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Hepatitis B virus-infected hepatocytes promote the secretion of collagen VI to the extracellular matrix

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Chronic hepatitis B virus (HBV) infection is a global health problem as it is the major cause of liver fibrosis and its complications cirrhosis and hepatocellular carcinoma. The role of virus–host interactions in liver fibrosis and progression to cancer remains poorly understood. Here we show that HBV infection of permissive cells trigger pathways relevant for extracellular matrix (ECM) remodeling, which is a hallmark of liver fibrosis. We demonstrate that collagen VI (ColVI) is secreted from infected cells and induces a profibrotic phenotype in patient-derived myofibroblasts and identified HBV-induced AKT signaling as a driver of ColVI expression in HBV-infected cells. Consistently, ColVI is upregulated in the liver of HBV patients with fibrosis. Our results suggest a role of ColVI as a driver of HBV-associated liver disease and highlight the potential of ColVI as a biomarker candidate and therapeutic target in HBV-infected patients.

Keywords Liver, Fibrosis, AKT signaling, Proteomics, Extracellular matrix

Chronic hepatitis B (CHB) represents the major risk factor for cirrhosis and hepatocellular carcinoma (HCC) worldwide[1,2]. The development of efficient antiviral strategies remains an unmet medical need. Hepatitis B surface antigen (HBsAg) loss, represented by a functional cure, is considered an ideal therapeutic outcome[3]. Moreover, one-third of the CHB population develops cirrhosis, liver failure, and HCC if left untreated[4]. Early detection of fibrosis and necroinflammation within the liver of patients with CHB is a key factor in disease management and cancer risk prognosis[5]. Although fibrosis regression may occur after long-term viral suppression, a sustained low level of Hepatitis B virus (HBV) DNA has been associated with fibrosis progression in patients treated with nucleos(t)ide analogues (NUCs)[6]. Moreover, the risk of HCC development can persist in both cirrhotic and non-cirrhotic patients, despite effective viral control[7].

During CHB, a combination of direct and indirect factors contributes to perturbed liver homeostasis, buildup of excessive extracellular matrix (ECM), and pro-oncogenic pressure[8,9]. At the cellular level, a hallmark of fibrosis is the transdifferentiation of hepatic stellate cells (HSCs), which lose their vitamin A-storing function and become ECM-deposing human liver myofibroblasts (HLMFs)[10]. HSCs activation is influenced by parenchymal and non-parenchymal liver cells such as hepatocytes, macrophages, Kupffer cells, and biliary epithelial cells[11–13]. In contrast to HCV, the role of HBV in HSC activation remains poorly understood, whereas its impact on HCC has been more widely investigated[14,15]. As urgently needed therapies targeting fibrosis are so far restricted[16], an understanding of hepatic intracellular communication and pathways that promote fibrosis is

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of fundamental importance to delineate novel therapeutic strategies against HBV-associated disease. Previous proteomics studies on HBV-related cirrhosis and HCC have led to the discovery of novel biomarkers and drug targets[17,18] and elucidated aspects associated with the natural history of chronic HBV infection[19].

To study the pathways dysregulated by HBV within hepatocytes, we aimed to profile proteomic changes in HepG2-NTCP cells, which is an HBV-permissive hepatoblastoma cell line expressing the NTCP receptor[20]. The HepG2 cell line retains features of normal hepatocytes and similar transcriptome and proteome profiles that differ at the level of drug-associated metabolism[21].

By assessing the entire proteome of HBV-infected cells, we revealed Collagen VI (ColVI) protein as a driver of HBV-associated liver fibrosis, which is pivotal for hepatocyte-myofibroblast interplay and ECM deposition in fibrotic livers.

Materials and methods

Cell lines, primary cells and virus

Production and purification of infectious HBV particles from the inducible human hepatoblastoma HepAD38 has been described[22,23]. HepaRG cells are immortalized liver progenitor cells that can differentiate to hepatocyte-like and cholangiocyte-like cells[24]. HepaRG cells were differentiated in 1.8% DMSO (Sigma, D2650)[25] and infected as previously described[26]. HepG2-NTCP and its infection with HBV have been described[27]. Primary HSCs were isolated as described[28]. Primary HSCs were obtained from four patients that underwent liver resection for colorectal liver metastasis, adenocarcinoma or alcoholic liver disease without cirrhosis or inflammatory infiltrates at the Department of Gastroenterology at the University Hospital of Strasbourg, France. All patients provided a written informed consent, the protocol followed the ethical principles of the declaration of Helsinki and was approved by the ethics committee of the University Hospital of Strasbourg and the local independent ethics committee (*comités de protection des personnes*). Isolated HSCs were cultured on collagen type 1-coated plate to minimize spontaneous differentiation[29]. HSCs were treated with ColVI or TGF-β, which is an inducer of HSC differentiation to HLMFs[30] (Fig. 4a,b). In a different experiment, patient-derived HSCs were treated with conditioned media from HepG2-NTCP transfected with pColVI and pDest (Fig. 4a,c).

Proteomic analysis

To provide greater depth and increase sensitivity for mass spectrometry analysis, we upscaled HBV infection to obtain 1.5 mg of protein yield per condition. To achieve temporal resolution of the complete HBV life cycle, protein expression was analyzed on day 2 and day 10 post-infection (pi) to define targets and pathways associated with early- and late-stage infection steps. HepG2 NTCP cells were plated in F175 flasks and infected after 24 h with HBV genotype D (MOI of 500, as measured in genomic equivalents by qPCR), as described[22,31], up to 10 days post-infection (pi). Protein lysates for total proteomics were obtained as described[32]. Samples for proteomic analysis were prepared at the Max Delbrück Center (MDC) for Molecular Medicine/Berlin Institute of Health (Berlin, Germany) using tandem mass tag (TMT) labeling[32]. Proteomics was performed on two biological replicates for each condition (HBV and NI). Each sample was labeled with 100 µg of peptide, randomly assigned to TMT10 channels, while channels 8 and 10 were left unlabeled. After pooling all channels, peptides were fractionated into 24 fractions using an UltiMate 3000 system (Thermo Fisher Scientific) and analyzed on a Q Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific) connected to an EASY-nLC 1200 system (Thermo Fisher Scientific). MaxQuant version 1.6.0.13[33] was utilized, employing MS2-based reporter ion quantitation and a PIF filter threshold of 0.5. Carbamidomethylation was designated as a fixed modification, while deamidation of asparagine and glutamine as well as oxidation of methionine were considered variable modifications. For protein quantitation, only non-contaminant proteins, and protein groups identified by at least two peptides, including at least one unique peptide, were retained. Corrected reporter ion intensities were log2 transformed and median-MAD normalized. Differential abundance analysis was conducted by applying two-sample moderated t-statistics using the limma package in R software (version R 4.3.3)[34].

Statistics and reproducibility

Proteomics was performed on two biological replicates for each condition (HBV and NI). Here, two-sample moderated t-statistics were applied using the limma package in R software (version R 4.3.3). Transcriptomics data from patients and from HBV-infected PHHs were retrieved from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. For the transcriptomic data, median-centered values for each sample were plotted to check if samples were cross-comparable. To make all the samples having the same value distribution, quantile normalization was applied (normalizeBetweenArrays function, limma package). Normality tests (D'Agostino-Pearson, Anderson–Darling, Shapiro–Wilk, Kolmogorov–Smirnov tests) were carried out as well as a homogeneity of variance test (Bartlett's test). For non-normal distributed data, Mann–Whitney test (between two groups) or Kruskal–Wallis test (for multiple comparison) were employed. When the assumptions for normality test and homogeneity of variance were met, one-way ANOVA was employed for multiple comparison (*COL6A1* expression across G0 to G4 groups of patients). Dunnett's Test was then used as post hoc analysis to compare multiple groups. For in vitro and ex vivo experiments, statistical analyses were performed with the Prism software (version 7.00; GraphPad Software Inc.). Except for HBV infection measurement (Fig. 1b), statistical analysis was performed on three biological replicates (n = 3), each consisting of three technical replicates per condition (all data points considered), unless otherwise specified.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Transcriptomic datasets were retrieved from the NCBI GEO database. HBV-infected PHH (GEO dataset GSE69590)[35] and HBV-associated fibrosis (GEO dataset GSE84044)[36] were used for bioinformatic

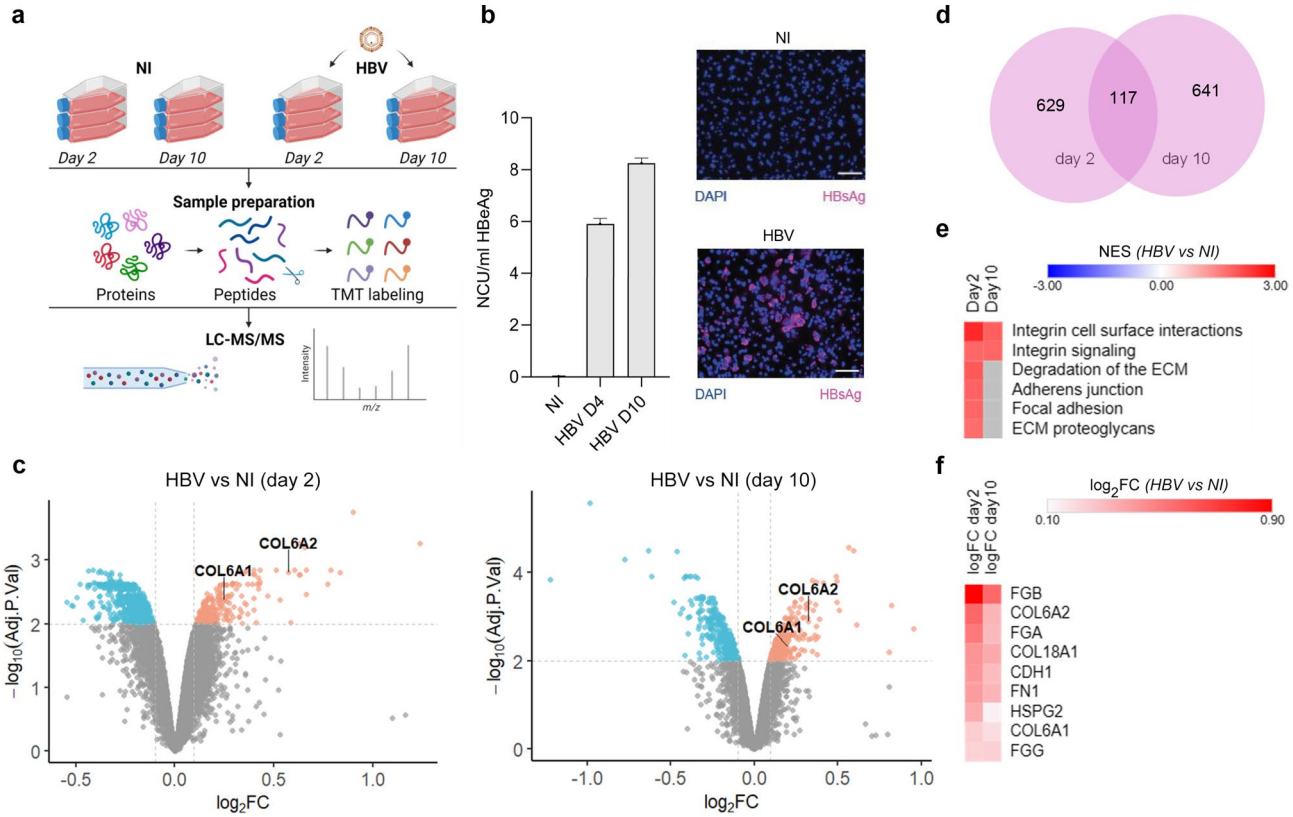

Fig. 1. HBV infection of permissive cells dysregulates pathways of cell adhesion and extracellular matrix remodeling. (**a**) Temporal proteomic analysis of HBV-infected HepG2-NTCP cells after days 2 and 10 pi using TMT labeling and LC–MS mass spectroscopy. Created in BioRender. Lupberger, J. (2024) *BioRender.com/q87k809*. (**b**) HepG2-NTCP cells infected with a MOI 500 with HBV genotype D from (a) led to a productive HBV infection according to HBeAg secretion (left panel) and HBsAg-positive cells after 10 days (right panel) according to HBsAg production. Scale bar: 100 µm. (**c**) Differentially expressed proteins in HBV-infected HepG2-NTCP vs non-infected cells (Mock) expressed in log2FC at day 2 (left) and day 10 (right) represented in volcano plot relative to adjusted *P* values (adj.P). (**d**) Overlap of significant differentially expressed proteins on day 2 and day 10 pi. (**e**) GSEA of pathways linked to cell–cell adhesion and ECM. Red = significantly upregulated (FDR < 0.05), grey = not significant. (**f**) Leading edge genes of the enriched gene set REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS in (**e**). NES: normalized enrichment score. Heatmaps created using *Morpheus heatmap* (Broad Institute).

analysis[37,38] after log2 transformation and normalization using RStudio. The clinical information of the 124 patients analyzed in GSE84044 can be retrieved from Supplementary Table S1 in the underlying study[36]. The generated mass spectrometry proteomics data were deposited to the ProteomeXchange consortium via the PRIDE[39] partner repository with the dataset identifier PXD051443. Additional data related to this paper may be requested from the authors.

Results

A proteomic analysis of HBV-infected cells reveals dysregulated components of cell–matrix communication

Chronic injury has a profound impact on cellular homeostasis and regulatory pathways in the liver[8,40]. To study HBV-induced dysregulation of cellular pathways, we assessed the proteomic profile of HBV-infected HepG2-NTCP cells using large-scale LC–MS-based proteomics (Fig. 1a). We used this cell line because it is suitable for robust and long-term culture, and it represents a reproducible model for HBV infection, supporting the complete life cycle of HBV infection[20]. Infection was confirmed by accumulating HBeAg levels in the supernatant (Fig. 1b, left) and by immunofluorescence analysis of intracellular HBsAg levels on day 10 post infection (pi) (Fig. 1b, right).

Proteomic analysis identified 7611 proteins (Table S1), demonstrating an excellent sensitivity comparable to published studies[32,41–43] and providing a valid protein atlas of persistent HBV infection within HepG2-NTCP. A cutoff of adjusted *P* value (adj.P) of < 0.01 was applied to identify targets significantly dysregulated throughout the infection period (Fig. 1c), identifying 746 and 758 significantly dysregulated proteins at day 2 and day 10 pi, respectively. 117 proteins were significantly dysregulated on both days 2 and 10 pi (Fig. 1d). Among the significant targets, we validated already known factors in the HBV life cycle, including apolipoprotein E (APOE)

(Table S1), which is a known HBV host factor[44]. In terms of viral pathogenesis, we observed that fibronectin 1 (FN1) and plasminogen (PLG) were upregulated by HBV infection (Table S1), which has been previously associated with HBV-related liver disease and liver failure[45,46].

HBV infection upregulates pathways linked to integrin-cell surface interaction and ECM remodeling

We applied gene set enrichment analysis (GSEA)[38,47–49] on the totality of 7611 detected proteins to identify pathways and associated biological functions perturbed by HBV infection. GSEA classifies differentially expressed genes according to their representation within a predefined gene set related to a phenotype. These dysregulated gene sets were both significantly (FDR < 0.05) upregulated and downregulated by HBV during the course of infection, as shown in the supplementary information (Table S2). Strikingly, we observed that HBV induces upregulation of pathways linked to ECM remodeling and integrin signaling within hepatocytes, indicated as REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS (integrin cell surface interaction) and REACTOME_INTEGRIN_SIGNALING (Integrin signaling) during the course of infection (Fig. 1e). Integrin cell surface interaction signaling appeared to be the most upregulated pathway in our analysis on day 2 pi (Table S2) and among the top upregulated hits at day 10 pi (Table S3). This finding is in line with previous studies that have uncovered integrin subunit β-like 1 (ITGBL1) as a key activator of fibrogenesis in the liver of patients with HBV[36]. ECM remodeling comprises proteins involved in the composition and degradation of the ECM and cell–matrix interactions, which are intricately linked to liver fibrosis progression and HSCs[50].

Collagen VI is upregulated in HBV-infected hepatocytes and associated with disease progression

Integrins are transmembrane receptors that facilitate adhesion between cells and the ECM, particularly collagen, which is a major component of the ECM[51]. Interestingly, our data indicate that HBV strongly induces the integrin cell surface interaction pathway in hepatocytes (Fig. 1e, Table S2-3), which was the most upregulated pathway in our analysis. To further dissect this observation, we identified nine leading-edge proteins driving the observed enrichment of the REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS at days 2 and 10 pi, including the alpha-1 and alpha-2 chains of ColVI (encoded by the genes *COL6A1* and *COL6A2*, respectively) (Fig. 1f).

As integrins are collagen receptors[51], we hypothesized a role for ColVI in HBV pathogenesis. First, we validated the significant ($p < 0.05$, unpaired t test) HBV-induced ColVI upregulation in HepG2-NTCP and differentiated HepaRG cells (dHepaRG) by flow cytometry analysis (Fig. 2a,b). To investigate whether ColVI is secreted by hepatocyte-like cells, we infected dHepaRG cells with HBV for 10 days and replaced the cell culture medium every 2–3 days. Consistent with our observations in HBV-infected cells (Fig. 2a,b), ColVI secretion was upregulated by HBV in the cell culture medium during the time course (Fig. 2c). Consistently, *COL6A1*

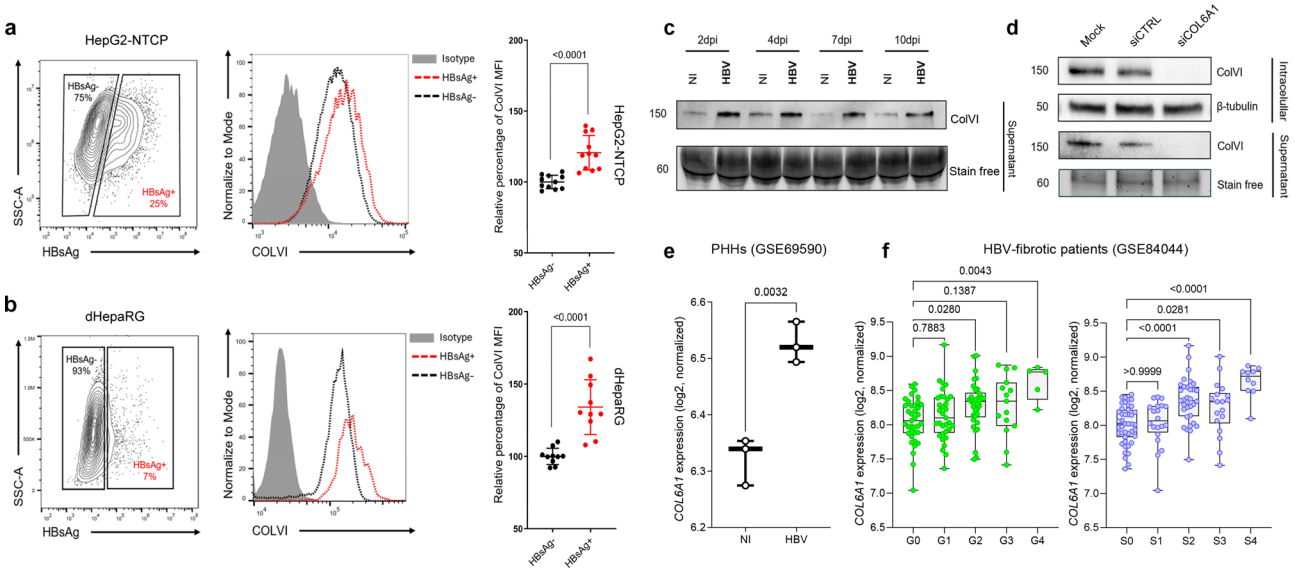

Fig. 2. ColVI is upregulated in HBV-infected hepatocytes and patient livers. (**a**, **b**) Flow cytometry analysis of permeabilized HepG2-NTCP (**a**) or dHepaRG (**b**). HBsAg-positive cells express significantly more ColVI compared to HBsAg-negative cells (Unpaired t test). (**c**) ColVI secretion by dHepaRG cells is promoted by HBV infection. Cell culture medium replaced every 2–3 days relative to stain free. (**d**) Knock-down of *COL6A1* in HepG2-NTCP cells abrogates ColVI secretion to the cell supernatant. (**e**) *COL6A1* transcripts are elevated in PHH infected for 40 h with a MOI of 50 vs NI (Unpaired t test). Data analyzed from GSE69590. (**f**) *COL6A1* is upregulated in patient livers with advanced fibrosis (S) (Kruskal–Wallis test) and inflammation (G) (Ordinary one-way ANOVA) according to Scheuer score[52] Data analyzed from GSE84044. Transcriptomic data in (**e**,**f**) were log2 transformed and normalized.

knockdown in uninfected HepG2-NTCP impaired the secretion of endogenous ColVI protein in cell lysates and cell culture supernatant 3 days after silencing (Fig. 2d). To investigate ColVI expression in primary cells and patients, we analyzed transcriptomic data. PHHs infected with HBV (GSE69590)[35] expressed significantly more ColVI than non-infected (NI) cells (log2 expression, unpaired t test) (Fig. 2e). To study the *in vivo* relevance of our findings, we analyzed 124 liver biopsies from CHB patients (GSE84044)[36]. Liver biopsies were classified into five classes (0 to 4) for grade (G) and stage (S) according to the Scheuer system[52]. Here, HBV-induced *COL6A1* expression was directly associated with higher fibrosis (S) and inflammation (G) Scheuer scores compared to S0 and G0 (ordinary one-way ANOVA), respectively (Fig. 2f), demonstrating an association between HBV-induced hepatic ColVI expression and liver disease progression in HBV patients.

HBV upregulated ColVI in hepatocyte-like cells via activation of AKT signaling

To identify the drivers of HBV-induced ColVI expression and secretion by hepatocytes, we analyzed the promoter region of the *COL6A1* gene. We identified a series of SP1 (110 sites), AP2 (45 sites), and AP1 (27) transcription factor-binding sites, all of which are transcription factors activated by AKT signaling[53–55]. To test this hypothesis, we used the AKT activator sc79, which specifically increased AKT phosphorylation at serine 473 (Fig. 3a). AKT activation induced ColVI expression in dHepaRG cells (Fig. 3b, Supplementary Fig. S1), which was attenuated by the AKT inhibitor MK-2206 (Fig. 3b). As HBx protein has been reported to activate AKT[56], we validated that HBV infection indeed induces AKT signaling in our infection models (Fig. 3c,d). Moreover, HBV-induced ColVI expression was partially attenuated by the AKT inhibitor, MK-2206 (Fig. 3c,e, Supplementary Fig. S2), suggesting that AKT-responsive transcriptional elements are responsible for HBV-induced ColVI expression in hepatocytes. Interestingly, while inhibitors of AKT signaling do not attenuate HBV entry or infection (Supplementary Fig. S3)[57,58], MK-2206 has been shown to reduce fibrosis and inflammation by inhibiting AKT[59,60].

Secreted ColVI promotes patient-derived myofibroblast differentiation

Crosstalk between hepatocytes and non-parenchymal cells (NPCs) is a major determinant of liver homeostasis and pathogenesis[61–63]. HSCs are characterized by an elongated morphology that allows the establishment of cell–cell contacts with adjacent hepatocytes through the Disse space[64]. In liver injury, HSCs activate and differentiate into HLMFs, which are largely responsible for ECM deposition during wound healing, chronic liver injury, and fibrosis[12]. Secreted ColVI may therefore contribute to the ECM and the activation of HLMFs via membrane receptor interaction. HBV-infected cells secrete not only ColVI but also a larger array of pro-inflammatory factors that impact stellate cells[65], as also indicated by our GSEA analysis showing upregulation of the TGB-β receptor complex pathway (REACTOME_SIGNALING_BY_TGF_BETA_RECEPTOR_COMPLEX) (Table S2). Therefore, we incubated serum-starved, patient-derived HLMFs with recombinant ColVI or conditioned medium from recombinant ColVI-expressing cells (pColVI) and assessed the expression of pro-fibrotic marker genes (Fig. 4a). Similar to TGF-β1, treatment of HLMF with recombinant ColVI promoted *ACTA2* and *COL1A1* expression (Kruskal–Wallis test), which are markers for HLMF activation[66,67] (Fig. 4b). To investigate whether secreted ColVI can also activate HLMFs, we collected conditioned cell culture medium from HepG2-NTCP cells expressing ColVI (pColVI) and incubated serum-starved HLMFs (Fig. 4c). Consistently, we observed

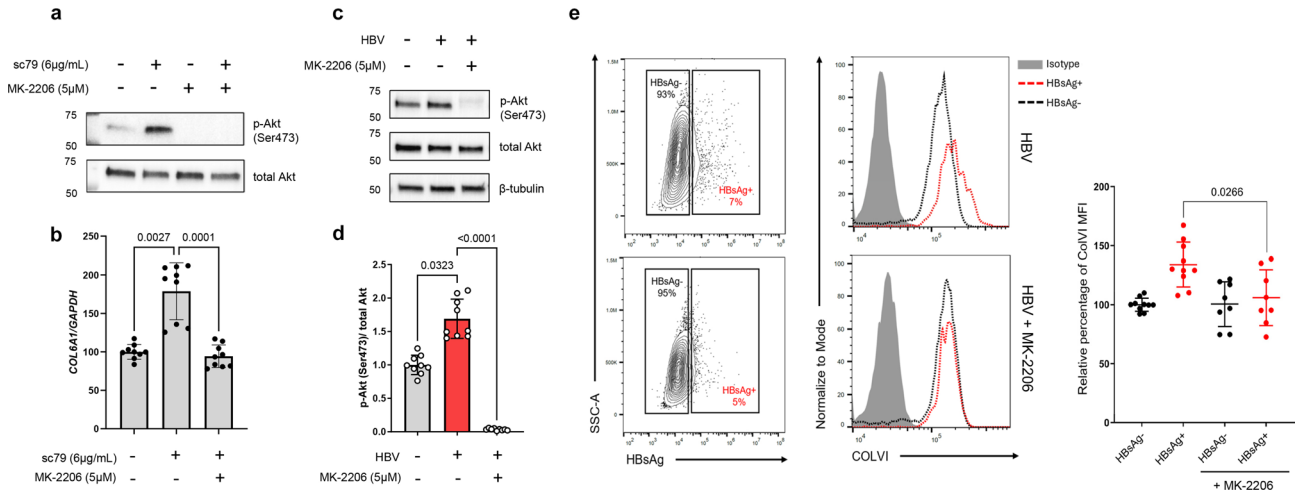

Fig. 3. HBV induces ColVI expression via AKT signaling. (**a**,**b**) sc79 phosphorylated Akt in HepaRG cells, which is inhibited by MK-2206. (Kruskal–Wallis test, multiple comparison) (**a**) Western blot of dHepaRG cells pre-treated for 1 h with MK-2206 prior stimulated for 1 h with sc79 or a combination of sc79 and MK-2206. (**b**) qPCR of dHepaRG cells pre-treated for 1 h with MK-2206 prior stimulated for 24 h with sc79 or a combination of sc79 and MK-2206. (**c**,**d**) p-AKT is induced in HBV-infected dHepaRG cells 2 days pi, which is attenuated in presence of MK-2206. (**e**) ColVI is induced by HBV infection in dHepaRG cells and attenuated by MK-2206 treatment (Mann–Whitney test). Cells have been infected with HBV for 10 days and treated at day 1 pi with MK-2206 prior FACS analysis and HBeAg ELISA (Supplementary Fig. S3).

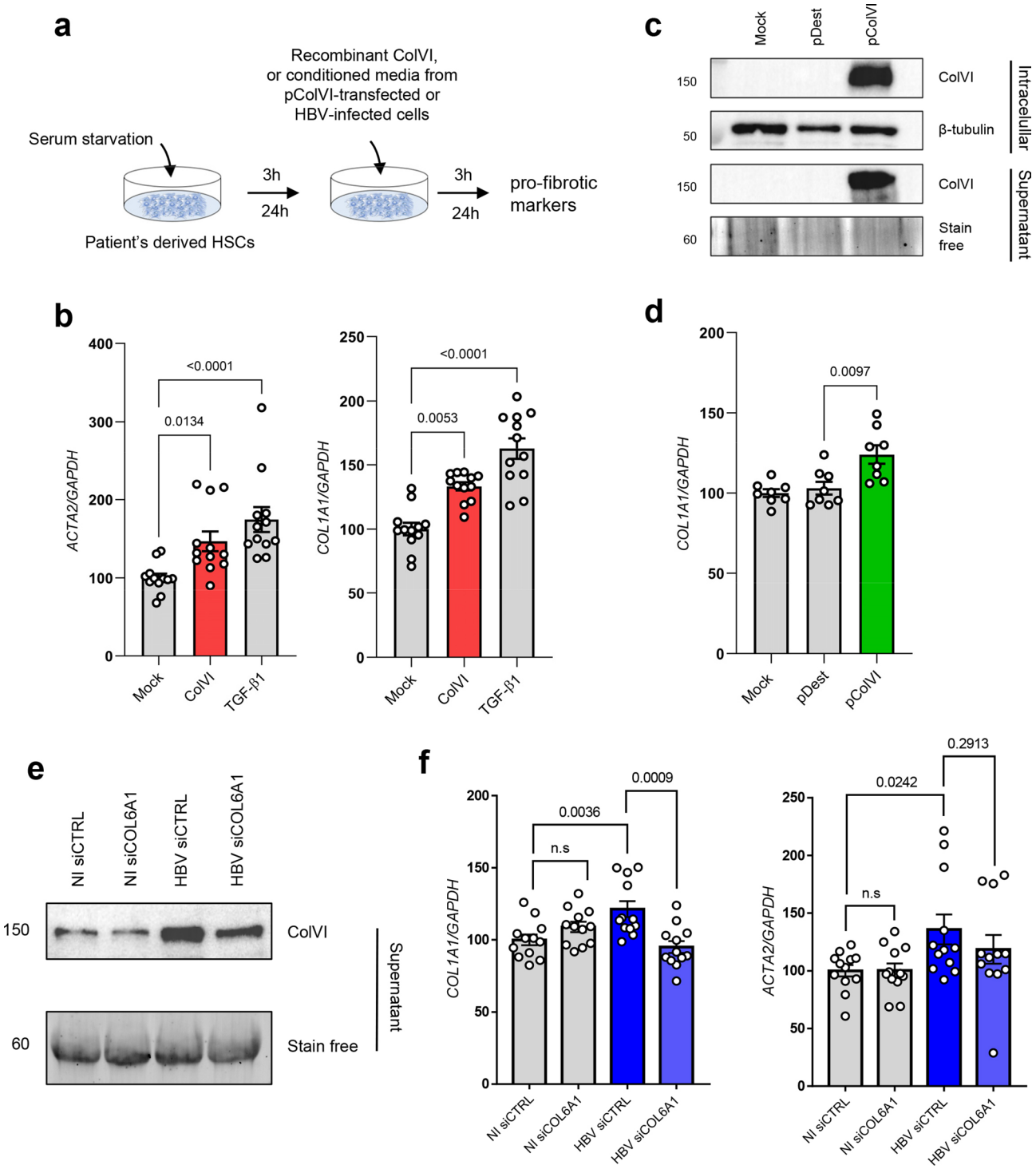

Fig. 4. Secreted ColVI induces activation of patients-derived liver myofibroblasts. (**a**) Patient-derived HLMFs were serum-starved and treated with ColVI and conditioned medium from pColVI-transfected HepG2-NTCP cells in separate experiments. After 3 h, pro-fibrotic markers were quantified by RT-qPCR. (**b**) Treatment for 4 h with recombinant ColVI (0.5 µg/mL) induces activation of HLMFs via *ACTA2* (Krustal-Wallis test) and *COL1A1* expression (Krustal-Wallis test). (**c**) pColVI-transfected HepG2 cells show an increase of ColVI in both cytosol and supernatant by western blot. (**d**) Supernatant from pColVI-transfected HepG2-NTCP cells increased *COL1A1* expression in the patient-derived HLMFs (Unpaired t test). (**e**) ColVI levels in supernatants of HepG2-NTCP cells infected with HBV for 7 days and transfected for 4 days pi with siCOL6A1 or siCTRL. (**f**) Supernatant from HBV-infected cells (**e**) increased *COL1A1* and *ACTA2* expression in the patient-derived HLMFs, while partially reduced in HLMF incubated with supernatants of ColVI-silenced cells (Mann–Whitney test).

a significant upregulation of the activation marker *COL1A1* in HLMFs exposed to the conditioned medium from ColVI-expressing HepG2-NTCP compared to the control supernatants (pDest) or mock (unpaired t test) (Fig. 4d). In the context of HBV-infected cells, we demonstrated the activation of patient-derived HLMF when exposed to HBV-infected cell supernatants (Fig. 4e,f). Because HBV-infected cells may harbor additional factors capable of activating HLFMs, we combined HBV infection with siRNA specific for ColVI (siCOL6A1). While silencing of ColVI in HepG2-NTCP with established HBV infection (4 days pi) had no effect on HBV replication (Supplementary Fig. S4), it strongly decreased ColVI levels in the cell culture supernatants (Fig. 4e). Importantly, however, exposure of HLMF to ColVI-silenced supernatants of HBV-infected cells, however, did partially reduced HLMFs activation compared to supernatants of control-silenced cells (Fig. 4f). Together, these results demonstrate that ColVI is secreted by hepatocytes and able to promote HLMF activation.

Discussion

Chronic HBV infection is the main cause of liver fibrosis and HCC worldwide. Despite being an effective vaccine, CHB can only be controlled but is rarely cured. Importantly, loss of HBsAg (functional cure) is hardly achieved with the current antiviral strategies[68] and viral control cannot fully prevent fibrosis progression in CHB patients[69]. To identify mechanisms related to HBV pathogenesis, we generated a temporal proteomic atlas of persistent HBV infection in permissive cells.

A key question in this study was whether HBV infection induces significant dysregulation of cellular pathways within hepatocytes, as only these cells are infected by HBV. PHH are considered the gold standard for *in vitro* experiments[70] however, they are limited by a low infection rate and donor-to-donor variability. To overcome this, we employed a well characterized cell model of HepG2-NTCP cells, which is permissive to HBV infection[20] and allows for high infection rates[25].

Using this model, we revealed that HBV infection causes both the upregulation and downregulation of a series of signaling pathways associated with clathrin-mediated endocytosis, integrin signaling, and extracellular matrix remodeling (Table S2-3, Fig. 1). The identification of pathways related to focal adhesion and adherens junctions strongly indicated that HBV manipulates the hepatocyte cytoskeleton and cell morphology to facilitate its own transport, as previously suggested[71,72]. Importantly, a strong upregulation of integrin-cell surface interactions and ECM remodeling characterizes HBV infection in our model. Although a plethora of studies have demonstrated that activated HSCs are the main effector cells of liver fibrosis and ECM deposition[73], stressed or injured hepatocytes also contribute to the establishment of a pro-fibrotic and pro-inflammatory environment by the secretion of damage-associated molecular patterns (DAMPs), IL-33, and other molecules that trigger the trans-differentiation process of HSCs, as previously described[74]. Moreover, upregulation of specific components associated with ECM has already been observed in rat hepatocytes[75]. Consistently, our data suggest that HBV-infected hepatocytes contribute to ECM formation by inducing ColVI expression.

One of the most remarkable findings of our study was the discovery of the pro-fibrotic role of ColVI, which is secreted by HBV-infected hepatocytes and trigger a pro-fibrotic phenotype in primary HLMFs. ColVI forms a tetrameric structure in the ECM composed of alpha-1 and alpha-2 chains as essential ColVI subunits, with a third subunit that can be alpha-3, alpha-4, alpha-5, or alpha-6, as described previously[76]. In addition to its role in fibrogenesis in several organs and tissues, ColVI is known to activate signaling pathways, such as AKT and ERK integrin-mediated signaling pathways[77,78]. We suggest that ColVI treatment may accelerate fibrosis progression by activating patient-derived HSCs into differentiated HLMFs, which is consistent with the effects of ColVI treatment observed on HSCs from rodents[79]. In addition, the role of ColVI as an activator of HSCs has been observed in cardiac tissues[80]. Consistent with this result, we demonstrated that the supernatant of HepG2-NTCP overexpressing ColVI acts as an inducer of HLMFs. Depending on the studied infection model we observed an increase of intracellular ColVI transcripts ranging from ~ 15% in HBV-infected PHHs (Fig. 2e) to 25–30% in infected HepG2-NTCP and dHepaRG (Fig. 2a,b), respectively. A limitation of our study is that even though the evident association of ColVI with liver fibrosis stages in patients (Fig. 2f), the effective local concentrations of secreted ColVI in HBV-infected patient livers are difficult to assess. While our in vitro HBV infection model combined with ColVI perturbation clearly suggests a functional role of ColVI in the activation of patient-derived HLMFs (Fig. 4e,f), infected cells do also secrete additional factors promoting liver fibrosis[81,82]. It remains thus the possibility that local ColVI levels around infected hepatocytes in patient livers may have an auxiliary effect on HLMF activation in the context of the generally pro-fibrotic microenvironment of HBV infection *in vivo*.

Our study reflects the impact of HBV infection on ECM remodeling and integrin-cell surface interaction in hepatocytes linked to dysregulation of circuits that converge on the activation of AKT signaling during the entire course of HBV life cycle infection. We found that the mechanism of action associated with ColVI expression in HBV-infected cells involves activation of the AKT signaling pathway, which we modulated using a specific activator and inhibitor. Although our perturbation studies (Fig. 3c,d) clearly highlight the important role of AKT signaling in HBV-induced ColVI expression, we cannot exclude that crosstalk with additional pathways may contribute to the elevated ColVI levels observed in patient livers, especially in the context of a pro-inflammatory microenvironment (Fig. 2f). The AKT pathway is central to many cellular mechanisms, including metabolism and survival, and its activation has been associated with HBV infection and interaction with HBV proteins[56,83]. The link between AKT signaling and fibrosis development in HBV infection is in line with the fact that an increased prevalence of fibrosis occurs in metabolic dysfunction-associated fatty liver disease (MAFLD) patients with HBV compared to those without HBV infection[84]. Altogether, these findings can pave the way for the design of new anti-fibrotic strategies that can target ColVI receptors in HSCs.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Transcriptomic datasets were retrieved from the NCBI GEO database. HBV-infected PHH (GEO dataset GSE69590) and HBV-associated fibrosis (GEO dataset GSE84044) were used for bioinformatic analysis after log2 transformation and normalization using RStudio. The clinical information of the 124 patients analyzed in GSE84044 can be retrieved from Supplementary Table S1 in the underlying study. The generated mass spectrometry proteomics data were deposited to the ProteomeXchange consortium via the PRIDE partner repository with the dataset identifier PXD051443. Additional data related to this paper may be requested from the authors.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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