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MicroRNA-181 expression regulates specific post-transcriptional level of SAMHD1 expression in vitro

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ABSTRACT

SAM domain and HD domain 1 (SAMHD1) is a newly discovered human immunodeficiency virus (HIV)-1 host restriction factor with high expression in HIV-1-non-permissive cells and low expression in HIV-1-permissive cells. The regulatory mechanism of SAMHD1 expression is still unclear. We examined the relationship between the expression levels of SAMHD1 mRNA and protein and microRNA-181 (miR-181) level in different cell lines. MiR-181 level was negatively correlated with SAMHD1 expression level. By examining the impact of miR-181 on SAMHD1 3′ untranslated region (UTR) reporter luciferase activity and on *SAMHD1* mRNA and argonaute RISC catalytic component 2 (AGO2) binding, we found that miR-181 acted directly on the SAMHD1 3′ UTR and regulated *SAMHD1* mRNA levels after transcription. MiR-181 over-expression significantly reduced the level of SAMHD1 expression in THP-1 cells; miR-181 inhibition up-regulated SAMHD1 expression in THP-1 and Jurkat cells. Our results suggest that miR-181 regulates the level of post-transcriptional SAMHD1 expression negatively by directly binding to the 3′ UTR in SAMHD1.

1. Introduction

Human immunodeficiency virus (HIV)-1 target cells include activated CD4+ T cells, dendritic cells (DCs), monocytes, and macrophages [1,2]. Activated CD4+ T cells are the major target cells; although DCs, monocytes, macrophages, and other myeloid cells can be infected, the rate of infection is very low and it is difficult to develop replicative infection effectively [2]. The reasons for this remain unclear. Intracellular dinucleotide triphosphate (dNTP) concentration can affect HIV-1 infection and replication. Diamond et al. [3] and Kennedy et al. [4] found that the dNTP concentration in myeloid cells was only 4.5% and 0.3% of that in peripheral blood mononuclear cells and CD4+ T cells, respectively. The low concentration of the dNTP pool may be one reason HIV-1 replication is inhibited in myeloid cells. It was believed that the lower dNTP concentration is related to a newly discovered dNTP triphosphohydrolase: SAM domain and HD domain 1 (SAMHD1). SAMHD1 is a diguanosine triphosphate (dGTP)-dependent phosphatase that mainly hydrolyzes dNTPs into deoxynucleoside and inorganic triphosphoric acid, thus reducing nuclear dNTP concentrations [5]. It is an essential nucleic acid metabolic enzyme in normal cells. The loss of SAMHD1 function can cause the accumulation of a large amount of intracellular dNTPs, contributing to strong immune activation and large amounts of secreted type I interferon (IFN) [6]. Congenital *SAMHD1* genetic mutation can cause a rare inherited autoimmune disease: Aicardi–Goutières syndrome [7]. Myeloid cells reduce the raw material required for HIV-1 reverse transcriptase through SAMHD1 hydrolyzation of intracellular dNTPs, limiting HIV-1 infection [6,8].

SAMHD1 expression is important for either normal nucleic acid metabolism or HIV-1 infection. However, our knowledge of the regulatory mechanism of SAMHD1 expression is poor. SAMHD1 expression differs in different tissues and cells; for example, a

Abbreviations: AGO2, argonaute RISC catalytic component 2; cDNA, complementary DNA; DCs, dendritic cells; dGTP, diguanosine triphosphate; DMEM, Dulbecco's modified Eagle's medium; dNTP, dinucleotide triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIV, human immunodeficiency virus; IFN, interferon; JAK, Janus kinase; NK, natural killer; NPM1, nucleophosmin; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; qPCR, quantitative PCR; RIP, RNA-binding protein immunoprecipitation; SAMHD1, SAM domain and HD domain 1; SIV, simian immunodeficiency virus; STAT, signal transducer and activator of transcription; UTR, untranslated region.

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study found high expression of *SAMHD1* mRNA in the peripheral blood and low expression in the brain, intestinal, and thymus tissues [9]. In addition, SAMHD1 expression in different HIV-1 target cell lines differs significantly. The level of SAMHD1 expression in non-permissive target cells is higher than that in permissive cells [5]. In addition, SAMHD1 expression in resting CD4+ T cells is significantly higher than that in activated CD4+ T cells [10]. However, the regulatory mechanism of specific cellular expression of SAMHD1 is unclear. De Silva et al. [11] found that epigenetic modifications to the SAMHD1 promoter exerted important effects on SAMHD1 expression. They also found that the SAMHD1 promoter has multiple CpG islands; the methylation level of the SAMHD1 promoter in THP-1 cells with high SAMHD1 expression was significantly lower than that in CD4+ T cell lines with low SAMHD1 expression. However, the above mechanism merely influences the transcriptional level of *SAMHD1* mRNA. The mechanism of post-transcriptional regulation has not been well studied. Although some studies have reported that SAMHD1 phosphorylation regulates the restriction of virus infection, but not catalytic dNTPase activity [12], it remains unclear how SAMHD1 expression is regulated post-transcriptionally.

MicroRNAs are important negative regulatory factor in the post-transcriptional regulatory network. The occurrence of many immune cell lines is related to microRNAs; different immune cells have unique microRNA expression profiles [13]. MicroRNAs play an important role in the development and function of the differentiation process in these cells [14]. The expression characteristics of microRNAs can be used as an important marker for distinguishing cell subtypes and functional status [15]. However, the relationship between differential expression of SAMHD1 and these immune cells is unclear. We predicted and detected the presence of miR-181 binding sites in the 3′ untranslated region (UTR) of SAMHD1. MiR-181 can promote T cell, B cell, and natural killer (NK) cell development and is essential for their development and differentiation [14,15]. We hypothesized that miR-181 may play an important role in the regulation of SAMHD1 expression. In the present study, we compared the expression levels of SAMHD1 and miR-181 in different cell lines. The level of miR-181 was negatively correlated with the SAMHD1 expression level; miR-181 acted directly on the SAMHD1 3′ UTR and regulated the post-transcriptional expression level of SAMHD1.

2. Materials and methods

2.1. Cells and reagents

Cells were obtained from American Type Culture Collection (Rockville, MD) and grown in a 5% CO_2 humidified incubator at 37 °C. THP-1, MT2, H9, Jurkat, and Raji cells were grown in RPMI 1640 while HEK293T and HEL cells were grown in DMEM. The cell growth medium contained 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. THP-1 cells were differentiated for 48 h with 50 ng/mL PMA (Sigma-Aldrich, St. Louis, MO). Anti-human SAMHD1 monoclonal antibody was obtained from R&D Systems (Minneapolis, MN). Anti-human AGO2 antibody was obtained from Abcam (Hong Kong, China). MiR-181 mimics and inhibitors were purchased from RiboBio (Guangzhou, China). Anti-miR-181a LNA was purchased from Exiqon (Vedbæk, Denmark). RIP kit was purchased from Millipore (Bedford, MA).

2.2. RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA was extracted with TRIzol (Life Technologies, Carlsbad, CA) and reverse transcribed into complementary DNA (cDNA) in a total volume of 20 μL. Real-time polymerase chain reaction (PCR) was performed using a DNA Engine Chromo 4 Real-time Quantitative PCR (qPCR) System (Bio-Rad, Hercules, CA) and a SYBR Green Kit (TAKARA, Dalian, China). Primer sequences are listed in Supplementary Table 1.

2.3. Quantitative analysis of microRNA

MicroRNA was isolated using a miRCURY™ RNA Isolation Kit (Exiqon); quantitative analysis of miR-181 was performed with real-time quantitative PCR using a miRCURY LNA™ Universal RT microRNA PCR kit and hsa-miR-181 LNA™ PCR primer sets (Exiqon). A U6 small nuclear RNA (snRNA) primer set was used as the internal control for the quantitative reverse transcription-PCR (qRT-PCR). We used a 10-μL reaction system. The experiments were performed according to the protocol provided in the kit.

2.4. Western blotting

Samples containing 1×10^6 cells were lysed in 200 μL cell lysis buffer (Cell Signaling Technology, Beverly, MA) for 30 min at 4 °C, and then clarified by centrifugation at 8000×*g* for 10 min. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL), using bovine serum albumin as the standard. Western blotting was performed as previously described [16]. GAPDH was used as the internal reference using an anti-human GAPDH polyclonal antibody (Cell Signaling Technology).

2.5. Plasmid construction, cell transfection, and luciferase activity assay

We prepared the wild type 3′ UTR reporter vector for *SAMHD1* mRNA by amplifying the full-length 3′ UTR of the gene and the mutant 3′ UTR reporter vector without miR-181 binding site by gene synthesis, and followed by insertion into the multiple cloning region between the humanized *Renilla* luciferase (hRluc) gene and synthetic poly (A) of the psiCHECK-2 vector (Promega, Madison, WI), which contains both the *Renilla* luciferase gene and firefly luciferase gene. Luciferase reporter plasmid for SAMHD1 3′ UTR was cotransfected with miR-181 mimics or inhibitors into HEK293T cells using Lipofectamine 2000 transfection reagent (Life Technologies). Luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer instructions. Data were normalized for transfection efficiency by dividing *Renilla* luciferase activity with that of firefly luciferase.

2.6. RNA-binding protein immunoprecipitation

Cells were lysed using complete RNA lysis buffer in an EZ-Magna RIP kit (Millipore). RIP experiments were performed according to the protocol provided by the kit.

2.7. MiR-181 over expression or inhibition

A pGPU6/GFP/Neo small interfering RNA (siRNA) expression vector (GenePharma, Shanghai, China) was used to construct a miR-181a over expression plasmid (p-181a). Double-stranded DNA for miR-181a hairpin was synthesized and cloned into the pGPU6/GFP/Neo vector downstream of the cytomegalovirus promoter and green fluorescent protein (GFP) gene. Hsa-miR-181a miRCURY LNA™ microRNA Power inhibitor (anti-miR-181a LNA; Exiqon) was used for the miR-181 inhibition. The p-181a vector and anti-miR-181a LNA were electrotransfected into THP-1 and Jurkat cells using a cell line transfection kit (Amaxa, Cologne, Germany) in an Amaxa Nucleofector electroporation apparatus using the V-010 electroporation procedure.

2.8. Statistical analysis

Every test was repeated thrice and data are reported as the mean ± SE. The statistical significance (*P*-values) of the results was calculated using SPSS v.20 software. Student's *t*-test was used to compare between two groups; one-way analysis of variance was used when comparing more than three groups.

3. Results

3.1. Differential expression of SAMHD1 in different cell lines

We examined the expression levels of SAMHD1 mRNA and protein to confirm specific SAMHD1 expression in different cell lines, especially in myeloid and CD4+ T cell lines. We used the CD4+ T cell lines MT2, H9, and Jurkat; THP-1 monocytes; and Raji B lymphocytes for the study, and used the human embryonic kidney cell line HEK293T and lens cell line HEL as controls. At both mRNA and protein expression level, SAMHD1 expression was highest in THP-1 cells. The expression levels in phorbol 12-myristate 13-acetate (PMA) stimulated differentiated and undifferentiated THP-1 cells were not significantly different; SAMHD1 expression was much lower in Raji, HEK293T, and HEL cells and the CD4+ T cell lines; in particular, the level of SAMHD1 expression in Jurkat cells was only 0.4-2.3% that of THP-1 cells (Fig. 1A and B). However, there was moderate SAMHD1 expression in MT2 cells, which was higher than that in the H9 and Jurkat cells. These results suggest that SAMHD1 expression was cell-specific.

3.2. Expression levels of miR-181 were closely related to SAMHD1 expression

We predicted and detected the presence of miR-181 binding sites in the SAMHD1 3′ UTR using TargetScan, miRanda, and other online software (Fig. 2A). There are four main members in the miR-181 family: miR-181a, miR-181b, miR-181c, and miR-181d, and they have the same 5′ end seed sequence and transcriptional regulatory function [17]. To determine the correlation between miR-181 and SAMHD1 expression, we examined the level of miR-181 expression in different cell lines using miRCURY LNA™ Universal RT microRNA PCR. Among the four miR-181 family members, miR-181a was highly expressed, and miR-181b expression was relatively lower. All cell lines expressed almost no miR-181c or miR-181d (Supplementary Fig. 1). MiR-181a and miR-181b expression in the T cell lines H9 and Jurkat was significantly higher than that in other cell lines. However, miR-181a and miR-181b expression in MT2 cells was very low. MiR-181a and miR-181b expression levels in THP-1 cells were the lowest, being only 3-12% of that expressed in the H9 and Jurkat cells (Fig. 2B). These results suggest that the level of miR-181 expression was negatively related to that of SAMHD1 and that miR-181 may play a negative regulatory role in SAMHD1 expression.

3.3. MiR-181 may act directly on the SAMHD1 mRNA 3′ UTR

The miR-181 mimics significantly inhibited wild type SAMHD1 3′ UTR luciferase reporter plasmid activity; inhibition by the miR-181a and miR-181c mimics was more significant (Fig. 3A). MiR-181a and miR-181c inhibitors significantly increased wild type SAMHD1 3′ UTR luciferase reporter plasmid activity (Fig. 3B). Deletion of miR-181 binding site in SAMHD1 3′ UTR increased the luciferase reporter plasmid activity about 2 times, and the miR-181 mimics and inhibitors didn't impact the mutant 3′ UTR luciferase reporter plasmid activity significantly (Fig. 3A and B). These results demonstrated that miR-181 can act directly on the SAMHD1 3′ UTR.

Typically, microRNA combines with argonaute RISC catalytic component 2 (AGO2) and complementary mRNA to form a functional RNA-induced silencing complex, downregulating the post-transcriptional expression of a gene [18]. To confirm whether miR-181 regulates the post-transcriptional expression level of *SAMHD1* mRNA, we transfected HEK293T cells with miR-181a mimics and examined the level of AGO2-bound *SAMHD1* mRNA using RNA-binding protein immunoprecipitation (RIP), using AGO2-bound nucleophosmin (*NPM1*) mRNA as an internal control. The miR-181a mimic promoted *SAMHD1* mRNA and AGO2 protein binding in an obvious manner (Fig. 3C and D), suggesting that miR-181 can promote the binding of *SAMHD1* mRNA and AGO2 protein into an RNA-induced silencing complex that inhibits the level of post-transcriptional SAMHD1 expression.

3.4. MiR-181 regulated SAMHD1 expression

To obtain direct evidence of miR-181 regulation in SAMHD1 expression, we used THP-1 and Jurkat cells as target cells, selected miR-181a, whose expression was higher and which had a significant regulatory effect on the SAMHD1 3′ UTR, and reconstructed a miR-181a expression vector (p-181a) and an anti-miR-181a locked nucleic acid (LNA) to up-regulate or down-regulate the level of intracellular miR-181a. The miR-181a level in p-181a-transfected cells was significantly higher than that in the control group; that in anti-miR-181a LNA-transfected cells was significantly lower (Fig. 4A). The *SAMHD1* mRNA level in p-181a-transfected THP-1 cells was significantly decreased; that in Jurkat cells was low and did not change significantly, while that in anti-miR-181a LNA-transfected THP-1 and Jurkat cells was significantly increased (Fig. 4B). Western blotting revealed that the SAMHD1 protein level was consistent with that of *SAMHD1* mRNA (Fig. 4C). MiR-181a inhibited the level of SAMHD1 protein expression significantly, while anti-miR-181a LNA increased the level of SAMHD1 expression by reducing the miR-181 protein level. These results suggest that miR-181 regulates SAMHD1 expression negatively.

4. Discussion

SAMHD1 is a newly discovered HIV-1 host restriction factor, which can be degraded by the viral accessory protein Vpx of HIV-2 and some simian immunodeficiency viruses (SIVs) [19]. However, HIV-1 lost the Vpx protein during the evolutionary process [20]. Therefore, SAMHD1 currently differs from other HIV-1 host restriction factors by being one of the host restriction factors that cannot be antagonized by HIV-1 [8]. Currently, many studies have reported that SAMHD1 expression is high in THP-1 cells, primary DCs, macrophages, resting CD4+ T cells, and other HIV-1-non-permissive cells, but is low in HIV-1-permissive CD4+ T cell lines [5,9,10]. In our study, we found that SAMHD1 expression was low in CD4+ T cell lines, whereas it was high in THP-1 cells, which is consistent with the findings of previous studies. We also found that SAMHD1 expression was higher in the B cell line Raji; expression was moderate in the somatic cell lines HEL and HEK293T. Surprisingly, the level of SAMHD1 expression in MT2 cells was significantly higher than that in the CD4+ T cell lines Jurkat and H9, and was closer to that in the HEL and HEK293T cells. This is possibly related to the different cell sources or cleavage. MT2 cells are hypertetraploid cells, whereas H9 and Jurkat cells are diploid cells. The specific mechanism requires further research. Our results suggest that SAMHD1 expression is significantly different between different cell types; in particular, the difference is more significant among myeloid and T cells and other immune

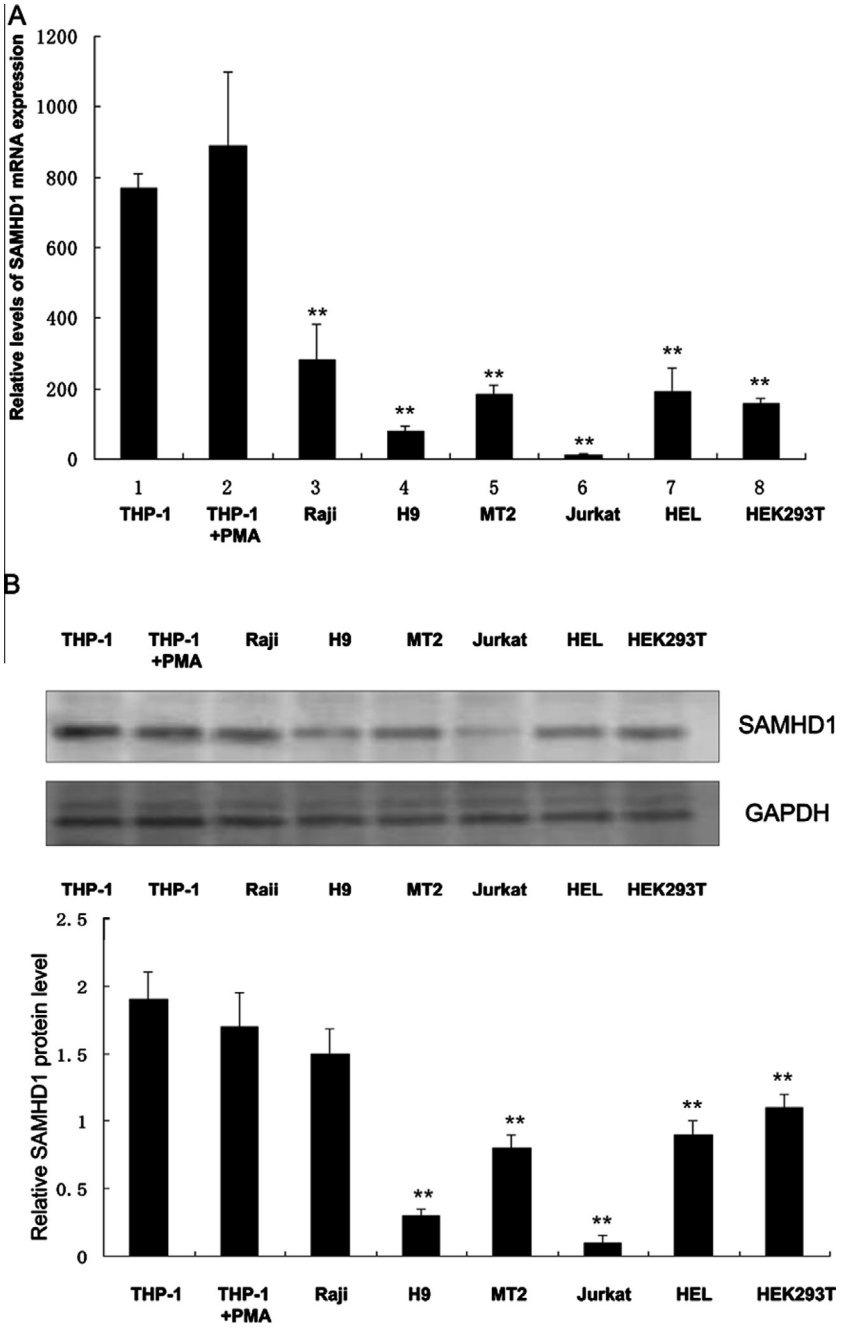

Fig. 1. Level of SAMHD1 expression in different cell lines. (A) Relative *SAMHD1* mRNA expression levels. (B) Level of SAMHD1 protein expression in whole cells. $^{**}P < 0.01$ vs. SAMHD1 expression in THP-1 cells.

cells. This difference may affect the immune function and HIV-1 susceptibility. As SAMHD1 is an important nucleic acid hydrolase involved in cellular nucleic acid metabolism, its level of expression is closely related to cell proliferation and level of activation. For example, SAMHD1 expression is high in resting CD4+ T cells, whereas it is low in activated CD4+ T cells [10]. The mechanism of SAMHD1 expression in primary cell functions should be investigated further.

The different SAMHD1 expression in different cells and tissues indicates that regulation of its expression is strictly controlled; however, we have no further information on this mechanism of expression regulation. De Silva et al. [11] found that epigenetic modification of the SAMHD1 promoter region is very important for the regulation of SAMHD1 expression. However, epigenetic modification of the SAMHD1 promoter may only affect SAMHD1 promoter activity and SAMHD1 transcription level. Regulation of the post-transcriptional level of SAMHD1 expression remains unclear. Moreover, De Silva et al. discovered that methyltransferase inhibitors and histone deacetylase inhibitors significantly increased the level of *SAMHD1* mRNA expression; that of protein did not change significantly. This indicated that SAMHD1 expression is subject to an important mechanism of post-transcriptional regulation. MicroRNA is a non-coding RNA whose essential function is regulating genetic expression [21]. Online software determined that miR-181 scored the highest among the microRNAs binding the SAMHD1 3′ UTR. Examining HIV-1-permissive and -non-permissive cells and somatic cells, we found that the miR-181 level was negatively related to the level of SAMHD1 mRNA

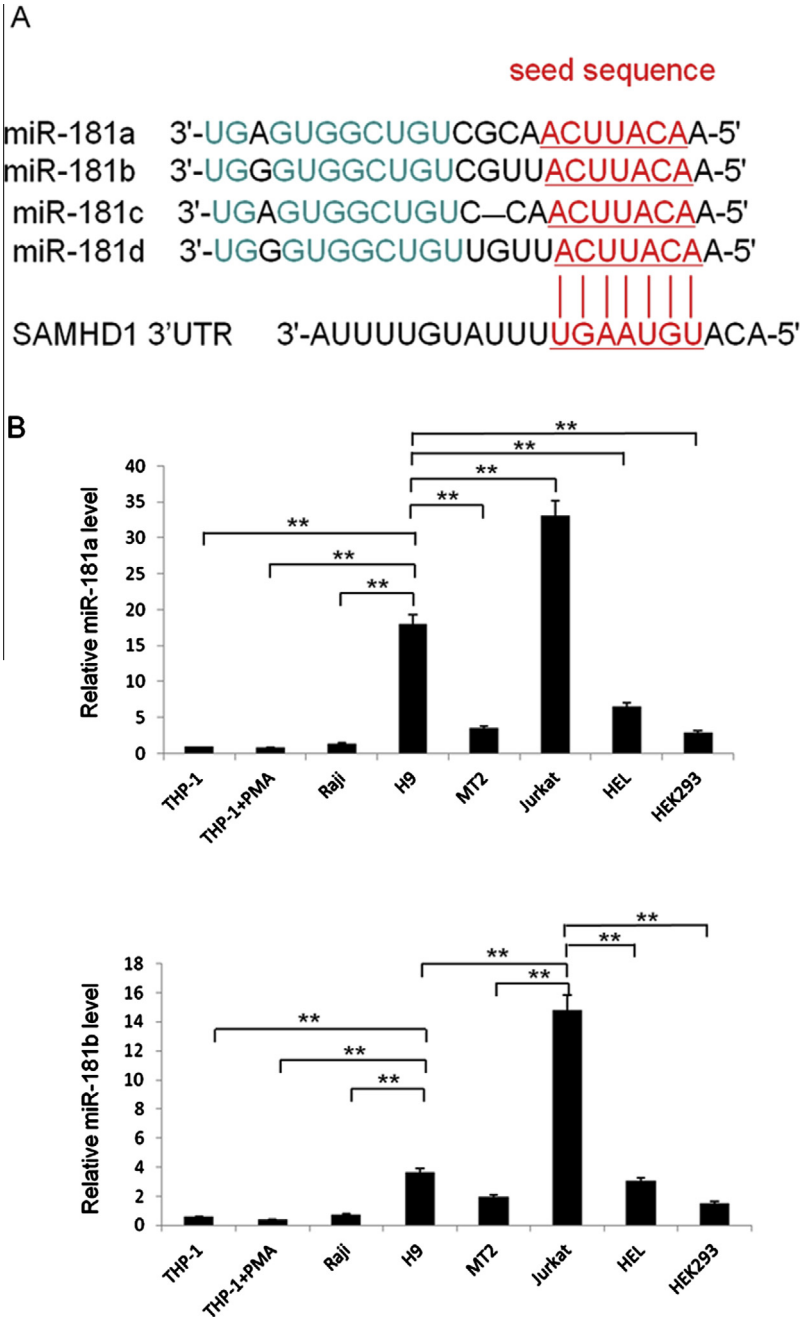

Fig. 2. Level of miR-181 expression in different cell lines. (A) MiR-181 seed sequence and binding site with *SAMHD1* mRNA 3′ UTR. (B) Relative miR-181a and miR-181b expression levels. MiRCURY LNA™ qPCR was used to detect the levels of the four miR-181. The level of miR-181a in THP-1 cells was defined as 1. $^{**}P < 0.01$.

and protein expression, suggesting that miR-181 plays an important role in SAMHD1 post-transcription regulation. We transfected HEK293T cells, which expressed moderate levels of SAMHD1, to study miR-181 regulation of the SAMHD1 3′ UTR, and found that miR-181a and miR-181c played a greater role than miR-181b and miR-181d. Considering the high miR-181a expression and low miR-181c expression, we believe that miR-181a may be the key microRNA regulating SAMHD1 expression in the cell lines. We verified the results using the levels of SAMHD1 mRNA or protein following miR-181a over expression and inhibition in THP-1 and Jurkat cells. In short, we demonstrated that miR-181 regulates the post-transcriptional level of SAMHD1 expression negatively. To our knowledge, this is the first report of microRNA regulation in SAMHD1 expression.

The miR-181 family is highly expressed in thymus, spleen, bone marrow, and other organs and tissues, and participate in some important biological processes including transcriptional and translational regulation, signaling transduction *etc.* [22]. MiR-181 plays important roles in the development, proliferation, and differentiation of T, B, NK, and other hematopoietic cells [14,15,22,23]. Since SAMHD1 is an important modulator of nucleic acid metabolism and cell cycle [24], the regulation of SAMHD1 by miR-181 may also be a vital approach for hematopoietic cell development. MiR-181 can influence T cell immune response through affecting the strength of some cell signals, such as T cell receptor signaling and Toll-like receptor signaling [25,26]. The signals are also important for SAMHD1 function and immune response to HIV-1 infection. Some studies reported that IFN-γ production in CD4+ T and NK cells is also

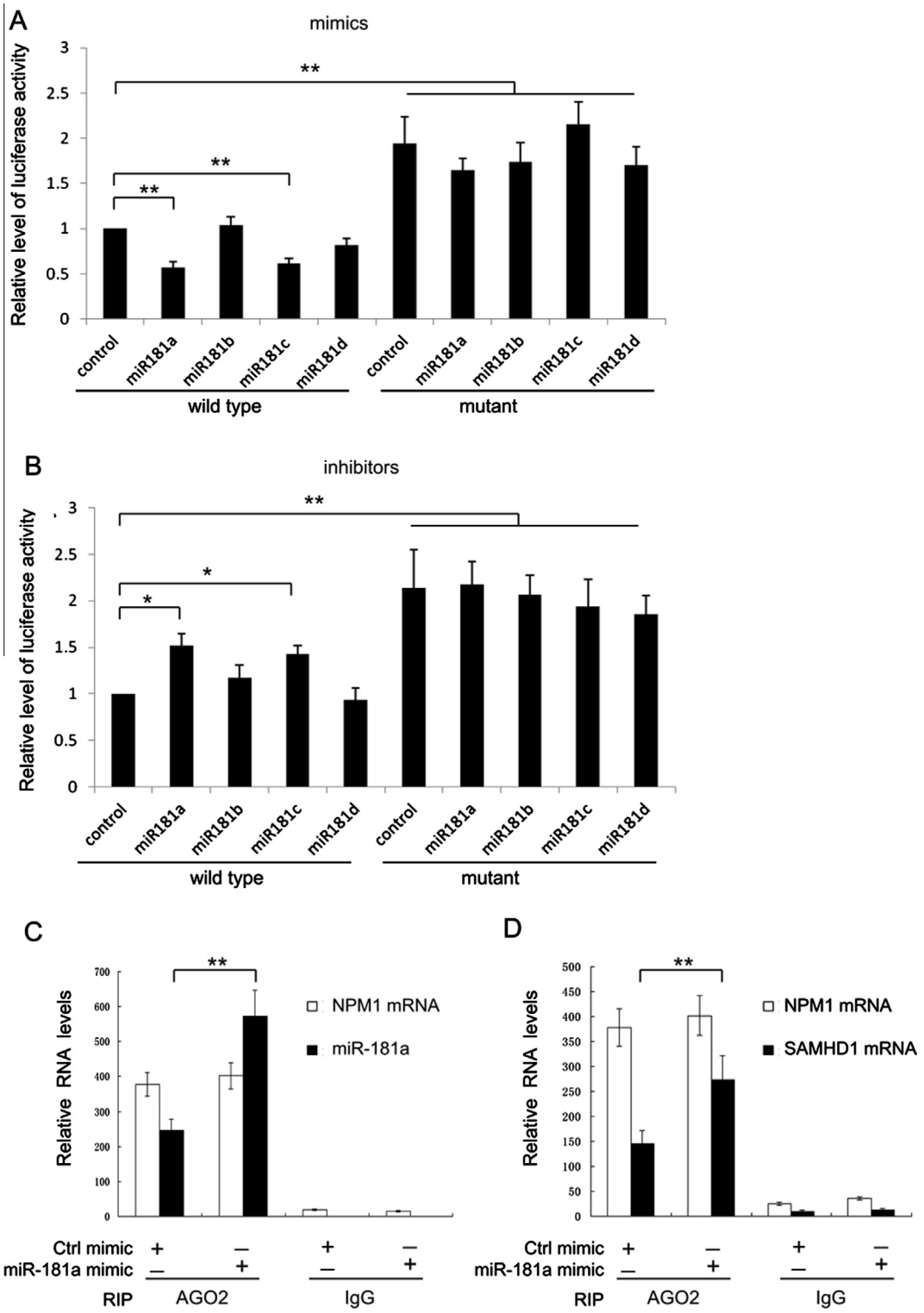

Fig. 3. Binding of miR-181 and *SAMHD1* mRNA 3′ UTR. To construct the luciferase reporter plasmid containing the wild type and mutant *SAMHD1* mRNA 3′ UTR, HEK293T cells were cotransfected with (A) miR-181 mimics or (B) inhibitors, lysed, and the relative luciferase activity determined. The relative activity of the luciferase reporter plasmid containing the SAMHD1 3′ UTR was defined as 1 in the control group. RIP was performed using anti-AGO2 antibody or immunoglobulin G (IgG) from lysates of HEK293T cells transfected with miR-181a mimics. *NPM1* mRNA, (C) miR-181a, and (D) *SAMHD1* mRNA levels were examined by qRT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$.

impacted by miR-181[27,28], which may also impact SAMHD1 expression since SAMHD1 is IFN-γ induced. SAMHD1 antiretroviral activity is modulated by post-translational modifications, cell-cycle-dependent functions and cytokine-mediated changes [24]. Recent studies have established a link among SAMHD1 restriction, innate sensing of DNA and protective immune responses. The relation among SAMHD1 post-transcriptional regulation by miR-181, innate immune responses and HIV-1 infection should be explored.

Our findings demonstrate that miR-181 regulates post-transcriptional SAMHD1 expression by degrading its mRNA. However,

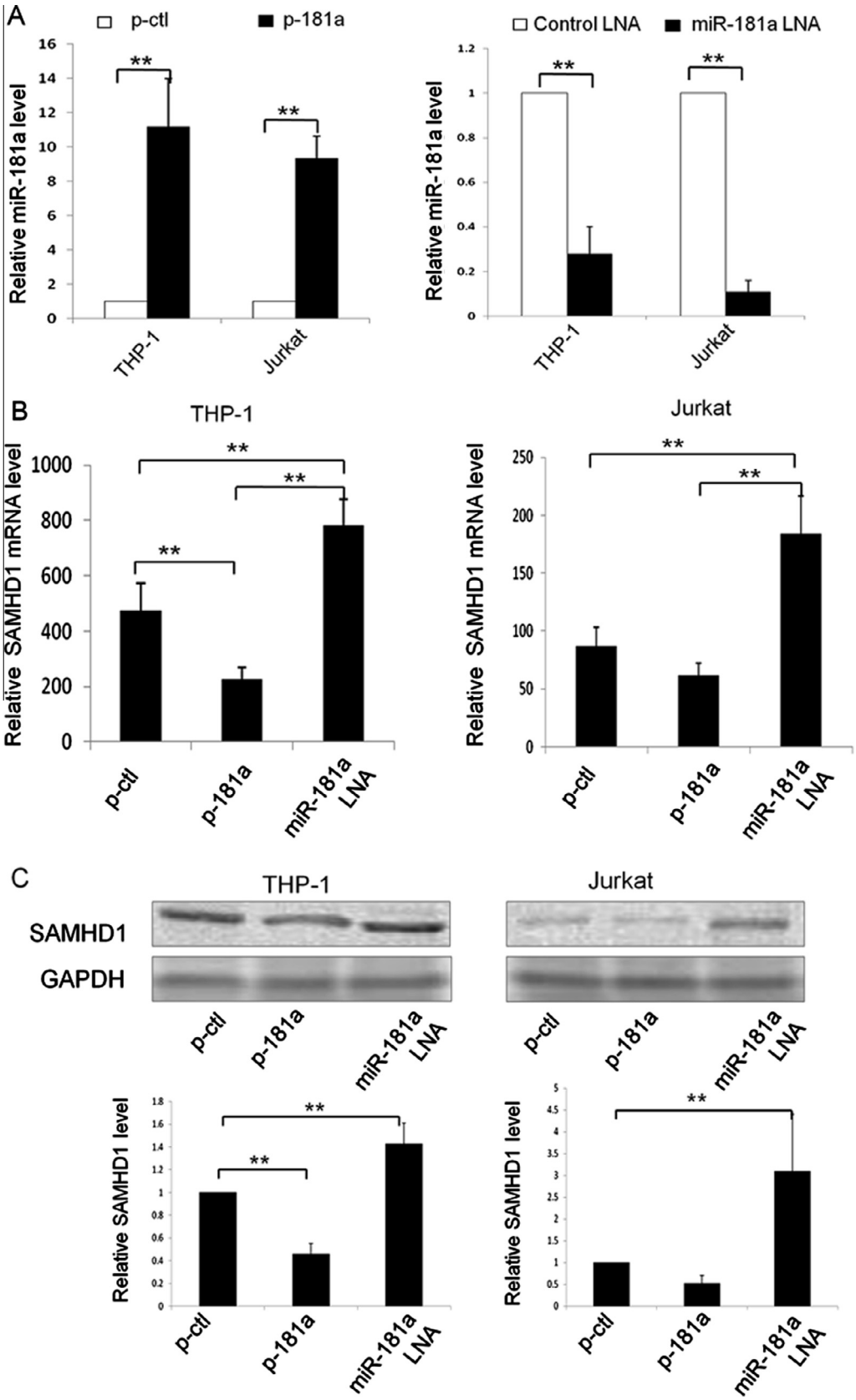

Fig. 4. Regulatory effect of miR-181 on SAMHD1 expression. THP-1 and Jurkat cells were electrotransfected with miR-181a overexpression plasmid (p-181a) or anti-miR-181a LNA. (A) QPCR detection of p-181a and anti-miR-181a LNA effect on intracellular miR-181a level. The miR-181a level in the control group was defined as 1. (B) QPCR determination of p-181a and anti-miR-181a LNA effect on cellular *SAMHD1* mRNA level; *GAPDH* mRNA was used as the control. (C) Western blots of p-181a and anti-miR-181a LNA effect on cellular SAMHD1 protein level with GAPDH as the control. SAMHD1 protein level in the control group was defined as 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

many factors, and not merely expression level, regulate SAMHD1 activity. Previous studies have suggested that phosphorylation of human SAMHD1 at threonine 592 negatively regulates its HIV-1 restriction activity [12]. Nuclear location and cell cycling can also affect the function of SAMHD1 [10,29]. Recently, GTP and dNTP were reported to be allosteric effectors of SAMHD1 [30]. DC–T lymphocyte talk also regulates SAMHD1 activity in DCs [31]. The role of miR-181 in the regulation of SAMHD1 bioactivity requires further study.

In summary, we demonstrated the miR-181 is involved in the post-transcriptional regulation of SAMHD1 expression; its mechanism of function is to bind directly with the SAMHD1 3′ UTR to degrade the mRNA, thus reducing SAMHD1 expression. Due to the important role SAMHD1 plays in cellular nucleic acid metabolism and HIV-1 infection, miR-181 may be an important target of many cytokines, such as IFN, for regulating SAMHD1 expression.

Conflict of interest

All authors declare no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.151.

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