Engineered zinc-finger transcription factors inhibit the replication and transcription of HBV *in vitro* and *in vivo*

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Abstract. In the present study, an artificial zinc-finger transcription factor eukaryotic expression vector specifically recognizing and binding to the hepatitis B virus (HBV) enhancer (Enh) was constructed, which inhibited the replication and expression of HBV DNA. The HBV EnhI-specific pcDNA3.1-artificial transcription factor (ATF) vector was successfully constructed, and then transformed or injected into HepG2.2.15 cells and HBV transgenic mice, respectively. The results demonstrated that the HBV EnhI (1,070-1,234 bp)-specific ATF significantly inhibited the replication and transcription of HBV DNA *in vivo* and *in vitro*. The HBV EnhI-specific ATF may be a meritorious component of progressive combination therapies for eliminating HBV DNA in infected patients. A radical cure for chronic HBV infection may become feasible by using this bioengineering technology.

Introduction

Hepatitis B virus (HBV) is a small (3.2-kb), partially double-stranded, relaxed circular, enveloped DNA virus, which specifically infects the hepatocytes of quadrumana (1,2). Worldwide, ~3.5 billion individuals are affected, among which >0.4 billion have chronic HBV infection (CHB); HBV is one of the most common pathogens in humans, which has a major global health impact (3-6). Persistent infection with HBV results in serious liver disease, including acute hepatitis, cirrhosis and hepatocellular carcinoma (7). It is estimated that 2% of patients with CHB are be likely to develop cirrhosis each year, and 15-25% of patients with CHB are likely to succumb to cirrhosis or hepatocellular carcinoma (8,9). Licensed drugs for the treatment of CHB are nucleos(t)ide analogues (NAs) and interferon α (IFN-α) (10). However, the emergence of side-effects poses challenges to the long-term administration of IFN-α. At the same time, the generation of drug-resistant strains of HBV with amino acid replacement in the YMDD motif of reverse transcriptase has been a severe problem in patients with lamivudine therapy, which may result in virological relapse and biochemical flare (11-13). Therefore, it is necessary to explore novel and more effective therapies for HBV.

Transcription factors comprise a deoxyribonucleic acid binding domain (DBD), and an effector domain (ED) or transcriptional regulation domain, which may provide a nuclear localization signal (NLS). The DBD is required to bind to the target sequence, including the enhancer (Enh) or promoter element, which is an upward or downward effect field regulating the target gene, and the NLS delivers a transcription factor into nuclei, as eukaryotic transcription occurs within the nucleus (14). The characteristics of eukaryotic transcription factors are the basis of artificial transcription factor (ATF) technology (15). The design of a specific recognition DBD is the most difficult and important part in the establishment of an ATF. Zinc finger proteins (ZFP) are the most common type of DNA-binding proteins in molecular biology. Engineered ZFPs bind to an extensive range of DNA sequences and the finger subunits may also be connected to bind to long, asymmetric DNA sequences (16-20). The novel Cys2His2 ZFPs are the most promising candidates for the DBD due to their high specificity and affinity (21,22). Their functions are diverse and include DNA recognition, RNA packaging, transcriptional activation, lipid binding, cell apoptosis and protein folding (23). Approximately 30 amino acids with a simple, ββα-fold stabilized by hydrophobic interactions and chelation of a single zinc ion constitute the one-fold ZF domain. A 3-bp DNA fragment is constantly and distinctively recognized by each ZF domain,

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and six-ZF proteins were reported to recognize unique 18-bp fragments due to the canonical TGEKP interfinger linker between the ZF units (24-26). While engineered ZFPs have been used in human immunodeficiency virus research (27,28), the potential of engineered ZFPs to inhibit HBV has remained to be investigated.

In the present study, based on advanced software development technologies and ZFP design tools on an online platform, the HBV EnhI-specific ATF was designed, an 18-bp sequence was selected as the ATF target sequence and corresponding ZF amino acid sequences were gained; the best ZFPs were fused to an NLS and an ED to generate the ATF. A series of ATF, ZFP and Kruppel-associated box (KRAB) eukaryotic expression vectors were constructed using genetic engineering methods. The fidelity was confirmed by restriction enzyme digestion and sequence analysis, and ATF, ZFP and KRAB eukaryotic expression vectors were transformed or injected into HepG2.2.15 cells and HBV transgenic mice. At the predetermined times, serum samples, culture supernatants, hepatic tissues and cells were gathered for serological and virological detection. The results of the present study demonstrated that only ATF significantly inhibited HBV transcription and replication at the viral RNA, protein and viral progeny level, without any obvious toxic effect *in vitro* and *in vivo*.

Materials and methods

Design and construction of the ATF expression vector of ZFP. Based on HBV DNA EnhI (1,070-1,234 bp) sequences as the template, an online platform (scripps.edu/mb/barbas/zfdesign/zfdesignhome.php) of a ZFP design tool was used (29) and the 'Search DNA Sequence for Contiguous or Separated Target Sites' and 'Design a Zinc Finger Protein' tools were explored for selecting the optimal target sequence and the corresponding ZF amino acid sequences. This meant that the specific target sites of the HBV DNA EnhI (1,070-1,234 bp) region were optimized, and were recognized to predict the ZF amino acid sequence. The N-terminal ZF domain was fused to KRAB repression domains from the human ZFP 10 gene (30). To facilitate the localization of ATF in the cell nuclei and detect the expression of ATF, an NLS from simian virus 40 large T-antigen (31) and the epitope Flag tag were separately added to the ATF's N-terminal and C terminus. The amino acid sequence of ATF was then reverse-transcribed into a nucleotide sequence and optimized by using Primer premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). Finally, the ATF nucleotide sequences were synthesized and cloned into the *Eco*RI and *Bam*HI restriction sites of pcDNA3.1(+) expression vector (Sangon Biotech Co., Ltd., Shanghai, China), Fidelity was confirmed by restriction enzyme digestion and sequence analysis. pcDNA3.1(+)-ATF (nls-ZFP-KRAB-Flag) was transformed into competent DH-5a *Escherichia coli* cells (Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University, Chongqing, China) and was purified with a TIANpure Midi Plasmid kit (Tiangen Biotech Co., Ltd., Beijing, China). Using pcDNA3.1(+)-ATF (nls-ZFP-KRAB-Flag) vector as a template, the individual primers were designed, and pcDNA3.1(+)-nls-ZFP-Flag and pcDNA3.1(+)-nls-KRAB-Flag were constructed according to the above methods.

Cell culture and transfection. The HepG2 and HepG2.2.15 cells were provided by the Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University. The HepG2 cell line, which is known to be a hepatoblastoma cell line, and HepG2.2.15 cells [clonal cells derived from HepG2 (32), which was transfected with a plasmid containing HBV DNA that secretes HB surface antigen (HBsAg) particles, nucleocapsids and virions] were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare, Little Chalfont, UK) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare), 100 U/ml penicillin, 100 μg/ml streptomycin (Beyotime Institute of Biotechnology, Haimen, China) and 380 μg/ml G418 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C in a humidified atmosphere containing 5% CO_2. Transient transfections of HepG2.2.15 were performed by using the X-treme GENE HP DNA Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions with a final plasmid concentration of 0.01 μg/μl. The HepG2.2.15 cells ($1.5x10^5$/well) were seeded into six-well plates and cultured overnight. On the second day, 100 ng (10 μl of a 10 ng/μl solution) pcDNA 3.1(+), pcDNA-nls-KRAB-flag, pcDNA-ATF or pcDNA-nls-ZFP-flag were added to the culture medium. At 24, 48 or 72 h post-transfection, the supernatants and cells were collected for analysis.

Animals. A total of 24 male C57BL/6-HBV-1.3 genome-eq transgenic mice (age, 6-8 weeks; weight, 18-24 g) were provided by the Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University. The Chongqing Medical University Medical Research Ethics Committee approved all animal experiments. All animals were provided sterile water and rat chow *ad libitum*, and were kept under a 12-h light/dark cycle at constant temperature and humidity (temperature, 18-22˚C; humidity, 50-60%). Mice (n=6/group) were injected with 8 μg pcDNA 3.1(+), pcDNA-nls-KRAB-flag, pcDNA-ATF or pcDNA-nls-ZFP-flag dissolved in 2 ml PBS via the tail vein within 5-8 sec. Serum samples were collected via the tail vein after intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg; cat. no. P3761; Sigma-Aldrich; Merck KGaA) on days 7, 14, 21 and 28. The post-anesthetic mice were sacrificed on day 28, and the serum and liver tissues were collected.

Extraction of HBV replicative intermediate-DNA (RI-DNA). Intracellular RI-DNA was extracted at 48 h post-transfection as previously described (33). In brief, HepG2.2.15 cells were harvested with trypsin washed twice with ice-cold PBS (pH 7.4). Cells were then lysed in 200 μl Nonidet P-40 cell lysis liquid with incubation at 37˚C for 15 min, and then centrifuged at 13,000 x g for 5 min. The supernatants were then incubated with 500 μl 35% polyethylene glycol 8000 (1.5 M) in an ice bath for 50 min, and samples were centrifuged again as described above. For virus precipitation, the sample (supernatants, serum or hepatic tissue) was incubated with 380 μl proteinase K digestion liquid, 20 μl proteinase K (Tiangen Biotech Co., Ltd.) in sterilized ultrapure water (50 μl) overnight at 45˚C

in a water bath. HBV DNA was extracted with isovolumetric phenol chloroform twice, and the supernatants were carefully collected. An equal volume of isopropyl alcohol was added and the sample was vortexed. The mixture was centrifuged for 30 min at 15,000 x g and 4˚C. After the precipitate was briefly washed with 75% ice-cold ethanol twice, it was resuspended in 10 μl sterilized ultrapure water.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from HepG2.2.15 cells using the RNAiso Plus kit (Takara Bio Inc., Otsu, Japan) at 72 h post-transfection following the manufacturer's instructions. A total of 1 μg RNA was reverse-transcribed to complementary (c)DNA using the PrimeScript™ RT reagent kit (Takara Bio Inc.). For analysis of HBV RNA levels, 2 μl of each cDNA were quantified by real-time PCR with SYBR-Green (Takara Bio Inc.) in a LightCycler CFX96 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR condition consisted of three steps as follows: Step 1, pre-degeneration at 95˚C for 2 min; step 2, 34 cycles of denaturation at 94˚C for 20 sec, annealing at 60˚C for 20 sec, and extension at 72˚C for 20 sec; and step 3, 4˚C forever. The following primers were used: HBV, forward 5'-ATACTGCACTCAGGCAAGC-3' and reverse 5'-TGCCTC GTCGTCTAACAAC-3'; and β-actin, forward 5'-GGGACC TGACTGACTACCTC-3' and reverse 5'-TCATACTCCTGC TTGCTGAT-3'. β-actin mRNA was used as an endogenous control, and the relative expression levels of HBV mRNA were determined using the $2^{-\Delta\Delta Cq}$ method (34-36).

Detection of HBsAg and HBe antigen (HBeAg). At 24, 48 and 72 h post-transfection, the culture medium was collected and centrifuged at 5,000 x g to remove cellular debris, followed by storage at -20˚C for analysis. At 7, 14, 21 and 28 days post-injection, blood was collected via the tail vein and then centrifuged at 1,300 x g to collect the serum, which was stored at -20˚C for analysis. The concentrations of HBsAg and HBeAg were detected with quantitative ELISA kits (Human HBsAg ELISA kit, E-EL-H1567c; Human HBeAg ELISA kit, CR-018; Elabscience Biotechnology Co., Ltd., Wuhan, China) following the manufacturer's instructions.

Quantitative analysis of HBV DNA. At 24, 48 and 72 h post-transfection, HBV DNA was extracted from the culture medium using a viral DNA extraction kit (Sangon Biotech Co., Ltd). HBV RI-DNA and HBV DNA from the culture medium of HepG2.2.15 cells was used as the template for real-time PCR and quantified using an HBV diagnostic kit (Da-An, Guangzhou, China) according to manufacturer's instructions.

Cell viability assay. HepG2.2.15 cells were seeded into 96-well plates at $2x10^4$/well and cultured. At 48 h post-transfection, 20 μl Cell Counting Kit-8 (CCK-8) reagent was added to each well, followed by incubation for 4 h at 37˚C with 5% CO_2. The amount of viable sell was determined by measurement of the absorbance at 450 nm.

Western blot analysis. At 48 h after transfection, the cells were lysed with 1% radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was determined using a BCA Assay kit (Beyotime Institute of Biotechnology). Proteins were separated by 8% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Beyotime Institute of Biotechnology). The membranes were incubated with polyclonal rabbit anti-HBxAg (cat. no. ab39716; 1:800; Abcam, Cambridge, MA, USA) and rabbit anti-HB core (c)Ag (cat. no. B0586; 1:1,000; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) overnight for 4˚C. Horseradish peroxidase-labeled goat anti-rabbit (cat. no. CW0240; 1:5,000; Beijing ComWin Biotech Co., Ltd., Beijing, China) was used as a secondary antibody and incubation was performed for 1 h for 37˚C. The signals were detected by the Enhanced Chemiluminescence Detection system (Pierce; Thermo Fisher Scientific, Inc.) and β-actin (anti-β actin antibody; cat. no. ab8226; 1:1,000; Abcam, Cambridge, MA, USA) was used to normalize the data (37).

Confocal microscopy. HepG2.2.15 cells transfected with ATF eukaryotic expression vector for 48 h (37˚C) were fixed with 4% paraformaldehyde for 30 min (room temperature), washed three times with PBS, and were permeabilized with 0.5% Triton X-100 for 5 min (room temperature). After washing with PBS for three times, the cells were incubated in 5% goat serum (1:20 in PBS dilution; cat. no. 16210064; Thermo Fisher Scientific, Inc.) for 60 min. This was followed by an incubation with anti-Flag polyclonal antibody (cat. no. YM3001; 1:1,000; ImmunoWay Biotechnology Company, Suzhou, China) at 4˚C for 14 h, followed by rinsing 3 times with PBS and incubation with fluorescein isothiocyanate-labeled goat anti-rat secondary antibody (cat. no. sc-2010; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37˚C for 1 h. Finally, the cells were stained with propidium iodide (Beyotime Institute of Biotechnology) for 1 min. The expression of ATF protein was visualized by using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

Hepatic immunohistochemistry (IHC). The location and expression of hepatitis B core antigen (HBcAg) in the hepatic tissues of mice at 28 days post inoculation was detected by IHC staining (38). The 4% paraformaldehyde-fixed (at room temperature for 24 h) paraffin-embedded tissue sections (4.5-μm thickness) were stained by IHC staining. Sections were submerged in citrate buffer (pH 6.0) at 95˚C for 15 min and then cooled at room temperature. HBcAg was determined in the hepatic sections by IHC staining with rabbit anti-HBcAg (cat. no. B0586; 1:150; Dako; Agilent Technologies, Inc.). Incubation was performed at 37˚C for 2 h. Horseradish peroxidase-labeled goat anti-rabbit (cat. no. CW0240; 1:500; Beijing ComWin Biotech Co., Ltd.) was used as a secondary antibody and incubation was performed for 30 min for 37˚C. The IHC images (original magnification, x400) were captured using a Leica light microscope (cat. no. DM3000; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Statistical analysis was performed using the one-way analysis of variance followed by Dunnett's post hoc test, which was used to assess the differences in numerical variables between the experimental and

LEPGEKP YKCPECGKSFS TSGELVRHQRTH TGEKP
YKCPECGKSFS RSDKLVR HQRTH TGEKP YKCPECGKSFS
TSGELVR HQRTH TGEKP YKCPECGKSFS RNDALTE
HQRTH TGEKP YKCPECGKSFS TSHSLTE HQRTH TGEKP
YKCPECGKSFS SKKHLAE HQRTH GKKTS

Figure 1. Amino acid sequence of zinc finger protein.

control groups, including HBV DNA, HBV mRNA, HBsAg and HBeAg. All analyses were calculated using SPSS v. 19.0.1 for Windows (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Target sites of ATF on HBV EnhI. Previous studies have demonstrated that the activity of EnhI was controlled by the complex interaction of hepatocyte-specific and immanent transcription factors, including the tumor suppressor protein p53, the activator protein-1 complex, retinoid X receptor, hepatocyte nuclear factor 3/4, CCAAT Enh binding proteins and regulatory factor X-1 (39-43). Transcription of the pre-genomic (pg)RNA was regulated through the EnhI region and the core promoter. Besides the pgRNA, EnhI region regulates transcription of the core and X genes, which has a predominant role in modulating the expression of the temporal and global HBV gene (44,45). Based on the important functions of EnhI, the present study demonstrated that inhibition to the transcriptional activity of HBV reduced the replication of HBV DNA. The 18-bp sequence 5'-CCC CCACTGGCTGGGGCT-3' was selected as the ZF target site and the corresponding amino acid sequence, which targets the HBV EnhI region and integrates with effector domains to confer transcriptional repression was successfully determined (Fig. 1). The resulting ATF was cloned into the mammalian expression plasmid pcDNA3.1(+). Homologous sequence alignment of the human genome using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) allowed for design of a ZFP with high specificity, and which did not combine with any other potential target sequence. The BLAST search enabled the comparison of the query sequence with a database of sequences, and enabled the identification of library sequences that resemble the query sequence above a certain threshold. This was used to align the homology to the human genome nucleotide sequence.

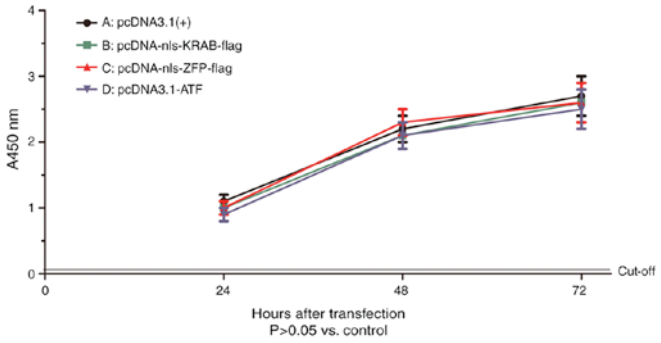

Figure 3. Effect of ATF on the viability of HepG2.2.15 cells. Cells were seeded into 96-well plates at $2x10^4$/well and cultured. After transfection with (A) pcDNA3.1(+), (B) pcDNA-nls-KRAB-flag, (C) pcDNA-nls-ZFP-flag or (D) pcDNA-ATF for 24, 48 or 72 h, the cell viability was determined by a Cell Counting Kit-8 assay. In terms of the viability of HepG2.2.15 cells, no statistically significant difference was present between the groups (P>0.05). The control group (A) value was set as 1. ATF, artificial transcription factor; ZFP, zinc finger protein; KRAB, Kruppel-associated box; nls, nuclear localization signal; A, absorbance.

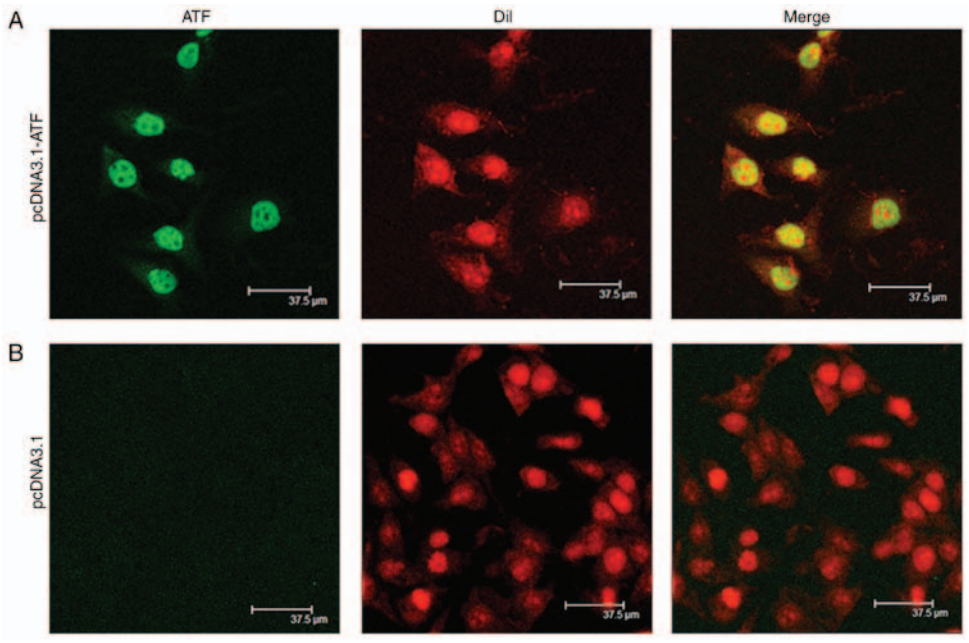

Figure 2. Expression of ATF in HepG2.2.15 cells. (A) HepG2.2.15 cells were transfected with pcDNA3.1-ATF (nuclear localization signal-zinc finger protein-Kruppel-associated box-flag) for 48 h. (B) HepG2.2.15 cells were transfected with pcDNA3.1(+) vector for 48 h. ATF protein was visualized by using scanning confocal microscopy (scale bar, 37.5 μm). ATF protein was located in the nuclei. ATF, artificial transcription factor; DiI, $DiIC_{18}(3)$, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.

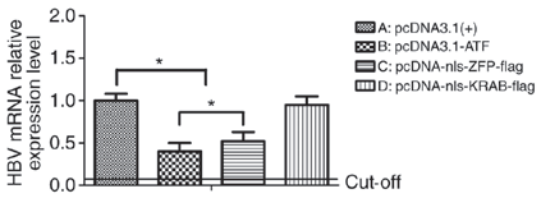

Figure 4. ATF causes a downregulation of total HBV mRNA. HBV mRNA levels in HepG2.2.15 cells after transfection for 72 h were analyzed using reverse transcription-quantitative polymerase chain reaction analysis. The HBV mRNA expression levels in the empty vector group were set as 1. (A) pcDNA3.1(+), (B) pcDNA-ATF, (C) pcDNA-nls-ZFP-flag, (D) pcDNA-nls-KRAB-flag. *P<0.05 as indicated. ATF, artificial transcription factor; ZFP, zinc finger protein; KRAB, Kruppel-associated box; nls, nuclear localization signal; HBV, hepatitis B virus.

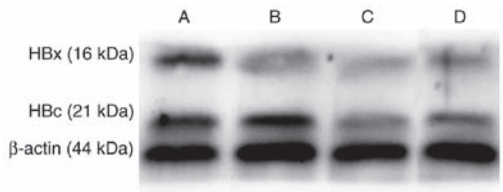

Figure 5. Effect of ATF on HB viral protein expression. HepG2.2.15 cells were transfected with (A) pcDNA 3.1(+), (B) pcDNA-nls-Kruppel-associated box-flag, (C) pcDNA-ATF or (D) pcDNA-nls-zinc finger protein-flag and the levels of HBx and HBc were detected by western blot analysis. *P<0.05 vs. the control group. ATF, artificial transcription factor; nls, nuclear localization signal; HB, hepatitis B; HBx/c, HB x/core protein.

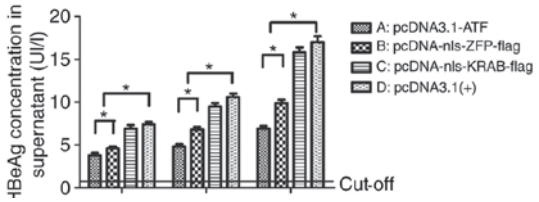

Figure 6. Level of HBeAg in the culture supernatant determined by ELISA at 24, 48 and 72 h post-transfection with (A) pcDNA-ATF, (B) pcDNA-nls-ZFP-flag, (C) pcDNA-nls-KRAB-flag or (D) pcDNA3.1(+). *P<0.05 as indicated. ATF, artificial transcription factor; ZFP, zinc finger protein; KRAB, Kruppel-associated box; nls, nuclear localization signal; HBeAg, hepatitis B e antigen.

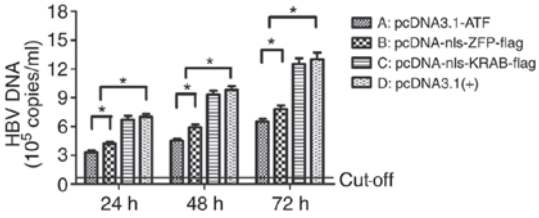

Figure 7. Detection the amount of HBV DNA copies. HBV replicative intermediates were measured at 24, 48 and 72 h post-transfection by polymerase chain reaction analysis. (A) pcDNA3.1-ATF, (B) pcDNA-nls-ZFP-flag, (C) pcDNA-nls-KRAB-flag and (D) pcDNA3.1(+). *P<0.05 as indicated. ATF, artificial transcription factor; ZFP, zinc finger protein; KRAB, Kruppel-associated box; nls, nuclear localization signal; HBV, hepatitis B virus.

Expression and localization of ATF. To determine the expression of the designed ATF eukaryotic expression vector after transfection, the ATF protein was visualized by scanning confocal microscopy. The results indicated that the designed expression vector normally expressed ATF, which was mainly located in the nuclei, whereas the control cells, which were transfected with empty vector pcDNA3.1(+), did not exhibit any ATF expression (Fig. 2).

Cell viability. To estimate the possibility that the lower protein expression in HepG2.2.15 cells may be attributed to a cytotoxic effect caused by ATF, HepG2.2.15 cells were subjected to a CCK-8 cell viability assay at 48 h post-transfection. The viability of HepG2.2.15 cells after transfection with ATF eukaryotic expression vector exhibited no difference compared with that of cells transfected with the empty vector pcDNA3.1(+) (Fig. 3). This result indicated that ATF had no cytotoxic effect on the HepG2.2.15 cells.

ATF reduces the HBV mRNA in vitro. To investigate the effects of the ATF on HBV mRNA *in vitro*, HepG2.2.15 cells were transfected with pcDNA3.1-ATF (nls-ZFP-KRAB-flag), pcDNA3.1(+)-nls-ZFP-flag, pcDNA3.1(+)-nls-KRAB-flag and pcDNA3.1(+) as the control. After 72 h, the HBV mRNA was collected and analyzed by RT-qPCR. In cells transfected with pcDNA3.1-ATF and pcDNA3.1(+)-nls-ZFP-flag, the HBV mRNA levels were respectively reduced by 63 and 49% compared with those in the empty vector control (P=0.03), and there was a significant difference between the expression levels in these two experimental groups (P=0.04). However, the pcDNA3.1(+)-nls-KRAB-flag-transfected cells only displayed a slight decrease in viral RNA production compared with that in the controls (P=0.24; Fig. 4).

ATF inhibits HBV protein expression in vitro. To further assess the effect of ATF on viral transcription *in vitro*, western blot analysis was performed to determine the effect of ATF on viral core and x protein expression (Fig. 5). The results indicated that pcDNA3.1-ATF and pcDNA3.1(+)-nls-ZFP-flag inhibited the expression of HBV core and x protein compared with that in the control group, while the *in vitro* inhibitory effect of pcDNA3.1-ATF was significantly stronger than that of pcDNA3.1(+)-nls-ZFP-flag.

ATF reduces the secretion of HBeAg, but not HBsAg in vitro. At 24, 48 and 72 h after transfection, the secretion of HBsAg in HepG2.2.15 cells was not different from that in the empty vector group (data not shown), while the secretion of HBeAg was time-dependently reduced in the pcDNA3.1-ATF group when compared with that in the empty vector group by 52.05, 54.19 and 60.37%, respectively (P<0.01). Furthermore, the inhibition in the pcDNA3.1(+)-nls-ZFP-flag group was significantly decreased when compared with that in the empty vector group (P=0.02), and there was a significant difference between the pcDNA3.1-ATF and pcDNA3.1(+)-nls-ZFP-flag groups; P=0.03), while no significant difference was present between the pcDNA3.1(+)-nls-KRAB-Flag and the empty vector group (P=0.08). This result suggested that pcDNA3.1-ATF inhibited the expression of HBeAg; while it had no effect on HBsAg. The inhibition was significantly decreased when the pcDNA3.1-ATF vector was voided of its effector domain KRAB (Fig. 6).

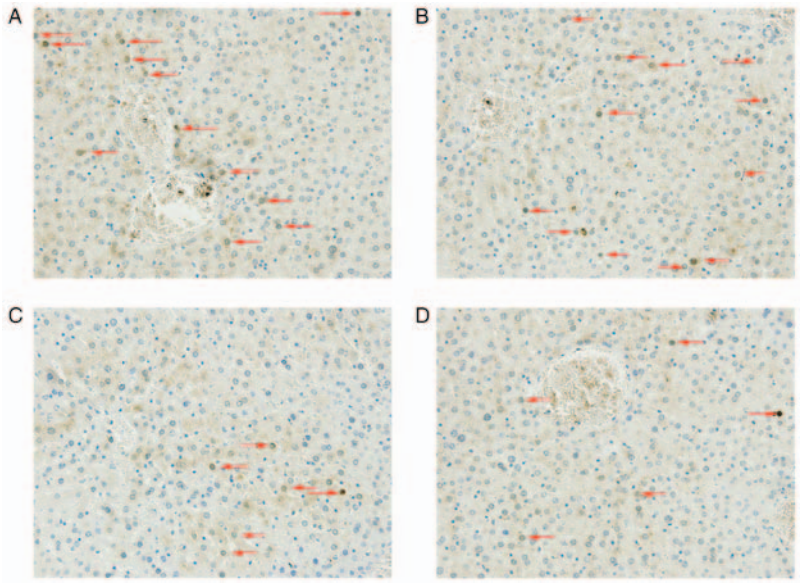

Figure 8. Detection of hepatitis B core antigen in hepatic tissue of mice at 28 dpi determined by immunohistochemistry (original magnification, x400). (A) pcDNA3.1(+), (B) pcDNA-nls-Kruppel-associated box-flag, (C) pcDNA-nls-zinc finger protein-flag and (D) pcDNA-artificial transcription factor. Red arrows indicate staining of core protein. nls, nuclear localization signal.

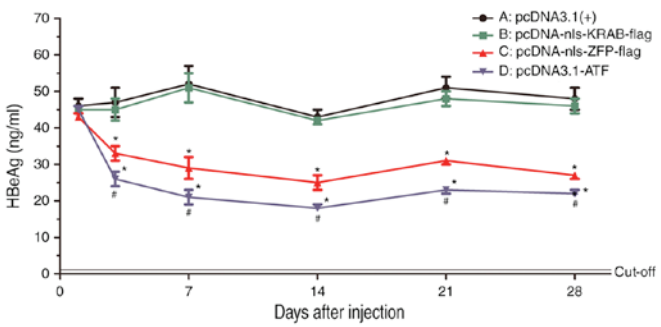

Figure 9. Serum levels of HBeAg were detected using a radioimmunoassay at 1, 3, 7, 14, 21 and 28 days after injection of (A) pcDNA3.1(+), (B) pcDNA-nls-KRAB-flag, (C) pcDNA-nls-ZFP-flag or (D) pcDNA-ATF. *P<0.05 vs. the control group; #P<0.05 vs. pcDNA-nls-KRAB-flag; ATF, artificial transcription factor; ZFP, zinc finger protein; KRAB, Kruppel-associated box; nls, nuclear localization signal; HBeAg, hepatitis B e antigen.

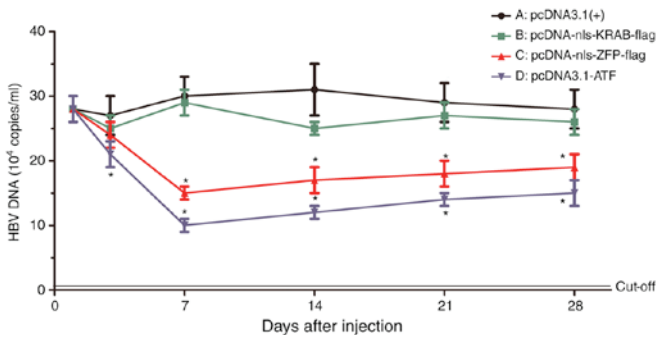

Figure 10. Expression of HBV DNA was detected *in vivo* by polymerase chain reaction analysis. HBV replicative intermediates were measured at 1, 3, 7, 14, 21 and 28 days after injection of (A) pcDNA3.1(+), (B) pcDNA-nls-KRAB-flag, (C) pcDNA-nls-ZFP-flag or (D) pcDNA-ATF. *P<0.05 vs. the control group. ATF, artificial transcription factor; ZFP, zinc finger protein; KRAB, Kruppel-associated box; nls, nuclear localization signal; HBV, hepatitis B virus.

ATF reduces HBV replicative intermediates in vitro. To determine whether vector-mediated ATF expression had an impact on HBV replication, the amount of HBV replicative intermediates was measured at 24, 48 and 72 h post-transfection by RT-qPCR. Similar to its effect on HBV transcription and protein expression, pcDNA3.1-ATF significantly inhibited HBV replication compared with pcDNA3.1(+)-nls-KRAB-Flag and empty vector (P=0.02), with the inhibitory rate (68.0%) being the highest at 72 h post-transfection (Fig. 7), which demonstrated a time-dependency of the inhibition of HBV replication. The inhibition was slightly decreased, although not significantly (P=0.27) when the ATF was voided of its effector domain KRAB, whereas pcDNA-nls-KRAB-flag exerted no inhibitory effect on HBV replication at 24, 48 and 72 h post-transfection, compared with the empty vector group (P=0.27).

ATF inhibits HBV protein expression in vivo. To further elucidate the effect of ATF on viral transcription *in vivo*, an immunohistochemical assay was performed to determine the effect of ATF on the expression of core protein. The results indicated that pcDNA3.1-ATF and pcDNA3.1(+)-nls-ZFP-flag inhibited the expression of core protein compared with that in the empty vector group. However, pcDNA3.1(+)-nls-KRAB-Flag did not inhibit HBV protein expression *in vivo* (Fig. 8).

ATF inhibits the secretion of HBeAg but not HBsAg in vivo. At 1, 3, 7, 14, 21 and 28 days after injection, the secretion of HBsAg in sera was not different from that in the empty vector group (data not shown), while the secretion of HBeAg was time-dependently reduced in the pcDNA3.1-ATF and pcDNA3.1(+)-nls-ZFP-flag groups when compared with that in the empty vector group (P=0.02). This result suggested that ATF and ZFP could inhibit the expression of HBeAg, but had no effect on HBsAg. The inhibition was significantly decreased when ATF lost its effector domain KRAB (Fig. 9), as the secretion of HBeAg was significantly lower in the pcDNA3.1-ATF group compared

with that observed in the pcDNA-nls-KRAB-flag group (P=0.03).

ATF reduces HBV replicative intermediates in vivo. The amount of HBV replicative intermediates was measured at 1, 3, 7, 14, 21 and 28 days after injection by using RT-qPCR (Fig. 10). Similar to its effect on HBV transcription and protein expression, ATF significantly inhibited HBV replication as compared with that in the pcDNA3.1(+)-nls-KRAB-Flag and empty vector groups (P=0.03). The inhibitory rate was highest at 7 days post-injection, and a time-dependency in the inhibition of HBV replication was observed. Of note, the pcDNA-nls-KRAB-flag had no inhibitory effect on HBV replication compared with that in the empty vector group (P=0.21).

Discussion

HBV is a small, coated virus containing a 3,200-bp partially relaxed circular double-stranded DNA genome (4). Once the hepatocyte is infected, the core nucleocapsid of HBV is released into the cytoplasm and the genomic DNA of HBV is transferred to the nucleus of the hepatocyte, where the partially relaxed circular double-stranded DNA is transformed into a closed covalently circular DNA (cccDNA) (46,47). The HBV cccDNA acts as a stencil for the transcription of all the viral RNAs, which include the subgenomic RNAs and pgRNA, which is pivotal for maintaining the replication and infection ability of progeny virus in the host (48). Transcription is activated by four promoters: The X, S1, S2 and preC/pg promoters; in addition, EnhI and EnhII have a decisive role in the regulation of viral gene transcription (2). Studies have demonstrated that transcription of pgRNA is regulated by the core promoter and the EnhI region. In addition, the transcription of the x and core genes is controlled by the pgRNA and EnhI region (49,50), which have a predominant role in modulating the expression of the temporal and global HBV gene (51). Based on the important functions of EnhI, inhibition of the transcription activity of HBV may be a novel strategy to reduce the replication of the progeny of HBV DNA.

The replication of HBV DNA is precisely controlled by the expression of the HBV gene. The S1, S2, X and preC/pg promoters are essential for the transcription of HBV sequences. In addition, EnhI and EnhII have an important role in the adjustment of HBV gene expression (2,52). In the HBV genome, there are partial overlaps between the EnhI region and X promoter, which may influence the activity of the promoter. Furthermore, the activity of the core promoter is regulated by the EnhI, and thereby, EnhI contributes to the high level of HBV replication (51). It is likely that inhibition of the transcriptional activity of EnhI may reduce the replication of HBV DNA.

Along with the rapid development of bioinformatics, a vast amount of biological information is available in various databases. Thus, by simulating the structure of natural transcription factors, artificial proteins that do not exist in nature, which may be generated to regulate specific genes, provides a modern tactical concept for the development of novel gene therapies (53,54). In fact, the conserved regions in the HBV genome, which included the HBV cccDNA, were specifically targeted and cleaved by the CRISPR/Cas9 system (55,56). The expression of endogenous target genes was modulated by the ATFs, which may be used as a potential powerful molecular tool in living organisms and cells. Numerous DNA-binding protein molecules have been designed as the DNA-binding motifs for the ATFs. Among them, the DNA-binding domain consists of ATFs, which include the Cys2His2-type ZFPs that have been extensively researched. The targeting sites are specifically recognized by the ZF-based ATFs in chromosomes, which not only effectively up- or downregulates the expression of their target genes by fusing functional domains, but may also be utilized for antiviral therapies (14,57,58).

In the present study, a genetic engineering method was applied to construct the pcDNA3.1-ATF eukaryotic expression vector, which efficiently expressed ATF *in vitro* and had no cytotoxic effects, and which was demonstrated to bind to the HBV Enh and inhibit the replication and expression of HBV DNA *in vivo* and *in vitro*. The DNA binding specificity of the ATF was unique, unmatched and unparalleled. The HBV EnhI-specific ATF was designed, constructed and then transformed or injected into HepG2.2.15 cells and HBV transgenic mice, respectively. The results demonstrated that the HBV EnhI-specific ATF significantly inhibited HBV transcription and replication of viral RNA, protein and viral progeny without any obvious toxic effect *in vitro* and *in vivo*. It is possible that the HBV EnhI-specific ATF is an important part of advanced combination therapies for eliminating HBV DNA in infected patients. An efficient treatment of chronic HBV infection may become feasible by using this bioengineering technology.

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Competing interests

The authors declare that they have no competing interests.

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