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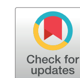
Research Article

LncRNA NKILA inhibits HBV replication by repressing NF-κB signalling activation



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ABSTRACT

Hepatitis B virus (HBV) infection results in liver cirrhosis and hepatocellular carcinoma (HCC). HBx/nuclear factor (NF)-κB pathway plays a role in HBV replication. However, whether NF-κB-interacting long noncoding RNA (NKILA), a suppressor of NF-κB activation, regulates HBV replication remains largely unknown. In this study, gain-and-loss experiments showed that NKILA inhibited HBV replication by inhibiting NF-κB activity. In turn, HBV infection down-regulated NKILA expression. In addition, expression levels of NKILA were lower in the peripheral blood-derived monocytes (PBMCs) of HBV-positive patients than in healthy individuals, which were correlated with HBV viral loads. And a negative correlation between NKILA expression level and HBV viral loads was observed in blood serum from HBV-positive patients. Lower levels of endogenous NKILA were also observed in HepG2 cells expressing a 1.3-fold HBV genome, HBV-infected HepG2-NTCP cells, stable HBV-producing HepG2.2.15 and HepAD38 cells, compared to those HBV-negative cells. Furthermore, HBx was required for NKILA-mediated inhibition on HBV replication. NKILA decreased HBx-induced NF-κB activation by interrupting the interaction between HBx and p65, whereas NKILA mutants lack of essential domains for NF-κB inhibition, lost the ability to inhibit HBV replication. Together, our data demonstrate that NKILA may serve as a suppressor of HBV replication via NF-κB signalling.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the fourth leading cause of cancer-related deaths worldwide. Chronic hepatitis B virus (HBV) infection is the predominant risk factor for HCC development that accounts for more than 90% of all HCC cases. To date, approximately 257 million people have been infected with HBV worldwide (Affo et al., 2017; Feng et al., 2017; Yoo et al., 2017; Lamontagne et al., 2016; Ayub et al., 2013; Li Z. et al., 2016). Although in recent years, advancements in molecular biology and oncology have led to the development of effective therapies for HBV-related HCC, the recurrence and metastasis of HCC after surgery are still the biggest obstacles to the survival of patients (Sohn et al., 2015; Urabe et al., 2017). Postoperative antiviral therapy for HBV-associated HCC can effectively alleviate the recurrence of liver cancer (Huang et al., 2018; Li Z. et al., 2016; Du et al., 2012). Therefore, further studies on the mechanisms of HBV infection and replication will provide important information for the development of new clinical therapeutic and prognostic strategies.

Nuclear factor (NF)-κB transcription complex is formed by the combination of five different monomers [RelA (p65), NF-κB1 (p50), NF-κB2 (p52), c-Rel, and RelB] and is involved in various biological processes, such as immunity, inflammation, cancer, and viral infection (Shokri et al., 2019; Hoesel and Schmid, 2013; Sunami et al., 2016). p65 and p50 heterodimers are responsible for NF-κB pathway activation (Oeckinghaus and Ghosh, 2009; Vallabhapurapu and Karin, 2009). In steady-state settings, p65 and p50 are sequestered in the cytoplasm by the inhibitor of NF-κB (IκB) protein. The central event of NF-κB activation is the singly induced phosphorylation of IκB molecules by IκB kinases (IKKs). IKKs consist of a regulatory subunit, IKKγ, and two homologous catalytic subunits, IKKα and IKKβ. Activated IKKβ induces the phosphorylation of

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IκB protein, leading to K48-linked ubiquitination of IκB and its subsequent degradation, which releases NF-κB dimers from the cytoplasm to the nucleus (Zhang et al., 2017; Mulero et al., 2019).

The state and extent of NF-κB activation play key roles in viral replication (Sunami et al., 2016; Yu et al., 2017). HBV genome has four open reading frames encoding HBx, core, and LHBs/MHBs/SMHBs which play important roles in virus replication, especially the HBx protein (Hsieh et al., 2013; Chong et al., 2017; Xiang et al., 2011). HBx induces the degradation of the Smc5/6 complex, which is involved in homologous recombination-mediated repair of damaged DNA (Sekiba et al., 2022) and promotes the expression of cellular factors to accelerate HBV replication and HBV-induced HCC development by NF-κB (Duan et al., 2018; Wu et al., 2021; Song et al., 2021; Lei et al., 2021). HBx directly affects NF-κB proteins, such as by causing their physical dissociation from IκB inhibitors (Shukla et al., 2011; Bui-Nguyen et al., 2010; Duan et al., 2018). Moreover, HBx stabilization is enhanced by NF-κB activation by interacting with p65 phosphorylation (Majano et al., 2001; Shukla et al., 2011). Therefore, the study of the NF-κB signalling pathway is helpful in providing a strategy for the regulation of immune balance and the development of new broad-spectrum HBV antiviral strategies.

Some long non-coding RNAs (lncRNAs) play significant roles in viral replication, cancer development, and immune responses (Liu et al., 2015; Zhang et al., 2020; Li J. et al., 2016; Wang et al., 2019; Jiang et al., 2018; Huan et al., 2018). NF-κB-interacting lncRNA (NKILA), 2570 bp in length, located on chromosome 20q13, acts as a suppressor of cancer development via abrogation of NF-κB signalling (Bird, 2018; Dijkstra and Alexander, 2015; Huang et al., 2016; Liu et al., 2015; Yang et al., 2018). NKILA interacts with NF-κB/IκB to form a stable complex, which directly masks phosphorylation motifs of IκB, thereby inhibiting IKK-induced IκB phosphorylation and NF-κB activation (Liu et al., 2015). Our recent study demonstrated that NKILA potentially inhibits HIV replication (Wang et al., 2020). However, whether NKILA regulates HBV replication has not yet been determined. In this study, we investigated the potential role of NKILA in HBV replication. Our data show that NKILA potentially inhibits HBV replication via NF-κB signalling in HBx-dependent manner, which provides important information for the development of new therapeutic tools against HBV.

2. Materials and methods

2.1. Cell culture and antibodies

HepG2 (77400), Huh7, HepAD38, HepG2.2.15, and HEK293T (CRL-11268) cells were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated foetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL) in a 5% CO_2 incubator at 37 °C. Stable cell lines with over- or low-expression lncRNA NKILA cultured with puromycin selection were constructed using a lentivector-mediated gene transfer system, and the cells with the vector pLVX or pLKO.1 were served as a blank control. The following antibodies were used: mouse anti-myc MAb (05-724; Millipore, Burlington, MA, USA), goat anti-human histone polyclonal (A0150240; Genscript, Piscataway, NJ, USA), rabbit anti-human IκBα polyclonal (9242; Cell Signalling Technology, MA, USA), mouse anti-phospho-IκBα (Ser32/36; 9246; Cell Signalling Technology, MA, USA), mouse anti-tubulin (ab11323; Abcam, Cambridge, MA, USA), mouse anti-FLAG (F1804; Sigma), rabbit anti-GFP (A-21311, Invitrogen), and anti-human GAPDH (G8795; Sigma) antibodies.

2.2. Plasmid construction

Full-length NKILA and its mutant (M1–M4) plasmids, pHBV1.3 (genotype D), pCMV-p65-flag/myc, p*Renilla*-luciferase, pNF-κB-luciferase, pLKO.1-NKILA, and pLVX-NKILA have been described previously (Wang et al., 2020; Zheng et al., 2020). To construct the HBx-myc plasmid, HBx was amplified from pHBV1.3 via PCR, and the resultant PCR products were cloned into the VR1012 vector.

2.3. RNA isolation and real-time RT-qPCR

RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. RNA samples were treated with RQ-1 DNase (Promega, Madison, WI) before reverse transcription and cDNA synthesis using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). A total of 250–1000 ng RNA was used as a template for each cDNA synthesis reaction, and samples containing only H_2O or no reverse transcriptase were used as blank samples. cDNA samples were stored at −80 °C until use. The FastStart Universal SYBR Green Master Mix (Roche) was used to perform the RT-qPCR assay on a Roche 480 instrument. To exclude contamination with genomic DNA, a blank sample without reverse transcriptase was used. RT-qPCR amplification of the target fragment was carried out as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was carried out at 90 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. To minimise the bias caused by housekeeping genes, the geometric mean of *GAPDH* and *β-actin* gene expression was used to characterise the expression stability, and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method, according to the MIQE guidelines. The primers used for RT-qPCR are listed below. *GAPDH*: forward sequence, CGTGCGTGACAT and reverse sequence, GTCAGGCAGCTCGTAGCTCTT; *NKILA*: forward sequence, AACCAAACCTACCCACAACG and reverse sequence, ACCACTAAGTCAATCCCAGGTG; *β-actin*: forward sequence, ACCGAGCGCGGCTACAG and reverse sequence, CTTAATGTCACGCACGATTTCC; HBV cccDNA: forward sequence, CTCCCCGTCTGTGCCTTCT and reverse sequence, GCCCCAAAGCCACCCAAG; HBV pgRNA: forward sequence, GCCTTAGAGTCTCCTGAGCA and reverse sequence, GAGGGAG TTCTTCTTCTAGG.

2.4. HBV cccDNA and pgRNA extraction

HepG2 and Huh-7 cells were transfected expression NKILA vector or control vector with monomeric linear full-length HBV expression genomes. 96 h after transfection, transfected cells were collected.

For quantification of HBV cccDNA, transfected cells were resuspended in a lysis buffer (20 mmol/L Tris,0.4 mol/L NaCl, 5 mmol/L EDTA, 1% SDS, pH = 8.0) in the presence of proteinase K (QIAGEN). Total DNA was extracted according to a standard phenol-chloroform extraction protocol. 500 ng of total DNA was digested with 0.5 μL plasmid-safe adenosine triphosphate (ATP)-dependent deoxyribonuclease DNase (PSAD) (Epicentre Technologies) in 25 μL reaction for 8 h at 37 °C to allow removal of linear genomic DNA and HBV replication intermediates (rcDNAs, single-stand DNAs, linear double-strand DNAs). DNase was inactivated by incubating the reactions for 30 min at 70 °C. 20 ng of digested DNA was used for quantification of HBV cccDNA by RT-qPCR assay.

For quantification of HBV pgRNA, the total RNA was extracted from transfected cells as described above and 2 mg of DNase-treated RNA were reverse transcribed and cDNA synthesis were using a Transcriptor First Strand cDNA Synthesis Kit. The FastStart Universal SYBR Green Master Mix (Roche) was used to perform the RT-qPCR assay on a Roche 480 instrument.

2.5. Transfection and Western blotting

Cells were seeded into a plate and transfected using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. The cells were harvested 48 h after transfection or stimulation. Cell samples were boiled in 1× loading buffer [0.08 mol/L Tris (pH 6.8), 2.0% SDS, 10% glycerol, 0.1 mol/L dithiothreitol, and 0.2% bromophenol blue) and subsequently separated on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes, which were probed with various primary antibodies against the proteins of interest. Primary antibodies were

diluted with 1% milk in phosphate-buffered saline (PBS) and incubated with primary antibodies followed by incubation with the corresponding AP-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA). Protein staining was performed using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium obtained from Sigma (St. Louis, MO, USA).

2.6. Luciferase assay

Luciferase reporters were used to detect whether NKILA affected NF-κB activation with or without HBx protein. A total of 3×10^5 HepG2 or HEK293T cells were seeded into a 12-well cell culture plate and transfected with Lipofectamine 2000, according to the manufacturer's instructions. After 48 h, the cells were collected and a dual luciferase reporter assay to examine the activities of firefly luciferase and Renilla luciferase was performed using the Promega Dual-Luciferase Reporter Assay System (Promega) using a GloMax 20/20 Luminometer (Promega), according to the manufacturer's instructions. The activity of firefly luciferase was normalised to that of Renilla luciferase.

2.7. Co-immunoprecipitation (co-IP)

HBx and p65 expression vectors were co-transfected with wild type NKILA or control vector into HepG2 cells. After 48 h, HepG2 cells were harvested and disrupted using a lysis buffer [PBS containing 1% Triton X100 and complete protease inhibitor cocktail (Roche)] at 4 °C for 1 h. Cell lysates were clarified via centrifugation at 10000×*g* for 30 min at 4 °C, precleared, mixed with anti-IgG antibody-conjugated agarose beads, and incubated with anti-flag antibody-conjugated agarose beads at 4 °C for 4 h on an end-over-end rocker. The mixtures were washed six times with cold wash buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 0.1 mmol/L EDTA, 0.05% Tween-20) and analysed via Western blotting or the extracted RNA was used for RT-qPCR analysis.

2.8. HBV production and infection

To obtain and purify HBV, stable HBV replicating HepAD38 cells were cultured without Tet and the cell culture medium was harvested and concentrated 100-fold via ultrafiltration. At 1000 HBV genome equivalents per cell, HBV was used to infect HepG2-NTCP-A3 cells seeded on cell plates coated with collagen, and the cells were cultured in the presence of 2.5% DMSO and 4% PEG 8000 (Sigma-Aldrich) for 6–8 h. Cell supernatants were removed by washing with PBS, and the infected cells were maintained in medium containing 2.5% DMSO. The medium was changed every three days, and samples were maintained until the preparation day at certain time points.

2.9. Statistical analysis

All data represent the results of three independent experiments and are presented as the mean ± standard deviation. Statistical significance was calculated using the Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate the significant differences.

3. Results

3.1. NKILA potently inhibits HBV replication

NF-κB plays a vital role in HBV replication and development of HBV-associated HCC. To investigate whether the NF-κB-interacting lncRNA NKILA, which represses NF-κB signalling, affects HBV replication, HEK293T cells were transfected with the pHBV1.3 expression vector and green fluorescent protein (GFP) plus the negative control vector, VR1012, or increased amounts of NKILA expression vector. After 48 h, cells were harvested for immunoblotting and RT-qPCR analyses. Increased ectopic NKILA decreased HBV core protein expression and had no effect on GFP expression (Fig. 1A). HBeAg and HBsAg expression levels in the viral supernatants were also decreased by NKILA in a dose-dependent manner (Fig. 1B), indicating that NKILA specifically suppresses HBV replication. The mRNA levels of NKILA were determined via RT-qPCR (Fig. 1C). In contrast, short interfering RNA against NKILA (siRNA NKILA) increased HBeAg and HBsAg expression levels compared to transfection with a short interfering negative control (siRNA NC) in HEK293T cells (Fig. 1D). Knockdown efficiency of NKILA was determined via RT-qPCR (Fig. 1E), and NKILA levels were decreased in cells transfected with HBV compared to those in cells transfected with the control vector. This result indicates that HBV replication may regulate NKILA expression.

To further confirm whether NKILA inhibits HBV replication in its target cells, we constructed NKILA overexpression and stable knockdown HepG2 cells and transfected them with increased amounts of HBV expression or control vector. Stable overexpression of NKILA was confirmed (Fig. 2A), and overexpression of NKILA inhibited the expression of HBeAg and HBsAg in the viral supernatants (Fig. 2B and C). In contrast, stable knockdown of NKILA with shRNA in HepG2 cells (Fig. 2D), led to increased levels of HBeAg and HBsAg (Fig. 2E and F) compared to transfection with increased amounts of HBV1.3 vector in the shRNA negative control. PMEPA1 protein encoded by the gene *PMEPA1*, running antisense to NKILA (Liu et al., 2015), was correlated with immunity pathways (Liu et al., 2011; Kakumani et al., 2021; Ji et al., 2020). To elevate whether PMEPA1 was associated with NKILA inhibition on HBV replication, we examined the PMEPA1 protein and mRNA levels in NKILA stably overexpressing or knockdown cells. Overexpression or knockdown of NKILA did not alter PMEPA1 expression (Fig. 2G and H), indicating that NKILA itself accounts for HBV replication. We further examined the effect of NKILA on HBV replication in stable HBV-producing HepG2.2.15 and HepAD38 cells. NKILA overexpression inhibited production of HBeAg and HBsAg in HepG2.2.15 and HepAD38 cells (Fig. 2I and K). The mRNA levels of NKILA were confirmed via RT-qPCR (Fig. 2J and L).

The HBV covalently closed circular DNA (cccDNA) plays a key role in viral persistence, reactivation after antiviral-treatment and drug resistance. HBV cccDNA is the template for transcription of the pregenomic RNA (pgRNA) and other subgenomic RNAs that are translated to produce the viral proteins (Chen et al., 2014; Levrero et al., 2009; Schiffer et al., 2012). To evaluate the effect of NKILA on HBV cccDNA production and the HBV pgRNA transcription, we co-transfected NKILA or control vector in HepG2 cells with the monomeric linear full-length HBV DNA. The mRNA levels of NKILA were confirmed via RT-qPCR (Fig. 2M). The expression of NKILA in cells transfected with monomeric linear full-length HBV DNA significantly reduced the viral production of HBeAg, HBsAg, and pgRNA, resulted in a decreased cccDNA level (Fig. 2N and O). Collectively, these data suggest that NKILA inhibits HBV replication.

3.2. HBV infection downregulates NKILA expression

As shown in Fig. 1E, NKILA expression decreased in cells transfected with the HBV expression vector. To determine the effect of HBV replication on NKILA expression, we examined NKILA mRNA levels in HBV-positive patients and healthy donors (Table 1). NKILA mRNA levels were lower in PBMCs from 16 HBV-positive patients than in those from 16 healthy donors (Fig. 3A). Additionally, NKILA mRNA level in HBV-positive patients with low viral loads was much higher than that with high viral loads (Fig. 3B). We further analysed the correlation of NKILA mRNA level with HBV viral loads. NKILA levels were found to be negatively correlated with HBV DNA levels in blood serum from HBV-positive patients (Fig. 3C). We also tested the mRNA level of NKILA in HepG2 and Huh-7 cells transfected dose of the HBV expression vector. At 48 h post-transfection, the cells were harvested and analysed using RT-qPCR. HBV transfection reduced NKILA expression in a dose-dependent manner compared to that in the control cells (Fig. 3D). We also examined the mRNA level of NKILA in HepG2-NTCP cells infected with HBV produced from HepAD38 cells without tetracycline treatment. Seven days post-infection, the cells were harvested and analysed for NKILA mRNA

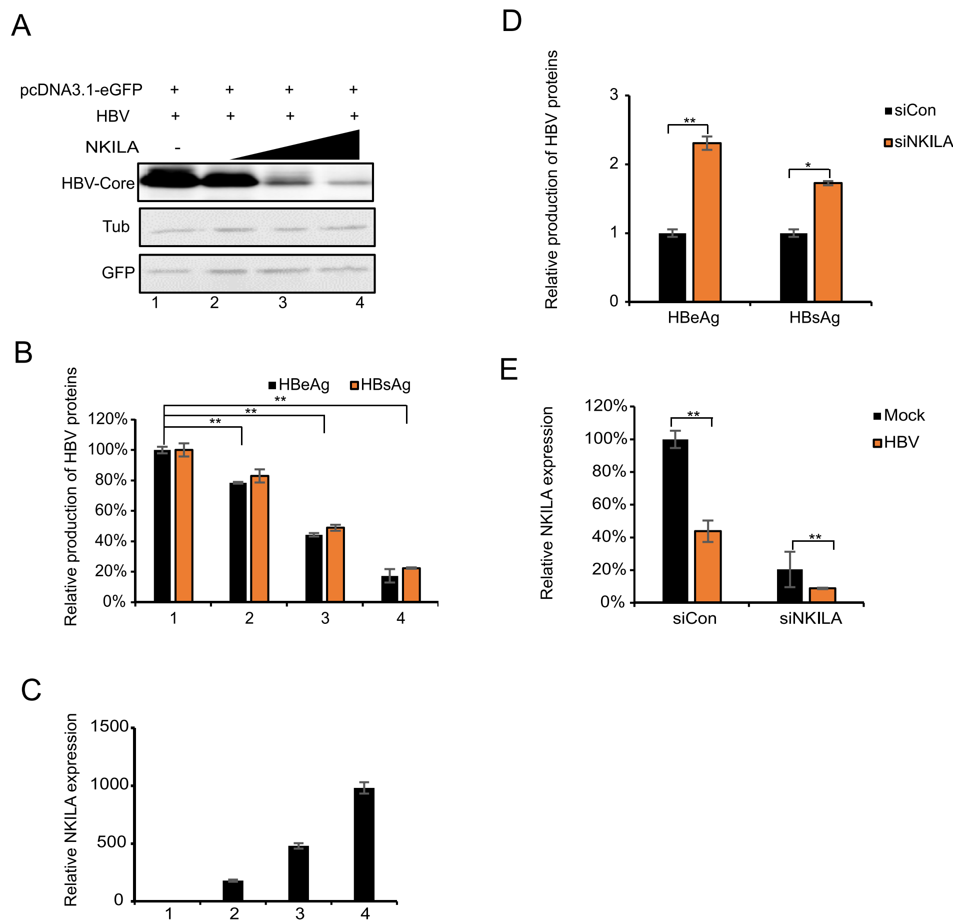

Fig. 1. NKILA inhibits HBV replication in HEK293T cells. **A–C** Overexpression of NKILA inhibits HBV replication. 100 ng, 300 ng, 900 ng of NKILA expression vector with green fluorescent protein (GFP) and HBV expression vector were co-transfected into HEK293T cells. At 48 h post-transfection, the cells and supernatants were harvested and analysed via Western blotting (**A**), ELISA to determine HBeAg and HBsAg expression levels, and RT-qPCR to determine NKILA mRNA levels (**C**). **D, E** Knockdown of NKILA increased HBV replication. Small interfering RNA (siRNA) NKILA or siRNA control with HBV expression or control vector was transfected into HEK293T cells. At 48 h post-transfection, the cells and supernatants were harvested and analysed via ELISA to determine HBeAg and HBsAg expression levels (**D**), and RT-qPCR to determine NKILA mRNA levels (**E**). Results were obtained from three independent experiments using the standard deviation (SD) method. Asterisk indicates that the difference between the groups evaluated by *t*-test is statistically significant (*$P < 0.05$, **$P < 0.01$).

levels. The results showed that HBV infection decreased NKILA expression compared with that in uninfected cells (Fig. 3E). In addition, the mRNA level of NKILA in HepG2.2.15, a cell that is an HBV stably transfected cell line constitutively producing HBV, was lower than that in HepG2 cells (Fig. 3F), which is the parental cell line of HepG2.2.15. Considering that the different cell lines may interfere with the gene expression profile, the mRNA level of NKILA was further examined in HepAD38 cell lines which is an HBV stably transfected cell line under the control of the tetracycline-responsive promoter. HepAD38 cell lines cultured without tetracycline treatment can continuously produce HBV (Ladner et al., 1997). The data showed that the mRNA level of NKILA was downregulated in HepAD38 cells without tetracycline treatment (HBV production) compared with that in cells treated with tetracycline (no HBV production) (Fig. 3G). These data show that NKILA expression is downregulated by HBV infection.

3.3. NKILA inhibits HBV replication by suppressing NF-κB activation

NKILA has been reported to suppress HCC metastasis by inhibiting the NF-κB/Slug pathway in SMMC-7721 and HCC-LM3 cell lines and by suppressing the phosphorylation of IκBα, p65 nuclear translocation, and NF-κB activation (Chen et al., 2020). To investigate the biological function of NKILA in HepG2 and Huh-7 cell lines, we first determined NKILA cellular localization in HepG2 and Huh-7 cells using nuclear and cytosolic fractionation assays. The cytosolic and nuclear fractions were analysed by immunoblotting to determine the efficiency of fractionation (Fig. 4A). RNA was isolated from the cytosolic and nuclear fractions, and NKILA expression was measured by RT-qPCR. Nucleic RNA, nuclear paraspeckle assembly transcript 1, cytosolic RNA, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were used as the positive controls. The results showed that the expression of NKILA was higher in the cytoplasm than in the nucleus in HepG2 and Huh-7 cells (Fig. 4B and C). A previous study found that NF-κB activation induced by TNFα affects NKILA expression in cancer cells (Majano et al., 2001; Shukla et al., 2011). To detect the effect of TNFα on NKILA expression, TNFα or dimethyl sulfoxide (DMSO) as a negative control was used to treat HepG2 and Huh-7 cells for 6 h, and then the cells were analysed by RT-qPCR. TNFα treatment led to a more than 2-fold increase in NKILA expression compared with DMSO treatment (Fig. 4D). We also transfected NF-κB and Renilla into HepG2 and Huh-7 cells, and the results showed that the luciferase activity of NF-κB (Fig. 4E) and NKILA mRNA expression (Fig. 4F) were upregulated by TNFα treatment. NKILA expression was regulated by NF-κB pathway. We next detected NKILA function in NF-κB pathway and found that NKILA inhibited the activity of NF-κB in a dose-dependent manner (Fig. 4G). NKILA expression was determined by RT-qPCR (Fig. 4H). As expected, we further confirmed that stable

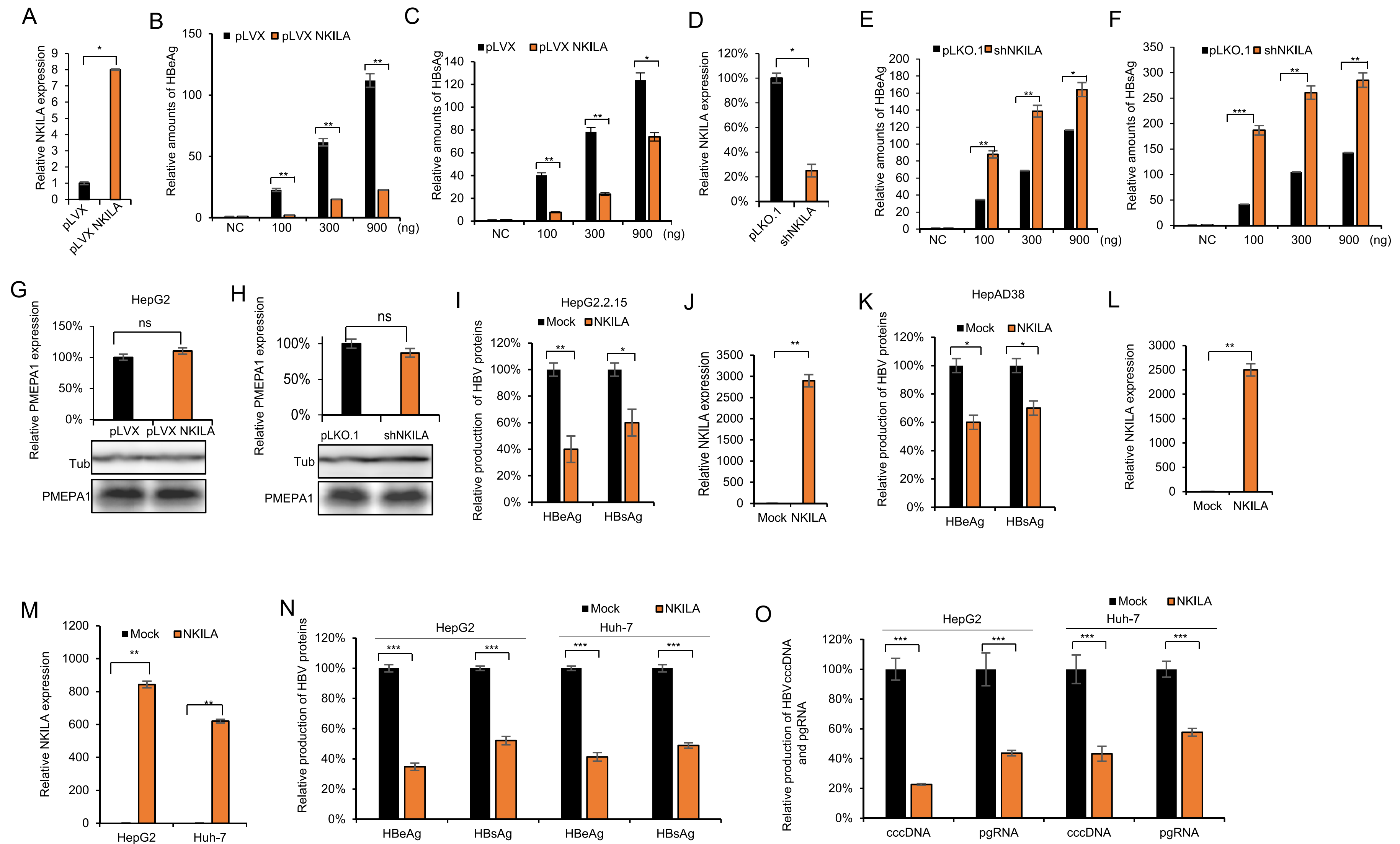

Fig. 2. NKILA inhibits HBV replication in HepG2 and HBV-stable expression cell lines. Overexpression (**A–C**) or knockdown (**D–F**) of NKILA regulated HBV replication. Cell lines were harvested for determining the mRNA levels of NKILA (**A, D**). 100 ng, 300 ng, 900 ng of HBV plasmids or control vector were transfected into stable expression or knockdown NKILA of HepG2 or mock cell lines. After 48 h, the supernatants were harvested and analysed via ELISA to determine the expression levels of HBeAg (**B, E**) and HBsAg (**C, F**). **G–H** Expression levels of PMEPA1 mRNA and protein in stable cell lines. HepG2 cells stable expressing or knockdown NKILA were harvested for detecting PMEPA1 mRNA or protein level. **I–L** NKILA inhibits HBV replication in HepG2.2.15 and HepAD38 cells. NKILA expression vector or control vector was transfected into Hep2.2.15 or HepAD38 cells. At 48 h post-transfection, the cells were harvested and analysed to determine the HBeAg or HBsAg levels via ELISA (**I, K**) and NKILA mRNA levels via RT-qPCR (**J, L**). **M–O** NKILA inhibits HBV cccDNA and pgRNA production. NKILA plasmids or control vector with monomeric linear HBV genomes were transfected into HepG2 cells. After 96 h transfection, the cells were collected for analysis. The expression of NKILA mRNA level was detected by RT-qPCR assay (**M**), HBeAg and HBsAg were analysed via ELISA (**N**), and HBV cccDNA and RNA were detected by RT-qPCR assay (**O**). Results were obtained from three independent experiments using the standard deviation (SD) method. Asterisk indicates that the difference between the groups evaluated by the *t*-test is statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant).

Table 1
Characteristic datum for HBV-infected (n = 16) and HBV-uninfected donors (n = 16).

Characteristic	HBV-infected donors			HBV-uninfected donors	P value
	HBV (low) (n = 8)	HBV (high) (n = 8)	Total (n = 16)	(n = 16)	
Gender					1.000
Female	3 (18.75)	6 (37.50)	9 (56.25)	10 (62.5)	
Male	5 (31.25)	2 (12.50)	7 (43.75)	6 (37.5)	
Age, years	43.125 ± 16.77	46.125 ± 14.88	44.625 ± 15.4	44.875 ± 15.8	0.964
HBV viral loads (IU/mL)	77318.75 ± 134699.1	5892500 ± 3290882	2984909.4 ± 3752347.6	<50	0.003
Antiviral therapy	No	No	No	No	

Values are expressed as the mean ± SD or n (%), unless otherwise indicated. Statistical analysis was performed by unpaired *t*-test.

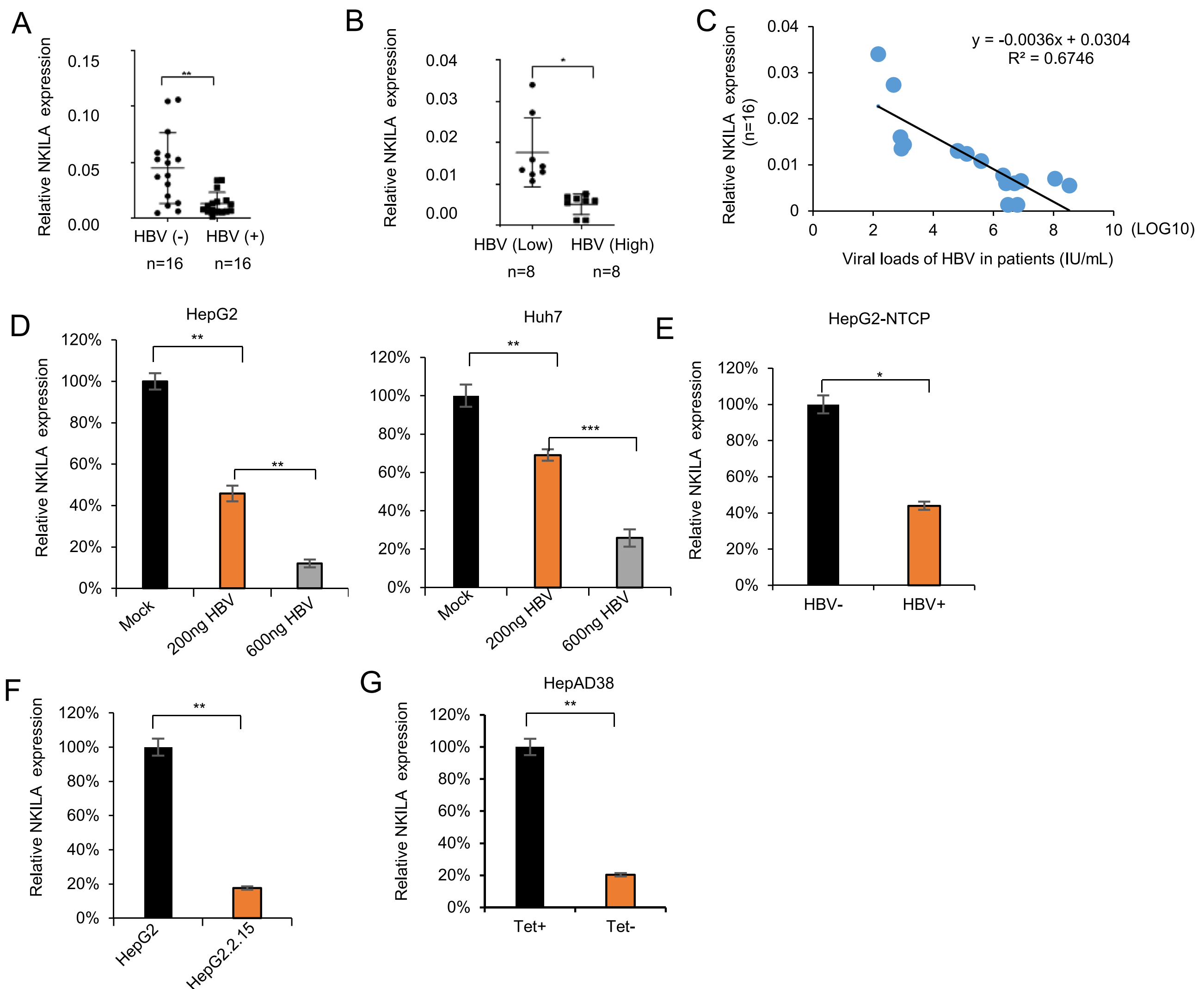

Fig. 3. NKILA expression is downregulated by HBV replication and infection in patient samples and liver cell line. **A–C** The mRNA levels of NKILA were lower in HBV-positive patients than in the healthy donors. PBMCs were purified from the blood samples of 16 HBV-positive patients or healthy donors and then NKILA mRNA level was analysed via RT-qPCR. **B** The mRNA levels of HBV-positive NKILA in patients with high viral loads (n = 8) were lower than that with low viral loads (n = 8). Those patients were divided into the two groups based on the median number of HBV loads. **C** The correlation between HBV viral loads and the NKILA mRNA level in HBV-positive patients were plotted, and linear regression analysis was performed. The X-axis represents log base 10 of HBV viral loads, and Y-axis represents relative NKILA mRNA level to GAPDH. **D** NKILA levels were decreased in HCC cells with HBV transfection. 200 ng, 600 ng HBV expression vector or control vector (mock) were transfected into HepG2 or Huh-7 cells. At 48 h post-transfection, the cells were harvested and analysed via RT-qPCR. **E** HBV infection decreased NKILA expression. The stable NTCP-expressing HepG2 cell line was constructed using the lentiviral package system. HBV obtained from HepAD38 cells was added to the NTCP-HepG2 cell lines. At seven days post-infection, the cells were harvested and analysed via RT-qPCR. **F, G** NKILA mRNA levels in HBV positive cell lines were lower than those in the mock cells. HepG2.2.15 cells were constructed from HepG2 cells. HepG2 and HepG2.2.15 cells were harvested and analysed via RT-qPCR to determine the NKILA mRNA levels (**F**). HepAD38 cells were cultured with or without tetracycline (Tet) and harvested to determine the NKILA mRNA levels via RT-qPCR (**G**). Results are reported as the mean ± SD of three independent experiments. Statistical analysis was performed by the *t*-test. ns, not significant; *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.

overexpression of NKILA decreased p65 translocation into the nucleus and reduced IκBα phosphorylation levels (Fig. 4I and J).

To explore the mechanisms through which NKILA inhibits HBV replication, we first investigated whether HBV viral proteins might be involved in this regulatory process. As reported, HBx plays a vital role in HBV replication via the NF-κB pathway (Salerno et al., 2020; Sekiba et al., 2022; Shukla et al., 2011). To examine the effect of NKILA on HBx-induced NF-κB activity, we transfected NF-κB and Renilla with increasing amounts of HBx or mock vector into HepG2 cells, and then the cells were harvested for analysis. RT-qPCR and immunoblotting analysis showed that the overexpression of NKILA had no effect on protein level of HBx (Fig. 4K), but NKILA decreased HBx-induced NF-κB activation in a dose-dependent manner (Fig. 4L).

To explore whether NKILA effects on HBx binding with p65, we transfected HBx and p65 expression vector with NKILA or empty vector into HepG2 cells. A part of the transfected cells was collected for immunoprecipitation assay, while the other part of cells was for nuclear and cytosolic fractionation assays. The cytosolic and nuclear fractions were then analysed by immunoblotting and immunoprecipitation assay. The result showed that NKILA strongly decreased the interaction between HBx and p65 in the whole cell lysates (Fig. 5A) and in the cytoplasm or nucleus (Fig. 5C), with the pattern more significant in cytoplasm than that in nucleus. mRNA level of NKILA was analysed by RT-qPCR in cells (Fig. 5B). These results demonstrate that NKILA inhibits HBx-induced NF-κB activation by regulating HBx/p65 complex assembly, which may be associated with HBV replication. In order to rule out the possibility that NKILA and HBx interferes with each other to influence HBV replication, we transfected dose of HBx expression vector, and found that increasing of HBx expression had on effect on NKILA mRNA level (Fig. 5D). Consisted the result showed in Fig. 4K, the result illustrated

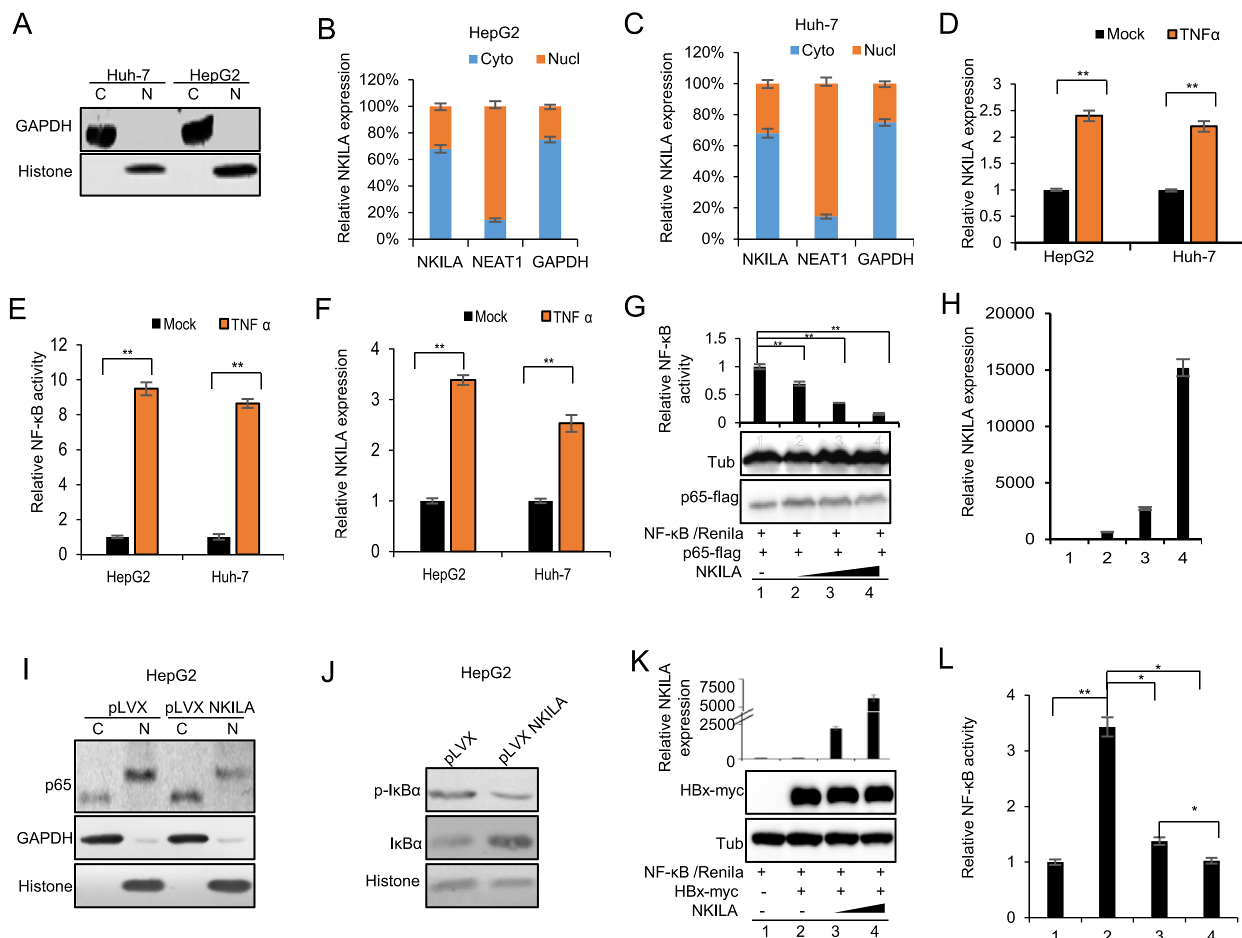

Fig. 4. NKILA is mainly located in the cytoplasm and regulates NF-κB activation. **A–C** NKILA is mainly located in the cytoplasm of HepG2 and Huh-7 cells. HepG2 and Huh-7 were subjected to nuclear (N) and cytoplasmic (C) separation experiments. Western blotting was used to detect GAPDH and histone protein as markers for the efficiency of separation (**A**). RT-qPCR was performed to determine the relative NKILA mRNA levels in HepG2 (**B**) and Huh-7 cell cytoplasm and nucleus (**C**). GAPDH and NEAT1 RNA were used as fractionation indicators. **D** NKILA expression was regulated by tumour necrosis factor (TNF)-α treatment. HepG2 and Huh-7 cells were treated with TNFα (20 ng/mL) for 6 h. RT-qPCR was performed to determine the relative NKILA mRNA levels in TNFα-treated HepG2 and Huh7 cells. **E, F** The enhanced transcription of NKILA caused by TNFα-induced NF-κB activity. Luciferase reporter assays were performed for HepG2 and Huh-7 cells transfected with reporter plasmids containing NF-κB and treated with PBS or TNFα (20 ng/mL) for 24 h. The cells harvested were analysed by luciferase assayfor detecting NF-κB activity (**E**) and RT-qPCR for detecting NKILA mRNA level (**F**). **G, H** NKILA inhibits NF-κB activity in HepG2 cells. Control vector (1) or 100 ng (2), 300 ng (3), 900 ng (4) of NKILA with NF-κB, Renilla, and p65 expression vector were co-transfected into HepG2 cells. At 48 h post-transfection, the cells were harvested to luciferase assay and Western blotting (**E**) and NKILA mRNA expression was tested via RT-qPCR (**F**). **I, J** p65 protein cellular localization and phosphorylated IkBα levels in HepG2 cells stably expressing NKILA. Cells were harvested and subjected to nuclear and cytoplasmic isolation. p65 and phosphorylated IkBα proteins were detected by via immunoblotting analysis. **K, L** NKILA inhibited HBx-induced NF-κB activity. 100 ng (3) or 300 ng (4) of NKILA or control vector, NF-κB, and Renilla were cotransfected with or without HBx expression vector in HepG2 cells. At 48 h post-transfection, the cells were harvested and tested via Western blotting (**K**) and luciferase assay (**L**). RT-qPCR was used to determine the mRNA levels of NKILA (**K**). Results are reported as the mean ± SD of three independent experiments. Statistical analysis was performed by the *t*-test. ns, not significant; $^*P < 0.05$; $^{**}P < 0.01$.

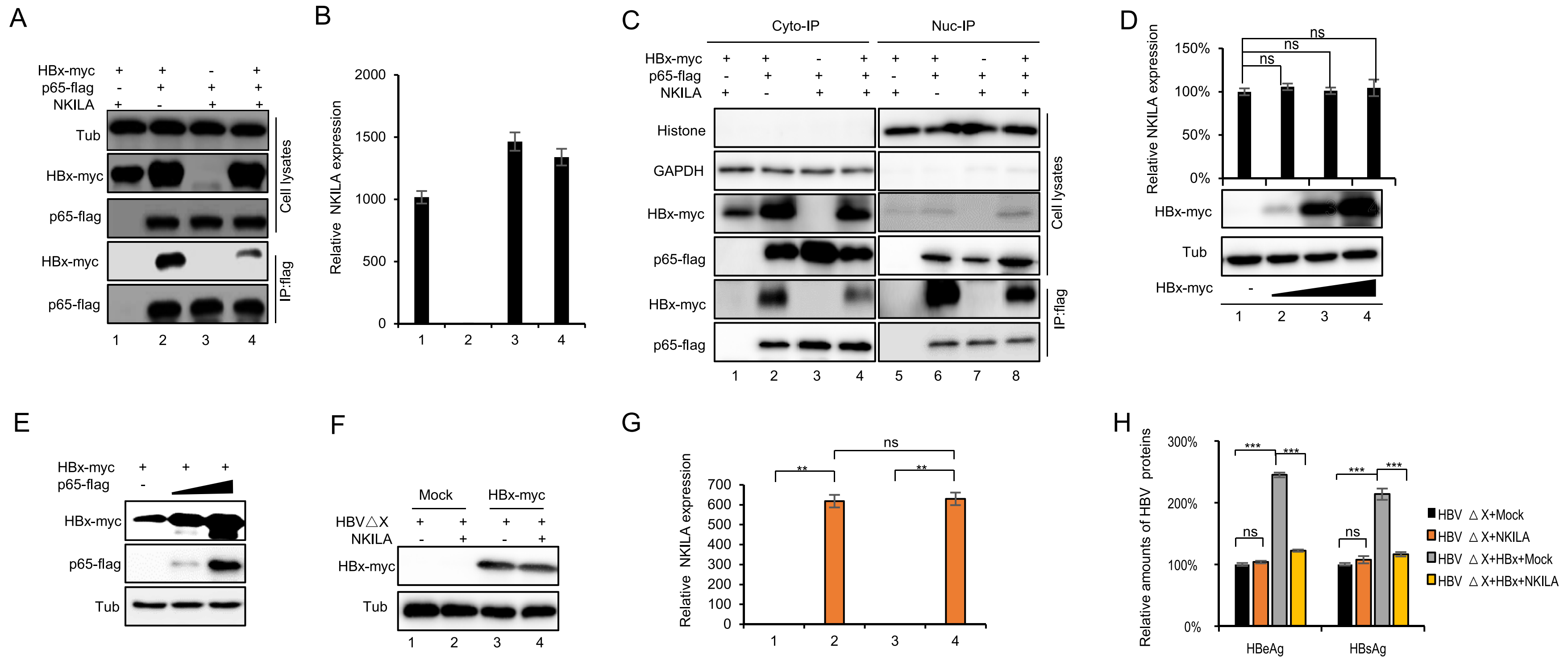

Fig. 5. NKILA affects HBV replication in an HBx-dependent manner through decreasing the interaction between HBx and P65 protein. **A–C** NKILA inhibits the interaction between HBx and p65 in the whole cell lysates. 500 ng HBx, 500 ng p65 with 1000 ng NKILA, and mock vectors were co-transfected into HepG2 cells, and harvested at 48 h post transfection. A part of transfected cells was immunoprecipitated with anti-flag antibody-conjugated agarose beads. HBx and p65 proteins were detected via immunoblotting analysis (**A**). The other part of cells was subjected to nuclear and cytoplasmic isolation. RT-qPCR was used to determine the mRNA levels of NKILA in cells (**B**). **C** NKILA decreased the binding of p65 with HBx in the cytoplasm as well as in the nucleus. Nuclear and cytoplasmic solutions from Fig. 4A were immunoprecipitated with anti-flag antibody-conjugated agarose beads. **D** HBx had no effect on NKILA mRNA expression. 100 ng (2), 300 ng (3), or 900 ng (4) of HBx expression vector were transfected into HepG2 cells and then detected via immunoblotting analysis for HBx protein and via RT-qPCR for NKILA mRNA. **E** HBx expression was increased by p65 in a dose-dependent manner. 300 ng or 900 ng of p65 expression vectors were transfected into HepG2 cells and then detected via immunoblotting analysis for HBx and p65 proteins. **F** NKILA lost its inhibitory activity in HBx-deficient HBV (HBVΔX) viruses. 500 ng of HBVΔX vector and 500 ng plasmids expressing HBx, with or without 900 ng NKILA, were co-transfected into HepG2 cells as indicated. At 48 h after transfection, the level of expression of HBx in cells was determined via immunoblotting analysis (**F**), NKILA mRNA was detected via RT-qPCR (**G**), and HBeAg or HBsAg were detected via ELISA (**H**). Results are reported as the mean ± SD of three independent experiments. Statistical analysis was performed by the *t*-test. ns, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

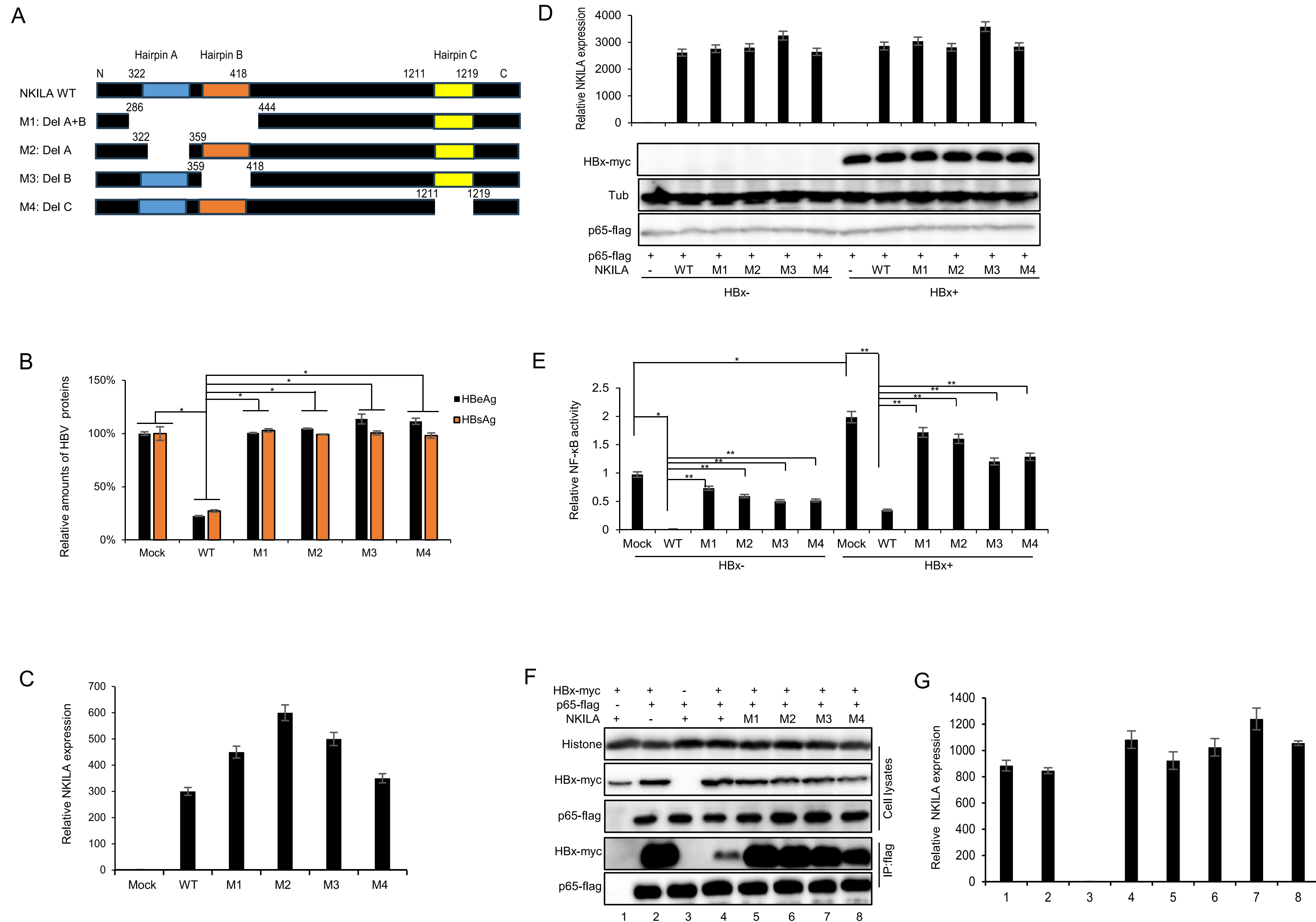

Fig. 6. NKILA truncations fail to inhibit HBV replication and NF-κB activation. **A** Schematic representation of NKILA mutants. 600 ng of negative control vector, NKILA wild-type (WT), or its mutants and 300 ng HBV expression vector were co-transfected into HepG2 cells. After 48 h, the cells were harvested and the mRNA levels of NKILA in cells were determined (**C**), and HBeAg and HBsAg levels in supernatants were determined via ELISA (**B**). **D, E** NKILA inhibited NF-κB activity with or without HBx expression. 600 ng WT or mutant NKILA with 100 ng NF-κB, 2 ng Renilla, and 300 ng p65 vectors were co-transfected with 300 ng HBx or control vector. At 48 h post-transfection, the cells were collected for RT-qPCR and immunoblotting (**D**), and the luciferase activity was determined via luciferase assay (**E**). **F, G** NKILA truncations fail to inhibit the interaction between HBx and p65 in the whole cell lysates. 500 ng HBx, 500 ng p65 with 1000 ng NKILA WT or mutants, and mock vector were co-transfected into HepG2 cells, and cells were harvested at 48 h post transfection. The transfected cells were immunoprecipitated with anti-flag antibody-conjugated agarose beads. HBx and p65 proteins were detected via immunoblotting analysis (**F**) and NKILA mRNA level was detected via RT-qPCR (**G**). Results are reported as the mean ± SD of three independent experiments. Statistical analysis was performed by the *t*-test. $^*P < 0.05$, $^{**}P < 0.01$.

that NKILA and HBx did not interfere with each other. It has been previously reported that p65 is a key player in HBx stabilization (Shukla et al., 2011). As showed in Fig. 5A lane 2 and Fig. 5E, HBx expression was increased by p65 in a dose-dependent manner.

To illustrate whether NKILA inhibits HBV through interfering the interaction between HBx and p65, the HBx-deficient HBV (HBVΔX) vector and HBx expression vector with or without NKILA, were co-transfected into HepG2 cells as indicated. At 48 h after transfection, HBx protein in the transfected cells were determined via immunoblotting analysis (Fig. 5F), NKILA mRNA level was detected via RT-qPCR (Fig. 5G), and HBeAg or HBsAg was detected via ELISA (Fig. 5H). The result showed that HBx and NKILA did not interfere with each other (Fig. 5F and G, lane 2 and lane 4). Moreover, NKILA had no effect on HBx-deficient HBV replication but strongly inhibited HBx-rescued HBVΔX replication (Fig. 5H). These results demonstrate that HBx was required for NKILA inhibition on HBV replication.

3.4. *NKILA truncations fail to inhibit HBV replication and NF-κB activation*

NF-κB pathway is responsible for HBV replication and HCC development. To facilitate HBV replication, HBx encoded by the HBV genome interacts with p65 to increase NF-κB pathway activation (Majano et al., 2001; Shukla et al., 2011). To investigate whether NKILA regulates HBV replication and HBx function via the NF-κB pathway, NKILA mutants lacking essential hairpins for NF-κB activation were generated (Fig. 6A) and transfected into HepG2 cells. NKILA mutants lost their inhibitory effect on HBV replication compared to wild type (WT) (Fig. 6B), indicating that each domain of NKILA required for NF-κB interference are essential for HBV restriction. The mRNA expression of WT and mutants were detected by RT-qPCR (Fig. 6C). Next, we examined the effects of WT NKILA and its mutants on HBx function. NF-kB, Renilla, and p65 expression vectors were transfected with or without the HBx vector. After 48 h of transfection, cells were harvested for analysis. The mRNA levels of WT and mutant NKILA with or without HBx expression were analysed via RT-qPCR (Fig. 6D). As expected, NKILA mutants lost the ability to inhibit HBx-independent and -dependent NF-κB activation compared to NKILA WT, whereas the protein levels of HBx and p65 were not affected (Fig. 6D and E). NKILA mutants also lost inhibitory effect on interaction between HBx and p65 (Fig. 6F). The mRNA levels of NKILA were detected via RT-qPCR assay (Fig. 6H). These data illustrate that NKILA abolishes basal or HBx-induced NF-κB activation and interrupted the HBx/p65 complex assembly to inhibit HBV replication.

4. Discussion

HBV infection has received much attention as the major cause of HCC. Due to inability of current antiviral therapy for HBV infection to eliminate HBV cccDNA and the primary matrix of HBV persistence, immunomodulatory treatments are still insufficient. Therefore, it is critical to develop new antiviral strategies for HBV-infected patients. There is increasing evidence to suggest the functional role of the host transcription factor, NF-κB, in HBV replication (Wu et al., 2021; Lei et al., 2021; Duan et al., 2018; Shukla et al., 2011). HBV utilises NF-κB to mediate transcriptional initiation and achieve high expression levels in the genome. The viral protein HBx is required for HBV replication, which relieves chromatin-mediated transcriptional repression of HBV cccDNA (Pollicino et al., 2006; Riviere et al., 2015; Belloni et al., 2009). Moreover, HBx impairs homologous recombination-mediated repair of damaged DNA by hijacking the cellular E3 ubiquitin ligase to degrade Smc5/6, and regulates other host factors to promote HBV infection (Salerno et al., 2020; Sekiba et al., 2022; Yuan et al., 2021). Therefore, HBx may be a promising antiviral target to repress HBV cccDNA transcription.

LncRNAs have been demonstrated to be involved in numerous biological and pathological processes (Tan et al., 2021; Nojima and Proudfoot, 2022; Bhan et al., 2017), including HBV infection and HBV-related diseases, including HCC (Salerno et al., 2020; Feng et al., 2019; Wong et al., 2018). NKILA is a negative regulator of NF-κB in multiple cancers, including breast cancer, melanoma, non-small cell lung cancer, and hepatocellular carcinoma (Liu et al., 2015; Bian et al., 2017; Lu et al., 2017; Yu et al., 2018). However, whether NKILA affects HBV replication has not been investigated. In the present study, we demonstrated that NKILA plays a key role in regulating HBV replication. Beginning with the overexpression or knockdown of NKILA in HEK 293T and HepG2 cells, we found that NKILA strongly inhibited HBV replication in a dose-dependent manner and that depletion of NKILA increased HBV replication. Moreover, NKILA suppressed HBV production in HBV-stable expression cell lines, such as HepG2.2.15 and HepAD38 cell lines. To elevate the effect NKILA on HBV cccDNA and pgRNA production, we transfected NKILA with monomeric linear full-length HBV into HepG2 cells. Our data showed that NKILA mediated inhibition of HBV cccDNA transcription, pgRNA production, and HBeAg and HBsAg expression in cells transfected with monomeric linear HBV plasmids, suggesting the importance of NKILA in the antiviral activity by regulating the life cycle of HBV DNA or RNA. Interestingly, we found that NKILA expression was downregulated during HBV replication and infection in cell lines and even in HBV-positive patients, and the mRNA NKILA in HBV-positive patients with low viral loads was significantly higher than that with high viral loads, which was correlated with their HBV viral loads, indicating that HBV decreased the expression of NKILA in cells.

NKILA has been demonstrated to form a stable ternary complex with p65 and retain NF-κB dimers in the cytoplasm to suppress NF-κB signalling (Bird, 2018; Liu et al., 2015). We observed that NKILA, mainly located in the cytoplasm, inhibited NF-κB activity mediated by p65 alone or even by p65 and HBx combined, decreased p65 translocation into the nucleus, and reduced IκBα phosphorylation levels. In addition, the interaction between HBx and p65 was significantly reduced in the presence of NKILA compared to that in the control. Meanwhile, our result showed NKILA inhibited HBV in a HBx-dependent way. However, HBV replication, the promoter activity of NF-κB, and the interaction between HBx and p65 were not affected by those NKILA mutants which lack functional domains that are responsible for p65 binding and masking NF-κB phosphorylation expression, compared to that of MKILA WT, indicating that the requirement of p65 binding and NF-κB phosphorylation is a prerequisite for NKILA to exert its antiviral activity. More importantly, HepAD38 contains a full length of pgRNA cDNA integrations, and HBV RNA, DNA and HBsAg could be independently expressed from the integrated HBV DNA. Our results showed that NKILA inhibited expression of HBV viral proteins in HepAD38 cells and HBV DNA or RNA level in HepG2 and Huh-7 cells. Those results illustrate NKILA may regulate the life cycle of HBV pgRNA. The regulation mechanism of NKILA on HBV life cycle need been investigated further.

5. Conclusions

To summazise, our study identifies NKILA acts as an inhibitor and has been shown potent anti-HBV activity via HBx-dependent NF-κB pathway. The information presented in this study may aid in the development of novel therapeutic strategies against HBV.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

This study was approved by the ethics review committee of the First Hospital of Jilin University, Changchun, China (No.2016-255). All patients signed informed consent forms prior to their participation in this study. HBV-positive patients and healthy donors were recruited from

the Changchun Center for Disease Control and Prevention (Jilin, China).

Author contributions

Xi Zhang: data curation, formal analysis, methodology, writing-original draft. Yuanyuan Li: data curation, methodology. Chen Huan: formal analysis, methodology, supervision. Yubao Hou: data curation, formal analysis, methodology. Rajia Liu: resources, software. Hongyun Shi: resources, software. Peng Zhang: resources, software. Baisong Zheng: methodology, resources, software, supervision, validation. Yingchao Wang: conceptualization, project administration, writing-original draft, writing-review, editing. Hong Wang: conceptualization, project administration, writing-original draft, writing-review, editing. Wenyan Zhang: conceptualization, project administration, writing-original draft, writing- review, editing. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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