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TAGLN2, a novel regulator involved in Hepatitis B virus transcription and replication

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ABSTRACT

Hepatitis B virus (HBV) infection is one of the major health problems in the world. Transgelin-2 (TAGLN2) expression has been revealed to be significantly altered in previous studies concerning HBV-host interaction. The present study investigated TAGLN2 expression patterns in HBV related hepatocellular carcinoma (HCC) tissues and its role in HBV transcription and replication. We collected 59 HBV related HCC tissue samples, their adjacent non-tumoral tissues and 16 normal livers to make the tissue microarray. TAGLN2 protein was detected by immunohistochemistry and the transcriptional levels of TAGLN2, HBc, HBs and HBx were detected by qRT-PCR. Then we investigated the function of TAGLN2 on HBV transcription and replication *in vitro* by ectopic expressing or knocking down TAGLN2 in HepG2 and HepG2.2.15 cell lines. We further studied the effect of HBx on TAGLN2 expression with a Tet-on HBx expressing cell line. TAGLN2 protein expression was lower in normal livers and HBV-HCC tissues comparing to adjacent non-tumoral tissues. The transcriptional levels of TAGLN2 in HBV-HCC tissues and their adjacent tissues were positively related to that of HBc, HBs and HBx ($P < 0.05$). Ectopic expression of TAGLN2 *in vitro* could enhance HBV transcription and replication while suppressing TAGLN2 had the contrary effect. TAGLN2 could be induced by HBx in a dose-dependent manner. Our data demonstrated that TAGLN2 might be an HBx induced positive host factor involved in HBV transcription and replication and HBx related liver fibrosis and tumorigenesis.

1. Introduction

Chronic Hepatitis B virus (HBV) infection is a major health problem and the most common cause for the development of cirrhosis, liver failure and hepatocellular carcinoma (HCC) [1,2]. Epidemiological studies have revealed that 2 billion people have ever been infected with HBV and over 350 million people are chronically infected with HBV worldwide [3]. HBV is a member of the Hepadnavirus family with the genome of 3.2 kb partially double-stranded circular DNA containing four partially overlapping open reading frames (ORFs), namely P, X, S and C [4]. HBx, a well-known oncoprotein, is the smallest protein encoded by HBV genome which is reported to be of great importance in HBV transcription and replication [5]. As a strong transactivator, HBx modulates various signaling pathways and affects host gene transcription, mutation and epigenetic modification, cell cycle, apoptosis and malignant transformation, by which plays an important role in HCC occurrence and development [6,7].

In the past decade, host protein alterations between different cellular models, animal models or samples from patients were identified by various approaches to investigate the roles of HBV or HBx in host cells [8–11]. The functions of most of these altered proteins in HBV life cycle remained elusive. In the previous study of Wei's group, they identified differentially expressed proteins between HBx or HBV-producing HepG2 cell line and their control cell lines by proteomic approach [9,10]. Thirty-four proteins were reported in both of the studies, among which they identified

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transgelin-2 (TAGLN2) as a significantly altered protein. Moreover, the upregulation of both genomic DNA copy number and transcriptional level of TAGLN2 were identified in HBV related HCC (HBV-HCC) tissues compared to 12 HCC cell lines [11], which also indicated a correlation between HBV and TAGLN2 in HBV-HCC tissues.

TAGLN2 belongs to calponin protein family with 64% amino acid sequence homology to transgelin (TAGLN) [12,13]. Unlike TAGLN, TAGLN2 is a newly identified protein and its function is poorly understood. Recently, TAGLN2 is proposed as a potential tumor marker in several tumors, including HCC [14,15] and is showed to be up-regulated in tumor tissues compared with non-tumor tissues and its expression increases with the progression of tumor [13]. However, though the altered expression of TAGLN2 has been observed in HBV or HBx producing cells as mentioned before, so far no evidence concerning its role in HBV life cycle has been reported.

In this study, we investigated the correlations of TAGLN2 expression with HBV proteins in HBV-HCC tissues and their paired adjacent non-tumoral tissues by immunohistochemistry (IHC) and quantitive real-time polymerase chain reaction (qRT-PCR). Then we studied the roles of TAGLN2 in HBV transcription and replication *in vitro* by ectopic expression or RNA interference followed by qRT-PCR, Southern blot and enzyme-linked immunosorbent assay (ELISA) analysis. At last, we revealed that TAGLN2 was one of the downstream proteins of HBx. Our subsequent investigations showed evidence to suggest that TAGLN2 is a positive factor in HBV transcription and replication.

2. Materials and methods

2.1. Tissue microarray and immunohistochemistry

We collected 59 HBV-HCC tissue samples and 16 normal liver samples from healthy people who donated the liver for transplantation between 2010 and 2012 in West China Hospital, Sichuan University, China. All the patients were confirmed by pathological diagnosis of HCC and had not received irradiation or chemotherapy prior to surgical operation. The study was approved by the Ethics Committee of West China Hospital, Sichuan University. Informed consents were obtained from all the patients or their relatives prior to analysis. Tissue microarrays (TMA) were made from 59 paraffin-embedded human HBV associated HCC tissues, their matched adjacent tissues and 16 normal liver tissues. Paraffin sections of TMA were incubated at 4 °C overnight with antibody against TAGLN2 (1:200, Abcam, USA). Semi-quantification of protein expression was assessed using Axiotis scoring method that consisted of an assessment for both positive staining intensity (scale: 0 to 3) and the percentage of positive cells (0–10% scored 0, 10%–25% scored 1, 26%–50% scored 2, 51%–75% scored 3, 76%–100% scored 4). The scores were multiplied to yield a final score (0–12). Two qualified pathologists analyzed the IHC data separately and the average score of each tissue was taken.

2.2. Plasmid construction

The plasmid payw1.2 was a replication-competent construct containing 1.2 copies of wild-type HBV genome (subtype ayw) and expressed HBV pregenomic 3.5-kb RNA under the control of the endogenous promoters of HBV [16]. The HBx-minus mutant vector payw*7 was derived from payw1.2 by site-directed mutagenesis and the FLAG-tagged full-length HBx expression plasmid, pNKF-HBX (subtype *adr*), was constructed as previously reported [17].

The full-length DNA sequence of TAGLN2 was amplified from the cDNA of HepG2 cells and cloned into the mammalian expression plasmid pEGFP-N1, generating the plasmid pEGFP-TAGLN2.

2.3. Cell culture and transfection

The human hepatoblastoma cell line HepG2, HepG2-derived HBV-producing cell line HepG2.2.15 and HEK 293T cells were obtained from American Type Culture Collection (ATCC) and cultured in Eagle's minimal essential medium (DMEM) (Thermo, USA) containing 10% fetal bovine serum (FBS) (Kangyuan, China) at 37 °C in 5% CO_2 in air. The Tet-on HBx expressing HepG2 cell line (HepG2-pTRE-HBx) and its parallel control cell line (HepG2-pTight) was established in our lab previously and cultured in DMEM containing 10% FBS, 1 μg/ml puromycin (Sigma, USA) and 200 μg/ml G418 (Amersco, USA) (He Z & Yu Y, 2015, in press). Cells were transfected with purified plasmids by X-tremeGENE HP DNA Transfection Reagent (Roche, Sweden) according to the manufacturer's instruction.

2.4. Real-time quantitative PCR analysis

Total RNA was isolated from cells using TRIzol (Invitrogen, USA) and reverse-transcribed into cDNA. qRT-PCR was performed with FastStart Essential DNA Green Master (Roche, Sweden) according to the manufacturer's instructions. The methods of detecting HBV DNA replication intermediates (RI), HBs and HBc mRNA have been described previously [18,19]. The primers used in qRT-PCRs were listed in Table 1.

2.5. Western blotting analysis

Proteins were extracted in RIPA buffer and quantified by G250 method (Beyotime, China). Samples were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were incubated with the primary antibodies respectively: rabbit-*anti*-TAGLN2 (1:1000, Proteintech, USA) and mouse-*anti*-GAPDH (1:5000, ZSBIO, China), then HRP-conjugated secondary antibody (1:10,000, Zen Bioscience, China). Target proteins were detected by ECL Western Blotting Substrate (Thermo, USA).

2.6. Immuno-fluorescent (IF)

Cells were seeded on the cover slides one day before experiment. Slides were fixed by 10% formalin and incubated with primary antibody rabbit-*anti*-TAGLN2 (1:100) and Alexa Fluor 488-Labeled Goat Anti-Rabbit secondary antibody (1:1000, ZSBIO, China) successively. Nuclears were stained with DAPI (1:1000, ZSBIO, China). Fluorescent signal was detected by EVOS imaging system (Thermo, USA).

2.7. RNA interference

Three small-interfere RNAs (siRNA#1, #2, #3) for TAGLN2 were designed and synthesized by RiboBio (China) and transfected into HepG2 cells with siRNA transfection reagent (RiboBio, China) respectively. Cells were harvested 72 h after transfection and the interfere efficiency of each siRNA were tested by western blot. Lentivirus system pSicoR, pxPAX2 and pMD2.G were preserved in our lab. The siRNA sequence with the highest efficiency was cloned into pSicoR to generate the plasmid pSicoR-shTAGLN2. pSicoR-shTAGLN2, pxPAX2 and pMD2.G were co-transfected into HEK 293T cells and the supernatant containing lentivirus were harvested 48 h and 72 h post transfection. HepG2 or HepG2.2.15 cells were incubated with the supernatant to silence TAGLN2.

2.8. Southern Blotting analysis

Transfected cells were harvested from 10 cm plates and used for extracting HBV RI as described previously [20,21]. The DIG-labeled

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Table 1
Primers for qRT-PCRs.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)
HBV [18]	CCTTCTTACTCTACCGTTCC	GACCAATTTATGCCTACAGCC
HBx	GACTCCCCGTCTGTGCCTTCTCATC	AGACCAATTTATGCCTACAGCCTCC
HBs [19]	CTCCAATCACTCACCAACCT	TCCAGAAGAACCAACAAGAAGA
HBc [19]	CTGGGTGGGTGTTAATTTGG	TAAGCTGGAGGAGTGCGAAT
TAGLN2	ATCACCACCCAGTGCCGAAAG	CATGGTGGAGGCCTGGATCTT
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCGT

full length HBV (subtype ayw) genomic DNA probe was used in Southern filter hybridization analyses. Results of filter hybridization were calculated by the ImageJ software.

2.9. HBV antigens assays

Cell supernatant was harvested and HBsAg and HBeAg were measured using ELISA kits as the manufacturer's instruction (Shanghai ShiyeKehua, China). Absorbance was measured with dual-wave length measurement (450/630 nm).

2.10. Statistical analysis

Receiver operating characteristic (ROC) curve analysis was used to determine the cut-off scores for TAGLN2 expression in HBV-HCC tissues and adjacent non-tumoral tissues separately as described previously [8,22]. The Chi-square test was used to analyze the correlation between TAGLN2 expression and clinicopathological characters of HBV-HCC patients. The Student t-test was applied to assess the IHC scores of TAGLN2 from TMA and HBV transcription and replication levels in transfected cells. Correlations of mRNA expression of TAGLN2 with HBcAg and HBsAg in HCC tumor tissues

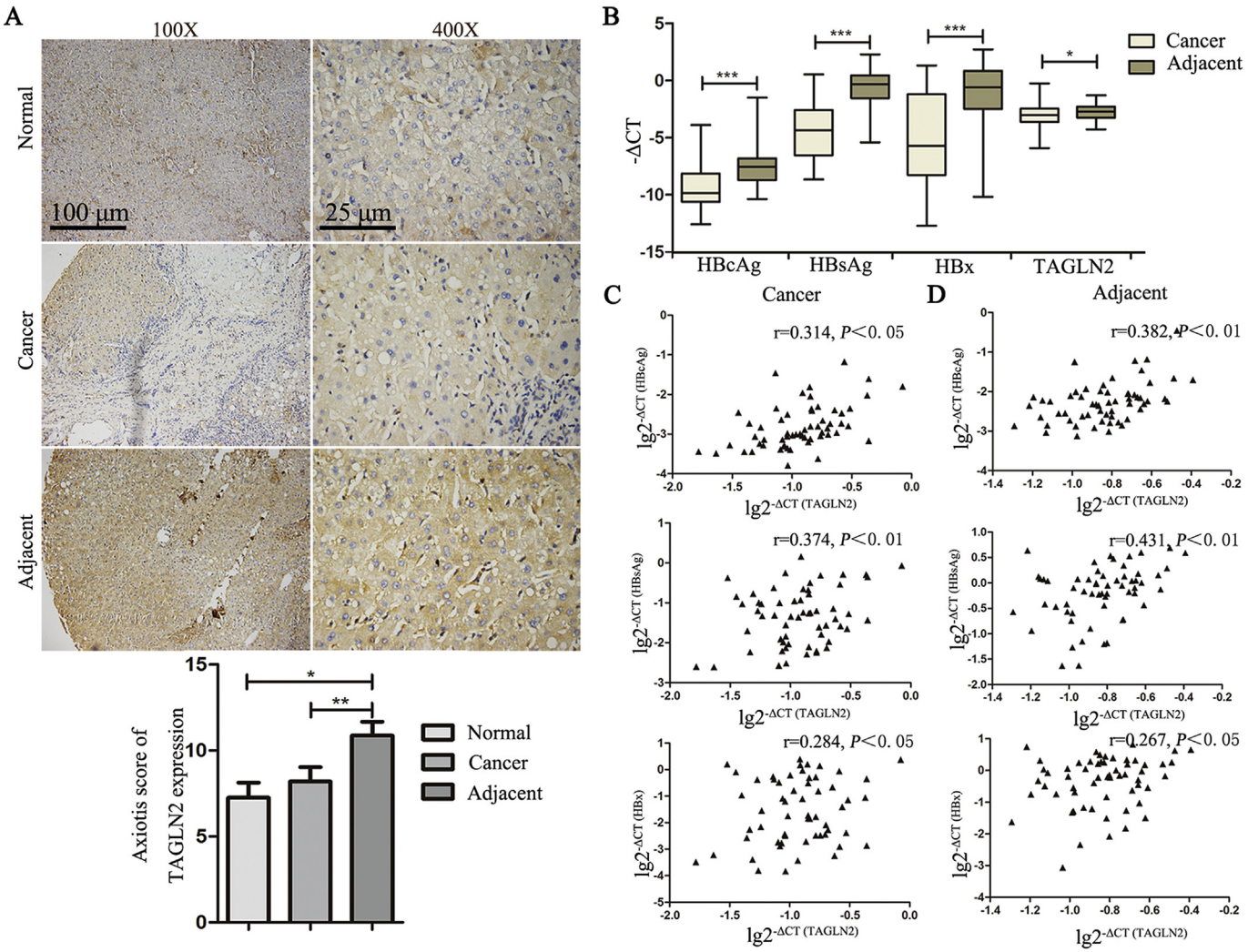

Fig. 1. Expression of HBc, HBs, HBx and TAGLN2 in normal, HBV-HCC and adjacent non-tumoral liver tissues. TAGLN2 expression in representative tissues and statistical expression of TAGLN2 in normal, HCC and adjacent non-tumoral tissues (A). Expression levels of HBc, HBs, HBx and TAGLN2 in HBV-HCC and adjacent non-tumoral tissues. GAPDH was used as internal reference (B). Correlation analysis between expression levels of TAGLN2 and HBc, HBs, HBx in HBV-HCC tissues (D) and adjacent non-tumoral tissues (E). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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were examined by Spearman's test. $P < 0.05$ was considered to be statistically significant. All statistical analysis was performed with SPSS 16 (IBM, Chicago, IL) and GraphPad Prism 5.0.

3. Results

3.1. TAGLN2 expression in HBV-HCC tissues, adjacent non-tumoral tissues and normal liver tissues

TAGLN2 expression in HBV-HCC tissues, their adjacent non-tumoral tissues and normal liver tissues were detected by IHC staining and assessed by Axiotis scoring method. Representative TAGLN2 protein staining in normal, HCC and adjacent non-tumoral tissues were illustrated in Fig. 1A. Statistical analysis showed that TAGLN2 scores of HCC adjacent non-tumoral tissues (10.86 ± 0.7913) were higher than that in HVC-HCC tissues (8.186 ± 0.8387) and normal livers (7.25 ± 0.8684). However, no significant difference of TAGLN2 protein expression was observed between normal livers and HBV-HCC tissues ($P > 0.05$) (Fig. 1A).

Areas under the ROC curves (AUC) and P values of ROC curves for all clinicopathological characters at different TAGLN2 scores were listed in Supplement Tables 1 and 2. The cut-off scores for TAGLN2 expression in HBV-HCC tissues and adjacent non-tumoral tissues were determined by the ROC curves for histological grade and ISHAK score respectively which showed the shortest distance from the point (0, 1). According to the cut-off scores, high expression was defined when the IHC score was greater than or equal to 2.83 in HBV-HCC tissues and 3.42 in adjacent non-tumoral tissues. TAGLN2 protein expression was found to be positively correlated with ISHAK scores in adjacent non-tumoral tissues ($P = 0.005$), but negatively correlated with histological grade in HBV-HCC tissues ($P = 0.019$).

3.2. Transcription level of TAGLN2 positively correlated to HBc, HBs and HBx in HBV-HCC tissues and adjacent non-tumoral tissues

To verify the correlation between TAGLN2 expression and HBV infection, we measured TAGLN2, HBc, HBs and HBx mRNA levels by qRT-PCR in 59 HBV-HCC tissues and their adjacent non-tumoral tissues. The results showed that mRNA levels of HBc, HBs, HBx and TAGLN2 in HCC tissues were lower than that in adjacent non-tumoral tissues (Fig. 1B). Furthermore, mRNA level of TAGLN2 was found to be positively related to HBc, HBs or HBx in both HCC tissues and their adjacent non-tumoral tissues (Fig. 1C and D). The above results suggested the involvement of TAGLN2 in HBV biological process and HBV related liver fibrosis.

3.3. Validation of differentially expressed TAGLN2 in vitro

Differential expression levels and subcellular localization of TAGLN2 in HepG2 and HepG2.2.15 cells were validated by qRT-PCR, Western blot and IF. Both mRNA and protein levels of TAGLN2 were higher in HepG2.2.15 cells comparing with HepG2 cells (Fig. 2A). IF showed that TAGLN2 localized in cytoplasm and its protein level was higher in HepG2.2.15 cells (Fig. 2B).

3.4. Silencing TAGLN2 suppressed HBV transcription and replication

TAGLN2 siRNA#2 transfection significantly reduced TAGLN2 protein expression in HepG2 cells comparing with the mock sequence transfection (Fig. 2C) and its sequence (5' CUGAGCG-CUAUGGCAUUAA dTdT 3') was chosen to generate the shTAGLN2 lentivirus. HBV transcription and replication in HepG2.2.15 cells after TAGLN2 knockdown were assessed by qRT-PCR and ELISA respectively. After 72 h of infection, HBV RI was reduced approximately 80% compared with the control group ($P < 0.05$) and HBeAg and HBsAg were significantly decreased (Fig. 2D). HepG2 cells were transfected with HBV construct payw1.2 or HBx-minus HBV construct payw*7 after 24 h of lentivirus infection, then harvested at 72 h post-transfection. Decreased HBV replication level was detected by Southern blot (Fig. 3A). These results demonstrated the important role of TAGLN2 in HBV transcription and replication.

3.5. Ectopic expression of TAGLN2 enhanced HBV transcription and replication in both the presence and absence of HBx

To further verify the function of TAGLN2 in HBV transcription and replication and whether this effect depended on HBx, HepG2 cells were co-transfected with pEGFP-TAGLN2 and payw1.2 or payw*7 with the ratio 2:1. After 72 h of transfection, HBV transcription and replication were detected by Southern blot, qRT-PCR and ELISA respectively. TAGLN2 were detected by Western blot after 48 h of pEGFP-TAGLN2 transfection (Fig. 2C). The results showed that TAGLN2 was able to enhance replication of HBV both with and without the presence of HBx (Fig. 3B and C). Meanwhile, TAGLN2 also promoted expression of HBV proteins at both mRNA level (Fig. 3D) and protein level (Fig. 3E) in both the presence and absence of HBx. However, much higher levels of HBV transcription and replication were detected after co-transfection with payw*7 and pNKF-HBx (Fig. 3C–E). The results indicated that TAGLN2 could partially restore the impaired transcription and replication of HBx-minus HBV and might enhance HBV transcription and replication by an HBx-independent pathway.

3.6. HBx could induce TAGLN2 expression in a dose-dependent manner

Since we have demonstrated the effect of TAGLN2 on HBV transcription and replication, we hypothesized that TAGLN2 would be a downstream protein of HBx. Thus, the effect of HBx on TAGLN2 expression was carried out using HepG2-pTRE-HBx cell line, which could stably express HBx under the control of doxycycline (Dox) in a dose dependent manner. TAGLN2 mRNA level in HepG2-pTRE-HBx cells was increased after 24 h treatment of gradual doses of Dox (0, 0.1, 0.25, 0.5 μg/ml) while no significant alteration was observed in the parallel control cells (HepG2-pTight) (Fig. 3F). The result indicated that TAGLN2 might be one of the downstream proteins in the signaling pathway induced by HBx (Fig. 3G).

4. Discussion

Chronic HBV infection is the leading risk factor of the development of HCC and causes 600,000 liver-related deaths annually [3,23]. HBx is the smallest protein in HBV genome, which plays a crucial role during HCC development by promoting HBV transcription and replication and modulating various cell signaling pathways and cell oxidative stress [24]. Researchers have been focusing on revealing its functions for years and a series of cell or animal models have been established. Among them, HepG2.2.15 cell line is a well-established cell model widely used in HBV related researches. It was derived from HepG2 cells and could stably express all viral RNAs and proteins and persistently secrete infectious viruses [25]. In previous studies, several groups have reported the proteomic differences between HepG2 and HepG2.2.15 cells, focusing on searching host factors which were either involved in HBV transcription and replication or that related to the carcinogenesis of HBV [10,26,27]. So far, the functions of very few of those differentially expressed proteins related to HBV have been well studied and TAGLN2 is one of those poorly studied

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Table 2
Correlations between TAGLN2 expression and clinicopathological features in 59 cases of HBV related HCC.

Characteristics	All cases	HCC tissues			Adjacent non-tumoral tissues		
		Normal	High	P value	Normal	High	P value
Age (y)				0.939			0.679
<50/≥50	34/25	14/9	20/16		14/11	20/14	
Gender				0.335			0.859
Female/Male	8/51	4/19	4/32		3/22	5/29	
AFP (ng/ml)				0.092			0.986
<8/≥8	16/41	4/19	12/22		8/16	8/25	
ALT (IU/L)				0.950			0.224
<55/≥55	33/26	15/8	18/18		17/8	16/18	
AST (IU/L)				0.384			0.981
<46/≥46	30/29	13/10	17/19		12/13	18/16	
HBsAg (S/CO)				0.460			0.637
<3000/>3000	14/45	7/16	7/29		5/20	9/25	
HBeAg (S/CO)				0.155			0.169
<1/≥1	47/12	21/2	26/10		22/3	25/9	
ISHAK score				0.828			0.019*
<4/≥4	15/44	6/17	9/27		11/14	4/30	
Tumor size (cm)				0.748			0.895
<5/≥5	18/41	7/16	11/25		7/18	11/23	
Capsule invasion				0.932			0.705
No/Yes	24/35	8/15	16/20		9/16	15/19	
Histological grade				0.005*			0.162
Moderate/High	36/23	8/15	28/8		13/12	23/11	
Metastasis				0.885			0.401
No/Yes	39/20	14/9	25/11		15/10	24/10	
HBV DNA				0.749			0.973
No/Yes	14/21	5/7	9/14		7/9	7/12	

* Statistically significant. P values were calculated using the $\chi 2$ test.

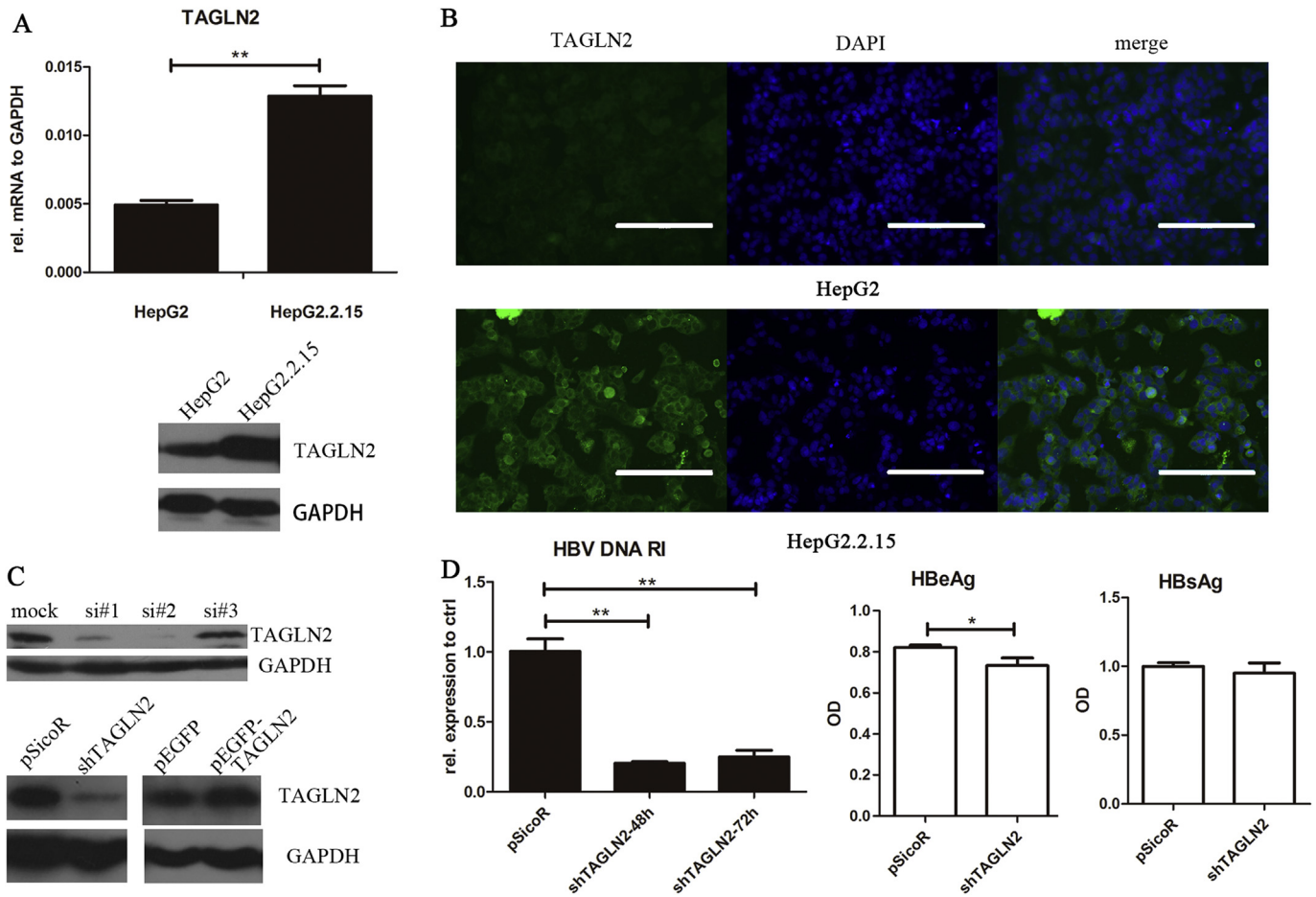

Fig. 2. Quantitative gene expression profile of TAGLN2 in HepG2 and HepG2.2.15 cells using qRT-PCR and Western blot. Data were presented after normalizing to GAPDH (A). TAGLN2 expression and localization were detected by IF. White bar: 200 μm (B). Knockdown efficiencies of siRNA or shTAGLN2 lentivirus and ectopic expression were detected by western blot (C). HBV replication in HepG2.2.15 cells after lentivirus mediated TAGLN2 knockdown for 48 h and 72 h was detected by qRT-PCR. HBeAg and HBsAg levels in the supernatant after 72 h of TAGLN2 knockdown or ectopic expression were detected by ELISA (D). $^{*}P < 0.05$, $^{**}P < 0.01$ by *t*-test.

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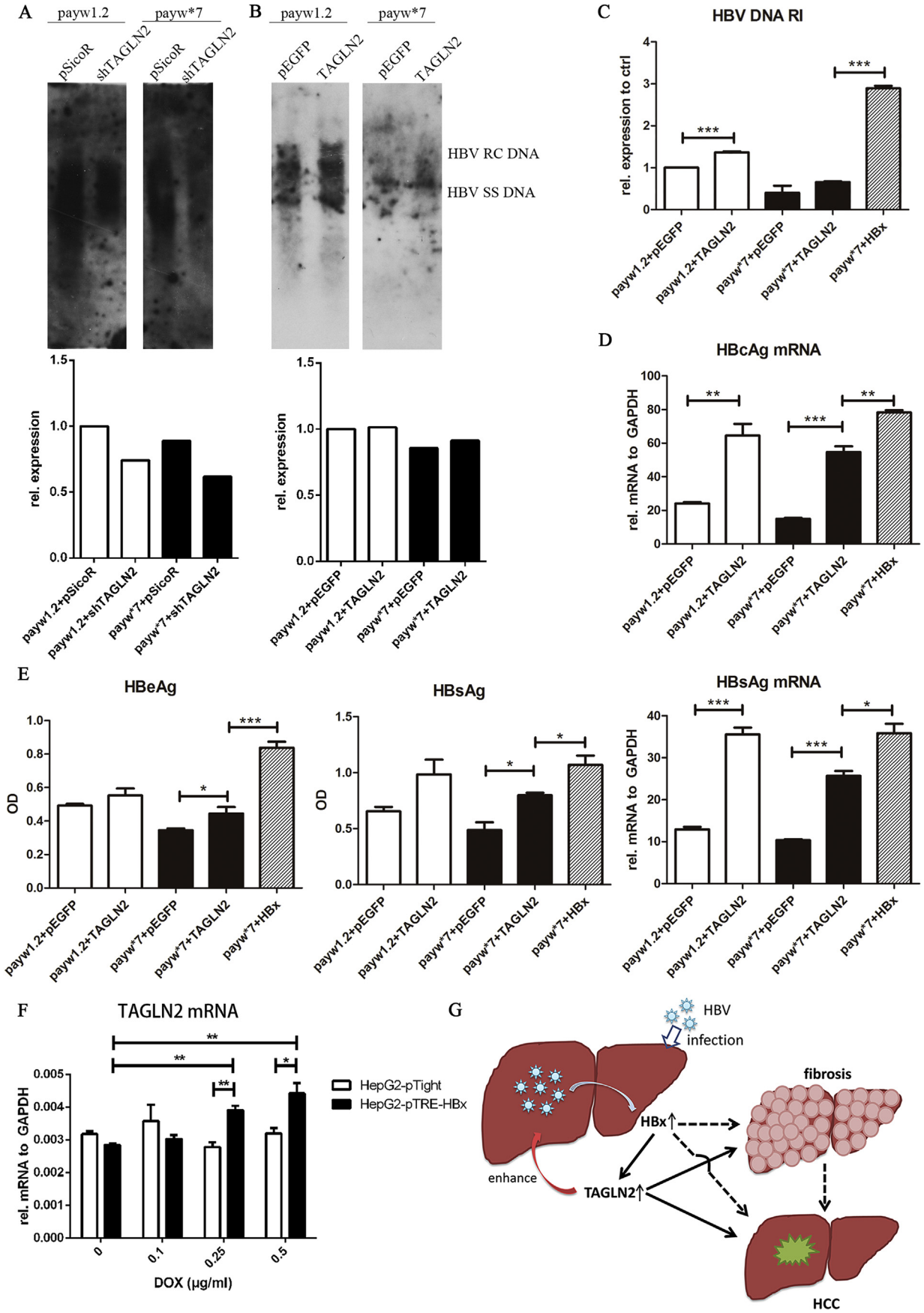

Fig. 3. Changes of HBV replication after TAGLN2 knockdown in HepG2 cells were detected by Southern blot (A). The effects of TAGLN2 on transcription and replication of HBV with or without HBx were analyzed by Southern Blot (B), qRT-PCR (C, D) and ELISA (E). HepG2 co-transfected with payw*7 and pNKF-HBx was used as the positive control (B—E). TAGLN2 mRNA levels in HepG2-pTRE-HBx cells and HepG2-pTight were detected by qRT-PCR after treated with gradual doses of Dox as indicated for 24 h (F). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test. The hypothetic positive feedback between HBx and TAGLN2. After HBV infection, HBx induces TAGLN2 expression in the liver and TAGLN2 in turn enhances HBV transcription and replication and promotes liver fibrosis and malignancy where HBx also plays a role (G).

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proteins.

TAGLN2 is the homolog of TAGLN (SM22α) which is identified as one of the earliest markers of differentiated smooth muscle [28]. TAGLN2 is considered to be a biomarker candidate for various tumors for its significantly higher abundance in malignant tumors though its oncogenic function remains elusive [29–31], and its role in tumor development is considered to be contradictory to TAGLN [13].

In our study, we demonstrated that TAGLN2 could enhance HBV transcription and replication independently of HBx, but this effect was not as efficient as HBx. We further clarified that TAGLN2 expression could be induced by increasing amount of HBx using the Tet-on HBx expressing HepG2 cell line we established previously. The mRNA level of TAGLN2 was induced by HBx in a dose-dependent manner. Therefore, given that HBx protein is a multi-functional *trans*-activator, we could infer that the effect of HBx on HBV transcription and replication might partially depend on TAGLN2, the transcription of which was activated by HBx itself. Thus, we concluded that there was a positive feedback loop between TAGLN2 and HBV transcription and replication. On the other hand, our results revealed that TAGLN2 might be one of the downstream proteins of the multi-functional protein HBx which acted as a pivotal tumor promoter in HBV-HCC development. We also found that TAGLN2 expression was negatively correlated with histological grade of HBV-HCC cancer tissues, which was consistent with the previous study [11]. Therefore, our results indicated the probable mediator role of TAGLN2 in HBx-dependent carcinogenetic processes (Fig. 3G).

Moreover, we observed that TAGLN2 expression levels were positively related to the ISHAK scores in HBV-HCC adjacent non-tumoral tissues, which indicated that TAGLN2 might be involved in the process of liver fibrosis, which could also be promoted by HBx [32–34]. From literatures, TAGLN2 was confirmed to be able to modulate cytoskeleton, regardless of the controversies about its functions. TAGLN2 suppressed cell migration by binding to ACTB to induce actin depolymerization [35] and its level was positively associated with metastasis and invasion in various tumor types [30,36,37]. It was confirmed to be a target of tumor-suppressive miRNAs miR-1 and miR-133a/b, whose functions involving suppressing cell migration [29,37,38]. Therefore, TAGLN2 might also participate in the development of HBV associated liver cirrhosis (Fig. 3G).

In summary, TAGLN2 expression is lower in HBV-HCC tissues than their paired adjacent non-tumorial tissues and is positively related to the transcriptional levels of HBV proteins in both tumorial and non-tumorial tissues. Ectopic expression of TAGLN2 enhances HBV transcription and replication independently of HBx, while suppression of TAGLN2 expression does the contrary way. HBx might enhance HBV transcription and replication partially via TAGLN2.

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Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.07.034.

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