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Original Article

Dopamine Inhibits the Expression of Hepatitis B Virus Surface and e Antigens by Activating the JAK/STAT Pathway and Upregulating Interferon-stimulated Gene 15 Expression

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Abstract

Background and Aims: Hepatitis B virus (HBV) infection is a major risk factor for cirrhosis and liver cancer, and its treatment continues to be difficult. We previously demonstrated that a dopamine analog inhibited the packaging of pregenomic RNA into capsids. The present study aimed to determine the effect of dopamine on the expressions of hepatitis B virus surface and e antigens (HBsAg and HBeAg, respectively) and to elucidate the underlying mechanism. ***Methods:*** We used dopamine-treated HBV-infected HepG2.2.15 and NTCP-G2 cells to monitor HBsAg and HBeAg expression levels. We analyzed interferon-stimulated gene 15 (ISG15) expression in dopamine-treated cells. We knocked down ISG15 and then monitored HBsAg and HBeAg expression levels. We analyzed the expression of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway factors in dopamine-treated cells. We used dopamine hydrochloride-treated adeno-associated virus/HBV-infected mouse model to evaluate HBV DNA, HBsAg, and HBeAg expression. HBV virus was collected from HepAD38.7 cell culture medium. ***Results:*** Dopamine inhibited HBsAg and HBeAg expression and upregulated ISG15 expression in HepG2.2.15 and HepG2-NTCP cell lines. ISG15 knockdown increased HBsAg and HBeAg expression in HepG2.2.15 cells. Dopamine-treated cells activated the JAK/STAT pathway, which upregulated ISG15 expression. In the adeno-associated virus-HBV murine infection model, dopamine downregulated HBsAg and HBeAg expression and activated the JAK-STAT/ISG15 axis. ***Conclusions:*** Dopamine inhibits the expression of HBsAg and HBeAg by activating the JAK/STAT pathway and upregulating ISG15 expression.

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Introduction

Hepatitis B virus (HBV) infection is a global public health challenge.[1] Over 350 million people worldwide have chronic hepatitis B virus (HBV) infection, 2.03% of whom are children under 5 years of age.[2–5] This disease causes approximately 686,000 deaths annually.[6–9]

Appropriate antiviral treatment can effectively prevent chronic complications of hepatitis B infection and improve survival.[10] Currently available medications for the treatment of chronic HBV infection include nucleos(t)ide analogs (NAs) such as lamivudine (LAM), tenofovir (TDF) and entecavir (ETV); however, LAM therapy can lead to the emergence of NA-resistant viruses.[11] Long-term high-dose TDF therapy (300 mg daily) can cause renal and skeletal adverse reactions such as proximal tubular dysfunction, osteomalacia, and hypophosphatemia.[12] *In vitro* and *in vivo* studies suggest that ETV can completely inhibit HBV DNA replication.[13] and suppress circulating HBV DNA, but not circulating HBsAg and HBeAg.[14]

The loss of detectable hepatitis B surface antigen (HBsAg) is considered a functional cure of chronic hepatitis B infection.[15] Agents such as pegylated interferon (IFN) can be used to attain HBsAg clearance. However, interferon-α treatment can clear HBV but is limited by systemic side effects.[16] Achieving a functional cure remains challenging. HBsAg and

Keywords: Hepatitis B virus; Dopamine; JAK-STAT/ISG15 axis; HBV surface antigen; HBV e antigen; HepG2.2.15 cell line; Human NTCP-expressing; HepG2 cell line; HepAD38 cell line.

Abbreviations: ccc, covalently closed circular; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ETV, entecavir; FBS, fetal bovine serum; GEI, genome equivalent of infection; HBV, Hepatitis B virus; IFN, interferon; IHC, immunohistochemistry; ISG, interferon-stimulated gene; JAK, Janus kinase; NA, nucleos(t)ide analog; NRTI, nucleotide reverse transcriptase inhibitor; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PMM, primary hepatocyte-maintaining medium; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; Ub, ubiquitin.

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HBV e antigen (HBeAg) play critical roles in various stages of the viral replication cycle and are potential targets for anti-HBV drug candidates.

We previously reported that the dopamine analogs 6-hydroxy-DL-DOPA and N-oleoyldopamine had specific effects in cell-free assays and inhibited viral replication and packaging in stable HBV production (HB611) and infection systems established with sodium taurocholate co-transporting polypeptide-expressing HepG2 (NTCP-G2) cells.[17] The clearance of HBV surface antigen (HBsAg) from the serum is a main goal of HBV infection treatment, and has been proposed to define the functional curing of this disease.[18–20] However, the long-term sustainability of such clearance, and the efficacy of HBsAg antibodies for its maintenance remain questionable.[18]

Type-I IFNs are cytokines that induce the expression of interferon-stimulated genes (ISGs) encoding various host-protecting proteins, and their production can be induced by tissue damage and infection.[21] These proteins include ISG15, a ubiquitin (Ub) homolog with a structure resembling that of di-Ub whose corresponding gene is most frequently upregulated upon viral infection in humans and other species.[21–24] ISG15 has been reported to have antiviral activities[25,26] and has been shown to generally protect against infection, as confirmed by the phenotypes of ISG15-deficient mice and humans,[21] but the mechanisms underlying its action remain unclear. As ISG15 exists freely and is bound to other proteins, these mechanisms may differ between intra- and extracellular environments.

The synthesis of mature ISG15 requires the removal of eight amino acids from its precursor protein's C-terminus, leaving the sequence motif LRLGG, which is shared with mature Ub.[23] In contrast to other Ub homologs, ISG15 has not been identified in lower organisms, implying that it has a highly specialized function in vertebrates.[23] It is secreted intracellularly and from monocytes and lymphocytes, and has cytokine-like activities such as the stimulation of natural killer cell activation and dose-dependent IFN-γ production by T cells; it also functions as a neutrophil chemotactic factor.[22–24] Extracellular ISG15 activates peripheral blood mononuclear cells by triggering the lymphocyte function-associated antigen 1 integrin receptor cluster of differentiation 11a, thereby enhancing IFN-γ and interleukin-10 secretion.[27] ISG15 expression, and thus antiviral activity, is regulated by the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway.[28–30]

In this study, we aimed to determine the effect of dopamine on the expressions of HBsAg and HBeAg, and to elucidate the underlying mechanism by exploring the key pathways and genes involved in the translation and expression of HBsAg and HBeAg.[31]

Methods

Cell lines, reagents, and cell treatment

The stable HBV-producing cell line HepG2.2.15[32] was cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Cellcook Biotechnology, Guangzhou, China), 1% antibiotic-antimycotic solution (Solarbio, Beijing, China), and 0.5 mg/mL G418 disulfate (Macklin, China) and kept in a humidified atmosphere containing 5% CO_2 at 37°C. HepG2.2.15 cells were seeded in 24-well plates with 500 μL culture medium (Nest Glass; 2,000 cells/well) and treated with dopamine (125, 62.5, 31.25, 15.6, and 7.81 μM). Cells were incubated with either ETV 20 nM or both 125 μM dopamine and 20 nM ETV for 12 days, with medium refreshment every 3 days.

NTCP-G2 cells[33] were maintained in a primary hepatocyte-maintaining medium (PMM; Williams' E medium (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic solution, 50 μM hydrocortisone (Sigma-Aldrich), 5 μM dexamethasone, 5 μg/mL transferrin (Wako, Japan), 10 ng/mL epidermal growth factor (Thermo Fisher Scientific), 5 μg/mL insulin (Sigma-Aldrich), 5 ng/mL sodium selenite, 2 mM L-glutamine (Nacalai Tesque, Japan), and 0.5 mg/mL G418 (Nacalai Tesque) and kept in a humidified atmosphere containing 5% CO_2 at 37°C. NTCP-G2 cells were seeded in 24-well plates (Nest Glass; 2×10^5 cells/well). The next day, they were infected with HBV from HepAD38.7 cells (~500 GEI) in 500 μL 4% PEG 8000 and 2% DMSO containing PMM. Uninfected cell cultures were subjected to mock treatment. Twenty-four hours after infection, the NTCP-G2 cells were washed twice with PMM and then treated by the addition of dopamine (125, 62.5, 31.25, 15.6, and 7.81 μM) and ETV (20 nM) to the culture medium for 9 days, with medium refreshment every 3 days.

HepAD38.7 cells[31] were maintained in DMEM/F12 (Nacalai Tesque) supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic solution (Nacalai Tesque), 0.5 mg/mL G418, and 400 ng/mL tetracycline and kept in a humidified atmosphere containing 5% CO_2 at 37°C. HBV was induced in confluent HepAD38.7 cells via the exclusion of tetracycline from the culture medium, which was collected weekly for 2 weeks, and precipitated by overnight treatment at 4°C with a final concentration of 6% polyethylene glycol (PEG) 8000 (Sigma-Aldrich). The precipitate was centrifuged to obtain pellets, followed by dissolution in phosphate-buffered saline (PBS) and concentration. HBV DNA was subjected to real-time quantitative polymerase chain reaction (qPCR), and HBV at the genome equivalent of infection (GEI) of 500 was used in subsequent experiments.[31]

Cell viability assay

HepG2.2.15 and NTCP-G2 cells were seeded in clear-bottomed white 96-well plates (Corning, USA; 800 and 3,000 cells/well, respectively) in 100 μL of their respective culture media. They were incubated with dopamine at various concentrations (1,000, 500, 250, 125, 62.5, 31.25, 15.6, 7.81, 3.9, and 1.95 μM) for 12 and 9 days. NTCP-G2 cells were cultured in 2% dimethyl sulfoxide (DMSO) containing PMM. Following the addition of 100 μL Cell Counting Kit-8 solution (Promega, USA) to the culture media and incubation at 37°C for 30 min, cell viability was assessed by measuring absorbance at 450 and 630 nm with a plate reader (GloMax, GM3000; Promega).

ISG15 silencing using small interfering RNA

An ISG15 small interfering RNA (siRNA) was designed and constructed by HanYi Biosciences Inc. (Guangzhou, China) using the following interference sequences: siRNA control, UUCUCCGAACGUGUCACGUTT and ACGUGACACGUUCGGAGAATT; *ISG15-1*, GAGCAUCCUGGUGAGGAAUTT and AUUCCUCACCAGGAUGCUCTT; and *ISG15-3*, UGUCGGUGUCAGAGCUGAATT and UUCAGCUCUGACACCGACATT. Lipofectamine 3000 (Thermo Fisher, USA) was employed as a transfection reagent. The siRNA was used to silence *ISG15* expression in HepG2.2.15 cells. The cells were seeded in a six-well plate (3×10^5 cells/well) for 24 h, then transduced with ISG15-specific siRNA or scramble control and incubated for an additional 24 h. ISG15 protein expression in two *ISG15*-knockdown clones was analyzed using western blotting.

Rescue experiment

We performed a rescue experiment to assess the role of dopamine in HBsAg and HBeAg inhibition via the upregulation of ISG15 expression. HepG2.2.15 cells were cultured in a six-well plate (3×10^5 cells/well) for 1 day, then transfected with scrambled control and *ISG15* siRNA for 24 h. The culture medium was then replaced, and one group of *ISG15* siRNA-transfected and control cells were treated with dopamine (125 μM). The cell lysates and culture media were collected 3 days after treatment, and HBsAg and HBeAg expression were examined by western blotting and ELISA, respectively. *ISG15* protein expression in two *ISG15*-knockdown clones was analyzed using western blotting.

Mouse infection model

The Animal Care and Use Committee of South China Agricultural University approved the animal experiments ([2020]d065). Male C57BL/6 mice aged 6–8 weeks were obtained from the Animal Center of the Guangdong Medical Laboratory. Mice were housed in the Special Pathogen-Free Laboratory Animal Center, with a temperature of 20–26°C, relative humidity of 40–70%, noise below 0 dB, nitrogen concentration under 14 mg/m^3, over 15 air changes per hour, and a 12-hour cycle of light and darkness. Five to six mice were placed in each cage and padded twice a week. Mice were provided filtered drinking water from the animal house and fed with CO60-irradiated feed that met the national standard.

The mice were given tail-vein injections of recombinant virus (adeno-associated virus [AAV] 8-HBV1.3, 2.5×10^{11} viral genome) or vector control (AAV8 [ssAAV.CAG.WPRE.SV-40pA]) diluted with PBS (total, 100 μL). After 4 weeks, mice with stable viremia were treated for 12 weeks with dopamine hydrochloride (10 μg/kg daily). In total, 10 mice were randomly allocated to treatment and placebo (normal saline) groups (n=5 each). Blood was collected every 3 weeks and serum HBV DNA, HBsAg, and HBeAg levels were determined using qPCR and ELISA as described above. At week 12, the animals were sacrificed, and intrahepatic HBV DNA was detected. Retro-orbital blood was collected at 0, 3, 6, 9, and 12 weeks for the monitoring of serum HBV genomic DNA. qPCR (AmpliPrep-COBAS TaqMan [CAP/CTM] assay, 20-IU/mL lower detection limit; Roche Molecular Systems, Branchburg, USA) was performed to quantify plasma HBV DNA. Serum HBsAg and HBeAg expression levels were determined by ELISA as described above.

Intracellular and extracellular DNA extraction

Extracellular (particle-associated) and intracellular (core-associated) HBV DNA in the treated cell supernatants were prepared for qPCR. To degrade DNA outside particles, the supernatants were incubated with 20 U/mL DNase I (Takara) with 5 mM $MgCl_2$ at 37°C for 2 h, followed by the addition of ethylenediaminetetraacetic acid (EDTA; pH 8.0, final concentration 10 mM). Virus particle precipitates were obtained with 6% (w/v) PEG 8000 (Sigma-Aldrich); suspended in buffer containing 10 mM Tris-HCl (pH 7.6), 5 mM EDTA (pH 8.0), 0.2 mg/mL proteinase K (Roche), and 0.5% sodium dodecyl sulfate; and incubated at 56°C for 3 h. RNase A (0.4 mg/mL; Roche) was then added, and the solutions were further incubated at 65°C for 30 min. Phenol:chloroform:isoamyl alcohol (25:24:1) containing sonicated salmon sperm DNA (10 μg) was used to extract complete particle (virion)-associated HBV DNA, which was precipitated with ethanol in 0.25 mg/mL glycogen (Nacalai Tesque). The pellets obtained were suspended in 20 μL Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). For the analysis of core-associated HBV DNA, harvested cells were washed with PBS and pelleted at 7,200 ×g and 4°C for 5 min, followed by suspension in a hypotonic buffer (20 mM Tris-HCl [pH 7.8], 50 mM NaCl, 5 mM $MgCl_2$, and 0.1% 2-mercaptoethanol) with vigorous vortexing. The cell debris was separated by centrifugation as in the previous step, and the clear lysate was taken to be virion-associated HBV DNA, which was suspended in 20 μL Tris-EDTA buffer.

Extraction of core particle-associated RNA

To assess packaged RNA, lysates from drug-treated NTCP/G2 and HepG2.2.15 cells were processed to obtain core-associated HBV DNA samples. Subsequently, TRIzol™ (Invitrogen) was added to the lysate in three volumes, and RNA extraction was conducted following the manufacturer's instructions. It is important to note that 1 μg of yeast tRNA was included prior to PCI extraction, and a 0.25 mg/ml glycogen solution (Nacalai Tesque) was introduced before isopropanol precipitation. The extracted RNA was then dissolved in 20 μL TE buffer and preserved as core-associated HBV RNA in a −70°C freezer until required.

Enzyme-linked immunosorbent assay (ELISA)

After treatment, both cell types and culture supernatants were collected. HBsAg and HBeAg levels in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA; Rapid-II [Beacle Inc.] and e-Antigen [Bioneovan Co.] kits, respectively).

Quantification of covalently closed circular HBV DNA

To study the HBV replication cycle, 2.1- and 2.4-kb mRNAs were transcribed from covalently closed circular (ccc) HBV DNA from HepG2.2.15 and NTCP-G2 cells.[34–36] A fluorescent probe qPCR kit (Supbio Biotechnology, Guangzhou, China) was used to quantify cellular HBV cccDNA. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and denatured at 85°C for 5 min. Relaxed circular, replicative double-stranded, and single-stranded HBV DNA were digested using plasmid-safe adenosine triphosphate-dependent DNase.[37] qPCR was performed with a primer pair and probe targeting the gap region of the HBV genome for cccDNA quantification in the range of 10–1,000,000 copies/μL. To normalize the cccDNA quantity, qPCR was performed with a primer set and probe to detect the human β-globin gene.[38]

Analysis of mRNA expression profiles

We assessed the mRNA expression profiles of dopamine (125 μM) and DMSO-treated HepG2.2.15 cells. After 12 days of culture with medium refreshment every 3 days, samples (n=3) were collected, and RNA was extracted using a TRIzol reagent kit (Invitrogen). RNA sequencing and library construction were performed using an Illumina HiSeq™ 2500/4000 device (Gene Denovo Biotechnology Co., Ltd., Guangzhou, China). Gene expression profiles were analyzed using DESeq2 software.[39] Enrichment pathway analyses, such as Gene ontology, Kyoto Encyclopedia of Genes and Genomes, and Bioinformatic analysis were performed using the OmicShare tools at (www.omicshare.com/tools).

qPCR and RT-qPCR

qPCR was performed with separate 20-μL solutions containing 1 μL of the two HBV DNA types, respectively, using Fast SYBR™ Green Master Mix (Applied Biosystems/Thermo Fisher Scientific), S region primers (forward, 5′-CTTCATC-

CTGCTGCTATGCCT-3′; reverse, 5′-AAAGCCCAGGATGATGGGAT-3′), and the QuantStudio™ 6 Flex system (Applied Biosystems by Life Technologies). The initial PCR cycle was performed at 95°C for 20 s, followed by 40 cycles at 95°C for 1 s and 60°C for 20 s and a final cycle at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. For reverse transcription, 10 μL core-associated HBV RNA was mixed with 0.5 mM deoxynucleotide triphosphates and 10 ng random primers, denatured by heat at 65°C for 5 min, and then chilled rapidly on ice. The reaction mixture (20 μL) containing 5 mM dithiothreitol, 40 units RNasin (Promega), and 200 units Superscript III (Invitrogen, USA) was incubated at 37°C for 30 min, 42°C for 30 min, and 50°C for 60 min, and then at 70°C for 30 min for reverse transcriptase inactivation. The copy number was determined with reference to the standard copy number in a plasmid containing a single HBV genome.

For reverse transcription, 10 μL of core-associated HBV RNA was mixed with 10 ng random primers and 0.5 mM dNTPs and quickly chilled on ice after heat denaturation at 65°C for 5 min. The solution was arranged in a 20 μL reaction mixture containing 5 mM DTT, 40 units of RNasin® (Promega), and 200 units of SuperScript III (Invitrogen). The mixture was then incubated at 37°C for 30 min, 42°C for 30 min, and 50°C for 60 min. Finally, the mixture was incubated at 70°C for 30 min to inactivate reverse transcriptase. A 1 μL aliquot of the reversely transcribed sample was subjected to qPCR for which primers were set at the HBV pgRNA (forward; 5′-CTCAATCTCGGGAACCTCAATGT-3′ and reverse; 5′-TGGATAAAACCTAGCAGGCATAAT−3′) , HBV total RNA (forward; 5′-ATGGCTGCTAGGCTGTGCTGC-3′ and reverse; 5′-ACGGTG GTCTCCATGCGACG−3′)[40] with the same condition as described above. qPCR data are presented as means with standard deviations relative to the control from at least three replicates.

Western blotting

We evaluated the signaling molecules in the JAK-STAT pathway to determine the mechanism underlying ISG15's effects on HBV HBsAg and HBeAg expression. HepG2.2.15 cells were treated with dopamine (125 μM) and DMSO for 12 days. We then lysed the cells, and examined JAK1, STAT1, and p-STAT1 expression by western blotting using specific antibodies. Antibodies used in this study included anti-ISG15 (1:1,000, Abcam, UK), anti-JAK1 (1:1,000, Absin, China), anti-STAT1 (1:1,000, CST, USA), and anti-p-STAT1 (1:1,000, CST, USA), with anti-β-actin serving as an internal control. Antibodies were used for protein detection by western blotting, immunofluorescence, and immunohistochemistry (IHC).

Immunohistochemical analysis

IHC was performed using a standard protocol. Paraffin-embedded block mouse liver tissue sections (3.5 μm) were dewaxed, rehydrated, and incubated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidases. Antigens were retrieved with EDTA (pH 8.0) by boiling in water for 2 min in a pressure cooker. After cooling at room temperature for 20 min, the sections were incubated with rabbit polyclonal anti-HBsAg antibody (1:50, Novus, USA) diluted with 1% bovine serum albumin at 37°C for 1 h. They were then washed with PBS and incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:50, ServiceBio, China). The stained sections were viewed under an Olympus Leica DM4000B microscope. The expression of HBsAg, ISG15, STAT1, phosphorylated (p)-STAT1, and JAK1 was calculated by Image J (FIJI), based on the DAB-positive area observed under a 20-fold magnification scope in 10 randomly selected fields.

Statistical analysis

Five animals were used per condition in each experiment. Cells were treated in triplicate. Data are expressed as means±standard deviation (SD). Comparisons between two groups were conducted using the unpaired Student's *t*-test, while comparisons among multiple groups were conducted using one-way ANOVA with a threshold. Differences were considered statistically significant at $p<0.05$.

Results

Dopamine inhibited HBV HBsAg, HBeAg, and DNA amplification, as revealed through cell-based assays

Dopamine-related cytotoxicity was evaluated. We used the maximum concentration, which resulted in over 90% cell viability (Fig. 1A). Dopamine decreased HBsAg and HBeAg levels in HepG2.2.15 cells dose-dependently (Fig. 1B and C). The combination of ETV and dopamine decreased HBsAg and HBeAg expression to the same levels obtained by dopamine monotherapy (Fig. 1D and E). Core-associated and extracellular particle-associated HBV DNA levels in HepG2.2.15 cells decreased after dopamine treatment in a dose-dependent manner (Fig. 1F and G). These results suggested that dopamine specifically inhibits HBV transcription and DNA amplification processes, such as packaging and genome DNA synthesis.

The effects of the nominated compounds were also tested in an HBV infection system using NTCP/G2 cells (Fig. 2A). We used the maximum concentration, associated with over 90% viability, in the infection assay. Dopamine decreased HBsAg and HBeAg levels dose-dependently (Fig. 2B and C). Similar to the results obtained in HepG2.2.15 cells, the addition of ETV to dopamine did not change HBsAg and HBeAg expression levels obtained with dopamine monotherapy (Fig. 2D and E). Core-associated and extracellular particle-associated HBV DNA levels in NTCP/G2 cells decreased dose-dependently following dopamine treatment (Fig. 2F and G). A wide range of drug concentrations was required for accurate determination; nonetheless, our results confirmed that dopamine inhibits HBV replication.

Dopamine decreased HBV HBsAg and HBeAg levels, but not cccDNA levels

To study the HBV replication cycle, 2.1 and 2.4 kb mRNAs were transcribed from HBV cccDNA.[34–36] We analyzed the expression levels of cccDNA in HepG2.2.15 and HepG2-NTCP cells to determine whether the dopamine-mediated downregulation of HBsAg and HBeAg expression was caused by an inhibition of cccDNA formation or transcription. We found that cccDNA formation was unaffected by dopamine when compared to DMSO (Fig. 3A and B). Our investigation focused on the levels of packaged pgRNA and total RNA expressions in HBV. Dopamine-treated HepG2.1.15 and NTCP-G2 cells exhibited decreased packaged pgRNA expression levels compared to DMSO-treated cells. This outcome aligns with our prior discoveries. Interestingly, total HBV RNA levels in dopamine-treated cells were marginally lower than those observed in DMSO-treated cells (Fig. 3C-F).

Dopamine altered mRNA profiles and increased ISG15 expression in cells

We assessed the mRNA expression profiles of dopamine (125 μM) and DMSO-treated HepG2.2.15 cells to identify the cel-

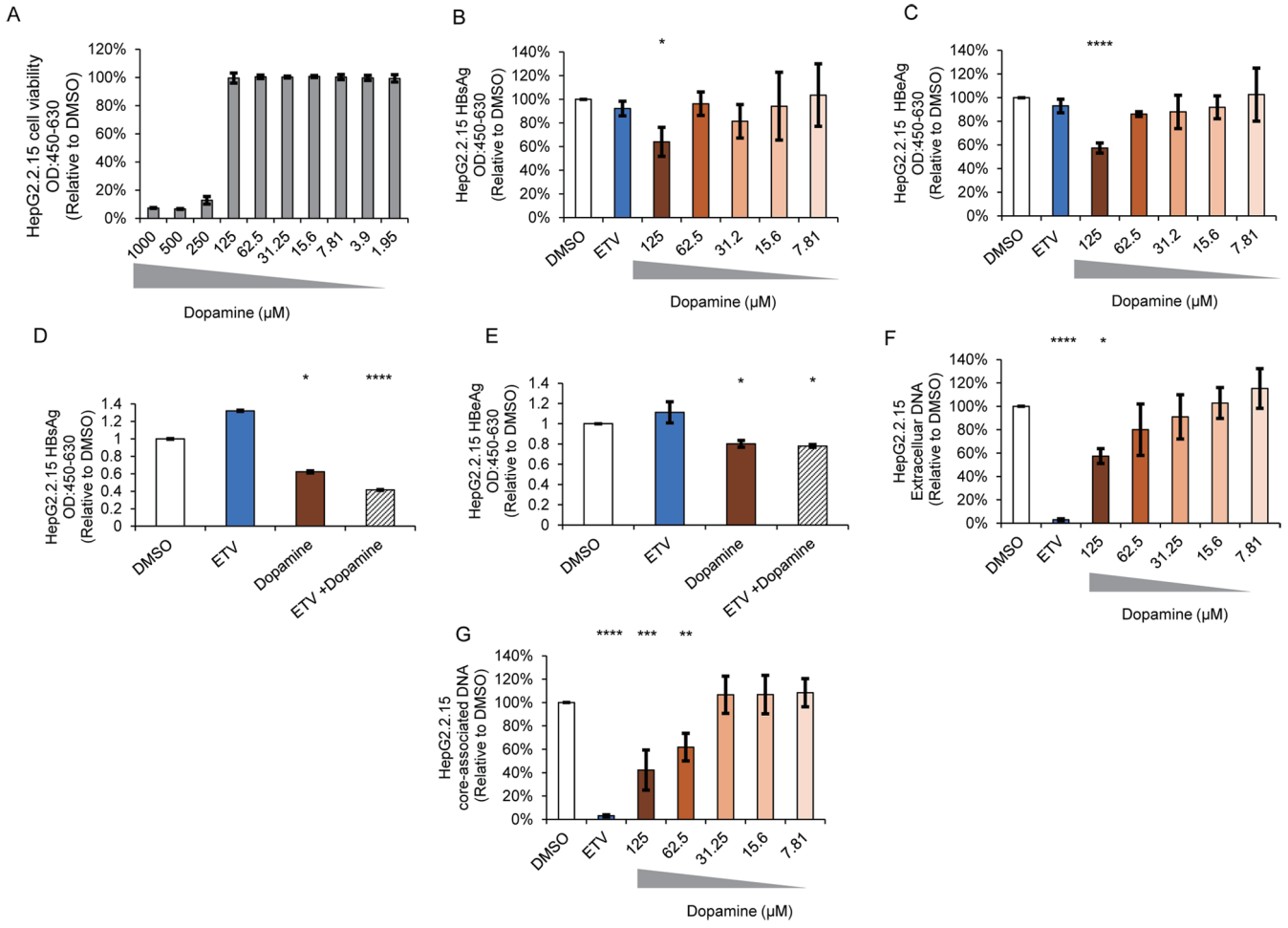

Fig. 1. Dopamine inhibits hepatitis B virus (HBV) amplification in a stable HBV-producing cell line. (A) HepG2.2.15 cell viability following dopamine treatment for 12 days. (B, C) Hepatitis B virus surface and e antigen (HBsAg and HBeAg, respectively) levels after dopamine and entecavir (ETV, 20 nM, control) treatment, measured by enzyme-linked immunosorbent assay. (D, E) HBsAg and HBeAg expression following ETV (20 nM) and/or dopamine (125 μM) treatment. (F, G) Core- and extracellular particle-associated HBV DNA levels following dopamine and/or ETV treatment, determined by quantitative polymerase chain reaction. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.005 ****p<0.001. OD, optical density; DMSO, dimethyl sulfoxide; ETV, entecavir.

lular components responsible for the dopamine-mediated inhibition of HBsAg and HBeAg expression. Compared with DMSO-treated cells, dopamine-treated cells presented 307 upregulated genes and 70 downregulated genes (Fig. 4A). The top seven mRNAs were upregulated in the dopamine-treated-cells compared to DMSO-treated HepG2.2.15 cells (Fig. 4B). We focused on ISG15 because of its reported antiviral activities. ISG15 expression was significantly increased in dopamine-treated HepG2.2.15 and HepG2-NTCP cells after infection (500 GEI) compared to that of DMSO-treated cells (Fig. 4C and D).

ISG15 inhibited in vitro HBV HBsAg and HBeAg expression

To determine the role of ISG15 in inhibiting HBsAg and HBeAg synthesis during HBV replication, we silenced ISG15 expression in HepG2.2.15 cells using specific small interfering RNAs (siRNAs). We also confirmed the relationship between ISG15 and HBsAg and HBeAg expression by conducting a rescue experiment (Fig. 5A and B). The two *ISIG15*-knockdown clones decreased ISIG15 expression by over 50% (Fig. 5C and D). HBsAg and HBeAg expression levels were significantly upregulated in HepG2.2.15 cells when ISG15 was silenced using siRNA (Fig. 5E and F).

We performed rescue experiments to confirm that dopamine inhibited HBsAg and HBeAg expression through the upregulation of ISG15. HepG2.2.15 cells were cultured at 3×10^5 cells/well in a 6 well plate. On the following day, cells were transfected with siRNA (control and ISG15) and incubated for 24 h. Subsequently, culture media were replaced in all the samples, and one sample was treated with dopamine (125μM). At 3 days post-treatment, cell lysates and culture media were collected. The results indicated that dopamine reversed the effect of ISG15 silencing in HepG.2.15 cells (Fig. 5G and H). HBsAg and HBeAg expression levels in the reversed cells were similar to those of controls (Fig. 5I and J).

Dopamine activated JAK-STAT pathway-mediated ISG15 upregulation in cells

Previous reports have revealed that the JAK-STAT pathway regulates ISG15 expression.[28,29] We assessed the signaling

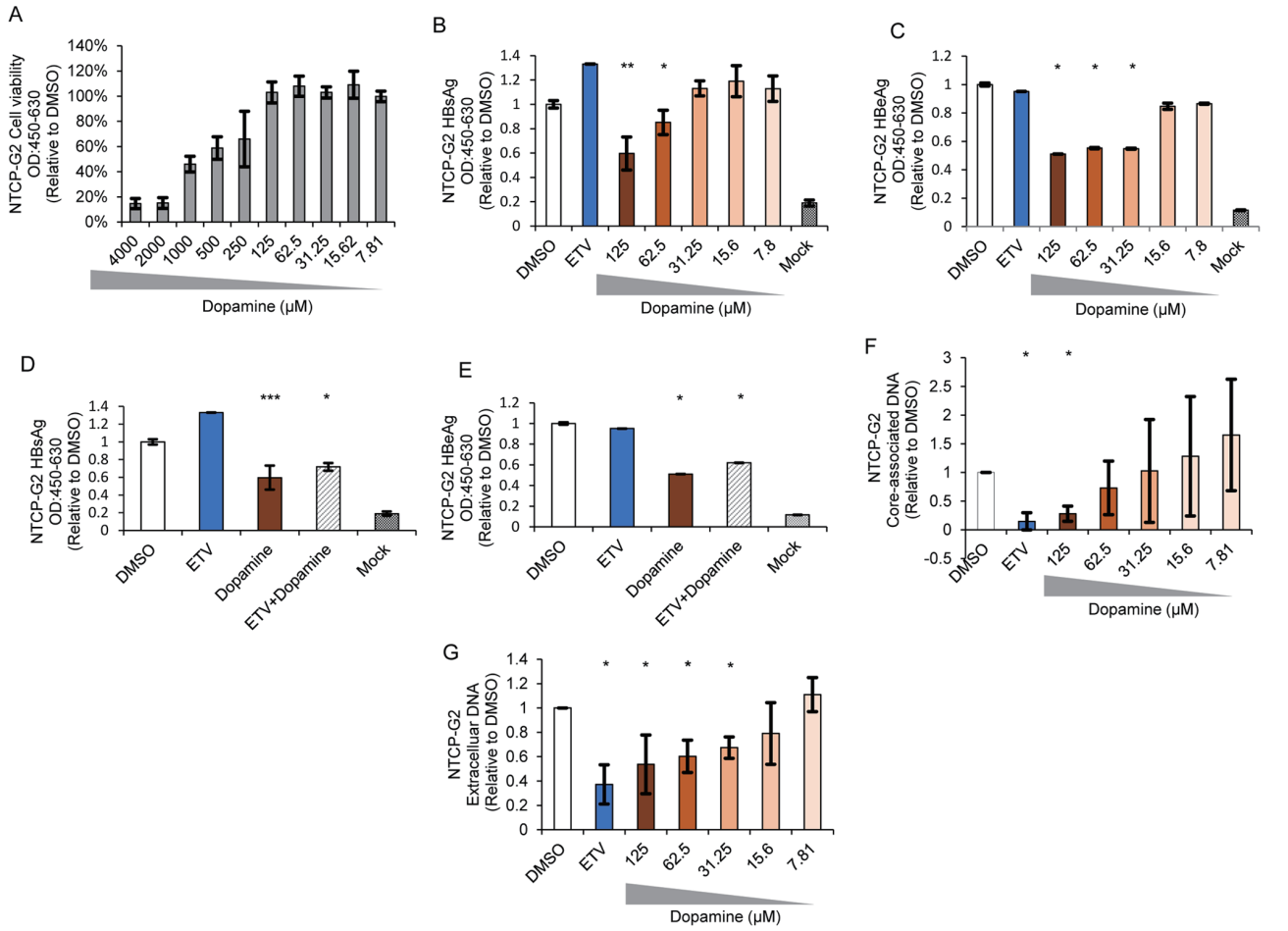

Fig. 2. Dopamine inhibits hepatitis B virus (HBV) amplification in an HBV infection system. (A) Sodium taurocholate co-transporting polypeptide-expressing HepG2 (NTCP-G2) cell viability following dopamine treatment for 9 days. (B, C) Hepatitis B virus surface and e antigen (HBsAg and HBeAg, respectively) levels in NTCP-G2 cells infected with supernatant from HepAD38 cells and treated with dopamine or entecavir (ETV; 20 nM), measured by enzyme-linked immunosorbent assay. (D, E) HBsAg and HBeAg expression following ETV (20 nM) and/or dopamine (125 μM) treatment. (F, G) Core- and extracellular particle-associated HBV DNA levels in HB-infected NTCP-G2 cells following dopamine and/or ETV treatment, determined by quantitative polymerase chain reaction. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.005. OD, optical density; DMSO, dimethyl sulfoxide; Mock, uninfected negative control; ETV, entecavir.

molecules in the JAK/STAT pathway to determine the mechanism by which ISG15 inhibits HBsAg and HBeAg expression. HepG2.2.15 cells were treated with either dopamine (125 μM) or DMSO. After 12 days, we lysed the cells and investigated the expression levels of JAK1, p-JAK1, STAT1, and P-STAT1 via western blotting using specific antibodies. The expressions of STAT1, p-STAT1 (Fig. 6A–D), JAK1, and p-JAK1 (Fig. 6E–H) were significantly upregulated in dopamine-treated cells compared to DMSO-treated cells.

Ruxolitinib inhibited the ISG15 expression level in cells

To measure the relationship between the JAK-STAT pathway and ISG15, we used Ruxolitinib, a JAK1/2 inhibitor to inhibit the JAK expression in the HepG2.2.15 cell line (Fig. 7A). We evaluated ruxolitinib-related cytotoxicity and used the maximum concentration resulting in over 90% cell viability (Fig. 7B). Treatment with ruxolitinib significantly downregulated JAK1, p-JAK1, STAT1, and p-STAT1 and ISG15 expression levels. Moreover, the expression levels of these proteins were rescued by dopamine treatment (Fig. 7C and D).

Dopamine inhibited HBV HBsAg expression in mice

To assess the long-term effects of the dopamine regimen, we treated AAV/HBV-infected mice with 10 μg/kg dopamine hydrochloride daily. HBsAg and HBeAg expression levels were examined every three weeks until the 12 week timepoint (Fig. 8A). HBsAg and HBeAg levels in mouse serum were reduced by 80% following dopamine hydrochloride treatment for 12 weeks (Fig. 8B and C). The HBV DNA level was reduced after dopamine hydrochloride treatment (Fig. 8D). Dopamine therapy did not change mouse weight during the 12 weeks of treatment (Fig. 8E). Similarly, HBsAg expression in hepatic tissue was downregulated after 12 weeks, as observed by IHC (Fig. 8F). We found that after 12 weeks, expression levels of ISG15, STAT1, p-STAT1, and JAK1 were considerably higher in the dopamine hydrochloride-treated group than in the placebo-treated group (Fig. 8G–J).

Discussion

Despite the demonstrated efficacy of antiviral therapy in halting the progression of liver disease and preventing he-

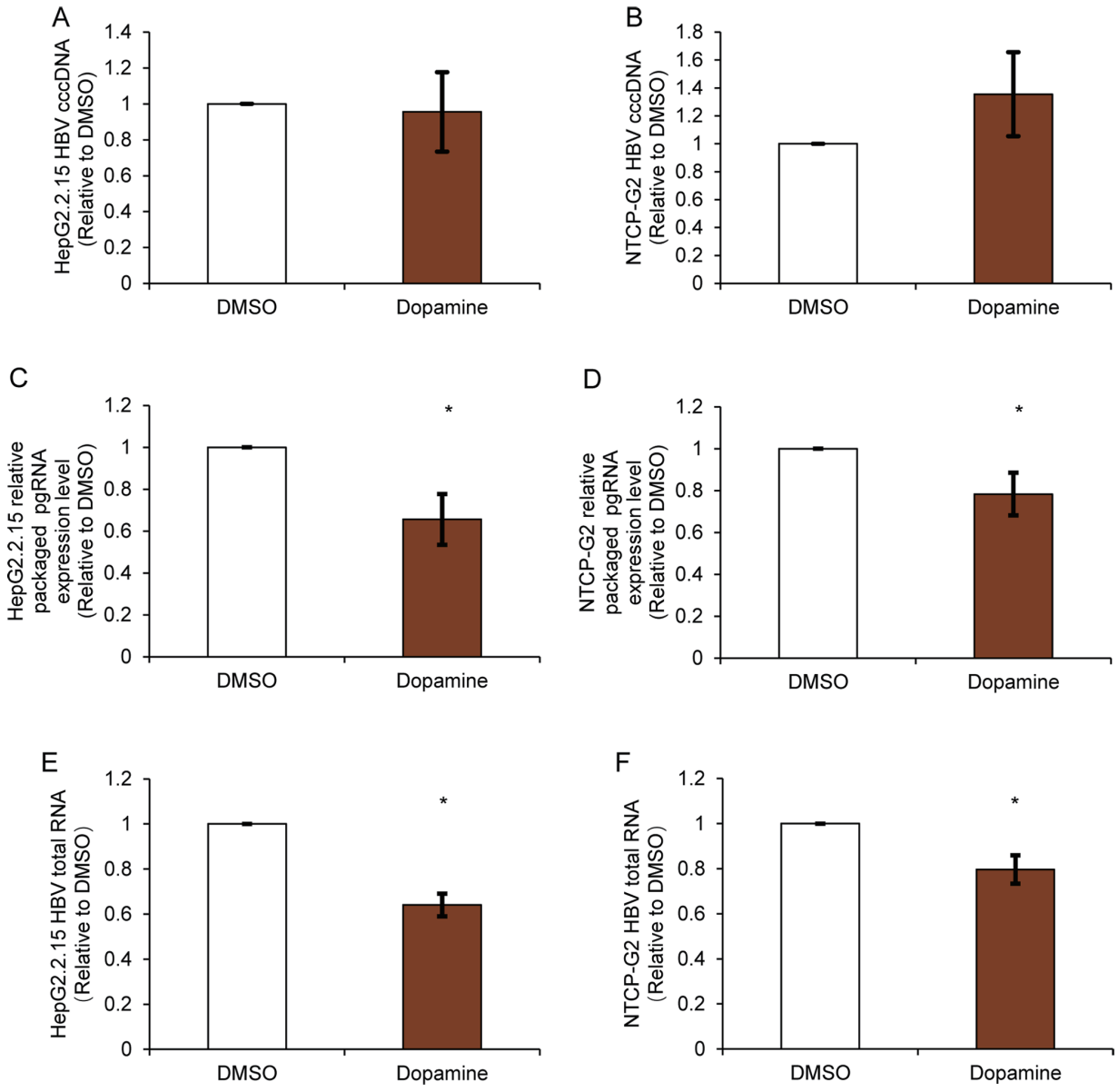

Fig. 3. Dopamine does not inhibit covalently closed circular DNA (cccDNA) formation. cccDNA, packaged pgRNA and HBV total RNA expression in (A, C and E) HepG2.2.15 cells and (B, D and F) HBV-infected sodium taurocholate co-transporting polypeptide-expressing HepG2 (NTCP-G2) cells following dopamine treatment for 12 and 9 days, respectively, as determined by quantitative polymerase chain reaction. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. *p<0.05. DMSO, dimethyl sulfoxide; HBV, hepatitis B virus.

patic failure in patients with chronic HBV infection, achieving a functional cure remains highly challenging due to the notably low success rate.[41] The goal of HBV therapy is to eliminate circulating HBsAg.[42]

Available therapeutic options for the management of chronic HBV infection include nucleos(t)ide analogs and PEGylated IFNs. Despite their administration over an extensive timeframe, these treatments exhibit a disappointing success rate (less than 10% following a 5-year evaluation) in achieving HBsAg clearance. Moreover, their inability to eliminate HBsAg from infected hepatocytes renders viral eradication unattainable.[43] Therefore, various antiviral strategies are being investigated to increase the HBsAg loss rate.[44] These include the use of HBV entry inhibitors and capsid assembly modulators. However, these drug candidates are still in preclinical or early clinical stages of development.[45,46]

In this study, we investigated the antiviral activity of dopamine, a small molecule compound. Our findings demonstrate that dopamine substantially reduced the level of HBV DNA, which was consistent with our previous study showing that dopamine reduced HBV DNA replication by inhibiting the binding of ε RNA with the HBV polymerase.[17] Surprisingly, in this study, we found that dopamine decreased the production of HBsAg and HBeAg in HBV-infected HepG2.2.15 and Hep-NTCP cell lines as well as the levels of HBsAg, HBeAg, and DNA in an AAV-HBV1.3 murine infection model.

We also explored potential mechanisms of action. We further found that dopamine considerably increased ISG15

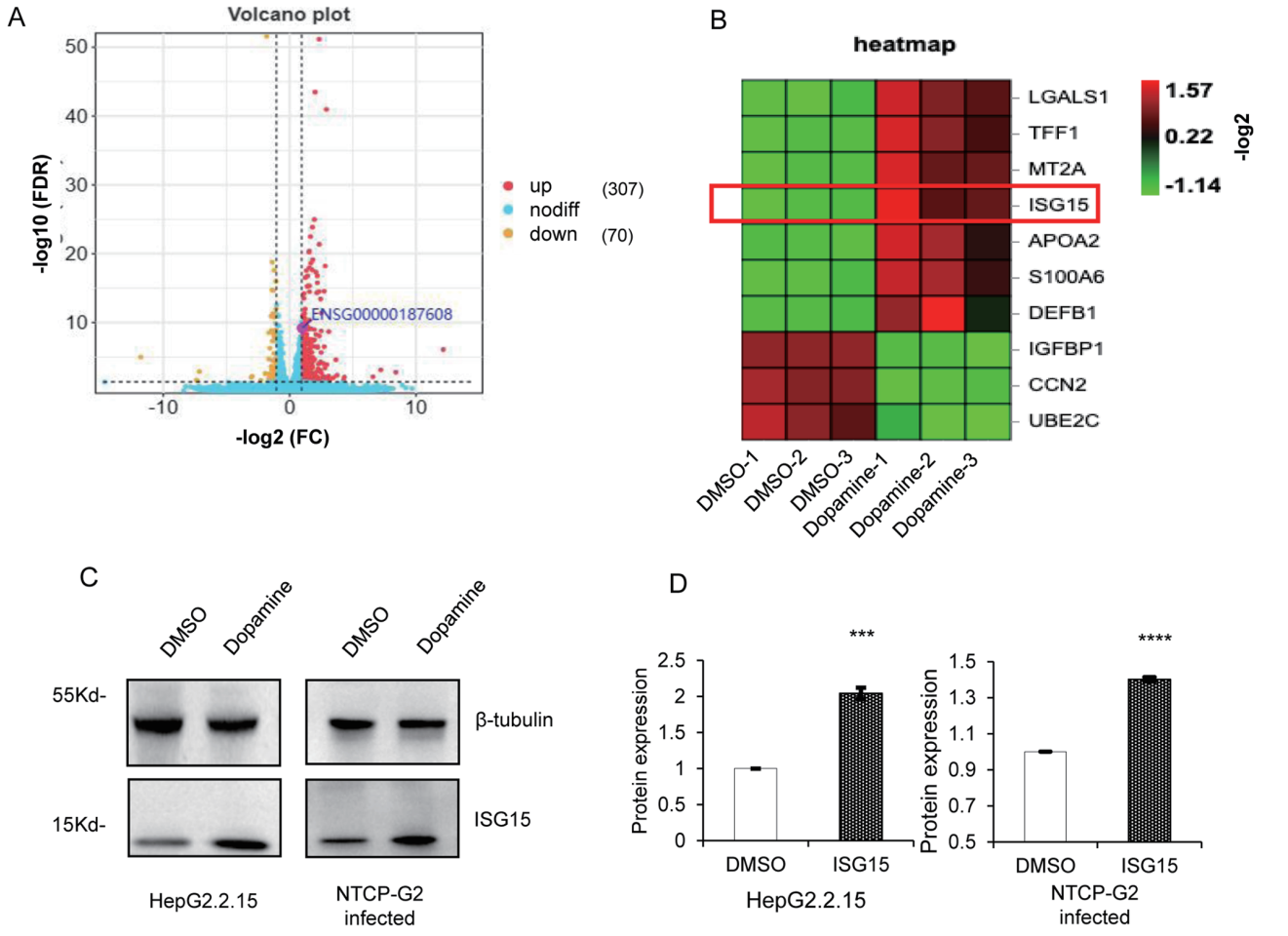

Fig. 4. Dopamine regulated interferon-stimulated gene 15 (ISG15) expression. (A) Volcano plots of differential gene expression between dimethyl sulfoxide (DMSO; control)- and dopamine-treated HepG2.2.15 cells. (B) Expression profiles of DMSO- and dopamine-treated HepG2.2.15 cells, determined by RNA sequencing. (C, D) ISG15 protein expression in HepG2.2.15 (left) and sodium taurocholate co-transporting polypeptide-expressing HepG2 (NTCP-G2, right) cells, determined by western blotting. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. ***p<0.005, ****p<0.001. FDR, false discovery rate; FC, fold change; DMSO, dimethyl sulfoxide.

expression in HepG2.2.15 and NTCP-G2 cells and in mice relative to control treatments, and that ISG15 knockdown increased HBsAg and HBeAg expression in HepG2.2.15 cells. These findings suggest that the antiviral activity of dopamine is related to its activation of ISG15 and regulation of HBsAg and HBeAg expression. In a previous study, ISG15 was characterized as a linear Di ubiquitin-like protein with interferon-stimulated antiviral activity, which is consistent with our finding.[47] The JAK/STAT signaling pathway induces the upregulation of several ISGs, such as MxA, ISG15, IFITM3, and ISG56, thereby inhibiting viral replication.[30] In our study, we additionally found that JAK1, STAT1, and p-STAT1 expression were considerably upregulated following dopamine treatment compared with that following DMSO treatment in *vitro*. Importantly, IHC of hepatic tissue taken from our AAV/HBV-mouse infection model demonstrated that JAK1, STAT1, and p-STAT1 expressions were significantly higher in the dopamine-treated mice than in the placebo group.

Recent studies have shown that dopamine and dopamine receptor D1 (DRD1) play important roles in cancer progression, and DRD1 expression is high in HepG2 cells,[48] explaining how dopamine entered the cells and activated the JAK/STAT pathway in this study. Although type-I and III IFNs signal through the JAK/STAT pathway to induce an antiviral state,[49] our results suggest that dopamine's activation of this pathway to induce ISG15 did not involve IFNs. These findings suggest that dopamine can be applied as a novel research tool to study the HBV replication cycle.

Our study has several limitations. First, we did not test other druggable DRD1 receptor agonists. Because dopamine therapy for HBV infection is not clinically feasible, we need to identify and test other druggable ISG15 agonists that may be suitable for long-term oral administration. Second, because we used the AAV-HBV1.3 mouse model, we were unable to observe *in vivo* changes in HBV cccDNA and pregenomic RNA, or chronic liver pathologies such as hepatitis, liver fibrosis, or oncogenesis. Third, this study focused only on hepatocytes and did not examine the effect of dopamine on immune effector cell function. In future studies, we plan to collect patient samples and construct liver-s pecific knockout and overexpressing mice to further elucidate the role of the JAK-STAT/ISG15 axis in suppressing HBV replication. Additionally, we intend to incorporate immune effector cells and a humanized mouse model to evaluate the effect of dopamine-mediated upregulation of the JAK-STAT/ISG15 axis on the anti-HBV immune response.

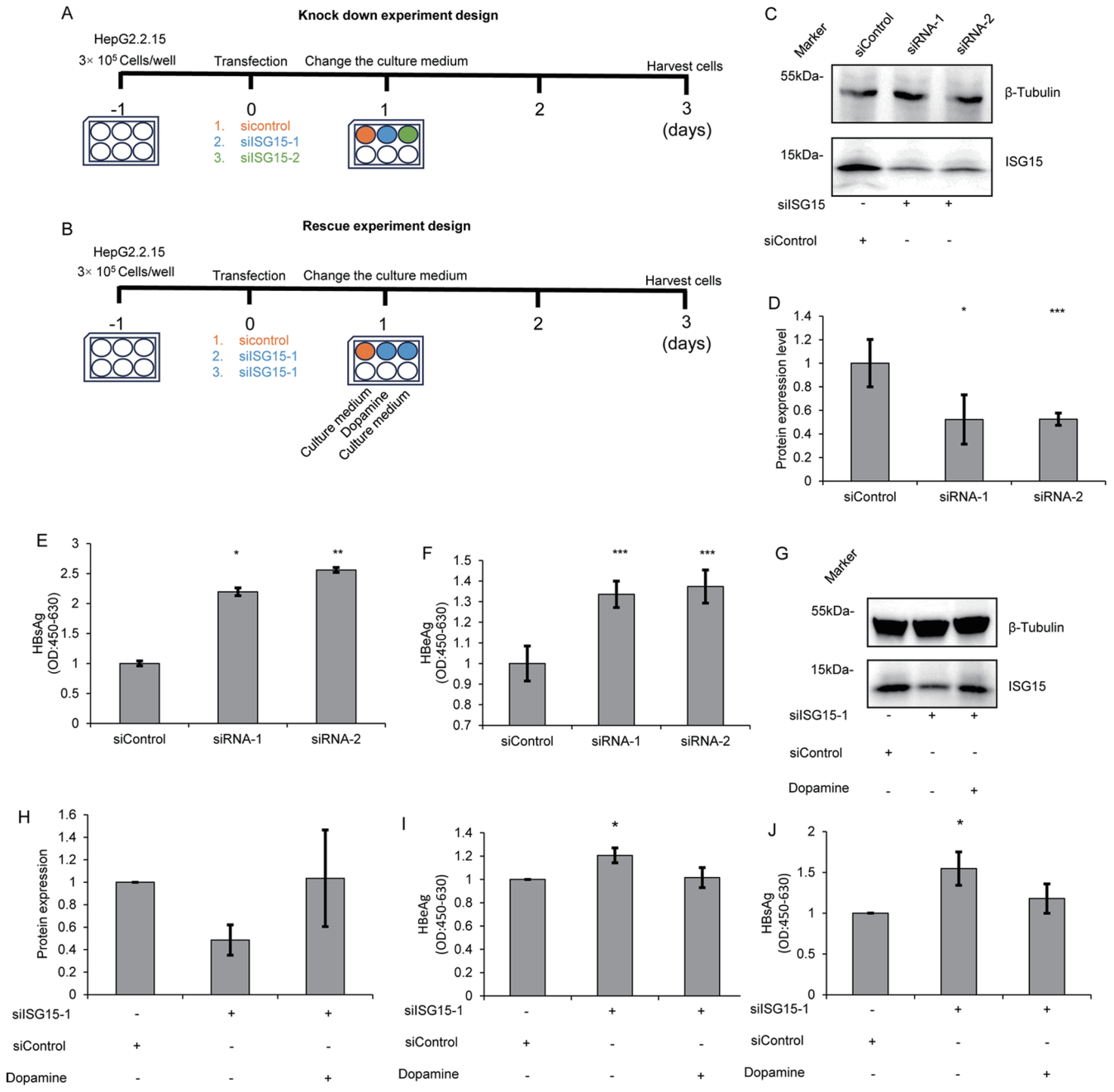

Fig. 5. Interferon-stimulates gene 15 (ISG15) regulated hepatitis B virus surface and e antigen (HBsAg and HBeAg) expression. (A, B) Cell culture and treatment in the knockdown and rescue experiments. (C, D) ISG15 protein expression in HepG2.2.15 cells with two *ISG15*-knockdown clones with (+) or without (−), determined by western blotting. (E, F) HBsAg and HBeAg expression in the *ISG15*-knockdown clones, determined by enzyme-linked immunosorbent assay (ELISA). (G, H) ISG15 gene and protein expression in the rescue experiment, in HepG2.2.15 cells treated siISG15-1 with (+) or without (−), dopamine with (+) or without (−), siControl with (+) or without (−), determined by western blotting. (I, J) HBsAg and HBeAg expression in the rescue experiment, in HepG2.2.15 cells treated siISG15-1 with (+) or without (−), dopamine with (+) or without (−), siControl with (+) or without (−), determined by ELISA. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001. siRNA, small interfering RNA; OD, optical density.

In summary, we successfully used dopamine to inhibit HBsAg, HBeAg, and HBV DNA expression both *in vitro* and *in vivo*. We identified a potential mechanism of action in which dopamine binding with DRD1 activated the JAK/STAT-ISG15 axis exerting an antiviral function, as depicted in Figure 9. Although dopamine therapy for HBV infection may not be feasible in the clinical context, the findings of this study increase the understanding of the regulatory relationship between the STAT/ISG15 signaling pathway and HBV replication, which may facilitate the discovery of therapeutic targets and inform the development of novel treatments for chronic HBV infection.

In conclusion, dopamine inhibits HBV DNA replication and the expressions of HBsAg and HBeAg, while activating the

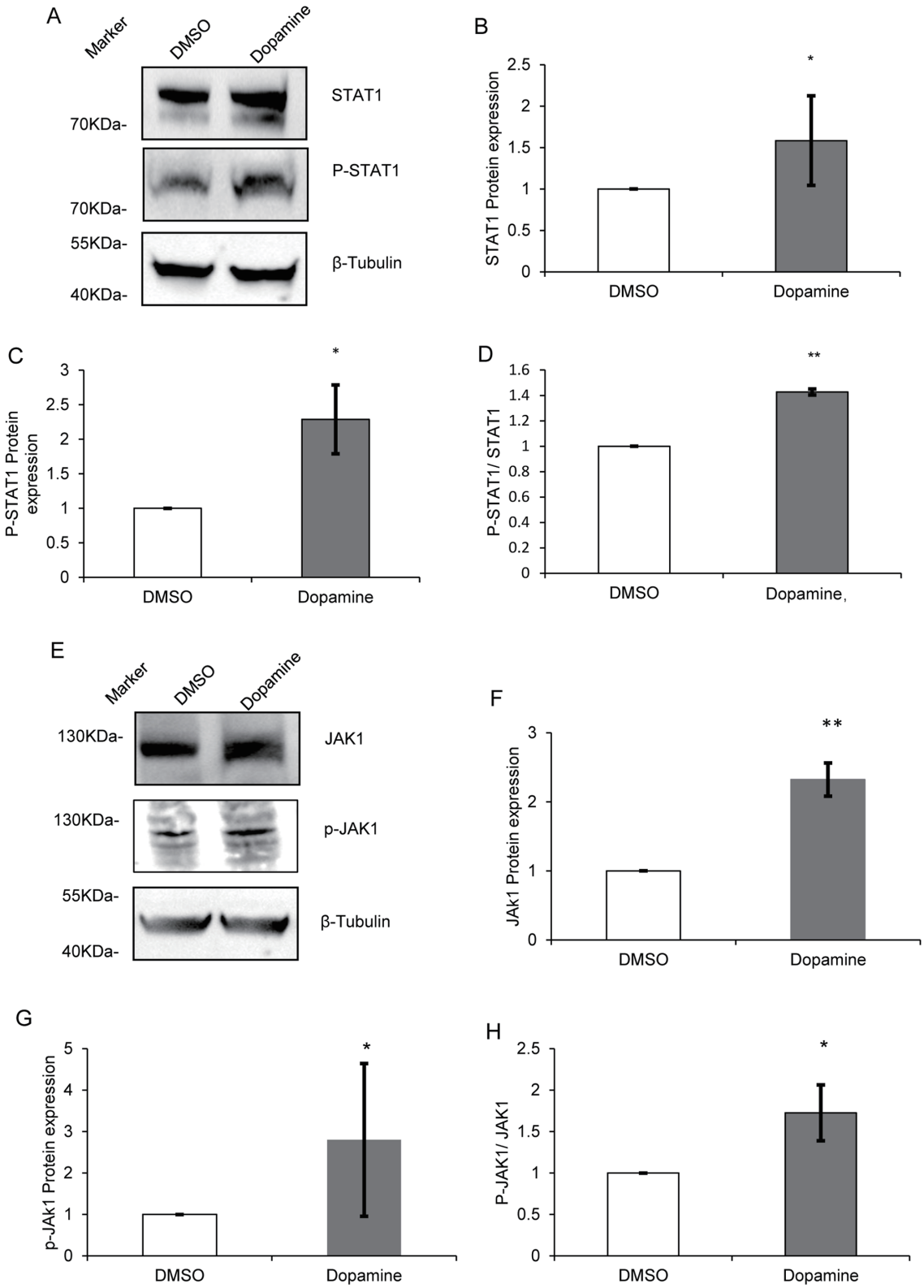

Fig. 6. Dopamine upregulates the expression of Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway molecules. STAT1, phosphorylated (p)-STAT1 (A-D), JAK1, and phosphorylated (p)-JAK1 (E- H) protein expressions in HepG2.2.15 cells treated with dopamine or dimethyl sulfoxide (DMSO, control) for 12 days, determined by western blotting. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. **p*<0.05, ***p*<0.01. DMSO, dimethyl sulfoxide

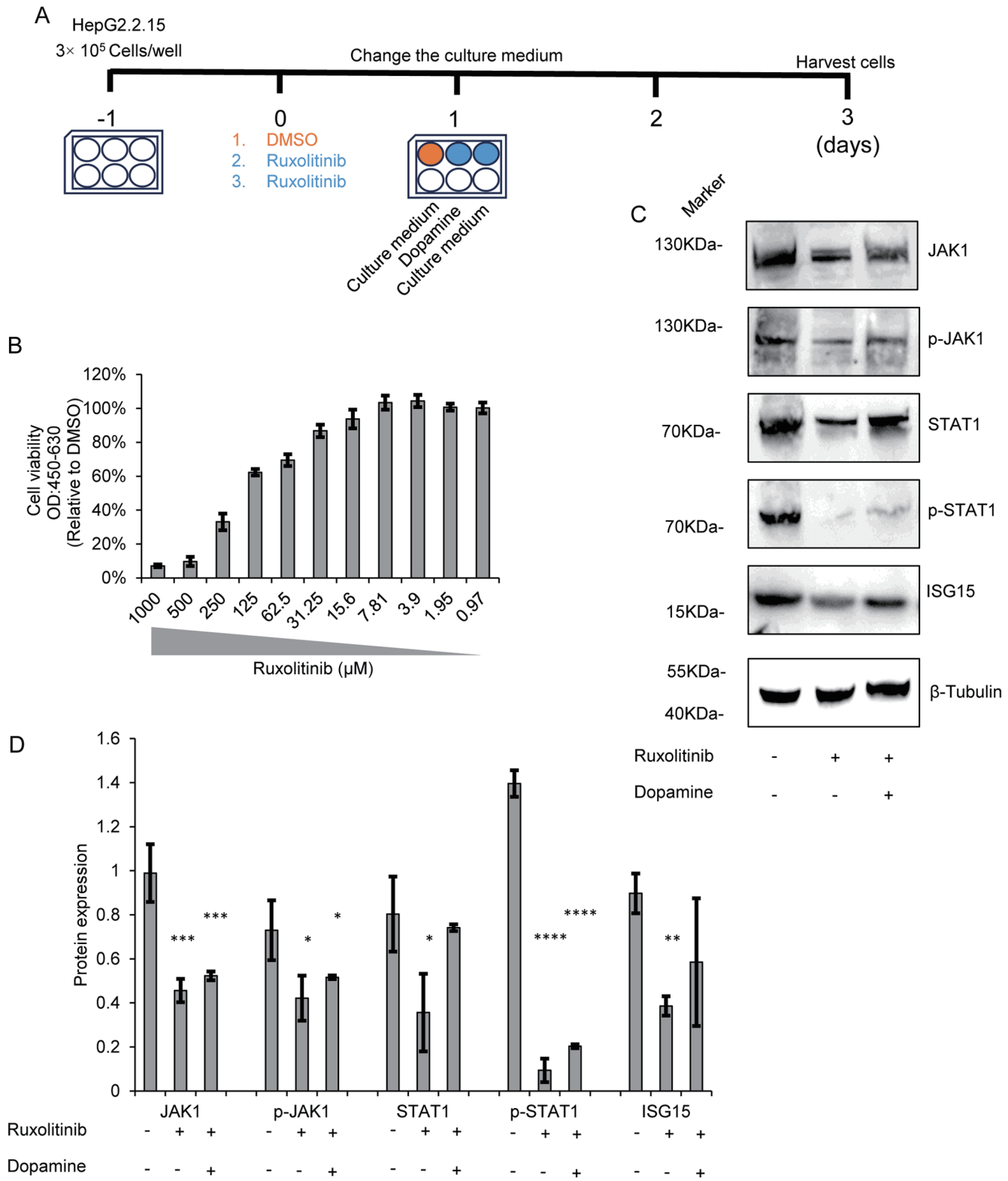

Fig. 7. ISG15 activation with JAK-STAT pathway in cells. (A) Cell culture and treatment in the ruxolitinib-treated experiments. (B) HepG2.2.15 cell viability following ruxolitinib treatment for 3 days. (C and D) JAK1, phosphorylated (p)-JAK1, STAT1, phosphorylated (p)-STAT1, and ISG15 expression in HepG2.2.15 cells treated ruxolitinib with (+) or without (−), dimethyl sulfoxide (DMSO, control) and combined dopamine with (+) or without (−) and ruxolitinib with (+) or without (−) for 3 days, determined by western blotting. Data are presented as mean values with standard deviations relative to the control from at least three independent experiments. *p<0.05, **p<0.01, *** p<0.005, **** p<0.001. OD, optical density; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

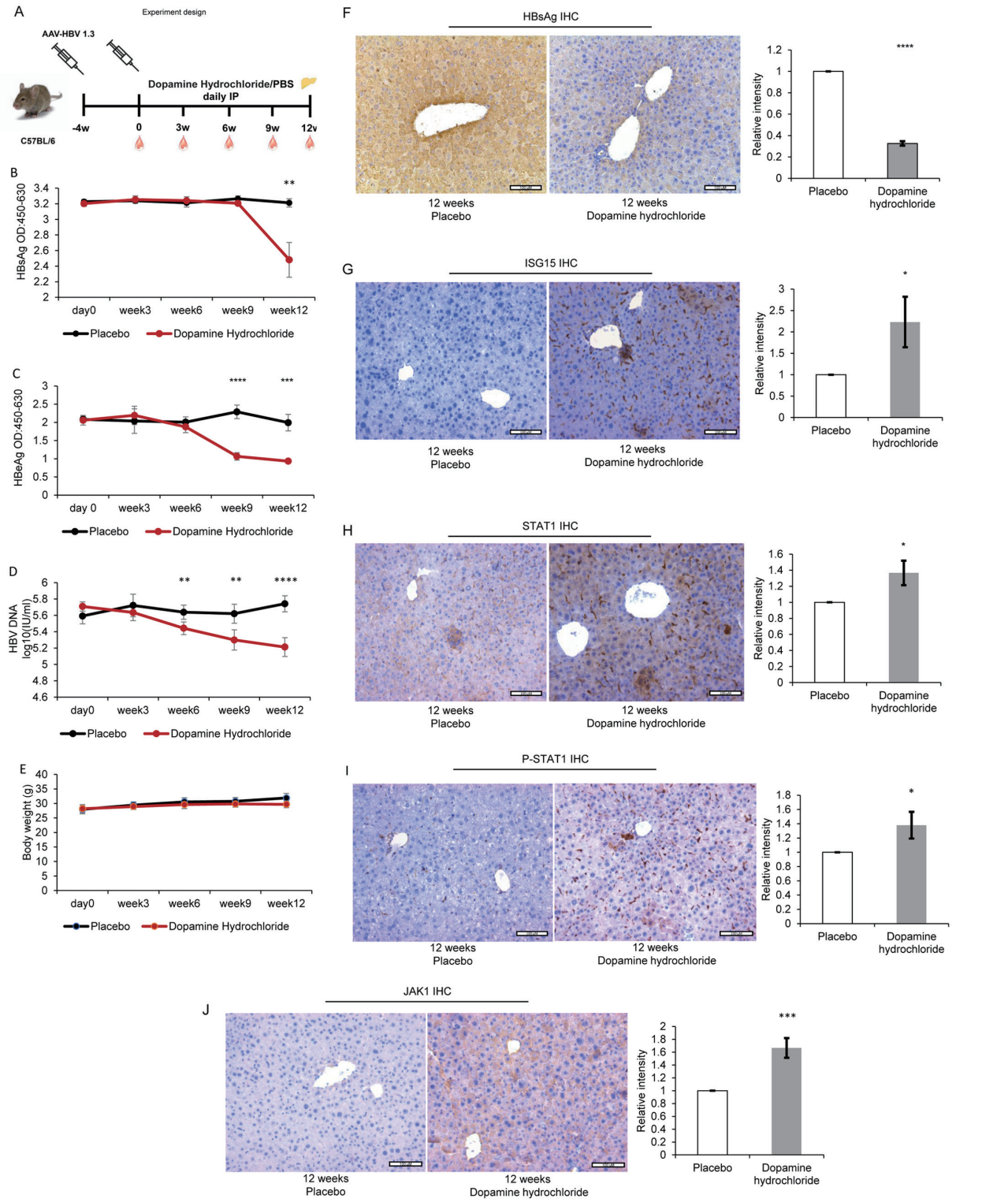

Fig. 8. Dopamine inhibits hepatitis B virus (HBV) surface antigen (HBsAg) expression in a mouse model. (A) Dopamine hydrochloride or placebo (normal saline; 10 μg/kg daily) treatment of adeno-associated virus (AAV)-HBV 1.3 mice. (B, C) Post-treatment HBsAg and hepatitis B virus e antigen (HBeAg) expression, determined by enzyme-linked immunosorbent assay. (D) Serum HBV DNA expression, determined by quantitative polymerase chain reaction. HBV DNA was quantified by qPCR (absolute quantification). The Y axis was adjusted to log10. (E) Mouse body weight. (F–J) HBsAg, interferon-stimulated gene 15 (ISG15), signal transducer and activator of transcription 1 (STAT1), phosphorylated (p)-STAT1, and Janus kinase 1 (JAK1) expression in mouse livers, determined by immunohistochemistry (IHC). 200× magnification, scale bar=100 μm. Data are presented as mean values with standard deviations relative to the control from at least five independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.005, *****p*<0.001. PBS, phosphate-buffered saline; IP, intraperitoneal; OD, optical density; IntDen, integrated density.

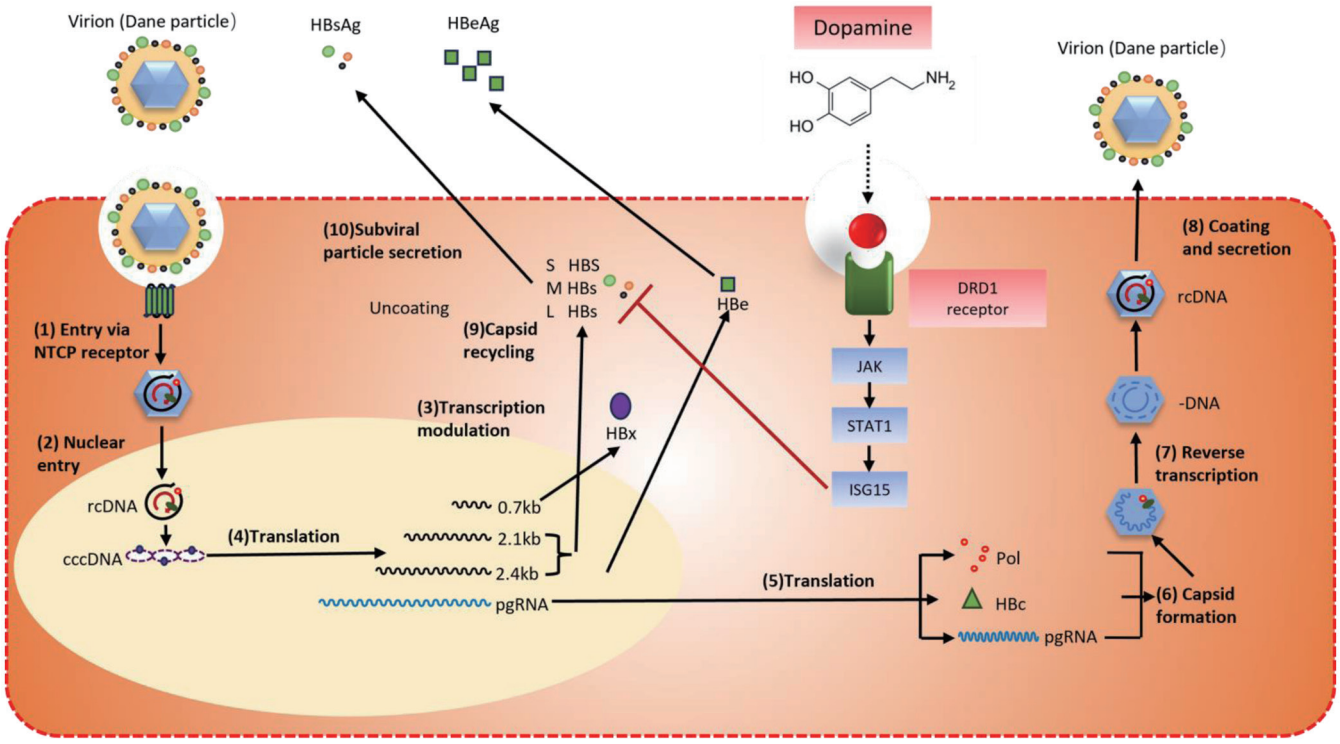

Fig. 9. Anti-hepatitis B virus mechanism of dopamine. Dopamine inhibits hepatitis B virus surface and e antigen (HBsAg and HBeAg) expression through increasing ISG15 expression via JAK/STAT pathway activation. rcDNA, relaxed circular DNA; cccNDA, covalently closed circular DNA; pgRNA, pregenomic RNA; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

JAK-STAT pathway and upregulating the expression of signaling molecules in this pathway to increase the expression of ISG15, which exerts antiviral activity. These findings enhance our understanding of targeting HBsAg and HBeAg. Although dopamine is not suitable for clinical use as an antiviral therapeutic, our study suggests that ISG15 agonists should be developed for the treatment of chronic HBV infection. The association of dopamine with the JAK1/STAT signaling pathway suggests its potential in regulating ISG15 activity or expression.

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Conflict of interest

The authors have no conflict of interest related to this publication.

Author contributions

XL: conceptualization, methodology, validation, formal analysis, and writing-original draft. XP: methodology, validation, and formal analysis. ZW and XC: validation and formal analysis. ZG: writing-original draft and funding acquisition. HD: investigation, conceptualization, formal analysis, writing-original draft, writing-review and editing, funding acquisition, resources, and supervision. All authors made significant contributions to this study and approved the final manuscript.

Ethical statement

All animal experiments were approved by the institutional Animal Care and Use Committee of South China Agricultural University ([2020]d065). All animals received humane care.

Data sharing statement

The data generated in this study will be made available on request.

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