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Hepatitis B virus suppresses the secretion of insulin-like growth factor binding protein 1 to facilitate anti-apoptotic IGF-1 effects in HepG2 cells

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ABSTRACT

Hepatitis B virus (HBV) infection is a major global health burden as chronic hepatitis B (CHB) is associated with the development of liver diseases including hepatocellular carcinoma (HCC). To gain insight into the mechanisms causing HBV-related HCC, we investigated the effects of HBV replication on global host cell gene expression using human HepG2 liver cells. By microarray analysis, we identified 54 differentially expressed genes in HBV-replicating HepG2 cells. One of the differentially-expressed genes was insulin-like growth factor binding protein 1 (IGFBP1) which was downregulated in HBV-replicating cells. Consistent with the gene expression data, IGFBP1 was suppressed at both the cellular and secreted protein levels in the presence of HBV replication. Transient transfection experiments with an inducible plasmid encoding the HBV X protein (HBx) revealed that HBx alone was sufficient to modulate IGFBP1 expression. Small interference RNA (siRNA)-mediated loss of function studies revealed that knockdown of IGFBP1 reduced apoptosis induced by either thapsigargin (TG) or staurosporine (STS). Treatment of cells with recombinant insulin-like growth factor 1 (IGF-1) decreased both TG- or STS-induced apoptosis. Interestingly, addition of recombinant IGFBP1 reversed the anti-apoptotic effect of IGF-1 on TG-induced, but not STS-induced, apoptosis. In conclusion, our results suggest an anti-apoptotic autocrine function of HBV-mediated downregulation of IGFBP1 in HepG2 cells. Such an effect may contribute to the development of HBV-mediated HCC by increasing pro-survival and anti-apoptotic IGF-1 effects.

1. Introduction

Hepatitis B virus (HBV) infection continues to be a global burden with more than 250 million chronic carriers worldwide causing nearly 1 million deaths each year due to complications such as cirrhosis and hepatocellular carcinoma (HCC) [1]. Especially, the small non-structural HBV X protein (HBx) is believed to contribute to the development of HCC by interfering with numerous host proteins including transcription factors [2]. In relation to HBV-related HCC, the effects of HBV and in particular HBx on apoptosis signaling have been extensively studied. However, the results have been contradictory and both inhibition and induction of apoptosis by HBx have been reported [3–7]. This is presumably due to differences in experimental conditions and/or specific HBV genotypes investigated. More specifically, HBx has been found to modulate p53 and NFκB signaling, the PI3K-Akt pathway as well as both the mitochondrial and the death receptor-mediated apoptotic pathways (reviewed in [8]). However, the exact molecular mechanisms responsible for the outcome of a chronic HBV infection are

Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; IGF-1, insulin-like growth factor 1; IGFBP1, insulin-like growth factor binding protein 1; STS, staurosporine; TG, thapsigargin; HBs, viral surface proteins

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still unclear. Insight into the possible effects of HBV on host cell gene expression may identify novel causative mechanisms.

Insulin-like growth factor 1 (IGF-1) is an important growth hormone, which activates pro-survival signaling via binding to the IGF-1 receptor (IGF-1R) [9,10]. The bioavailability of IGF-1 in the bloodstream is modulated by insulin-like growth factor binding proteins (IGFBPs) such as IGFBP1 [11,12] and altered IGF-signaling has been associated with HCC [13]. Hence, IGFBP1 inhibits the growth of human HCC cell lines [14] and low expression of IGFBP1 in HCC tissue is associated with a poor prognosis [15]. Hepatitis B virus infection has previously been found to modulate IGF-signaling e.g. by HBx-mediated upregulation of IGF-2 and IGF-1R expression [16–18]. Further, changes in IGFBP1 expression levels have been associated with HBV infection although results have been conflicting [19–24].

In this study, we used human HepG2 liver cells to identify genes with altered expression in the presence of HBV replication in vitro with particular focus on genes engaged in pathways associated with cancer development, such as proliferation and cell death.

We found that HBV replication led to decreased cellular and secreted IGFBP1 protein levels. Functional experiments provided evidence for an important autocrine role of IGFBP1 in the regulation of the pro-survival and anti-apoptotic actions of IGF-1 in HepG2 cells – a mechanism which may contribute to the development of HCC.

2. Materials and methods

2.1. Cell lines and cell culture

The Human Hepatoma HepG2 tet-on control cell line (HepG2 Tet-On Advanced cell line, #631150, CloneTech Laboratories, Mountain View, California, USA) containing the Tet-on inducible gene expression system [25,26] was stably transfected with the 3091 HBV genome (genotype D, serotype ayw3) [27]. The doxycycline-responsive promoter controls transcription of the preC/Core (including HBeAg) and polymerase genes, while HBs and HBx genes are transcribed continuously. Addition of doxycycline (1 μg/ml) to the media activates HBV replication and mature virus particles are produced. The untransfected HepG2 tet-on cell line was used as control.

Cells were maintained in Dulbecco Modified Eagle Medium (DMEM) (#41966–029, Gibco® by Life Technologies, Carlsbad, California, USA) with 10% Fetal Bovine Serum Premium (#p30–3302, PAN Biotech, Aidenbach, Germany), 1% Penicillin/Streptomycin (P/S) and G418 (100 μg/ml) at 37 °C with 5% CO_2. Puromycin (0.5 μg/ml) was added to HBs/HBx and HBV cells.

2.1.1. Verification of HBV replication

HBV DNA was isolated from the growth medium using the High Pure Viral Nucleic Acid kit (#11858874001, Roche, Basel, Switzerland) following the manufacturer's instructions and detected by qPCR (Forw.: 5′-actaggaggctgtaggcata-3′, rev.: 5′-agactctaaggcttcccg-3′). HBsAg and HBeAg secretion was verified using specific immunoassays.

2.1.2. Treatments and stimulations

HepG2 tet-on cells were exposed for 48 h to 0.1 μg/ml recombinant human IGF-1 (#AFL291-200, R&D Systems, Minneapolis, Minnesota, USA) and/or 1 μg/ml recombinant human IGFBP1 (#871-B1–025, R&D Systems). To inhibit the function of IGFs experimentally, IGFBP1 must either be phosphorylated or present in excess (4:1) [12]. In our experiments we added IGFBP1 to IGF-1 in a ratio of 10:1. Apoptosis was induced by a 24-h treatment with thapsigargin (#A2229,0001, PanReac AppliChem, Darmstadt, Germany) or staurosporine (Sigma Aldrich, St. Louis, Missouri, USA).

2.2. Transfection with siRNA

HepG2 tet-on cells were reverse-transfected with 10 nM ON-TARGETplus SMARTpool siRNA (mixture of 4 siRNA) (GE Healthcare Darmacon Inc., Lafayette, Colorado, USA) using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen, Carlsbad, California, USA). In short, siRNA and Lipofectamine RNAiMAX (0.03 μl/μl OptiMEM) was dissolved separately in Gibco® Opti-MEM® I Reduced Serum Media (Life Technologies) and incubated for 5 min at RT before mixing in a 1:1 ratio. After 20 min of incubation at RT, the siRNA – Lipofectamine RNAiMAX solution was added to each well. HepG2 tet-on cells in DMEM with 10% FBS without P/S was then added to each well (30000–37500 cells/cm^2) and the plate gently rocked to mix. After 24 h of incubation the transfection medium was changed to DMEM with 10% FBS and 1% P/S.

2.3. Transfection with plasmids

HepG2 tet-on cells were transfected in suspension using the SF Cell Line 4D-Nucleofector™ X kit (Lonza, Basel, Schweiz) and the Amaxa™ 4D-Nucleofector™ X Unit (Lonza) following the manufacturer's instructions. In short, 1 million cells were resuspended in 100 μl of Nucleofector™ SF solution containing 1 μg plasmid and transferred to a Nucleocuvette to be transfected using the EH-100 program. Post transfection, 200 μl RPMI growth medium was added and cells left for 10 min at 37 °C to recover before plating in pre-warmed DMEM with 10% FBS without P/S. The next day, the medium was changed to medium with or without doxycycline to induce HBx expression. Cells were harvested after another 48 h.

2.4. Microarray analysis

Microarray analysis was performed by AROS Applied Biotechnology A/S using the HumanHT-12 v4.0 Expression BeadChip (Illumina® Whole-Genome Gene Expression Direct Hybridization Assay system). This microarray includes 47,000 probes to detect the expression of 34,695 transcripts including known splice variants across the human transcriptome.

Cells were cultured in DMEM with only 2% serum to reduce the risk of FCS RNA interference [28]. Lysates were colleted after 48 h and stored at − 80 °C until RNA isolation using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentrations were measured on the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific™, Waltham, Massachusetts, USA). RNA integrity (RIN > 1.8) was evaluated on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano-kit (Agilent Technologies, Waltham, Massachusetts, USA). Microarray analysis was performed on three independent replicates.

2.4.1. Statistical analysis

Raw gene-based signal intensities were extracted from GenomeStudio Gene Expression module. Raw data were corrected for background noise using the normexp method [29] and quantile normalized [30] using both negative and positive control probes. Genes that were not expressed were filtered using a criteria of expression in atleast three samples according to the detection p-value of 5%. Differential expression analysis was performed using LIMMA [31]. P-values were adjusted for multiple comparisons using the Benjamini Hochberg method to control for the false discovery rate (FDR). Differentially expressed genes were identified using the double-filtering criterion: adjusted P-value (FDR) < 0.05 and an absolute Log fold change (absolute Log2(FC)) > 1. The statistical analyses were performed with Bioconductor in the R statistical environment [32].

2.5. RNA extraction

Total RNA was extracted from cell lines using RNeasy Mini Kit (Qiagen). Manufacturer's instructions were followed. RNA concentrations were determined and evaluated using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific™). RNA samples were

stored at − 80 °C.

2.6. cDNA synthesis and quantitative Real-Time PCR

cDNA synthesis was performed using iSqript™ cDNA Synthesis kit (BioRad, Hercules, California, USA) in accordance with the manufacturer's instructions on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). Between 250 and 1000 ng total RNA was used as template in each cDNA synthesis, and blank samples with H_2O as template were included. cDNA was stored at − 20 °C until further use.

Relative quantification of gene expression was performed using SsoFast™ EvaGreen Supermix (BioRad). Template corresponding to 5 ng RNA input was used in each reaction and running was performed in accordance with Manufactorer's instructions on a CFX384 Real-Time thermal cycler (Biorad). Expression cut-off was set to Ct < 36. The geometric mean of *GAPDH* and *ATCB* gene expression was used for normalisation and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers: *IGFBP1* (Forw.: 5′-atcctttgggacgccatcag-3′, rev.: 5′-attccaagggtagacgcacc-3′), *HBx* (Forw.: 5′-cttctcggggtcgcttgg-3′, rew.: 5′-cacacggtccggcagatga-3′), *GAPDH* exon 10–10 (Hs. PT.58.589810.g, Integrated DNA Technologies, Coralville, Iowa, USA), *ACTB* exon 6–6 (Hs. PT.56a.40703009.g, Integrated DNA Technologies).

2.7. Western blotting

Cells were lysed directly in wells using M-PER® Mammalian Protein Extraction Reagent (#78501, Thermo Fisher Scientific™) with Halt™ Protease & Phosphatase inhibitor Cocktail (Thermo Fisher Scientific™) following manufacturer's instructions. Lysates were centrifuged at 14,000 × *g* for 10 min and the supernatants (whole cell lysates) collected and stored at − 80 °C. Protein concentrations were determined using the DC™ Protein Assay (BioRad) according to the manufacturer's instructions. Loading: 20 µg protein per lane. Secondary HRP-conjugated antibodies were used and immune complexes were detected by chemiluminescence. Images were captured digitally using a FUJI LAS4000 imaging system (Fujifilm) and band intensities quantified using ImageQuant TL. GAPDH was used for normalisation.

Primary antibodies: IGFBP1 (EPR14472(B)) (ab181141, Abcam, Cambridge, United Kingdom), p-AKT1/2/3 (Ser 473)-R (#sc-7985-R, Santa Cruz Biotechnology), GAPDH (6C5) (#ab8245, Abcam).

Secondary antibodies: Anti-rabbit IgG, HRP-linked Antibody (#7074 S, Cell Signaling Technology, Danvers, Massachusetts, USA) and Anti-mouse IgG, HRP-linked Antibody (#7076 S, Cell signaling Technology). Protein size was verified using MagicMark™ Prestained Protein Ladder (#10748–010, Invitrogen).

2.8. IGFBP1 ELISA

IGFBP1 levels were measured in the culture medium collected after 48 h using a Human IGFBP-1 ELISA Kit (#EHIGFBP1, Thermo Fisher Scientific™) according to the instructions of the manufacturer. Prior to performing the assay, virus was inactivated by adding Triton-X to a 10% final concentration and samples had been stored at − 80 °C.

2.9. Cell death measurements

Caspase 3/7 activity was measured in duplicates in 96 well plates using the Caspase-Glo® 3/7 Assay (#G8093, Promega, Madison, Wisconsin, USA). In short, Caspase-Glo solution was added to the media in a 1:1 ratio, shaken for 30 s and incubated for 15 min at RT. Luminescence was measured on a TECAN infinite M200PRO. Measurement of total cellular protease activity using the CytoTox-Flour assay (Promega) was performed after the caspase assay to normalize for differences in cell numbers. In short, CytoTox-Flour solution was added in a 1:3 ratio, shaken for 30 s and incubated 40 min in darkness at 37 °C. Fluorescence was measured on a TECAN, infinite M200PRO. Background values measured in cell culture medium with Caspase-Glo solution was subtracted all values.

Cell Death detection ELISA^{PLUS} (#11920685001, Roche), determining the cytoplasmic fraction of mono- and oligonucleosomes in cell lysates, was performed according to the instructions of the manufacturer. In short, cells were lysed directly in wells and lysates from duplicates collected and pooled. Each sample was measured in duplicates on a TECAN infinite M200PRO. The ELISA results were normalized to the DNA levels in the lysates measured after ultrasound sonication using the QuantiFlour dsDNA System kit (E2670, Promega, Madison, Wisconsin, USA) following the manufacturer's instructions.

2.10. Statistics

Unless stated otherwise, the statistical analyses were performed in Prism version 7.02 (Graphpad Software Inc, La Jolla, California, USA). One-way ANOVA (no pairing) was used to analyze data from the three cell lines, while one-way ANOVA with repeated measures (RM) was used to analyze data from siRNA transfection and IGF-1/IGFBP1 incubation experiments in the HepG2 tet-on cell line. All ANOVA analyses were corrected for multiple comparisons using the Bonferroni method. In case of comparison of only two conditions two-tailed paired *Student's t-test* was applied. All data is presented as means ± SEM. P-values ≤ 0.05 were considered statistically significant ($p \leq 0.05$ *, $p \leq 0.01$ ** and $p \leq 0.001$ ***).

3. Results

3.1. HBV replication in vitro alters host cell gene expression

To investigate the effect of HBV on host cell gene expression, we used human liver HepG2 cells with doxycycline-inducible HBV replication. Addition of doxycycline to the culture medium leads to the production of mature virus particles (designated “HBV cells” in the following), while viral surface proteins (HBs) and HBx are transcribed continuously in the absence of doxycycline (designated “HBs/HBx cells”). Induction of HBV replication upon doxycycline treatment of the cells was verified by measuring the levels of HBsAg, HBeAg and HBV DNA in the growth medium (Table 1).

Microarray gene expression analysis was performed on total RNA to measure differences in gene expression in HBs/HBx and HBV cells compared to HepG2 tet-on control cells. After normalisation and background correction 16,146 genes were found to be expressed in the

Table 1
Verification of HBV-replicating cells. Growth medium was harvested after 48 h and HBV DNA detected by qPCR. Fold changes (FC) were calculated using the $2^{-\Delta Ct}$ method. Data are means ± SEM of 4 independent experiments. HBsAg and HBeAg secretion to the media were detected as signal to cut-off (S/CO) by specific immunoassays.

	HBV DNA (FC) (Mean ± SEM)	HBsAg (S/CO) (Cut-off: 0.13)	HBeAg (S/CO) (Cut-off: 0.1)
HepG2 tet-on cells	1.00 ± 0.00	0.01	0.00
HBs/HBx cells	1.00 ± 0.00	1.83	0.01
HBV cells	92.31 ± 21.56	1.62	1.14

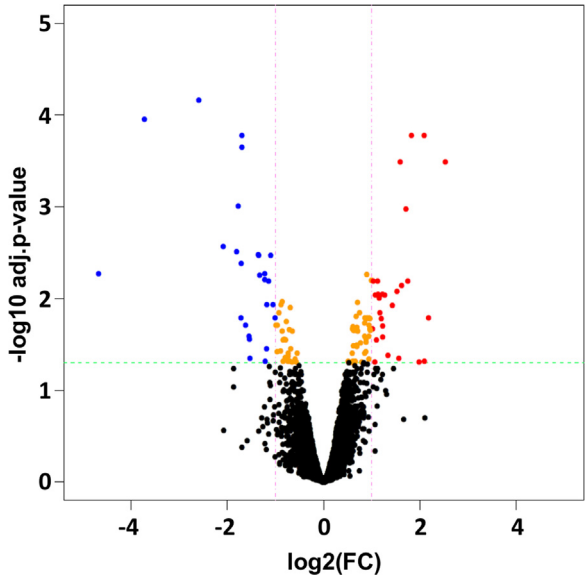

Fig. 1. Gene expression in HBV-replicating cells.
Volcano plot of differential gene expression in HBV cells compared to HepG2 tet-on control cells. Cut-off was set at FDR ≤ 0.05 and an absolute log2 fold change (FC) > 1. Data are means of 3 independent experiments.

cell lines using a detection p-value cut-off < 0.05. We found 54 genes significantly upregulated (≥ 2-fold) or downregulated (≤ 0.5-fold) in the presence of full HBV replication compared to the control after correction for multiple comparisons (FDR < 0.05) (Fig. 1 and Table 2). Interestingly, 32 of these genes were also differentially expressed in HBs/HBx cells. In total, 42 genes were significantly differentially expressed in HBs/HBx cells compared to the HepG2 tet-on control (Table 2). No genes were found differentially expressed between the HBs/HBx and HBV cells after correction for multiple comparisons (FDR ≤ 0.05). These results suggest that the presence of HBs and HBx, and not full virus replication, is sufficient to cause the majority of the host cell gene expression changes.

3.2. HBV decreases the cellular and secreted levels of IGFBP1

Among the identified genes found to be differentially expressed in HBV cells were insulin-like growth factor binding protein 1 and 7 (IGFBP1 and IGFBP7). These genes seem particularly interesting with regard to the development of HBV-related liver cancer, as IGFBPs are known to bind to and modulate the action of Insulin-like growth factors (IGFs), which are important regulators of cell survival, growth, and apoptosis through the PI3K/AKT signaling pathway [9]. According to The Human Protein Atlas [33,34], IGFBP1 is mainly expressed in the liver, while IGFBP7 is expressed in many different tissues. This prompted us to examine the role and function of IGFBP1 in HepG2 cells in the presence or absence of HBx/HBs or HBV replication.

In line with the microarray results, RT-qPCR and western blotting confirmed downregulation of IGFBP1 in both HBs/HBx and HBV cells as compared to HepG2 tet-on control cells (Fig. 2A and B). Accordingly, a significantly reduced secretion of IGFBP1 to the culture medium was observed in both HBV cells and HBs/HBx cells as compared to control cells (Fig. 2C).

The effect of HBV and particularly HBx on IGFBP1 expression has previously been studied in different HBV and HBx model systems, however, the results have been contradictory [19–24]. One factor that could account for the reported discrepancies between different models is differences in the expression level of HBx. We therefore in more detail investigated the relationship between the level of HBx and IGFBP1 expression. HepG2 tet-on cells were transiently transfected with either a doxycycline-inducible HBx-expression plasmid or an empty plasmid as control. High transfection efficiency was verified by GFP expression 48 h after transfection with a GFP plasmid (Suppl. Fig. 1). A concentration-dependent doxycycline-inducible HBx expression was confirmed by RT-qPCR (Fig. 2D). Due to leakiness of the tet-on system [35], a low level of HBx was also detected without doxycycline induction. Interestingly, we found that both the mRNA and protein levels of IGFBP1 decreased in response to induction of HBx expression by doxycycline (Fig. 2E and F). At a low HBx level the expression level of IGFBP1 was increased compared to the empty vector control cells, while the level of IGFBP1 decreased at higher levels of HBx. These data indicate that the impact of HBx on the expression of IGFBP1 depends on the exact expression level of HBx.

3.3. Knockdown of IGFBP1 reduces apoptosis in HepG2 cells

IGF-1 protects HepG2 cells from apoptosis induced by doxorubicin [13], but to our knowledge the potential apoptosis-regulatory role of IGFBP1 has not previously been investigated in HepG2 cells. To investigate the effect of decreased IGFBP1 expression on apoptosis, we transfected HepG2 tet-on cells with an IGFBP1-specific siRNA pool (siIGFBP1) or non-targeting siRNA (siCTRL) as a negative control. The IGFBP1 knockdown efficiency by the siRNA pool was verified at both the mRNA and protein levels after 48 h (Fig. 3A and B). To induce apoptosis, we used thapsigargin (TG) and staurosporine (STS), two well-known apoptosis inducers. TG is an inhibitor of the sarcoendoplasmic reticulum calcium transport ATPase (SERCA) pump, leading to endoplasmic reticulum (ER) stress and subsequent apoptosis [36]. STS is a protein kinase C inhibitor causing mitochondrial dysfunction leading to apoptosis [37]. Treatment of HepG2 cells with TG or STS lead to an upregulation of IGFBP1 expression (Fig. 3B and Suppl. Fig. 2), which is consistent with what other studies have reported [38–41]. Interestingly, we found that both STS- and TG-induced caspase-3/7 activity were significantly lower in cells with knockdown of IGFBP1 (Fig. 3C). Although statistically significant, the apoptosis-lowering effects of IGFBP1 knockdown were modest. However, focusing on the induced apoptosis and calculating the percentage of inhibition after subtraction of basal levels of caspase activity, knockdown of IGFBP1 inhibited caspase activity up to 35% (Fig. 3D). These data were confirmed measuring cell death as determined by the level of cytoplasmic nucleosomes (Fig. 3E and F). These results indicate that IGFBP1 has a pro-apoptotic function, possibly by inhibiting the pro-survival effects of IGF-1.

3.4. IGFBP1 inhibits IGF-1-mediated protection against TG-induced apoptosis

IGFBP1 is known to have both IGF-dependent and -independent functions [11]. To investigate whether the protective effect following knockdown of IGFBP1 is IGF-dependent, HepG2 tet-on cells were pre-stimulated with recombinant human IGF-1 and/or IGFBP1 for 24 h before inducing apoptosis. Since serum contains IGFs, these experiments were performed under serum-free or low serum (2% FBS) conditions. Furthermore, because IGF-1 is known to stimulate cell growth, caspase activity was normalized to cell numbers by measuring cellular protease activity using a cytotox assay found to have a linear correlation to the cell count (Suppl. Fig. 3A). As expected, cell growth was affected by both IGF-1 and/or IGFBP1 especially under serum-free conditions (Suppl. Fig. 3B and C). We found that TG-induced caspase-3/7 activity was slightly, but significantly reduced by IGF-1 – an effect that was only present under serum-free conditions (Fig. 4A and B). The protective effect of IGF-1 was more prominent when using a lower TG concentration (Fig. 4C). The combination of IGF-1 and IGFBP1 negated the protective effect of IGF-1 alone (Fig. 4A and C). These data suggest that the protective effect of knockdown of IGFBP1 on TG-induced apoptosis is mediated via increased IGF-1 anti-apoptotic effects.

To confirm active IGF-1 signaling upon treatment with IGF-1, we

Table 2
Gene expression in HBV-replicating and HBs/HBx cells. Differentially expressed genes in HBV cells and HBs/HBx cells compared to HepG2 tet-on cells after correction for multiple comparisons. Cut-off was set at FDR ≤ 0.05 and an absolute log2 fold change (FC) > 1 (black font). For genes only significantly differentially expressed in either HBV cells or HBs/HBx cells, the corresponding data is in grey font. Mean of 3 independent experiments.

	HBV - HepG2 tet-on		HBs/HBx - HepG2 tet-on	
GENE SYMBOL	log2(FC)	adj. p-value	log2(FC)	adj. p-value
AKR1B10	-1.22	6.24E-03	-0.92	2.41E-02
AKR1B15	-1.14	6.45E-03	-0.88	2.41E-02
ALDH3B1	0.54	1.20E-01	1.01	9.38E-03
ARHGAP10	1.12	6.45E-03	1.48	1.46E-03
BEX1	-1.70	2.26E-04	-1.70	2.23E-04
BGN	-1.53	4.49E-02	-1.68	2.82E-02
BRSK1	0.85	2.58E-02	1.02	1.26E-02
C3ORF70	-2.59	6.92E-05	-2.59	7.12E-05
CP	-1.13	5.51E-02	-1.47	1.79E-02
CYP3A7	1.34	4.19E-02	1.08	8.27E-02
DHCR7	1.53	8.37E-03	1.19	2.72E-02
DKK4	1.62	7.23E-03	1.03	5.53E-02
DNAJC12	-1.77	9.83E-04	-1.44	4.80E-03
DTX3	-1.01	1.62E-02	-0.63	9.98E-02
DYSF	1.71	1.06E-03	1.23	8.73E-03
EMX1	1.59	3.26E-04	1.40	1.25E-03
FADS1	1.17	1.43E-02	0.96	3.32E-02
FADS2	2.18	1.62E-02	1.73	4.16E-02
FST	-1.36	3.35E-03	-1.41	3.15E-03
FYN	-1.70	1.68E-04	-1.64	2.23E-04
GNG4	-1.62	1.96E-02	-1.78	1.34E-02
GPR44	1.10	2.82E-02	0.93	5.58E-02
HS.510638	-0.66	1.44E-01	-1.03	2.42E-02
HS.581580	0.96	1.67E-02	1.24	5.79E-03
HSD17B14	1.23	2.65E-02	1.30	2.27E-02
IGFALS	1.08	5.84E-02	1.26	2.87E-02
IGFBP1	-1.55	2.57E-02	-1.33	4.93E-02
IGFBP7	-1.54	2.78E-02	-1.71	1.84E-02
KANK3	1.20	1.67E-02	1.12	2.41E-02
LCN15	-1.22	4.84E-02	-1.09	6.50E-02
LCN2	-4.68	5.43E-03	-3.53	2.06E-02
LIPG	2.10	4.88E-02	1.76	8.17E-02
LOC100133545	1.13	9.02E-03	0.76	5.06E-02
LOC440030	2.09	1.68E-04	2.15	1.50E-04
LOC653458	-1.10	3.39E-03	-0.87	1.24E-02
LOC731486	-1.72	1.63E-02	-1.45	3.32E-02
LOX	-1.81	3.10E-03	-1.77	3.89E-03

(*continued on next page*)

Table 2 (*continued*)

LPPR1	-0.92	1.43E-02	-1.08	6.89E-03
LRRC56	1.07	9.18E-03	1.12	7.66E-03
LSS	1.23	2.01E-02	0.96	5.58E-02
MGC61598	-1.05	1.16E-02	-0.97	1.84E-02
MIR1974	-0.91	6.17E-01	-3.31	3.22E-02
MVD	1.56	4.52E-02	1.07	1.45E-01
NR5A2	1.83	1.68E-04	1.90	1.50E-04
NT5E	-2.08	2.72E-03	-2.02	3.69E-03
P2RY8	1.75	6.45E-03	1.54	1.33E-02
PCSK9	1.98	4.95E-02	1.35	1.58E-01
PCOLCE2	-0.89	6.76E-02	-1.14	2.46E-02
PDGFRB	-1.19	3.52E-02	-1.27	2.46E-02
RENBP	-1.22	5.43E-03	-0.92	2.15E-02
RTN4R	1.03	6.45E-03	0.76	2.80E-02
SC5DL	1.27	9.21E-03	0.97	3.22E-02
SIGLEC14	1.12	5.87E-02	1.45	1.92E-02
SLC22A15	-1.33	5.60E-03	-1.20	9.85E-03
SLC29A2	1.02	2.16E-02	0.70	9.17E-02
SLC7A10	2.53	3.26E-04	2.13	1.46E-03
SLCO4C1	1.22	9.11E-03	0.96	2.80E-02
SRY	1.15	9.89E-03	1.11	1.26E-02
TM7SF2	1.06	4.95E-02	0.88	9.18E-02
TRIM2	1.43	1.19E-02	1.66	6.06E-03
TRMT12	-1.71	4.14E-03	-1.59	6.18E-03
TSPAN7	-3.72	1.12E-04	-3.37	1.50E-04
ZDHHC14	-1.35	3.39E-03	-1.09	1.14E-02
ZNF185	-1.18	1.17E-02	-0.82	5.58E-02

examined the protein level of phosphorylated AKT (p-AKT) by western blotting after IGF-1 treatment with and without TG exposure (Fig. 4D). A significantly increased level of p-AKT was detected both under serum-free and low serum conditions (Fig. 4D) confirming the stimulatory effect of IGF-1 treatment on HepG2 tet-on cells. Notably, TG exposure caused a marked decrease in the level of p-AKT (Fig. 4D).

Interestingly, as opposed to that observed for TG-induced apoptosis, IGFBP1 did not negate the protective effect of IGF-1 on STS-induced caspase activity (Fig. 4E), suggesting that the protective effect of knockdown of IGFBP1 on STS-induced apoptosis is IGF-1-independent.

4. Discussion

Chronic hepatitis B infection is associated with a high risk of developing severe liver disease including cirrhosis and HCC. However, little is known about the molecular mechanisms involved in the development of these diseases. In this study, we investigated the impact of HBV replication on global host cell gene expression by microarray analysis and identified 54 differentially expressed genes in HBV-replicating HepG2 cells. These findings support the concept that HBV contributes to altered host cell gene expression that may contribute to the pathogenesis of liver disease such as HCC.

Two of the differentially expressed genes were IGFBPs, including IGFBP1, known to be regulators of IGF activity. We chose to focus on IGFBP1 for several reasons; i) it is specifically expressed in the liver [33,34], ii) altered IGF signaling is associated with HCC [13], iii) IGFBP1 inhibits the growth of human HCC cell lines [14], and iv) low expression of IGFBP1 in HCC tissue is associated with a poor prognosis [15]. We confirmed that IGFBP1 is downregulated at both the cellular and secreted protein levels in HBs/HBx and HBV cells compared to control cells. Specifically, the effect of HBx on IGFBP1 expression was found to depend on the HBx expression level. Knockdown of IGFBP1 in HepG2 tet-on cells reduced both TG- and STS-induced apoptosis as revealed by decreased caspase 3/7 activity and lower levels of cytoplasmic levels of nucleosomes, both of which are characteristics of apoptosis. Furthermore, addition of recombinant IGFBP1 to the culture medium reversed the protective effect of IGF-1 on TG-induced apoptosis, but not STS-induced apoptosis. Altogether, this suggests a pro-apoptotic effect of IGFBP1 in HepG2 tet-on cells partly through the inhibition of IGF-1 signaling.

The decreased level of IGFBP1 found in the HBs/HBx cells indicates

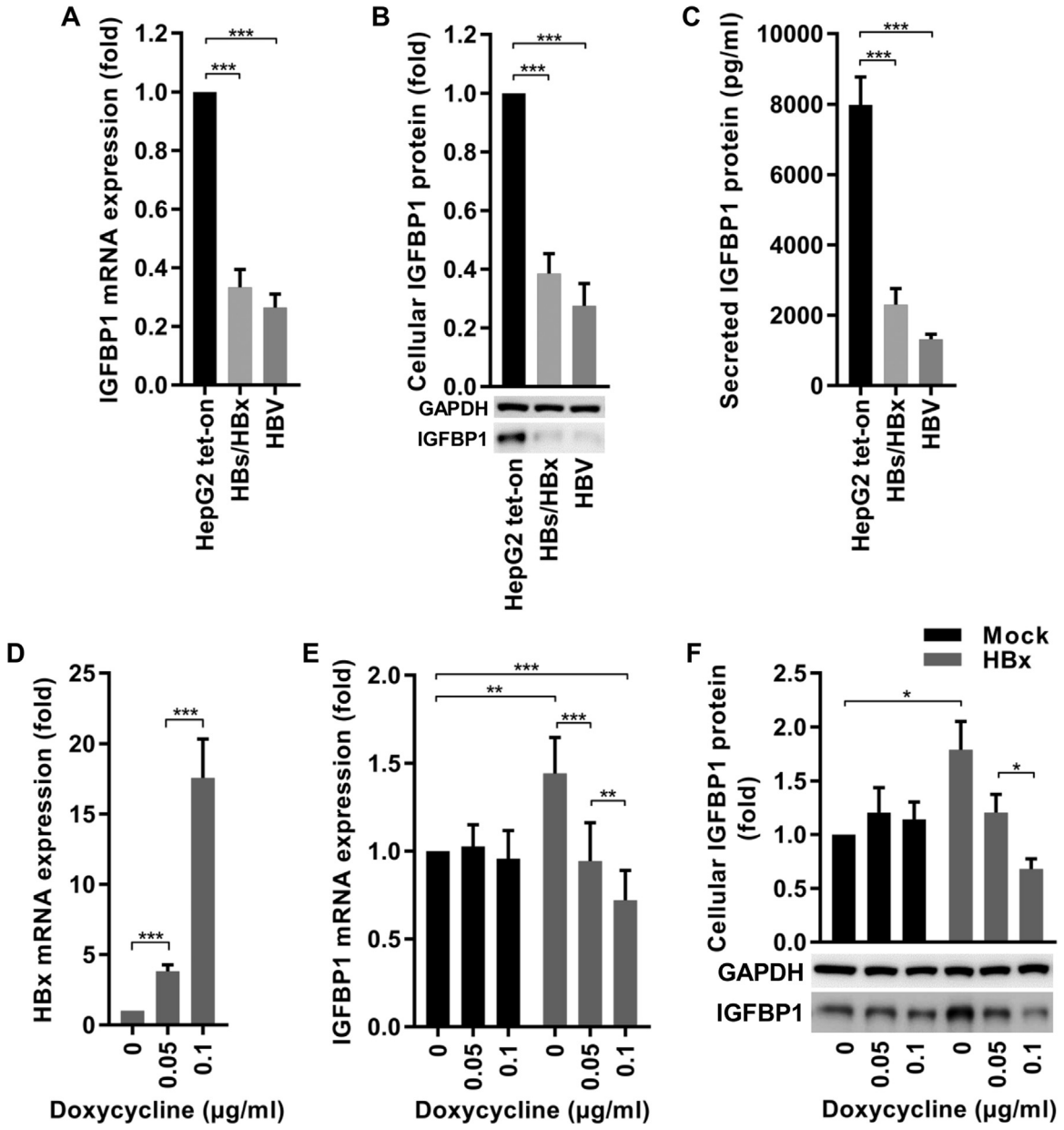

Fig. 2. IGFBP1 expression and secretion in HBV-replicating and HBs/HBx cells Growth media and cell lysates were collected after 48 h. A: IGFBP1 mRNA level was measured by RT-qPCR. Data are means ± SEM of 7 independent experiments. Statistics: One-way ANOVA, corrected for multiple comparisons (Bonferroni). B: IGFBP1 protein level was measured by western blotting and quantified (normalized to GAPDH). Data are means ± SEM of 4 independent experiments. Statistics: One-way ANOVA, corrected for multiple comparisons (Bonferroni). C: IGFBP1 protein secretion to the culture medium detected with ELISA. Data are means ± SEM of 4 independent experiments. Statistics: One-way ANOVA, corrected for multiple comparisons (Bonferroni). HepG2 tet-on cells were transiently transfected with a doxycycline-inducible HBx-expression plasmid (grey bars) or an empty plasmid (mock) (black bars). D: HBx mRNA expression was measured by RT-qPCR. Data are means ± SEM of 6 independent experiments. Statistics: RM One-way ANOVA, corrected for multiple comparisons (Bonferroni). IGFBP1 mRNA (E) and protein (F) levels were measured by RT-qPCR and western blotting, respectively. Data are means ± SEM of 6 independent experiments. Statistics: Two-way ANOVA, corrected for multiple comparisons (Bonferroni).

that the presence of HBs and HBx proteins is sufficient to suppress IGFBP1 expression. Both HBs and HBx have previously been shown to modulate the host environment [42,43]. The effect of HBV infection on IGFBP1 expression has been investigated previously, but the results have been contradictory. In primary human hepatocytes (PHH) infected with HBV, the expression of IGFBP1 has been found upregulated, however, this could not be confirmed in tissue from HBV patients [19]. In HBx-transgenic mice, IGFBP1 has been found both up- and down-regulated [21,22]. In hepatocytes from humanized chimeric mice transfected with HBV, IGFBP1 expression was increased [20]. In plasma from HBV-infected children with cirrhosis, IGFBP1 was upregulated [23], while IGFBP1 was downregulated in HBV-related HCC tissue [24]. Interestingly, we found that in the inducible HBx-expressing cells the IGFBP1 expression level inversely correlated with the HBx expression level. However, compared to Mock cells, the HBx-expressing cells had higher IGFBP1 expression even in the absence of doxycycline. This suggests a bell-shaped association between HBx and IGFBP1 expression. This finding may explain the discrepancy between results in the literature as different HBV models might have different HBx expression levels. Furthermore, it could be speculated that changes in HBx expression levels during the pathogenesis of chronic hepatitis B could affect IGFBP1 expression.

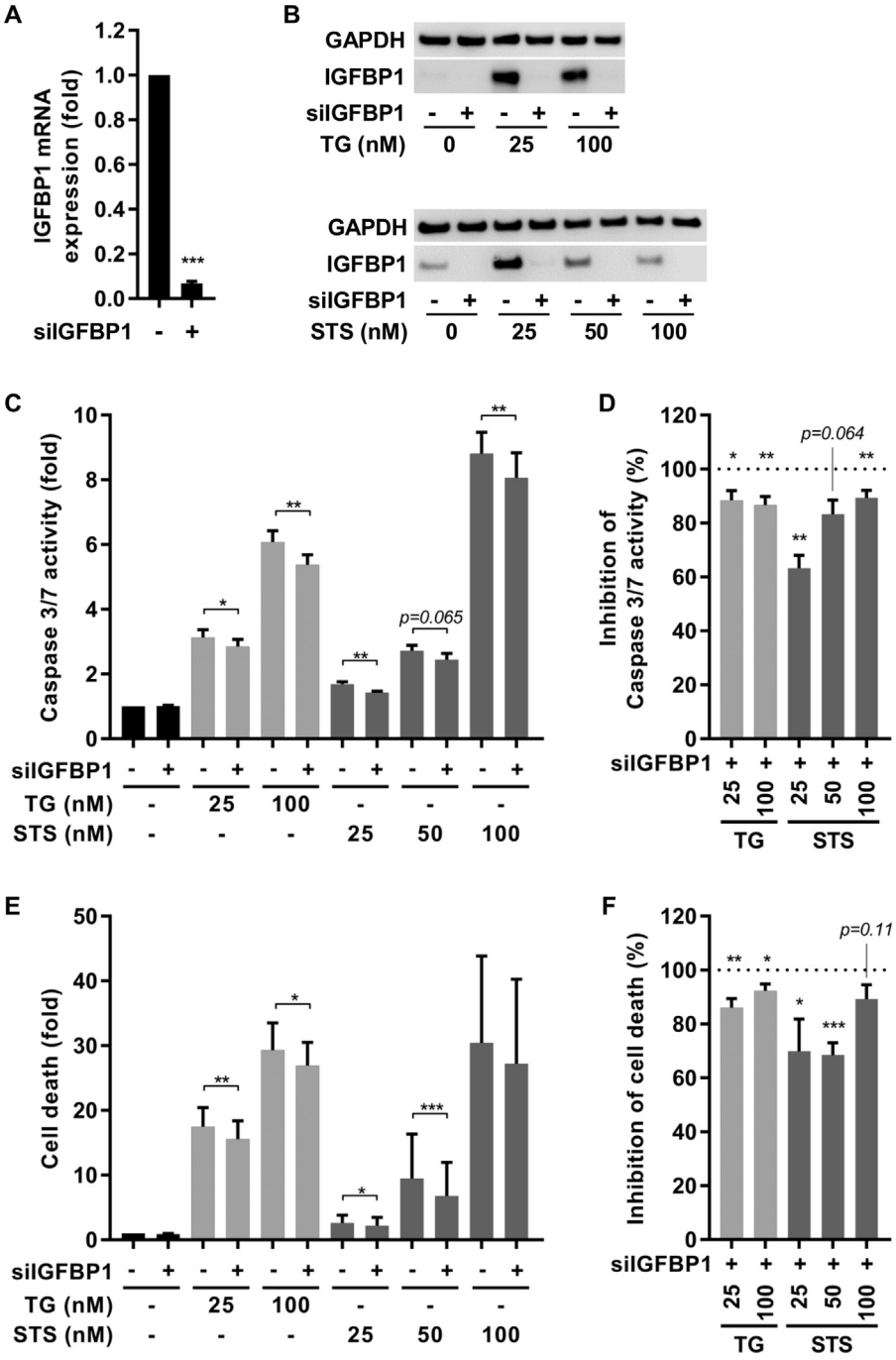

Fig. 3. Knockdown of IGFBP1 reduces apoptosis HepG2 tet-on cells were transfected with siIGFBP1 or siCTRL. Lysates were collected and apoptosis measured after 48 h. A: Verification of siRNA knockdown on mRNA level by RT-qPCR. Data are means ± SEM of 6 independent replicates. Statistics: two-tailed paired *Student's t-test*. B: Verification of siIGFBP1 knockdown by western blotting. Representative blot of 4 independent experiments are shown. C: Caspase-3/7 activity after 24 h of treatment with different concentrations of thapsigargin (TG) or staurosporine (STS). Data are means ± SEM of 7–8 independent experiments. D: Percent caspase-3/7 activity to controls after subtraction of basal caspase activity level. E: Cell death after 24 h treatment with different concentrations of TG or STS. Data are means ± SEM of 7–8 independent experiments. F: Percent cell death to controls after subtraction of basal cell death level. Statistics: two-tailed paired *Student's t-test* to individual controls.

It has been reported that the Tet-on system, even in the absence of doxycycline, causes low transcription from the tetracycline-inducible promoter [35]. In the case of the HBs/HBx cells this would give a low level of HBV replication. This fact could explain why no significant changes in gene expression were found between HBs/HBx and HBV cells. Thus, we cannot exclude the possibility that the observed changes in gene expression in the HBs/HBx cells are caused by a low level of HBV replication and not solely by the HBs and HBx proteins. However, the negative inverse expression correlation found between IGFBP1 and HBx nevertheless indicates that HBx alone is sufficient to modulate IGFBP1 expression in HepG2 cells.

We found that IGF-1 significantly reduced TG- and STS-induced apoptosis under serum free conditions, although the effect was only modest after treatment with TG. An explanation for this could be that the level of IGFBP1 was strongly increased in response to TG and STS treatment, potentially leading to a higher level of IGFBP1 in the media counteracting the effect of IGF-1 stimulation. Consistent with our results, IGFBP1 expression has previously been shown to dynamically change in response to ER stress [41] and pro-inflammatory cytokines [39]. In HepG2 cells upregulation of IGFBP1 expression may therefore be a general cellular response to inducers of stress and apoptosis. The fact that co-incubation with IGFBP1 and IGF-1 negated the protective effect of IGF-1 alone on TG-induced apoptosis suggests that in HepG2 tet-on cells, IGFBP1 can inhibit the anti-apoptotic effects of IGF-1. However, as co-incubation with IGFBP1 and IGF-1 failed to reverse the protective effect of IGF-1 alone on STS-induced apoptosis, IGFBP1 likely also has IGF-1-independent functions in HepG2 cells.

Although the observed reductions in TG- and STS-induced apoptosis mediated by knockdown of IGFBP1 or addition of IGF-1 may not appear pronounced, it is important to keep in mind that the development of

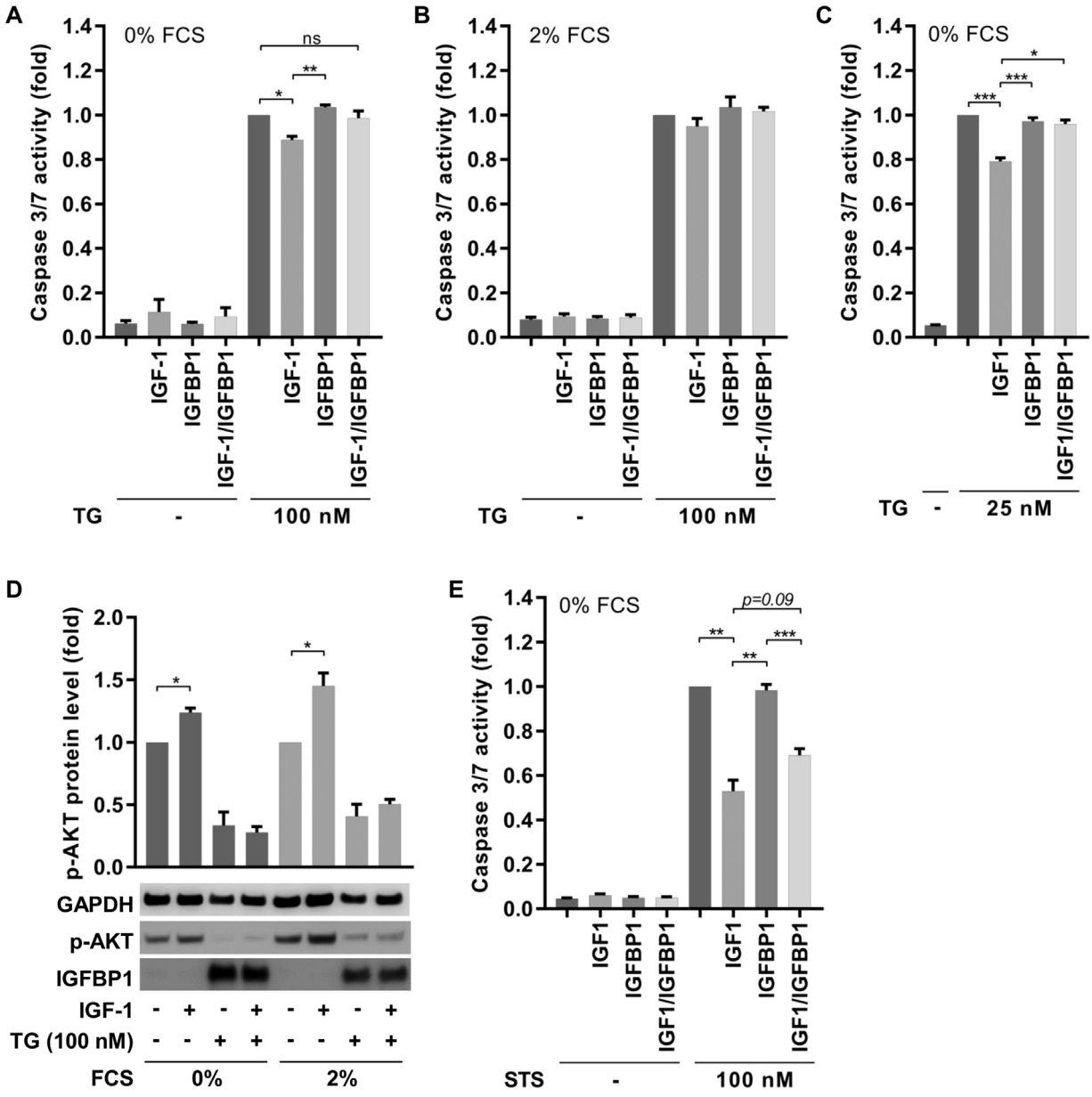

Fig. 4. IGFBP1 reverses the anti-apoptotic effect of IGF-1 Caspase 3/7 activity in HepG2 tet-on cells was measured after 48 h stimulation with recombinant human IGF-I and/or IGFBP1 in 0% FCS (A) and 2% FCS (B). Apoptosis was induced with 24-h thapsigargin (TG) (100 nM) treatment. Data are means ± SEM of 5 independent experiments. Statistics: RM one-way ANOVA, corrected for multiple comparisons (Bonferroni) C: Caspase 3/7 activity after 24 h of treatment with 25 nM TG. Data are means ± SEM of 7 independent experiments. Statistics: RM one-way ANOVA, corrected for multiple comparisons (Bonferroni). D: Protein levels of p-AKT and IGFBP1 after 48 h of stimulation with recombinant human IGF-I. TG (100 nM) was added to the media after 24 h. Representative blots of 3 independent experiments are shown. Quantification of p-AKT protein levels after IGF-I stimulation in 0% FCS (dark bars) and 2% FCS (light bars) (normalized to GAPDH). Data are means ± SEM of 3 independent replicates. Statistics: two-tailed paired *Student's t-test* between each set of -/+ IGF-I. D: Caspase 3/7 activity in HepG2 tet-on cells after 48 h of stimulation with recombinant human IGF-I and/or IGFBP1 in 0% FCS and staurosporine (STS) (100 nM)-induced apoptosis. Data are means ± SEM of 7 independent experiments. Statistics: RM one-way ANOVA, corrected for multiple comparisons (Bonferroni).

HBV-related liver diseases like HCC is a slow process going on for years. Therefore, even small changes in activity of the IGF1-signaling pathway may well contribute to the malignancies caused by CHB.

In conclusion, using a HepG2 HBV model system, our findings suggest that HBV via HBx suppresses host cell expression and secretion of IGFBP1 thereby allowing increased IGF-1-dependent and -independent pro-survival and anti-apoptotic effects that may contribute to HBV-associated liver malignancies including HCC.

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

KON, KSJ, TNW, DG, FP, BH and JS conceived and designed the

study. DG provided the doxycycline-inducible HBV-replicating hepatoma cell lines. KON performed the experiments. KON, AHM, SK and JS analyzed the data. AHM and SK performed the bioinformatics analyses. KON wrote the first draft of the manuscript and JS commented on this. KON revised it following significant scientific input from all authors.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2018.07.002.

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