Biochemical and Biophysical Research Communications 430 (2013) 1228–1233

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-501 promotes HBV replication by targeting HBXIP

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ARTICLE INFO

Article history:
Received 24 November 2012
Available online 22 December 2012

Keywords:
miRNA
HBV replication
miR-501
HBXIP

ABSTRACT

MicroRNAs (miRNAs) can negatively regulate gene expression and also induce or inhibit viral replication. In the present study, we found 10 miRNAs were differentially expressed in a stable HBV-producing cell line (HepG2.2.15) compared with its control cell line (HepG2) by miRNA array analysis. miR-501 was significantly up-regulated in HepG2 cells and tissues with high-HBV replication. miR-501 expression was significantly up-regulated in hepatocellular carcinoma tissues, where HBV replication kept high. Down-regulating miR-501 could significantly inhibit HBV replication, but not influence the growth of HepG2.2.15 cells. Luciferase reporter and western blot assays revealed that HBXIP, an inhibitor of HBV replication, was a potential target of miR-501. Moreover, knockdown of HBXIP rescued the inhibition of HBV that occurred after the loss of miR-501 in HepG2.2.15 cells, suggesting that miR-501 induced HBV replication partially by targeting HBXIP. Thus, knockdown of miR-501 might provide a new mechanism and therapeutic target for inhibiting HBV replication.

1. Introduction

Hepatitis B Virus infection is a major health problem around the world, particularly in China. Every year, around 300,000 Chinese people die of HBV-related diseases, including around 180,000 HCC (hepatocellular carcinoma) patients [1]. The use of interferon or nucleoside analogs can partially inhibit but not entirely eradicate HBV replication [2]. Moreover, adverse effects, escape mutants and recurrence are often observed following long-term treatments in addition to drug withdrawal [3,4]. Therefore, it is necessary and urgent to establish new strategies for treating HBV infection.

Recently, several studies demonstrated that miRNAs played critical roles in regulating the viral life cycle [5]. Liver-specific miR-122 can interact with the 5′ noncoding region of HCV genes and may facilitate viral RNA replication [6]. miRNAs are also involved in HBV replication and HBV-related disease. miR-141 was identified as a repressor of HBV expression while screening a small miRNA library. Furthermore, synthetic miR-141 mimics also significantly suppressed HBV expression and replication in HepG2 cells that were transfected with pHBV1.3 [7]. It was noted that miR-1 transfection augmented farnesoid X receptor α expression, resulting in a marked increase in HBV replication through enhancing transcriptional activity of the HBV core promoter [8]. Hua Tang et al. reported that miR-199a-3p and miR-210 suppress HBV replication by targeting HBV gene transcripts [9]. However, whether cellular miRNAs affect HBV replication in host cells remains largely unknown.

In the present study, using a miRNA array analysis, we identified differentially expressed miRNAs in a stable HBV-producing cell line (HepG2.2.15) compared with the control cell line (HepG2). miR-501 expression was significantly higher in HCC cells and tissues which HBV replication was high. The effect and mechanism of miR-501 regulation of HBV replication was further investigated.

2. Materials and methods

2.1. Cell lines and clinical samples

The human HCC cell line HepG2 and the HBV stable replication cell line HepG2.2.15 was maintained in our laboratory [10]. GenBank accession number U95551, was obtained from the Academy of Military Medical Science, Beijing, China. The cells were cultured in RPMI-1640 media supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. To maintain stable HBV replication, HepG2.2.15 cells were cultured in the presence of G418 (300 μg/ml, Sigma–Aldrich).

Fresh surgical HBV-related HCC tissues from 10 patients with high HBV replication (>10^6 copies/ml) and 9 patients with low

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http://dx.doi.org/10.1016/j.bbrc.2012.12.071

HBV replication (<10^3 copies/ml) were obtained from the Department of General Surgery at Xijing Hospital. All of the tissues were clinically and pathologically confirmed. All of the study patients signed informed consent forms. The Hospital's Protection of Human Subjects Committee approved all of the protocols.

2.2. miRNA microarrays

miRNA microarrays were performed as described [11]. Microarray images were acquired using a Genepix 4000B scanner (Axon Instruments, Union City, CA) and then processed and analyzed with Genepix Pro 6.0 software (Axon Instruments) and Excel. The medians of four background-corrected replicas for each miRNA capture probe were normalized to the 50th percentile of the positive controls (U6-snRNA-1 and U6-snRNA-2) and to the median of the 50th percentile of the total signal intensities on the array.

2.3. Quantitative real-time PCR for miRNAs and quantitative PCR for HBV DNA

SYBRGreen-based real-time PCR was used to quantitate the cellular miRNA following the manufacturer's protocol, and the data were normalized to U6 snRNA [12]. The fold-change for each miRNA between the HepG2.2.15 cells compared with the control HepG2 cells was calculated using the $2^{-\Delta\Delta CT}$ method [13], where $\Delta\Delta Ct = \Delta Ct$ HepG2.2.15/HepG2 and $\Delta Ct = Ct$ miRNA-Ct U6.

The cell lysate DNA was extracted, and quantitative PCR (qPCR) was performed to quantify the HBV DNA levels as described previously [7]. qPCR was performed to quantify the HBV DNA levels with an Applied Biosystems Prism 7000 instrument and an Applied Biosystems SYBR® green master mix reagent according to the manufacturer's protocol (Finnzymes, Finland). The sequences of oligos used in this reaction were: 5′-AGGAGGCTGTAGGCATAAATTGG-3′ (sense) and 5′-CAGCTTGGAGGCTTGAA CAGT-3′. Each PCR assay was performed in triplicate.

2.4. Plasmids and transfection

Pre-miR-503, pre-miR-448, pre-miR-320, pre-miR-501, pre-miRNA control, anti-miR-501, anti-let-7a and anti-control miRNA were purchased from Ambion (Invitrogen). The siRNA sequence (5′-GCAGCUAAGCUAACCUCUGTT-3′) targeting HBXIP mRNA, and scramble sequence (5′-CCAGAAGAGCAATCTGTAC-3′) targeting none of the known gene, were constructed following a previous study [14]. The miRNA mimics, miRNA antisense oligonuleotides (ASOs), miRNA controls and siRNAs were transfected into HepG2.2.15 cells at the indicated concentrations using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Generally, HepG2.2.15 cells were transfected 24 h after seeding with 40 nm sequence-specific 2′-O-methyl miR-501 ASO (Ambion) or anti-control, and some cells were transfected with 1 μg HBXIP-siRNA. The cells and the supernatant were collected for qRT-PCR.

2.5. Luciferase reporter experiments

Luciferase reporter experiments were performed according to a previous study [15]. The following primer set was used to generate specific fragments: HBXIP-UTRF1, 5′-ACTAGT-gatgctcatatctgttcttc-3′and HBXIP-UTRR1, 5′-AAGCTT ttcctccaaacagattt attg-3′. Firefly and *Renilla* luciferase activities were measured consecutively with the dual-luciferase assay (Promega) 24 h after transfection.

2.6. Microparticle enzyme immunoassay

HBsAg and HBeAg in HepG2.2.15 cell supernatants were detected by microparticle enzyme immunoassay (MEIA) (7A40-22, 7D52-20, Abbott Laboratories, USA) at 1, 3, 5, and 7 days after transfection following the manufacturer's instructions(Lucifora et al., 2011).

2.7. Methyl thiazolyl tetrazolium (MTT) assay

Methyl thiazolyl tetrazolium (MTT) assays were performed as described previously [16]. Briefly, the cells were trypsinized and seeded into 96-well plates at a density of 2×10^3 cells/well in a volume of 200 μl. The cells were incubated with 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml; Sigma, St. Louis, MO) solution for 4 h in a humidified atmosphere containing 5% CO_2 at 37 °C. The supernatant was removed, 150 μl DMSO was added to dissolve the crystals, and an ELISA reader (Bio-Rad Laboratories, Richmond, CA) was used to determine the absorbance values at 490 nm each day. Viable cells were tested at 1, 2, 3, 4, 5, 6 and 7 days after plating, and each experiment was repeated three times.

2.8. Western blot

Cell protein preparation and western blot analyses were performed according to standard procedures as described previously [16]. Total proteins were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Expression of HBXIP was detected with anti-HBXIP (1:500, sc-134791, Santa Cruz, USA).β-actin was used as a loading control.

2.9. Statistical methods

All statistical analysis were performed using the SPSS 17.0 software package (SPSS, Chicago, IL), and the data were considered statistically significant when $P < 0.05$. All graphs represented the mean ± SD from three independent experiments. Variance analysis and Student's *t*-test were used for data analysis.

3. Results

3.1. Differentially expressed microRNAs in HepG2.2.15 and HepG2 cells

Among 342 human miRNAs tested, 10 were significantly different between the two cell lines (Table 1). Among them, hsa-miR-18a, hsa-miR-431, hsa-miR-302b, hsa-let-7a and hsa-miR-501 were up-regulated, whereas hsa-miR-148a, hsa-miR-320, hsa-miR-503, hsa-miR-519e and hsa-miR-448 were down-regulated more than 2-fold in HepG2.2.15 cells compared with HepG2 cells.

qRT-PCR analyses were used to verify the results from the comprehensive miRNA profiling assay; miR-18a, miR-431, miR-302b, let-7a and miR-501 were statistically up-regulated, whereas miR-148a, miR-503, miR-519e and miR-448 were significantly reduced in HepG2.2.15 cells compared with HepG2 cells. However, there was no significant difference of miR-302b expression between the two cell lines (Fig. 1).

3.2. Functional identification of differential miRNA regulation of HBV replication

miR-431, 18a and 148a were excluded from further studies because the expression of these three miRNAs was very low in HepG2.2.15 cells; thus, we chose let-7a, miR-501, miR-320, miR-503 and miR448 as potential targets. HepG2.2.15 cells were transfected with miR-320, miR-503, miR-448, miR-501, anti-miR-501, anti-let7a, mock or an anti-miRNA control. Seventy-two hours after transfection, qRT-PCR analyses demonstrated that miR-320, miR-503, miR-501 (transfected with pre-miR-501 mimic), and

Table 1
miRNAs differentially expressed inHepG2.2.15 compared with HepG2 cell line.

miRNA	Tendency	Chromosome location	Putative targets	*P*-value	Fold change
let-7a	Up	9q22.2; 11q24.2	RAS	0.006	5.2
miR-18a	Up	13q31.3		0.023	2.7
miR-431	Up	14q32.2		0.028	2.4
miR-302b	Up	4q25		0.008	3.3
miR-501	Up	Xp21.1	HBXIP	0.002	7.3
miR-148a	Down	7p15.2	HSP 90	0.018	2.9
miR-448	Down	Xq23	AKT3	0.007	4.2
miR-320	Down	8p21.3	MAPK1	0.002	2.5
miR-503	Down	Xq26.3	AKT3, RAF1	0.012	3.0
miR-519e	Down	19q13.4		0.023	2.2

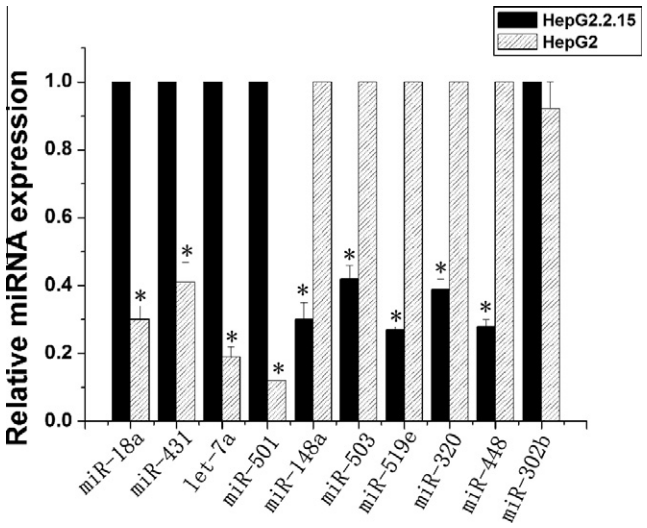

Fig. 1. Validation of microarray data using qRT-PCR. qRT-PCR analysis of differentially expyressed miRNAs was performed to validate the microarray results. The data (means ± SD) represent the relative changes in miRNA expression in HepG2.2.15 and HepG2 cells. Assays were performed in triplicate for each RNA sample, and the relative amount of each miRNA was normalized to U6 snRNA. $^{*}p < 0.05$ compared with each control.

miR-448 were significantly up-regulated, whereas miR-501(transfected with anti-miR-501) and let-7a were significantly down-regulated in transfected cells compared with the controls (Fig 2A). HBV DNA levels in the HepG2.2.15 cell culture media were determined by qPCR on the 5th day post-transfection. As shown in Fig 2B, miR-501 mimic obviously increased the HBV DNA levels, and anti-let-7a and anti-miR-501, significantly down-regulated the HBV DNA levels in the HepG2.2.15 tissue culture media three days post-transfection.

3.3. miR-501 expression correlated with HBV replication in HBV-related HCC tissues

miRNA profiling and real time-PCR assays showed that the difference of miR-501 expression between HepG2.2.15 cells and HepG2 cells are the most significant. Therefore, miR-501 was selected for our further study. The results of qRT-PCR analyses results showed that miR-501 expression was significantly higher in the group with high HBV replication (>10^6 copies/ml) compared with the low replication group (<10^3 copies/ml) (Fig 3A). Further analysis showed that miR-501 expression also correlated with HBsAg levels ($P = 0.03$). There was no statistical effect of miR-501 on the levels of anti-HBs, HBeAg, anti-HBe and anti-HBc (Table 2).

3.4. Suppression of miR-501 inhibited HBV replication

Anti-miR-501 was transfected into HepG2.2.15 cells to determine whether down-regulation of miR-501 influenced HBV replication. The results obtained from qRT-PCR showed that miR-501 was significantly down-regulated after 72 h (Fig. 3B). MTT assays revealed that down-regulating miR-501 expression did not markedly influence HepG2.2.15 cell growth (Fig. 3C). The cell lysates

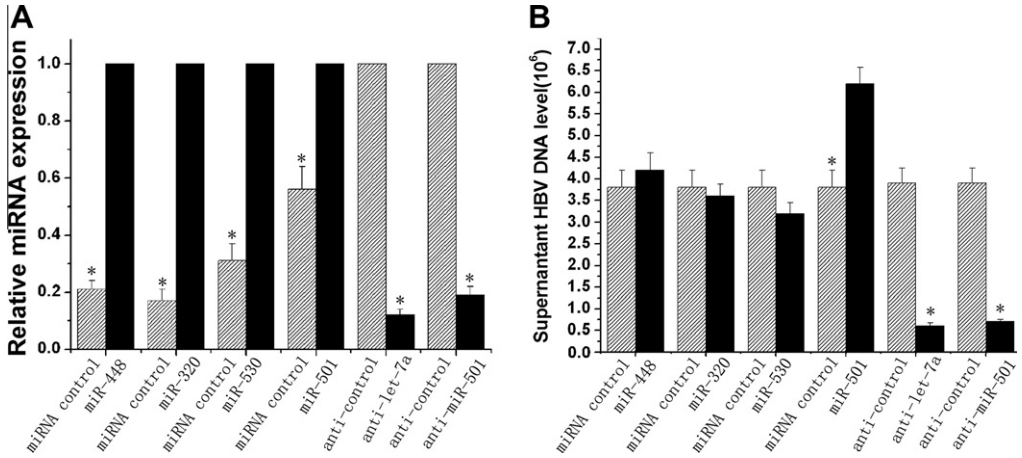

Fig. 2. Functional identification of differentially expressed miRNAs and their effects on HBV replication. HepG2.2.15 cells were transfected with miR-320, miR-503, miR-448, miR-501, anti-miR-501, anti-let7a, mock or the anti-miRNA control. (A) Seventy-two hours post-transfection, qRT-PCR analysis showed that the expression levels of miR-320, miR-503, miR-501 and miR-448 were significantly up-regulated, whereas miR-501 and let-7a were significantly down-regulated in the transfected cells compared with the matched controls. (B) qPCR analysis showed that miR-501 mimic obviously increased the HBV DNA levels, and anti-let-7a and anti-miR-501 transfections significantly down-regulated HBV DNA levels in the HepG2.2.15 cell tissue culture media 5 days post-transfection. The data represent the mean of three independent experiments. $^{*}p < 0.05$ compared with each control.

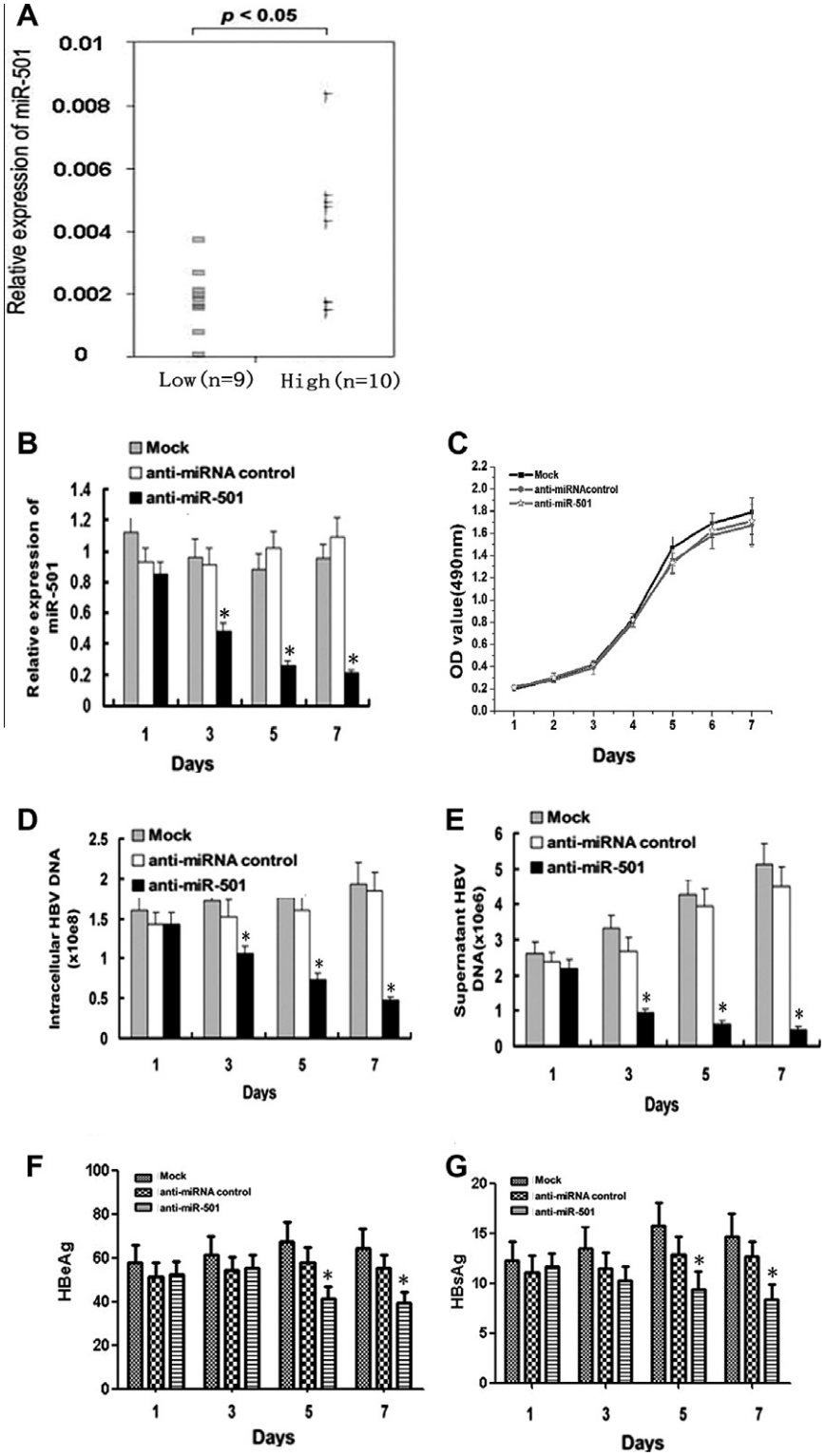

Fig. 3. Down-regulating miR-501 significantly inhibited HBV replication. qRT-PCR analysis of miR-501 was performed to validate the correlation between miR-501 expression and HBV replication in tissues from HBV-related liver cancer patients. miR-501 expression was significantly correlated with HBV replication. (B) qRT-PCR analysis demonstrated that miR-501 was significantly inhibited 3 days post-2′O-metha-miR-501 transfection. (C) MTT assays showed that down-regulating miR-501 did not significantly influence HepG2.2.15 cell growth. (D and E) HBV DNA levels in both the cell lysates and tissue culture media were significantly decreased 3 days post-transfection. (F and G) HBsAg and HBeAg levels in the tissue culture media were significantly decreased 5 days post-transfection. The data represent the mean of three independent experiments. $^{*}p < 0.05$ compared with each control.

and culture media were collected for further analysis at 1, 3, 5 and 7 days post-transfection. HBV DNA levels in the cell lysates and the tissue culture media were significantly decreased 72 h post-transfection ($P < 0.05$, Fig. 3D and E). HBsAg and HBeAg levels were also significantly reduced five and seven days post-transfection ($P < 0.05$; Fig. 3F and G). These data suggest that anti-miR-501 re-

Table 2
Correlations of miR-501 expression with HBV replication.

Factor	Group	miR-501		*P* value
		Low	High	
Age	⩽	4	5	0.84
	>	5	5	
Gender	Male	2	4	0.41
	Female	7	5	
HBV	Low	7	2	0.03*
	High	2	7	
HBs	Low	7	2	0.03*
	High	2	7	
Anti-HBs	Low	6	3	0.18
	High	3	7	
HBe	Low	6	3	0.18
	High	3	7	
Anti-HBe	Low	3	6	0.36
	High	6	4	
Anti-HBc	Low	6	3	0.18
	High	3	7	

The associations of miR-501 expression with the ability of HBV replication were detected by Kruskal–Wallis test.
* $P < 0.05$ was considered statistically significant.

duced HBV replication but no impact on cell proliferation in HepG2.2.15 cells.

3.5. HBXIP is a miR-501 target

Analysis of the ViTa database showed that HBV mRNA was not a potential target of miR-501, indicating that miR-501 might target host HBV replication-related gene(s), thus inducing HBV replication. Therefore, we used miRNA target prediction programs to find possible direct targets of miR-501. The results revealed that HBXIP, which can interact with the HBV x protein and repress HBV replication [17], was a potential miR-501 target (Fig 4A). Dual luciferase reporter assays demonstrated that expression of the HBXIP 3′-UTR reduced luciferase activity (Fig 4B). qRT-PCR and western blot analysis demonstrated that HBXIP mRNA and protein expression were markedly up-regulated in HepG2.2.15 cells that had been transfected with anti-miR-501 (Fig 4C and D). These data demonstrated that miR-501 significantly down-regulated HBXIP expression by binding to the 3′UTR of HBXIP mRNA.

3.6. HBXIP down-regulation rescued the miR-501 knockdown-induced inhibition of HBV replication

To investigate whether anti-miR-501 suppressed HBV replication through HBXIP regulation, HepG2.2.15 cells were transfected with a miRNA-501 inhibitor; 72 h after transfection, qRT-PCR demonstrated significant down-regulation of miRNA-501 (Fig 3A). These cells were then transfected with HBXIP siRNA for an additional 4 days, and western blot assays demonstrated a significant reduction in HBXIP expression (Fig 4G). As shown in Fig 4H, HBV DNA in the culture media was significantly increased in the siRNA-transfected cells compared with the anti-miR-501 transfected cells. However, there was no significant difference between the siRNA-transfected and the anti-miRNA control transfected cells ($P > 0.05$), indicating that down-regulating HBXIP rescued the inhibition of HBV replication that was induced by miR-501 knockdown.

4. Discussion

Recent studies showed that human liver-specific miR-122 induces hepatitis C virus (HCV) replication [6]. The genetic interactions between human miR-122 and the 5′-NCR of the HCV genome were analyzed by mutating the predicted miRNA target sites and ectopically expressing miR-122 with compensatory mutations. Human miR-122 likely facilitates viral RNA replication and may be a target for antiviral intervention. In addition, HIV is also predicted to be a miRNA target. Human miRNAs that are expressed in T-cells, the natural HIV-1 infection site, target HIV-1 genes and related clade sequences at highly conserved target sites. These studies implied that human miRNAs potentially affect HIV-1

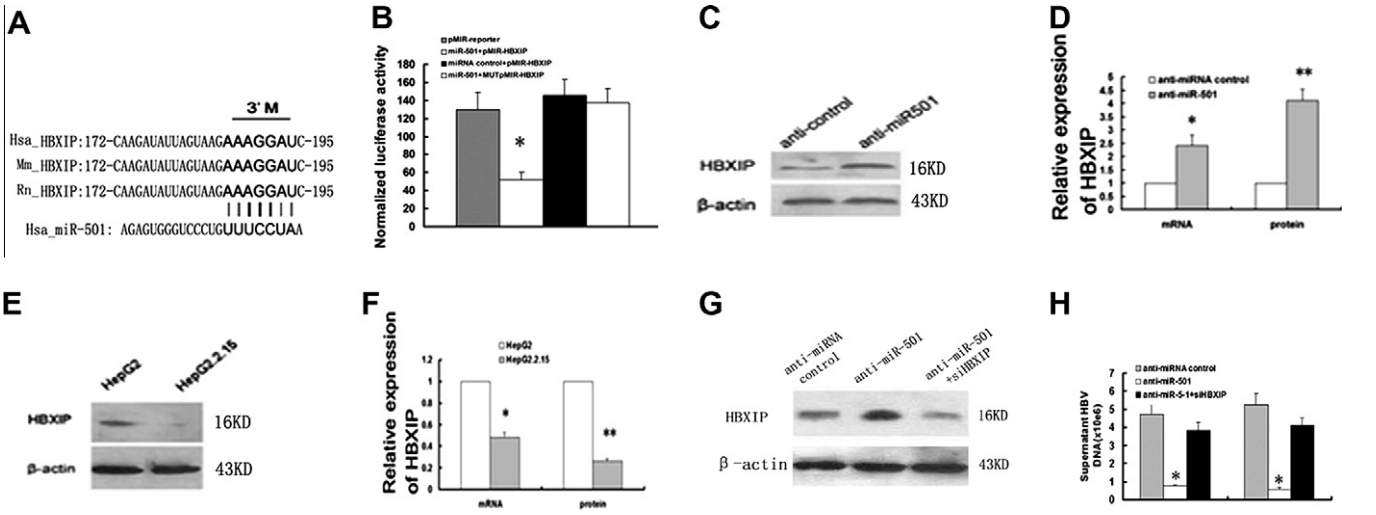

Fig. 4. HBXIP is a target of miR-501. (A) Putative binding sites of miR-501 in the HBXIP 3′UTR are conserved in humans, mice, and rats. The sites that were targeted by mutagenesis are indicated. (B) Dual luciferase reporter assays were performed in HepG2.2.15 cells that were transfected with the luciferase construct alone or were co-transfected with the miR-501 precursor or the precursor control. The firefly luciferase construct containing the mutant (MUTPMIR-HBXIP) HBXIP 3′UTR target site was generated and transfected as indicated. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample. (C and D) Repressing miR-501 significantly increased HBXIP protein and mRNA levels in HepG2.2.15 cells. (E and F) HBXIP protein and mRNA levels were significantly lower in HepG2.2.15 cells compared with HepG2 cells. (G and H) Down-regulating HBXIP rescued miR-501 knockdown-induced inhibition of HBV replication. (G) Western blot analysis demonstrated that the HBXIP siRNA transfection markedly inhibited HBXIP expression, which was induced by the anti-miR-501 transfection. (H) HBXIP siRNA transfection significantly increased HBV DNA levels in the tissue culture media. The results represent mean of three independent experiments. *$p < 0.05$ compared with the anti-miRNA control or anti-miR-501 and siHBXIP.

gene expression and could be utilized to develop future anti-HIV-1 therapies [18]. Therefore, by recognition of foreign nucleic acids, cellular miRNAs have direct antiviral effects in addition to regulatory functions. The relationships between viruses and miRNAs are worthy of further investigation.

Our present study began by comparing the miRNA profile between the HBV-stable replication cell line HepG2.2.15 and its parental cell line HepG2, using a miRNA array system. Ten miRNAs were differentially expressed in the two cell lines. By screening viral mRNA transcription and DNA replication levels, miR-501, a non-conserved miRNA among species, was identified to effectively induce viral replication. miR-501 was highly expressed in HepG2.2.15 cells. Anti-miR-501 2′-O methyl-antisense oligonucleotides were transiently transfected into HepG2.2.15 cells. As expected, miR-501 was down-regulated by qRT-PCR after transfection with the miRNA inhibitor. We collected cell lysates and tissue culture media 1, 3, 5 and 7 days post-transfection to measure viral DNA, HBsAg, HBeAg and perform a protein assay. The experimental data showed that miR-501 down-regulation significantly inhibited HBV DNA replication, but that was not observed at the protein level.

The Vita website analysis indicated that no miR-501-binding sites were found in the HBV genome. Therefore, miR-501 is not likely to regulate HBV replication by directly affecting HBV mRNA, but rather other host proteins. Then we used miRBas, miRNAMap, TargetScan, Miranda, RNAhybrid and PICTAR to predict miRNA targets by determining the most energetically favorable miRNA hybridization sites, identified HBXIP as a putative miR-501 target gene.

HBXIP, originally isolated as a human protein that binds to the viral HBX protein, may interact with a domain necessary for HBX transactivation to reduce HBV replication [17]. Our results indicate that HBXIP expression was high in nonmalignant human liver tissue with high HBV replication compared with patients that have low or no HBV replication. Furthermore, HBXIP expression has an inverse relationship with miR-501 in cells and tissues. By western blot and luciferase assays, we validated that miR-501 negatively regulated the HBXIP expression. To investigate whether HBXIP was responsible for miR-501 induced HBV replication, a HBXIP-targeting siRNA was constructed, which effectively suppressed HBXIP expression. HBXIP expression was necessary for the miR-501-induced HBV replication. Therefore, we conclude that miR-501 induced HBV replication in vitro partly by repression of HBXIP expression.

In conclusion, our results demonstrated that miR-501 induces HBV replication partly through negative regulation of HBXIP expression in HepG2.2.15 cells. These data are somewhat suggestive because lack of ideal animal models to study the HBV replication in vivo, however, it also suggests the potential roles of miRNAs in the regulation of HBV replication, and implicates the potential application of miRNAs in antiviral therapy.

Acknowledgments

This work was supported by National Basic Research Program of China (Nos. 2010CB732400; 81071368) and National Natural Science Foundation of China (Nos. 30973428, 30900674 and 30970149). We thank Tai-dong Qiao, Guang-Bo Tang and Jian-Hua Dou in our lab for excellent technical assistance.

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