy besides highly active antiretroviral therapy (HAART), which can significantly slow the progression of the disease. However, HAART treatment still cannot fundamentally cure people infected with HIV. Since HIV-1 infects host cells, its viral genome can be integrated into the genome of host cells to cause a latent infection. Silent pre-viruses do not transcribe or replicate at very low levels, thus avoiding the attack of the host immune system and antiretroviral drugs, thereby allowing long-term latency. Once the conditions are correct, the latent virus can resume the ability of replication, resulting in rapid deterioration of the condition of HIV-infected persons.^{17–21} A previous study demonstrated an interaction between microRNAs and latent HIV infection, and those microRNAs play an important role in HIV infection and disease progression. On the one hand, HIV infection affects the microRNA expression profile in cells; on the other hand, the high expression of anti-HIV microRNA in cells is not conducive to the replication of cells but is conducive to the formation and maintenance of viral latency.^{22–24}

In this study, we explored the functions of lncRNAs in latent HIV-1 infection by bioinformatics approaches and gene expression arrays. We used bioinformatics approaches to obtain critical lncRNAs and potential key signaling pathways for HIV-1 latency. Subsequently,

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Correspondence: Yaokai Chen, Department of Infectious Disease, Chongqing Public Health Medical Center, #109 Baoyu Road, Shapingba District, Chongqing 015000, China.

E-mail: ykchenmc@163.com



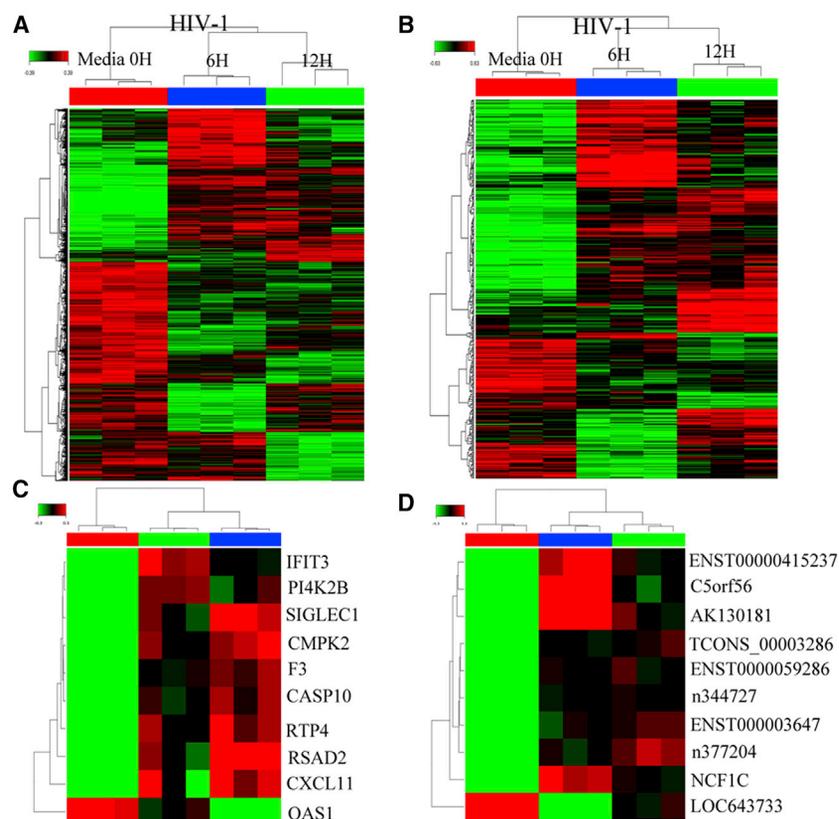


Figure 1. Heatmaps of Differentially Expressed Genes and lncRNAs that Were Identified

The heatmap of differentially expressed genes and lncRNAs were identified. up-regulated genes are shown in red, and downregulated genes are shown in blue. Then, the differentially expressed genes (A) and lncRNAs (B) were visualized in a hierarchical clustering heat map. The top ten genes (C) and lncRNAs (D) were visualized in the hierarchical clustering heat map.

we performed further enrichment analysis to obtain final hub genes and key signaling pathways. In this way, we can further understand the pathogenesis of HIV-1 latency at the molecular level.

RESULTS

Identification of Differentially Expressed Genes (DEGs) and lncRNA Profiles in PBMCs Infected with HIV-1

By comparing the peripheral blood mononuclear cells (PBMCs) infected with HIV-1 at 6 and 12 h to the media control group, we identified 449 differentially expressed coding protein genes, including 217 upregulated genes and 232 downregulated genes, with a p value < 0.05 and a $|\log_2FC$ (fold change) > 2 as the screening criteria. A total of 413 lncRNAs were differentially expressed under the conditions of “ $Q < 0.001$ and fold change > 1.3 .” Then, the differentially expressed genes and lncRNAs were visualized in a hierarchical clustering heatmap (Figures 1A and 1B). The top 10 genes and lncRNAs were visualized in the hierarchical clustering heatmap (Figures 1C and 1D).

GO and KEGG Functional Analyses of the DEGs

With the help of the R language package mentioned above, we obtained 349 DEGs, among which the number of upregulated DEGs was 158, and the number of downregulated DEGs was 191. Gene Ontology (GO) analysis classifies terms into three GO categories: biological process (BP), molecular function (MF), and cellular component (CC). All DEGs were significantly enriched in the BP terms virus-host interaction,

signal transduction, innate immune response, defense response to virus, inflammatory response, and cytokine-mediated signaling pathway; the MF terms protein binding, ATP binding, protein kinase activity, phospholipid binding, and cytokine activity; and the CC terms cytoplasm, plasma membrane, integral to membrane, cytoskeleton, and actin cytoskeleton. The top 15 GO terms of BP-based counts are shown in Figure 2. For Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, the upregulated DEGs were significantly enriched in metabolic pathways, cytokine-cytokine receptor interaction, herpes simplex infection, Toll-like receptor signaling pathway, the chemokine signaling pathway, and human T cell leukemia virus type 1 (HTLV-1) infection. The results of the functional annotation of KEGG pathways are shown in Figure 2. IFIT3, MLA3, MOV10, NT5C3A, PER1, PI4K2B, PML and IFI27 participate in the pathways associated with the virus-host interaction. COMP, THBS2, and THBS4 are significantly enriched in all three pathways.

A Newly Identified lncRNA Could Be an Independent Factor for HIV-1 Latency

We identified the top 20 high-node-degree genes for the whole DEGs, upregulated DEGs, and downregulated DEGs using STRING, Cytoscape, and the Cytoscape plug-in NetworkAnalyzer (Figure 3A). The potential hub genes were identified as IFIT3, MLA3, MOV10, NT5C3A, PER1, PI4K2B, PML, PPM1K, PYCO2, TRIM5, TRIM8, UBA7, USP18, YEATS2, ACAP1, BTG2, and ANF569, and the potential lncRNAs were identified as AK130181, AK126253, ENST00000546482, AL360159, NR_037670, and AL831948. We observed that the genes in the cluster were mainly associated with the virus-host interaction, innate immune response, defense response to virus, cytokine-cytokine receptor interaction, JAK (Janus kinase)-STAT (signal transducer and activator of transcription) signaling pathway, and nuclear factor κ B (NF- κ B) signaling pathway. The KEGG results for cluster 1 were compared with the gene set enrichment analysis (GSEA) results for the whole samples and with GO/KEGG analysis for the upregulated and downregulated DEGs. We concluded that the virus-host interaction pathway and the NF- κ B signaling pathway were the key signal transduction pathways involved in HIV-1 latency, and the potential hub genes in the two final key

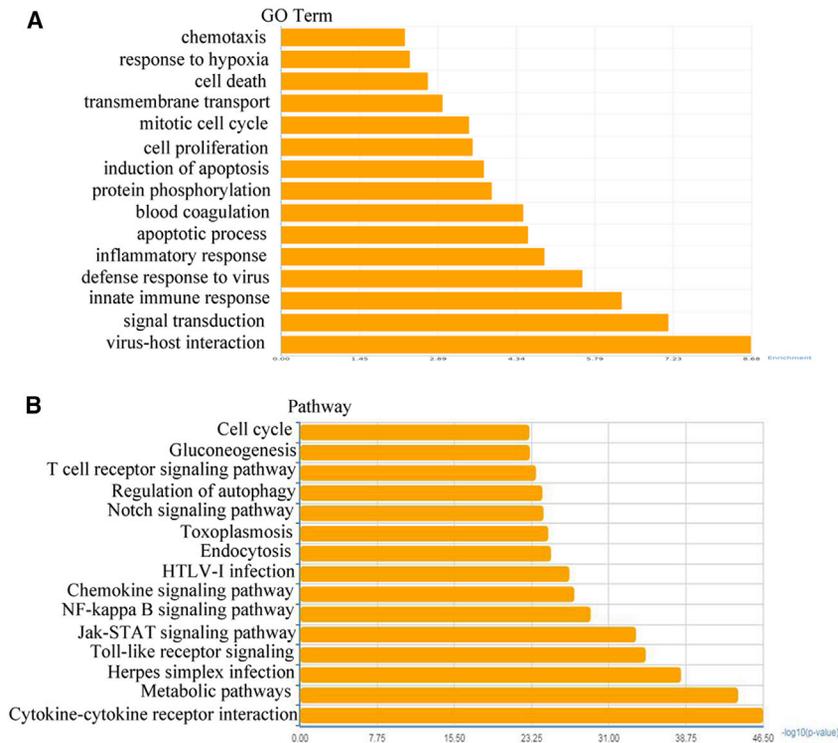


Figure 2. GO and KEGG Analysis

(A and B) Gene Ontology (A) and KEGG pathway (B) analyses of the DEGs are shown.

signaling pathways were regarded as the final hub genes (IFIT3 and lncRNA AK130181) for this microarray analysis (Figure 3B).

lncRNA AK130181 Expression Is Elevated in Resting CD4⁺ T Cells and Suppresses HIV-1 Replication

To investigate whether these lncRNAs and genes are involved in HIV-1 replication and latency, we selected several lncRNAs (AK127609, AK126253, NR037670, AL360159, and AK130181) and genes (TPCN1, IFIT3, and GFPT2) to determine their expression in human primary CD4⁺ T lymphocytes by quantitative real-time polymerase chain reaction (qRT-PCR). AK130181 and IFIT3 were highly expressed in human primary CD4⁺ T lymphocytes compared to the control gene TBET at the mRNA level. The lncRNA AK130181 showed significantly higher expression levels in resting CD4⁺ T lymphocytes than in activated cells (Figure 4B). To investigate the function of the endogenous lncRNA AK130181 on HIV-1 replication, the replication-competent HIV-1_{NL4-3} strain was used to infect primary CD4⁺ T cells. Silencing endogenous AK130181 using a specific small interfering RNA (siRNA) and a scrambled siRNA in activated primary CD4⁺ T cells (Figure S1) and AK130181 knockdown increased HIV-1_{NL4-3} replication in primary CD4⁺ T cells (Figures 4C and 4D). These data confirm that the overexpression of AK130181 inhibits HIV replication in primary CD4⁺ T cells.

lncRNA AK130181 Specifically Represses HIV-1 Transcription and Participates in Maintaining HIV-1 Latency

To investigate the mechanism by which AK130181 inhibits HIV-1 replication, we transfected HEK293T cells with the provirus plasmid

pNL4-3 and found that HIV-1 total RNA expression increased significantly upon silencing AK130181 expression (Figure 5A). Conversely, the expression of HIV-1 total RNA decreased significantly after overexpression of AK130181 compared with the control group (Figure 5A), with these data suggesting that the transcription of HIV-1 is affected by AK130181. Because lncRNAs can regulate downstream gene transcription and the HIV-1 long terminal repeat (LTR) promoter plays a key role in viral transcription, we hypothesized that AK130181 affects HIV-1 LTR promoter activity to inhibit HIV-1 replication in primary resting CD4⁺ T lymphocytes. We found that HIV-1 promoter activity was significantly increased after AK130181 was silenced in Jurkat cells. The HIV-1 promoter reporter system plasmid, the full-length LTR promoter plasmid, the scrambled siRNA, or the AK130181 siRNA was nucleofected into resting CD4⁺ T cells. Dual-luciferase reporter gene assays confirmed that HIV-1 promoter activity was significantly increased after AK130181 was silenced (Figure 4A). We overexpressed endogenous AK130181 using pcDNA3.1-AK130181 (Figure 5B) and found that AK130181 overexpression increased promoter activity in resting CD4⁺ T lymphocytes. To investigate whether AK130181 functions in latent HIV-1 infection, we successfully constructed a latent HIV-1-infected Jurkat cell line as described in [Materials and Methods](#).

To evaluate the effect of AK130181 on HIV-1 reactivation from the latency status, we knocked down endogenous AK130181 in HIV-1 latency model cells using specific short hairpin RNAs (shRNAs). HIV-1 reactivation was measured by GFP expression, and AK130181 knockdown increased HIV-1 reactivation. Furthermore, silencing AK130181 combined with treatment with suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, resulted in a much higher reactivation level than treatment with the scrambled siRNA in combination with SAHA (Figures 5C and 5D). These results confirm that AK130181 inhibits the transcriptional activity of HIV and contributes to viral latency in CD4⁺ T cells.

lncRNA AK130181 Suppresses HIV-1 LTR Promoter Activity by Inhibiting NF-κB

LTFRs play an important role in the regulation of HIV-1 expression. There are binding sites with various transcription factors in the LTR region of the HIV-1 genome. Various regulatory proteins form

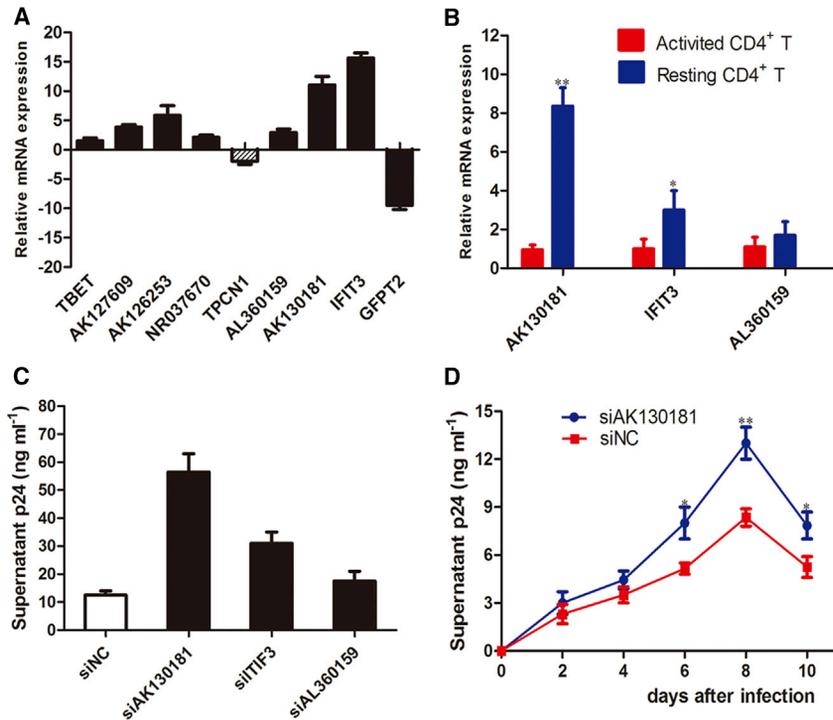


Figure 4. IncRNA AK130181 Inhibits HIV-1 Replication

(A) The expression levels of genes and lncRNAs in human primary CD4⁺ T lymphocytes by qRT-PCR. (B) AK130181, IFIT3, and AL360159 expression level differences between resting and activated primary CD4⁺ T lymphocytes from the same donor by qRT-PCR (n = 3). (C) Silencing AK130181 increases HIV-1 replication. Phytohemagglutinin (PHA)-activated primary CD4⁺ T cells were transfected with an AK130181-targeting siRNA or a control siRNA, and the cells were infected with replication-competent HIV-1_{NL4-3}. (D) The levels of HIV-1 p24 gag in the supernatants were quantified using a human p24 ELISA. All results are shown as the mean \pm SD (n = 3), and the data presented are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

Silencing of AK130181 Promotes Viral Reactivation in Primary Resting CD4⁺ T Cells from HIV-1-Infected Subjects

To investigate the role of lncRNA AK130181 in maintaining HIV-1 latency *in vivo*, we examined the effect of silencing of AK130181 on HIV-1 latency in resting CD4⁺ T lymphocytes from HIV-1-infected individuals who underwent a combination antiretroviral therapy for more than 3 years with an undetected plasma viral load by using a standard RT-PCR-based assay. After trans-

fection of the resting cells with AK130181-specific siRNAs, the expression of AK130181 was significantly decreased (Figure 7A). Knocking down AK130181 significantly increased HIV-1 reactivation upon T cell activation by phytohemagglutinin (PHA)-P, as a positive control by detecting HIV-1 particles released into the supernatants (Figure 7B), or quantifying the levels of HIV-1 *gag* or *tat-rev* mRNA (Figures 7C and 7D). In conclusion, these data demonstrated that AK130181 contributed to HIV-1 latent infection.

DISCUSSION

Highly effective antiretroviral therapy has been widely used in the clinic, as it has been shown to significantly improve the survival of AIDS patients, but it has not been able to completely eradicate HIV. HIV in treated patients shows persistent inactive transcription in resting cells and is reactivated after cell activation. This latent state makes the virus exist for a long time even under treatment, and patients cannot be cured. Latency is usually described as a reversible and unproductive state of infection of the virus, and there is usually no release of the virus from the offspring. It was found that latent reservoirs are established in the acute infection stage after the invasion of host cells. The actual latent state can also occur under the baseline transcription of a small amount of transcription, incomplete synthesis, and many other situations. Compared with infected cells, the frequency of breaking through the host defense to establish latency is

a complex regulatory network by interacting with DNA and proteins to regulate the expression of viral structural proteins and nonstructural proteins. Because the lncRNA AK130181 specifically represses HIV-1 promoter transcription and the AK130181 sequence contains NF- κ B binding sites (Figures 6A and 6B), we speculated that it suppresses NF- κ B-dependent HIV-1 LTR-driven gene expression. We have confirmed that lncRNA AK130181 had significantly higher expression levels in resting CD4⁺ T lymphocytes than in activated cells (Figure 4B). To confirm this hypothesis, we coexpressed pcDNA3.1 full-length AK130181 and a pNF κ B-TA-luc reporter in HEK293T and Jurkat cells, and transfection efficiency was determined using the pRL-TK vector expressing Renilla luciferase. qRT-PCR analysis demonstrated that AK130181 mRNA expression was significantly overexpressed at 48 h posttransfection, whereas the expression of GAPDH was unchanged (Figure S2). Then, we treated the cells with tumor necrosis factor (TNF)- α to deactivate the activity of NF- κ B. In the absence of TNF- α stimulation, the transient expression of AK130181 reduced NF- κ B reporter activity, while AK130181 overexpression suppressed TNF- α -induced NF- κ B activation (p < 0.001) (Figures 6C and 6D). The interaction of AK130181 with NF- κ B components was further validated by RNA immunoprecipitation and RNA pull-down assays (Figures 6E and 6F). In conclusion, these data suggest that AK130181 suppresses NF- κ B-dependent HIV-1-LTR-driven gene expression.

Figure 3. Construction of the lncRNA-mRNA Network and Module Analysis

(A) The differentially expressed genes were selected as candidate genes as a function of HIV-1 latency by constructing a gene coexpression network with the k-core algorithm. (B) IFIT3 and AK130181 were identified as the key genes in the gene network. Node size represents degree of centrality.

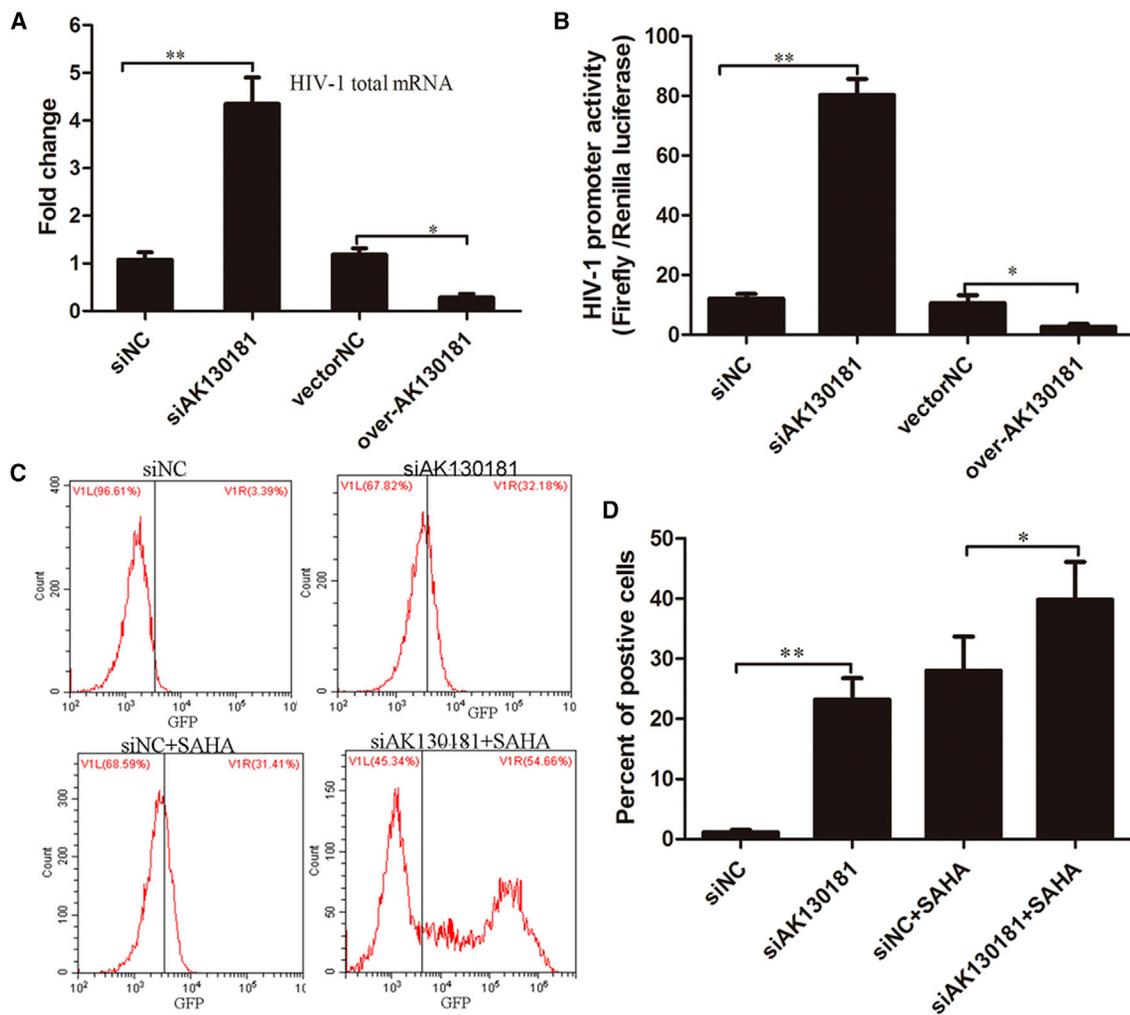


Figure 5. AK130181 Represses HIV-1 Transcription and Participates in HIV-1 Latency

(A) Total HIV-1 mRNAs, including spliced and unspliced mRNAs, were detected with real-time qRT-PCR in HEK293T cells that were co-transfected with the pNL4-3 provirus plasmid and siRNAs against AK130181 or the scrambled control. Conversely, the expression of HIV-1 total RNA decreased significantly after overexpression of AK130181 compared with the control group. (B) Resting CD4⁺ T lymphocytes were co-transfected with pHIV-Pro-luc reporter plasmids, pRL-TK (as a transfection normalization reporter), and the AK130181 siRNA or the scrambled control siRNA. The promoter activity was determined by dual-luciferase reporter assays at 48 h after transfection (n = 3). (C) The latently infected Jurkat T cells were transfected with AK130181 siRNAs or the scrambled siRNA control or transfected with siRNAs and treated with SAHA and then detected by flow cytometry at 48 h post-transfection. (D) The GFP⁺ ratio indicated the reactivation level. All results are shown as the mean \pm SD (n = 3), and the data presented are representative of at least three independent experiments. *p < 0.05, **p < 0.01.

rare. However, the persistent infectious characteristics of immune surveillance escape, attack, inheritance, and reactivation are the most challenging obstacles to current antiviral clearance strategies. The systematic excavation of latency-related cells and molecules is a prerequisite to further reveal the latency mechanism and provides a scientific basis for future radical cures. It has great application value. Among the reservoirs contributing to the long-term persistence of HIV-1 in the face of an effective therapy is a pool of latently infected memory CD4⁺ T cells.^{25–27} These cells that harbor integrated but transcriptionally inactive proviruses can persist for decades while retaining the potential to activate viral production and reseed systemic infection when antiviral therapy is discontinued.^{28–30} We have sought

to understand in molecular terms the basis of HIV latency to identify novel strategies to purge the latent reservoir.

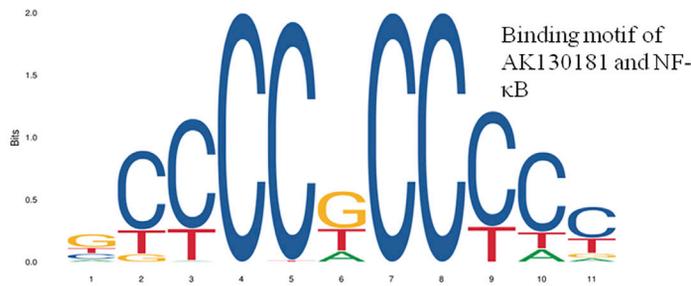
In this study, we used bioinformatics approaches to obtain critical lncRNAs and potential key signaling pathways related to HIV-1 latency. We concluded that the virus-host interaction pathway and the NF- κ B signaling pathway are the key signal transduction pathways involved in HIV-1 latency, and the potential hub genes identified in the two final key signaling pathways were IFIT3 and the lncRNA AK130181. The lncRNA AK130181 showed significantly higher expression levels in resting CD4⁺ T lymphocytes than in activated cells. Furthermore, silencing endogenous AK130181 using a

lnc-AK130181

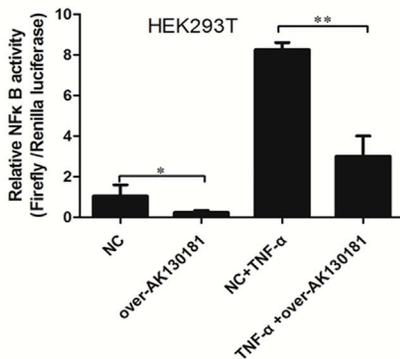
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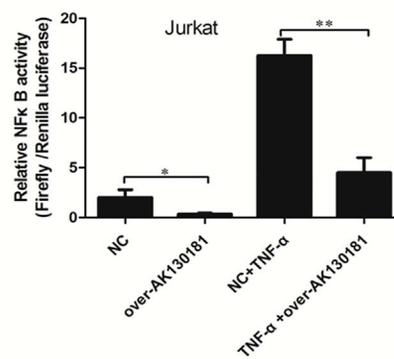
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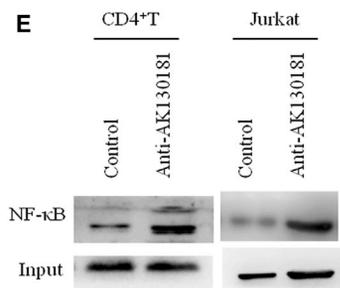
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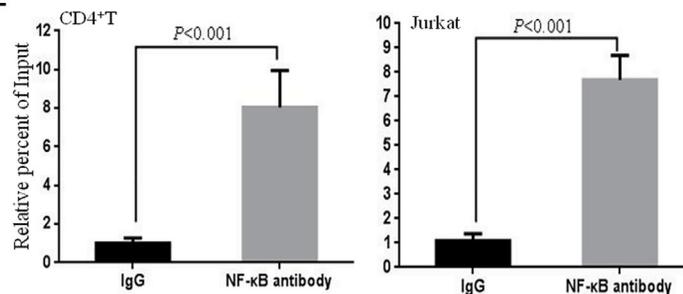
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lnc-AK130181

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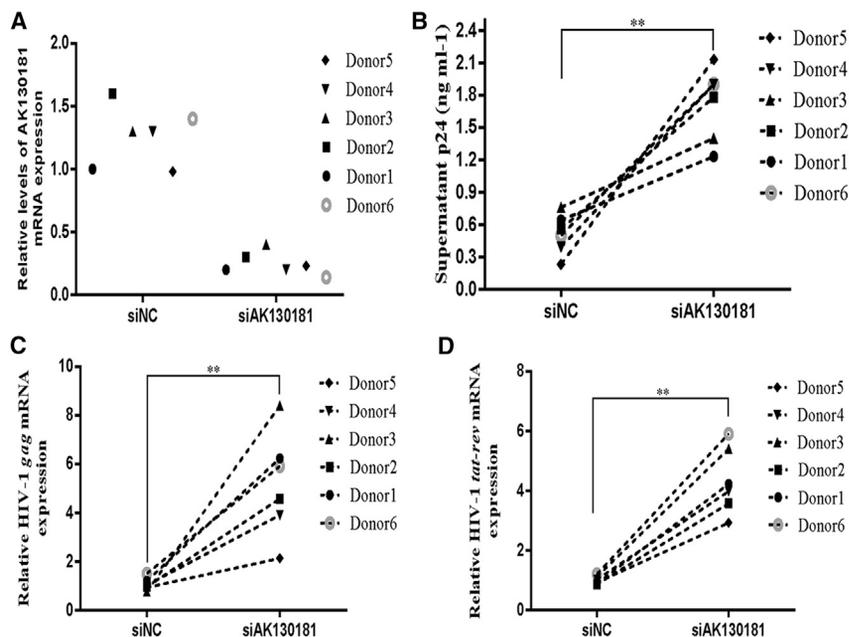


Figure 7. Silencing AK130181 Increases HIV-1 Reactivation in Primary Resting CD4⁺ T Cells from Patients

Resting CD4⁺ T cells were isolated from three antiretroviral-treated patients and co-infected with the AK130181-specific siRNA or the negative control siRNA. (A) AK130181 expression levels were detected by qRT-PCR. (B) HIV-1 reactivation in CD4⁺ T cells upon PHA-P stimulation was detected by either quantifying p24 gag levels in the supernatants with a human p24 ELISA or by quantifying cell-associated HIV-1 gag (C) and *tat-rev* (D) mRNAs, respectively. * $p < 0.05$, ** $p < 0.01$, according to an unpaired *t* test.

cultured with media alone, HIV-1MN, HIV-2NIH-Z, or the TLR9 agonist CpG-A (four conditions), and cells were harvested at 6 and 12 h (two time points) for RNA extraction. In this study, six samples from the HIV-1 group and three samples from the media alone group were analyzed.

Identification of DEGs and the lncRNA Profile

The limma (linear models for microarray data) package is a full-featured package that contains

specific siRNA and a scrambled siRNA in activated primary CD4⁺ T cells and AK130181 knockdown increased HIV-1-NL4-3 replication in primary CD4⁺ T cells. AK130181 knockdown and treatment with SAHA, a well-known histone deacetylase (HDAC) inhibitor, resulted in a much higher reactivation level than did treatment with the scrambled siRNA in combination with SAHA.

In conclusion, our data demonstrate that a novel lncRNA, AK130181, maintains HIV-1 latency by suppressing NF- κ B activity and thereby hinders the initiation of transcriptional translation to maintain the latency status of HIV-1.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Clinical Research & Ethics Committee at Chongqing Public Health Medical Central, Chongqing, China. Before the investigation, informed consent was obtained from each patient and volunteer and approved by the Hospital Ethics Committee.

Reprogramming of the Research Microarray Data

The gene expression profile of Gene Expression Omnibus (GEO) series 58994 (GEO: GSE58994)³¹ was downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), which is based on the GEO platform GPL16686 (Affymetrix Human Gene 2.0 ST Array). Raw samples from 24 patients were analyzed. PBMCs from three donors were

both raw data input and a preprocessing (normalization) function for cDNA chips. The limma package can be used to analyze differentially expressed genes, especially for a “multifactor designed experiment.” The limma package was applied to screen the differentially expressed genes and lncRNAs between 6 h and 12 h of HIV-1 infection. The cut-off criteria were an adjusted *p* value < 0.001 and a logFC ≥ 4 or a logFC of -4 or less. Hierarchical clustering and visualization were performed with the heatmap package of R.

GO and KEGG Analyses of the DEGs

Functional enrichment and pathway enrichment analyses of the differentially expressed genes co-expressed with lncRNAs were analyzed with the online DAVID tool. Biological process and KEGG pathway results were obtained. The threshold value of significance used in this study was a *p* value of 0.1 or a modified Fisher’s exact count > 2 of the enrichment score.

Analysis of the lncRNA-mRNA Interaction Network

A co-expression analysis of the significant differentially expressed RNAs and lncRNAs was carried out. Pearson correlation coefficients between the differentially expressed lncRNAs and each differentially expressed mRNA were calculated with R software. The Pearson coefficient threshold of highly correlated lncRNAs-mRNAs was a correlation greater than or equal to 0.80. The obtained lncRNA-RNA relationship was used to construct the network using Cytoscape.³²

Figure 6. AK130181 Suppresses NF- κ B Activity

AK130181 overexpression inhibits NF- κ B activation. (A) The AK130181 sequence contains NF- κ B binding sites. (B) Binding motif of AK130181 and NF- κ B. (C and D) pcDNA3.1-AK130181 or the mock vector and the pNF κ B-TA-luc reporter plasmid were co-transfected into HEK293T (C) and Jurkat (D) cells, and a Renilla luciferase expressing vector was used to normalize transfection efficiency. (E and F) At 24 h posttransfection, cells were treated with or without TNF- α for an additional 24 h, and then cells were harvested and the reporter gene expression was assessed. The interaction of AK130181 with NF- κ B was verified with RNA immunoprecipitation (RIP) and RNA pull-down assays.

Cell Culture and siRNA Transfection

HEK293T and Jurkat cells were derived from typical culture preservation centers in the United States. HEK293T cells were cultured in DMEM, while Jurkat and primary CD4-positive cells were cultured in RPMI 1640 medium. Additional CD3, CD28, and interleukin (IL)-2 were added to human primary CD4T cells (all diluted at 1:1,000). For adherent cell lines, Lipofectamine 2000 was used for plasmid DNA transfection, Lipofectamine RNAiMAX was used for siRNA transfection, and 4D-Nucleofector (Lonza) transfection was used for cells in suspension, in which the kit used for Jurkat cells was the Amaxa SE cell kit. For human primary CD4 T cells, the kit used was the Amaxa P3 primary cell kit (V4XP, Lonza). The transfection methods were strictly operated in accordance with the instructions for the use of reagents.

Assays for HIV-1 Reactivation from Latently Infected Cells

Using the expression of green fluorescent protein (GFP), we analyzed mock-infected HIV-1 Jurkat T cells with flow cytometry. The GFP-positive cells were sorted, and after 20 days of treatment, the number of GFP-negative cells was obtained by activating the agent after kojic acid bacteriostatic (trichostatin A [TSA]) stimulation. GFP and p24 antigen expression was detected, and the HIV-1 monoclonal cells with latent infections were selected. Latently infected cells were transfected with AK130181 siRNA or a scrambled control siRNA to detect latency reactivation with flow cytometry.

RNA Isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was extracted and retranscribed into DNA. The retranscribed DNA was diluted 10 times as a template, and three replicates were set for each group of samples. The SYBR Premix Ex Taq kit was used for amplification. The conditions used were as follows: 95°C for 2 min, denaturation at 94°C for 30 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s, for a total of 40 cycles. qPCR results were analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

All measurements are expressed as the mean \pm SD. GraphPad Prism 6 software was used for statistical analysis. A Student's *t* test was used to compare the mean between two groups. One-way ANOVA was used to compare differences between two groups, and the Pearson correlation coefficient was used for the correlation analysis. $p < 0.05$ indicated a significant difference (* $p < 0.05$, ** $p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.04.011>.

AUTHOR CONTRIBUTIONS

H.L. and Y.C. performed the experiments, were major contributors in writing and submitting the manuscript, and drafted the manuscript. X.C., R.L., and J.O. retrieved the data and performed the statistical analysis. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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