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Inhibition of Hepatitis B virus replication by SAMHD1

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ABSTRACT

Sterile alpha motif and HD-domain containing protein 1 (SAMHD1) is a newly identified intracellular antiviral factor. By depleting the dNTPs pool of host cells to a low level that cannot support the efficient synthesis of viral cDNA, it restricts replication of some retroviruses. As a DNA virus, Hepatitis B virus (HBV) experiences a process of reverse transcription in its life cycle akin to that of retroviruses. However, whether SAMHD1 can restrict HBV replication in liver cells is unknown. Here, we reported that SAMHD1 expression was detectable in four liver cell lines. Exogenous expression of SAMHD1 in SMMC-7721 cells restrained HBV replication. Similarly, SAMHD1 impeded HBV replication in another liver cell line, BEL-7402. Remarkably, the catalytically inactive mutant, SAMHD1 HD/AA also hampered HBV replication. Additionally, HBV replication reduced SAMHD1 expression in HepG2 cells. Moreover, it was found that IFN-α induced expression of SAMHD1 in liver cells. Together, these findings suggested that IFN-α-inducible SAMHD1 inhibited HBV replication in liver cells.

1. Introduction

Hosts have evolved diverse mechanisms to defend themselves against viral infection. Intrinsic immunity mediated by constitutively expressed restriction factors is the recently recognized way to block viral replication, which is somewhat different from the conventional innate immunity [1]. In 2011, Sterile alpha motif and HD-domain containing protein 1 (SAMHD1) was identified as a novel intracellular antiviral factor in intrinsic immunity [2–4]. By depleting the deoxynucleoside triphosphates (dNTPs) pool of host cells to a low level that cannot support the efficient synthesis of viral cDNA, SAMHD1 restricts replication of Human immunodeficiency virus type 1 (HIV-1) [5–7]. Besides HIV-1, SAMHD1 has been found to inhibit replication of other retroviruses [8–10]. Recent studies further demonstrated SAMHD1 restricted DNA viruses in non-dividing myeloid cells by limiting DNA replication [11,12].

Hepatitis B virus is a DNA virus whose infection accounts for approximately 1 million deaths per year from cirrhosis, liver failure and hepatocellular carcinoma (HCC) [13]. As a nonretroviral virus, HBV experiences a process of reverse transcription in its life cycle akin to that of lentiviruses [14]. SAMHD1 was found to be expressed in human liver [15]. However, whether HBV replication can be inhibited by human SAMHD1 in hepatocytes is unknown.

In the present study, we examined the role of SAMHD1 in HBV replication in liver cells. We found IFN-α-inducible SAMHD1 could inhibit HBV replication in liver cells without depleting intracellular dNTPs.

2. Materials and methods

2.1. Plasmids

pHBVwt, which contained HBV1.3L (replication-competent overlength 1.3-fold HBV), and its mutant pHBV ΔX (HBx defective) were generous gifts from Dr. James Ou (University of Southern California), and had been described previously [16]. pcDNA3.1-HA-SAMHD1wt (pSAMHD1wt) and pcDNA3.1-HA-SAMHD1 HD/AA (pSAMHD1 HD/AA) were kind gifts from Dr. Klaus Strebel (National Institutes of Health). pRL-TK (Promega) was used to correct for transfection efficiency and luciferase activities were measured using Renilla Luciferase Assay System (Promega) according to its technical manual. The empty vectors pcDNA3.1 and pUC19 were purchased from Invitrogen and Sangon respectively and were stored in our laboratory.

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2.2. Cell culture, transfection and IFN-α stimulation

HepG2, LO2, BEL-7402, SMMC-7721 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum in a 37 °C incubator with 5% CO_2. BEL-7402, SMMC-7721, HepG2 cells were plated in the appropriate amount per well in 96-well plates (for MTT assay), 6-well plates (for Western blot and immunofluorescence assay) and 12-well plates (for Western blot and determination of HBV antigens and DNA levels) respectively and grown to be 80–90% confluent. Then cells were transfected (or cotransfected) with the corresponding plasmids using Lipofectamine™ 2000 (Invitrogen) according to its recommended protocol. 48 h post-transfection, cells were harvested for next analysis. In IFN-α stimulation experiments, BEL-7402, SMMC-7721 cells were treated with 0, 10, 100, 1000, 10,000 IU/ml IFN-α (AnkeBio) for 8 h respectively, then cells were harvested for Western blot. Meanwhile, BEL-7402, SMMC-7721 cells were stimulated with 1000 IU/ml for 0, 2, 4, 6, 8, 10, 12 h and then harvested for Western blot.

2.3. Western blot analysis

After washing with ice-cold phosphate-buffered saline, cells were lysed in RIPA lysis buffer. The protein concentrations of the samples were determined using BCA kit (Beyotime). The prepared cell lysates were separated by SDS–PAGE and transferred to PVDF membranes. After blocking, membranes were incubated with the primary antibodies: anti-SAMHD1 (Proteintech) or anti-HA (Proteintech) or anti-β-actin (ZSGB-Bio). Following extensive washing of the sheets in TBS including 0.1% Tween-20, membranes were incubated with the secondary antibodies: horseradish peroxidase conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG (Biosharp). Immunoreactive bands were finally visualized by chemiluminescence using Super Signal West Femto (Thermo Scientific) and images were captured using digital imaging system (Tanon). In IFN-α stimulation experiments, Western blot strips were quantified by densitometric analysis and showed as fold change relative to untreated control in bar graphs respectively.

2.4. Immunofluorescence

HepG2, LO2, SMMC-7721, BEL-7402 cells were plated on coverslips and fixed with Immunol Staining Fix Solution (Beyotime) for 15 min at room temperature, then cells were washed three times for 5 min with Immunol Staining Wash Buffer (Beyotime) containing Triton X-100 for permeabilization. The fixed cells were incubated with anti-SAMHD1 primary antibody (1:200 dilution; Proteintech) for 45 min at room temperature. After washing with Immunol Staining Wash Buffer (Beyotime), cells were incubated at room temperature with rhodamine labeled secondary antibody (1:100; Proteintech) for 1 h. Cells were further washed with Immunol Staining Wash Buffer (Beyotime) and incubated with DAPI (Beyotime) for 5 min. Then cells were washed again with Immunol Staining Wash Buffer (Beyotime) and coverslips were mounted on glass slides with Antifade Mounting Medium (Beyotime). Finally, mounted coverslips were examined and images were taken by Laser Scanning Confocal Microscope (Leica). pSAMHD1wt-transfected BEL-7402 cells were regarded as a positive control for SAMHD1 expression.

2.5. Cytotoxicity assay

MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime) was used to test the cytotoxicity after introducing exogenous SAMHD1. Briefly, BEL-7402 and SMMC-7721 cells were seeded in the 96-well plate and transfected with pcDNA3.1, pSAMHD1wt or pSAMHD1 HD/AA, 48 h later, 10 μl 5 mg/ml MTT solution (Beyotime) were added into each well. Then cells with MTT solution were incubated in a 37 °C incubator with 5% CO_2 for 4 h. Subsequently, 100 μl Formazan solution (Beyotime) were added into each well and incubated with cells in the 37 °C incubator with 5% CO_2 until the crystal dissolved. Finally, the absorbance of the cell solution was measured with a universal microplate reader (Bio-tek) at 570 nm wavelength. Results were presented as relative values of cell viability and OD value of pcDNA3.1-transfected cells was set as 100.

2.6. ELISA for determining HBsAg and HBeAg levels

48 h after transfection, the culture supernatants were collected and centrifuged at 2000 rpm for 10 min to remove cell debris. The concentrations of HBsAg and HBeAg in the supernatants were determined respectively by the commercial ELISA kits for HBsAg and HBeAg (Kehua) according to the manufacturer's instructions. The absorbance was measured at 450 nm wavelength using a universal microplate reader (Bio-tek). Data were normalized to Renilla luciferase activities in the corresponding wells. Results were shown as ratio of the value from pSAMHD1wt or pSAMHD1 HD/AA-transfected cells to that from pcDNA3.1-transfected cells which was set as 1.

2.7. Quantitative analysis of core-associated HBV DNA levels

The cell culture supernatants were collected 48 h post-transfection and centrifuged at 13,000 rpm for 1 min to remove cell debris. The supernatants were adjusted to 1 × DNA buffer ($MgCl_2$) and 0.1 U/μl DNase I (Thermo Scientific) and incubated for 2 h at 37 °C. Then 4.5 mM EDTA were added into the reactants and incubated for 10 min at 65 °C to stop the reaction. After centrifuging at 13,000 rpm for 1 min, the supernatants were used for quantification of core-associated HBV DNA by Fluorescence Quantitative PCR Detection Kit for Hepatitis B virus DNA (Aconlab) according to the kit's protocol. The copy numbers of core-associated HBV DNA were normalized to Renilla luciferase activities in the corresponding wells. Results were shown as ratio of the value from pSAMHD1wt or pSAMHD1 HD/AA-transfected cells to that from pcDNA3.1-transfected cells which was set as 1.

2.8. Statistical analysis

Data were presented as the mean ± SD of triplicate wells. Each group was compared with pcDNA3.1-transfected group respectively. The statistical significance of differences between two groups was analyzed with Student's t test. A p value (p) < 0.05 was considered statistically significant.

3. Results

3.1. SAMHD1 expression is detectable in four liver cell lines

To investigate whether SAMHD1 restricted HBV replication in liver cells, we firstly detected endogenous expression of SAMHD1 in four liver cell lines: HepG2, LO2, BEL-7402, SMMC-7721. BEL-7402 cells transiently expressing exogenous SAMHD1 was regarded as a positive control for SAMHD1 expression. As showed in Fig. 1A, SAMHD1 expression was detectable in all four liver cell lines using Western blot whilst it was over-expressed in transfected BEL-7402 cells. These results were further confirmed by immunofluorescence (Fig. 1B). Moreover, similar to previous reports [17,18], we discovered that SAMHD1 was localized in the nucleus of liver cells (Fig. 1B).

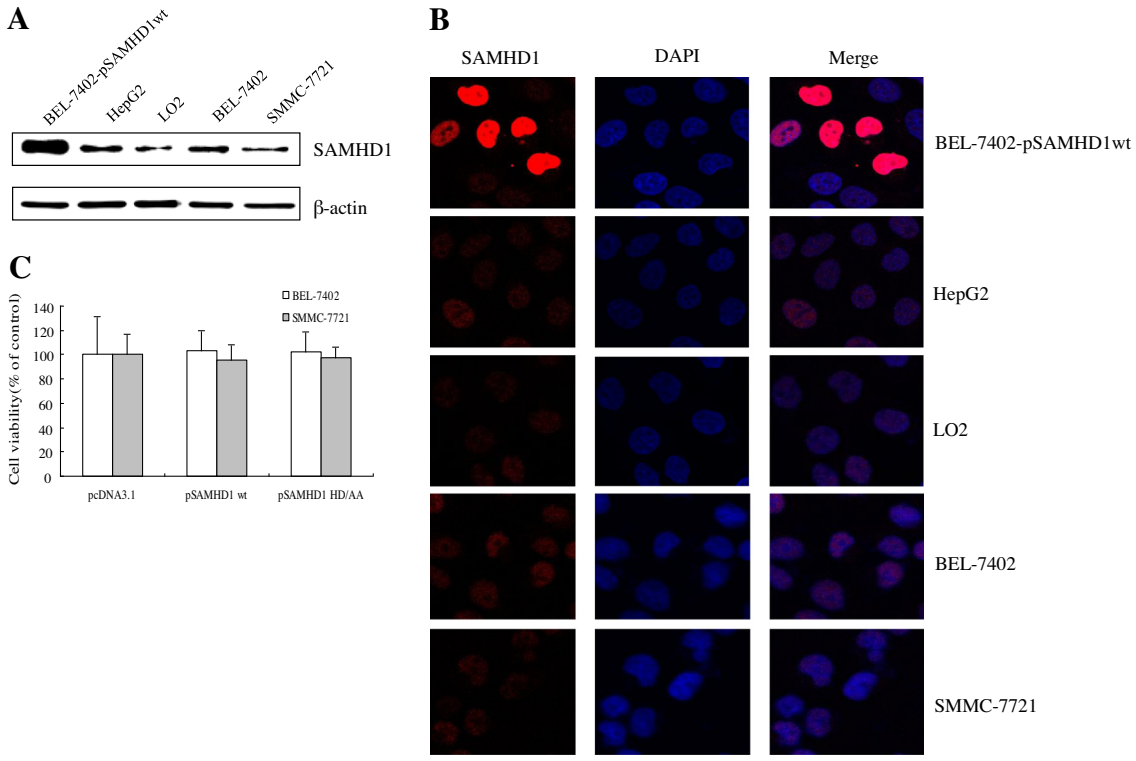

Fig. 1. Expression status of endogenous SAMHD1 in human liver cell lines and effects of exogenous expression of SAMHD1 on cell viability. (A) HepG2, LO2, SMMC-7721, BEL-7402 cells were harvested for Western blot using anti-SAMHD1 primary antibodies. BEL-7402 cells transiently transfected with pSAMHD1wt (pcDNA3.1-HA-SAMHD1wt) were set as a positive control for SAMHD1 expression. (B) HepG2, LO2, SMMC-7721, BEL-7402 cells and pSAMHD1wt-transfected BEL-7402 cells were plated on coverslips and observed by confocal laser scanning microscopy after immunofluorescence staining. (C) pcDNA3.1 (empty vector), pSAMHD1wt and pSAMHD1 HD/AA (pcDNA3.1-HA-SAMHD1 HD/AA) were transfected into BEL-7402 and SMMC-7721 cells respectively, cell viability was measured by MTT method after 48 h. Results were presented as relative values and OD value of pcDNA3.1-transfected cells was set as 100. Data were representative of three independent experiments and shown as mean ± SD ($n = 3$).

3.2. Exogenous expression of SAMHD1 has no cytotoxic effect on liver cells

Exogenous expression of SAMHD1 has been reported to have no obvious effect on cellular proliferation and cellular β-actin mRNA splicing in HEK293T cells [19]. However, whether SAMHD1 overexpression has cytotoxic effect on liver cells is unclear. We utilized MTT assay to evaluate the cytotoxicity of exogenous SAMHD1 expression in BEL-7402 and SMMC-7721 cells. It was found that exogenous expression of SAMHD1 had no effect on cell viability of these two liver cell lines (Fig. 1C). And no apparent alteration of cell morphology between pcDNA3.1-transfected cells and pSAMHD1wt or pSAMHD1 HD/AA-transfected cells was observed under the light microscope (data not shown). These results suggested exogenous expression of SAMHD1 had no cytotoxic effect on liver cells.

3.3. SAMHD1 restricts HBV replication in SMMC-7721 cells

Next we examined the effect of SAMHD1 in HBV replication in liver cells. Firstly, we transiently co-transfected pHBVwt with pcDNA3.1 (empty vector), pSAMHD1wt, pSAMHD1 HD/AA into SMMC-7721 cells respectively, then HBV antigens and core-associated HBV DNA levels in cell supernatants were determined respectively by ELISA and Realtime PCR. Because pSAMHD1wt and pSAMHD1 HD/AA expressed HA-tagged proteins, overexpression of SAMHD1 and SAMHD1 HD/AA were clearly detected in transfected-SMMC-7721 cells by Western blot using anti-HA antibody (Fig. 2A). Importantly, we found HBsAg and HBeAg concentrations in cell supernatants were significantly decreased in pSAMHD1wt and pSAMHD1 HD/AA-transfected cells in comparison with pcDNA3.1-transfected cells, suggesting SAMHD1 possessed inhibition activities against HBV replication (Fig. 2B). And these inhibition activities were elevated gradually with increasing doses of plasmids expressing SAMHD1 (Fig. 2C). HBV core-associated DNA levels were also reduced significantly in the supernatants of pSAMHD1wt and pSAMHD1 HD/AA-transfected cells as compared with pcDNA3.1-transfected cells (Fig. 2D). These results suggested SAMHD1 restricted HBV replication in SMMC-7721 cells.

SAMHD1 in which histidine and aspartate residues were substituted with alanine (HD/AA) is catalytically inactive and cannot hydrolyze dNTPs [3,7]. Interestingly, SAMHD1 HD/AA also suppressed HBV replication in SMMC-7721 cells although its inhibition function was less than wild-type SAMHD1 protein (Fig. 2B and D). These results indicated that SAMHD1 could inhibit HBV replication in liver cells without depleting intracellular dNTPs.

3.4. SAMHD1 suppresses HBV replication in BEL-7402 cells

We further examined whether SAMHD1 restricted HBV replication in another liver cell line, BEL-7402. Similar to in SMMC-7721 cells, exogenous expression of SAMHD1 and SAMHD1 HD/AA were distinctly detected in BEL-7402 cells by Western blot using anti-HA antibody after co-transfection (Fig. 3A). Both wild-type SAMHD1 and SAMHD1 HD/AA mutants significantly reduced the production of HBsAg and HBeAg in the cell supernatants of transfected BEL-7402 cells (Fig. 3B). Furthermore, HBV core-associated DNA levels in the cell supernatants were significantly decreased by wild-type SAMHD1 and SAMHD1 HD/AA mutants (Fig. 3C). These results suggested SAMHD1 restricted HBV replication in BEL-7402 cells.

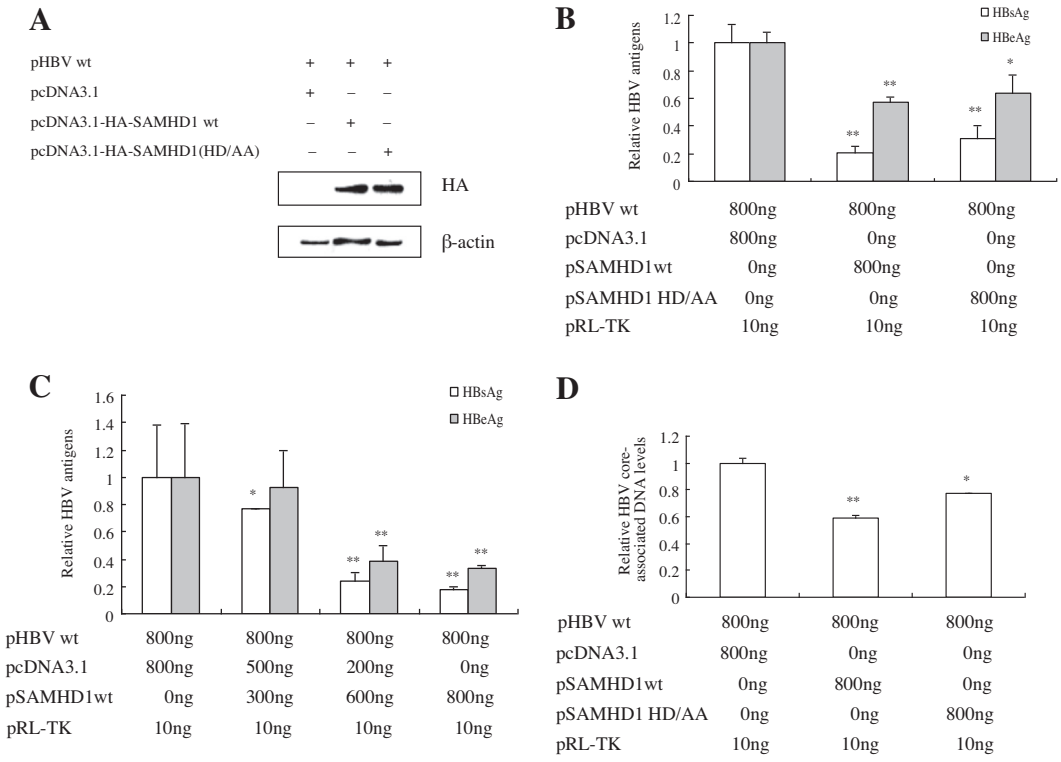

Fig. 2. SAMHD1 restricts HBV replication in SMMC-7721 cells. (A) HBV1.3L (wild type) plasmids (pHBVwt) were co-transfected transiently into SMMC-7721 cells with pcDNA3.1, pSAMHD1wt (pcDNA3.1-HA-SAMHD1wt), pSAMHD1 HD/AA (pcDNA3.1-HA-SAMHD1 HD/AA) respectively. 48 h later, exogenous SAMHD1 expression was detected by Western blot using anti-HA antibody. (B) pHBVwt were transiently co-transfected into SMMC-7721 cells with pRL-TK (transfection efficiency control) and pcDNA3.1, pSAMHD1wt, pSAMHD1 HD/AA respectively according to doses indicated in the legend. 48 h later, the levels of HBsAg and HBeAg in cell supernatants were determined by ELISA. (C) pHBVwt were transiently co-transfected into SMMC-7721 cells with pRL-TK, pcDNA3.1 and pSAMHD1wt according to doses indicated in the legend. 48 h later, the levels of HBsAg and HBeAg in cell supernatants were determined by ELISA. (D) pHBVwt were transiently co-transfected into SMMC-7721 cells with pRL-TK and pcDNA3.1, pSAMHD1wt, pSAMHD1 HD/AA respectively according to doses indicated in the legend. 48 h later, HBV core-associated DNA levels in the cell supernatants were measured by real time PCR. The values from pcDNA3.1-transfected cells were set as 1. Data were representative of at least three independent experiments. The error bars were shown as mean ± SD ($n = 3$). ($^{*}p < 0.05$;$^{**}p < 0.01$.)

Taken together with the results from SMMC-7721 cells, we concluded that SAMHD1 possessed the anti-HBV functions in liver cells.

3.5. HBV replication down-regulates SAMHD1 expression in HepG2 cells

HIV-2 and SIV (simian immunodeficiency virus) have evolved Vpx to counteract SAMHD1-mediated restriction of retroviral infection [3,4]. Whether HBV possesses similar antagonism is unknown. HBx is the sole regulatory protein of HBV and it mediates many functions that may facilitate HBV replication. SAMHD1 is expressed at relatively high levels in HepG2 cells (Fig. 1A). We examined whether the replication of HBVwt (wild type) and HBV ΔX (HBx defective) affected SAMHD1 expression in HepG2 cells. After transfection with pHBVwt, SAMHD1 protein levels were decreased to a low level, while SAMHD1 expression was not altered in pHBV ΔX-transfected cells (Supplementary Fig. 1). These results showed HBV replication down-regulated SAMHD1 expression in HepG2 cells and HBx played the important role in this process.

3.6. IFN-α induces SAMHD1 expression in liver cells

IFN-α is widely used for treatment of HBV infection in the clinical practice. Previous evidence showed IFN-α induced SAMHD1 expression in monocytes [20], astrocytes [21], U87-MG cells [22], HEK293T and Hela cells [23]. However, it is unclear whether SAMHD1 is upregulated by IFN-α in liver cells. We found IFN-α treatment increased SAMHD1 protein levels in BEL-7402 and SMMC-7721 cells in a dose and time dependent manner (Fig. 4). Moreover, IFN-α was easier to induce SAMHD1 expression in SMMC-7721 cells than BEL-7402 cells (Fig. 4A and C). Time course analysis indicated that up-regulation of SAMHD1 protein levels by IFN-α reached to the peak at 10 h and then declined at 12 h (Fig. 4B and D) in both BEL-7402 and SMMC-7721 cells. We also found SAMHD1 was induced by IFN-α in HepG2 cells, albeit modestly (data not shown).

4. Discussion

It has been well known that SAMHD1 restricts replication of retroviruses [8–10]. Recent studies showed SAMHD1 also blocked replication of two double stranded DNA viruses, vaccinia virus and herpes simplex virus 1, by depleting dNTPs in non-dividing myeloid cells [11,12]. Here we provided evidence that SAMHD1 inhibited replication of HBV, another DNA virus, in liver cells. Remarkably, the catalytically inactive mutant, SAMHD1 HD/AA also hampered HBV replication, suggesting SAMHD1 had additional antiviral function except for depleting intracellular dNTPs. We found the total inhibition activities against HBV mediated by SAMHD1 HD/AA was less than wild-type SAMHD1 protein (Figs. 2B, D, and 3B, C). These results implied that both dNTPs

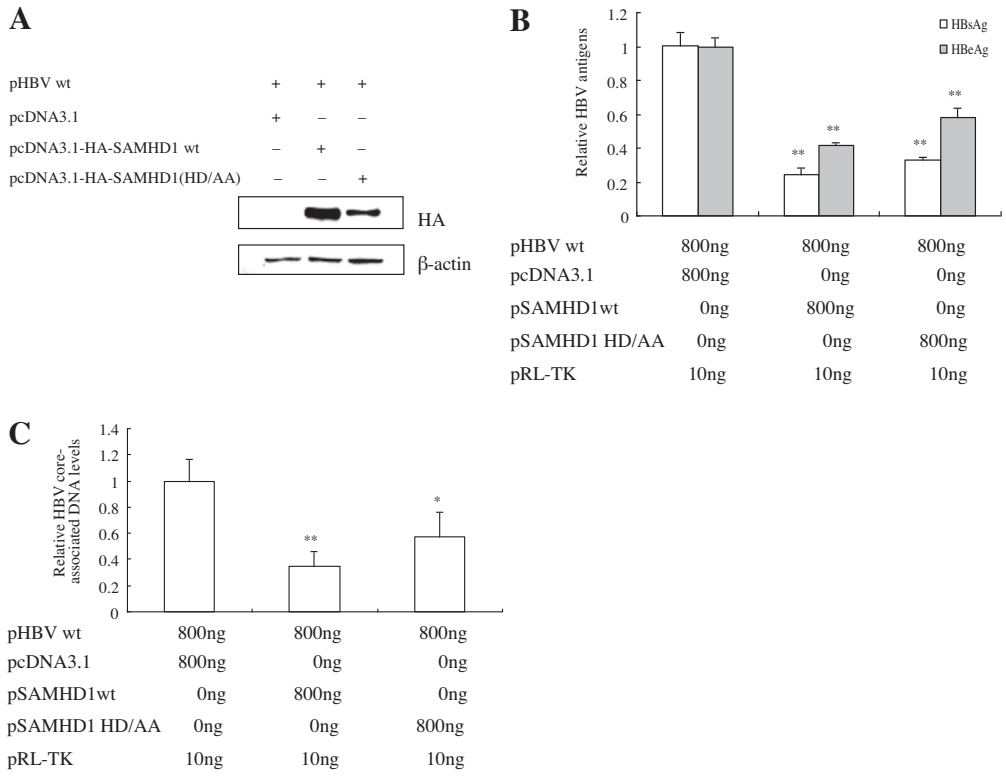

Fig. 3. SAMHD1 suppresses HBV replication in BEL-7402 cells. (A) pHBVwt were transiently co-transfected into BEL-7402 cells with pcDNA3.1, pSAMHD1wt, pSAMHD1 HD/AA respectively. 48 h later, exogenous SAMHD1 expression was detected by Western blot using anti-HA antibody. (B) pHBVwt were transiently co-transfected into BEL-7402 cells with pRLTK and pcDNA3.1, pSAMHD1wt, pSAMHD1 HD/AA respectively according to doses indicated in the legend. 48 h later, the levels of HBsAg and HBeAg in cell supernatants were determined by ELISA. (C) pHBVwt were transiently co-transfected into BEL-7402 cells with pRL-TK and pcDNA3.1, pSAMHD1wt, pSAMHD1 HD/AA respectively according to doses indicated in the legend. 48 h later, HBV core-associated DNA levels in the cell supernatants were determined by real time PCR. The values from pcDNA3.1-transfected cells were set as 1. Data were representative of at least three independent experiments. The error bars were shown as mean ± SD ($n = 3$). ($^{*}p < 0.05$;$^{**}p < 0.01$.)

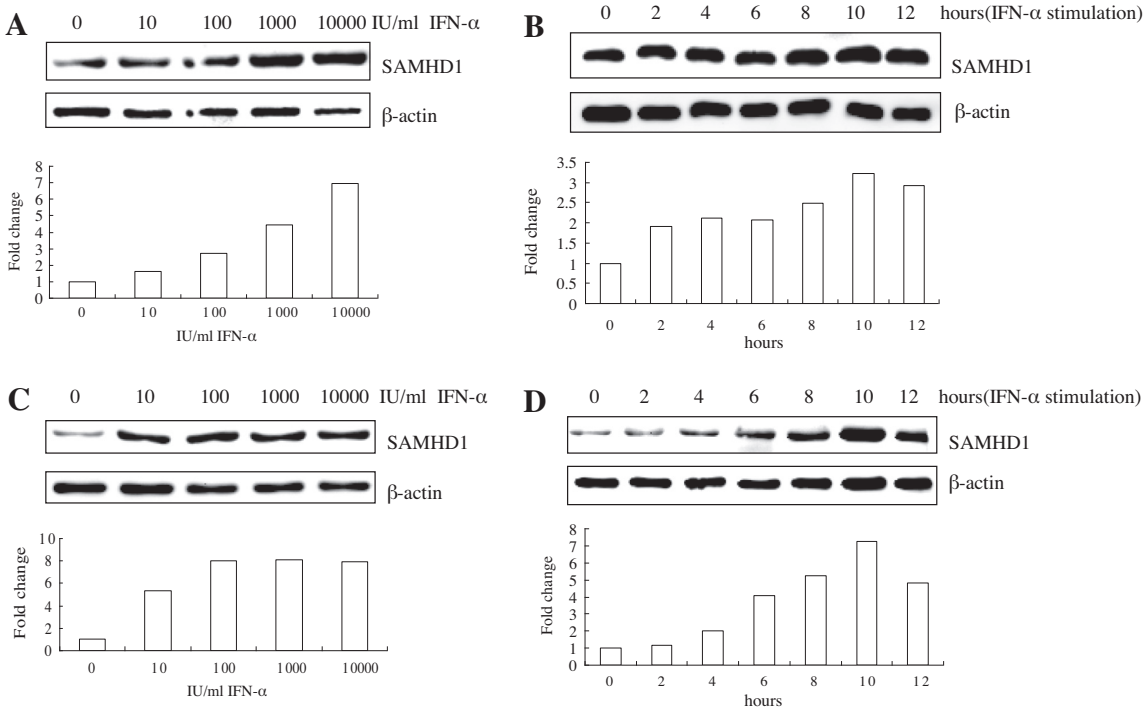

Fig. 4. IFN-α induces SAMHD1 expression in liver cells in a dose and time dependent manner. BEL-7402 (A) and SMMC-7721 (C) cells were treated with 0, 10, 100, 1000, 10,000 IU/ml IFN-α respectively, 8 h later, cells were harvested for Western blot using anti-SAMHD1 antibody. BEL-7402 (B) and SMMC-7721 (D) cells were stimulated with 1000 IU/ml IFN-α for 0, 2, 4, 6, 8, 10, 12 h, then cells were harvested for Western blot using anti-SAMHD1 antibody. Western blot strips were quantified by densitometric analysis and showed as fold change relative to untreated control in bar graphs respectively. Data were representative of at least three independent experiments.

depleting and non-dNTPs depleting mechanism might be involved in anti-HBV activities of SAMHD1 in liver cells.

We found exogenous expression of SAMHD1 had no cytotoxicity on BEL-7402 and SMMC-7721 cells. Thus, inhibition of HBV replication after co-transfection in our studies was not due to cytotoxic effect of SAMHD1 on liver cells. Most recently, Zhao et al. reported that SAMHD1 barely affected the production of HBV in HEK293T cells [19]. The discrepancy between their and our results might be due to different usage of cell lines. Unexpectedly, the depletion of endogenous SAMHD1 gene expression in HepG2 cells by RNA interference did not increase HBV products and HBV antigens in the supernatants were at low levels. This might be because of the release of IFN-β after silencing SAMHD1 in HepG2 cells (unpublished data).

Our results demonstrated HBV replication down-regulated SAMHD1 expression in HepG2 cells and HBx was involved in this process (Supplementary Fig. 1). Previous evidence showed HBx enhanced HBV replication in HepG2 cells in vitro and in vivo [24,25]. Interestingly, HBx may bind DDB1 to redirect the ubiquitin ligase activity of the CUL4-DDB1 E3 ligase [26,27]. This is reminiscent of Vpx which recruits SAMHD1 to the CUL4-DDB1 E3 ubiquitin ligase for proteasome-dependent degradation, thereby relieving SAMHD1-mediated inhibition of HIV-1 infection [3,4]. It is very likely that HBx recruits SAMHD1 to the same E3 ubiquitin ligase for protein degradation in HepG2 cells.

IFN-α could not induce SAMHD1 expression in monocyte-derived macrophages (MDMs), monocyte-derived dendritic cells (MDDCs) and primary CD4+ T cells [22,23], probably because of high levels of endogenous SAMHD1 that were already existed. Consistent with this phenomenon, we found IFN-α induced less SAMHD1 expression in HepG2 cells with relatively high levels of endogenous SAMHD1 than in SMMC-7721 and BEL-7402 cells. By immunofluorescence, we found SAMHD1 localized in the nucleus of liver cells (Fig. 1B), where HBV cccDNA and RNAs were synthesized. It was possible that SAMHD1 interacted with and degraded HBV nucleic acids in the nucleus of liver cells.

In conclusion, we find IFN-α-inducible SAMHD1 inhibits HBV replication in liver cells. The anti-HBV mechanisms of SAMHD1 are currently under deep investigation.

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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.2014.07.023.

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