Targeted HBx gene editing by CRISPR/Cas9 system effectively reduces epithelial to mesenchymal transition and HBV replication in hepatoma cells

Preety Rawal
Gautam Buddha University https://orcid.org/0000-0003-4177-5139
Dinesh M Tripathi
Institute of Liver and Biliary Sciences
Hamed Hemati
Institute of Liver and Biliary Sciences
Jitendra Kumar
Institute of Liver and Biliary Sciences
Shiv K Sarin
Institute of Liver and Biliary Sciences Department of Hepatology
Vikrant Nain
Gautam Buddha University
Savneet Kaur (savykaur@gmail.com)
Institute of Liver and Biliary Sciences https://orcid.org/0000-0002-9863-2772

Research Article

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Abstract

Background and Aims: Hepatitis B virus X protein (HBx) play a key role in pathogenesis of HBV-induced hepatocellular carcinoma (HCC) by promoting epithelial to mesenchymal transition (EMT). In this study, we hypothesized that inhibition of HBx is an effective strategy to combat HCC.

Methodology and Results: We designed and synthesized novel HBx gene specific single guide RNA (sgRNA) with CRISPR/Cas9 system and studied its *in vitro* effects on tumor properties of HepG2-2.15. Full length HBx gene was excised using HBx-CRISPR that resulted in significant knockdown of HBx expression in hepatoma cells. HBx-CRISPR also decreased levels of HBsAg and HBV cccDNA expression. A decreased expression of mesenchymal markers, proliferation and tumorigenic properties was observed in HBx-CRISPR treated cells as compared to controls in both 2D and 3D tumor models. Transcriptomics data showed that out of 1159 differentially expressed genes in HBx-CRISPR transfected cells as compared to controls, 70 genes were upregulated while 1089 genes associated with cell proliferation and EMT pathways were downregulated.

Conclusion: Thus, targeting HBx by CRISPR/Cas9 system effectively downregulates HBx gene expression, HBsAg production and mesenchymal characteristics of HBV-HCC cells and holds potential as a therapeutic approach for HBV-HCC.

Introduction

Chronic hepatitis B virus (HBV) infection is the major risk factor for the development of hepatocellular carcinoma (HCC) owing to several factors and mechanisms governing tissue inflammation, cirrhosis and direct viral oncogenesis [1–2]. The hepatitis B virus X gene (HBx), one of the four open reading frames of HBV, encodes a 17 kDa protein which is strongly associated to the development of HCC [3]. HBx is a multifunctional transactivator protein that activates cellular signaling pathways and has been reported to be essential for HBV infection [4–5] and transcription [6]. HBx has been shown to modulate a wide range of cellular functions, including proliferation, the cell cycle, apoptosis, autophagy, metastasis, and metabolism, which lead to the development of HCC [7–9]. In our previous study, we have documented that mesenchymal genes, Thy-1 and CDH2 expression were significantly enhanced in TGF-β1 treated HBx-Huh7 cells [10]. HBx in presence of TGF-β1 has also been reported to cause the activation of normal liver cells into liver cancer stem cells (CSCs), promote epithelial to mesenchymal transitions (EMT) and progression of HCC in chronic HBV patients [11]. Recent studies have also shown that interaction of HBx with chromatin remodelling proteins (Par14/Par17) stabilizes HBx and promotes its translocation to the nuclear and mitochondrial fractions, and increases HBV replication including cccDNA formation, HBV RNA and DNA synthesis, and virion secretion [12]. Given the pleiotropic roles of HBx in HCC progression, an inhibition of HBx expression might constitute an effective strategy to stall the development and progression of HCC. In the present study, we designed specific single guide RNAs (sgRNAs) against HBx and developed a HBx-specific CRISPR/Cas9 system to establish HBx knockdown in HCC cell lines. We

evaluated the effects of HBx knockdown on viral replication and key tumor attributes in HBV-hepatoma cell lines in both two- and three- dimensional (2D and 3D) tumor models.

Material And Methods

2.1 Designing of HBx-CRISPR

In silico, we first designed sgRNA against HBx by CRISPOR web based tool with minimal off target activity. SgRNA was synthesized by GenScript, cloned in non-viral eSpCas9-2A-Puro(PX459)V2.0 vector (HBx-CRISPR) and confirmed by the molecular digestion. For control, we used non-targeting control gRNA (Addgene# 80205) CRISPR vector (Control vector).

2.2 Cell cultures: HepG2.2.15 and HepG2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (GIBCO) with 10% FBS (Hyclone) and 100µg/ml streptomycin and 100 IU/ml penicillin (GIBCO) at 37℃ in incubator containing 5% CO_2.

2.2.1 *Transfection in Hepatoma Cell Lines*

HepG2-2.15 cells were transfected with HBx-CRISPR (HepG2.2.15-HBx-CRISPR) and Control vector (HepG2.2.15-Ct) and HepG2 cells were co-transfected with HBV plasmid (HepG2-HBV) (Addgene# 65462, received as gift from Prof. Vijay Kumar) and HBx-CRISPR plasmid (HepG2-HBV-HBx-CRISPR). For transfection, lipofectamine 2000 (ThermoFisher Scientific, Invitrogen #11668-019) was used according to manufacturer's instructions. As a control, untreated HepG2 cells were used in experiments. Forty-eight hours after transfection, the cells were seen under an inverted microscope (Nikon ECLIPSE Ti).

2.3 HBV cccDNA expression: To examine the knockdown effects of HBx-CRISPR on the formation of HBV cccDNA, cccDNA was extracted using the Hirt's protein-free DNA extraction procedure with minor modifications [13]. DNA samples were digested with T5 Exonuclease (New England Biolabs, cat no# M03635) (reaction mixture containing 5µl eluted DNA, 1µl 10X reaction buffer, 0.5µl T5 Exo, and 3.5µl water) at 37°C for 1 h and 70°C for 20 min were subjected to cccDNA qPCR [14]. The forward primer was 5'-TGCACTTCGCTTCACCT-3', and the reverse primer was 5'-AGGGGCATTTGGTGGTC-3'. The following cycling parameters were used: start at 95℃ for 5 min, denaturing at 95℃ for 10s, annealing at 62℃ for 25s, elongation at 72℃ for 30s, and a final 5 min extra extension at the end of the reaction and repeated for 45 amplification cycles.

2.4 Three-dimensional spheroid cultures: Tumor spheroids were generated by seeding 4000 cells/well (Control-transfected and HBx-CRISPR transfected) in 6 well ultra-low attachment culture plates (Corning Inc. New York, NY, USA), culturing them in complete media, after 3 days complete media replaced by serum free media. Spheroids were further grown on ultra-low attachment plates for 2 weeks to make 3D anchorage-independent models which were imaged by NIKON Eclipse Ti. The average size of the spheroids was measured using the ImageJ Software. The hanging drop method was utilized to assess

tumorigenic potential of HCC tumor spheroids [15–16]. For full methodology, please refer supplementary file.

Detailed methodology of transwell assay, scratch assay, colony formation assay, ELISA assay, immunophenotyping assays, quantitative RT-PCR assay and RNA Seq studies are mentioned in supplementary data sheet.

2.5 Statistical Analysis: All quantitative data were expressed as mean ± standard deviation. Statistical significance was considered when $p \leq 0.05$(*), $p \leq 0.01$(**) or $p \leq 0.001$(***). Student's unpaired t-tests were used to analyze and compare the groups.

Results

3.1 Selection and synthesis of HBx-specific sgRNAs and in vitro efficacy: To generate HBx knockdown in HBV-positive HCC cell lines, we first analyzed sequences in the viral genome (HBx ORF) to identify potential sgRNAs adjacent to CRISPR associated protein 9 (Cas9) target sequences plus proto-spacer adjacent motifs. Initially, 69 potential sgRNAs were identified. Out of these, sgRNA that showed maximum specificity to X gene sequences and had minimum off-target mismatches in the human genome was selected for target sequence knockdown (Figure S1A and S1B). This region was synthesized and cloned in non-viral eSpCas9-2A-Puro (PX459)V2.0 vector (HBx-CRISPR) (Fig S1C). HBx-CRISPR plasmid was isolated, digested with restriction enzymes and checked on agarose gel (Fig. 1A and S2A). Next, we evaluated the efficacy of sustained Cas9/sgRNA expression in inhibiting HBx using a model that more reliably recapitulated HBV life cycle components. For these studies, we used the HepG2.2.15 hepatoblastoma cell line, which harbors both a functional HBV integrated form and cccDNA. After 48 h of co-transfection (HBx-CRISPR and pcDNA3-EGFP plasmid) in HepG2.2.15 cells, transfection efficiency was around 60% (Fig. 1A and S2B). An optimum concentration of the plasmid (1.5 µg), which resulted in low amount of plasmid usage as well as effective knockdown of HBx and less cell death in culture was used for transfection in further experiments (Fig. 1a and S2C-S2D). We excised the full length, 465 bp HBx gene using our designed HBx-CRISPR that resulted in a significant knockdown of the HBx gene expression in the HepG2-2.15 cells after 96 hours (18-fold decrease, $p < 0.01$ Fig S3A). We also validated our HBx-CRISPR plasmid in another cell system, where we co-transfected HepG2 cells with whole HBV plasmid and then with the HBx-CRISPR plasmid. We observed a 4-fold decrease in HBx gene expression in these double transfected cells ($p < 0.05$ Fig. 1b).

3.2 CRISPR/Cas9-mediated disruption of HBx decreases HBV replication in HBV-integrated-hepatoma models: We examined whether knockdown of HBx affected hepatitis B surface antigen. HBsAg ELISA assays indicated that HBx-CRISPR transfection significantly decreased the levels of HBsAg in the HepG2.2.15 cells supernatants after 96 hours (2-fold, $p < 0.05$, Fig. 1c and Fig S3B) and in HepG2-HBV model, (4.5-fold, $p < 0.01$, Fig. 1c). HBx knockdown also significantly reduced HBV cccDNA expression in the HepG2-2.15 cells after 96 hours (2-fold, $p < 0.01$, Fig. 1d and Fig S3B). Simultaneously, in HepG2-HBV model, results showed significant reduction in the expression of HBV cccDNA (5-fold, $p < 0.05$, Fig. 1d).

The two variables i,e. HBx and HBV-cccDNA showed a similar line of expression pattern, whereas significant reduction was clearly seen at 96 hrs in the HBx-CRISPR cells (Fig S3B).

3.3 Knockdown of HBx inhibits cell proliferation, migration and invasion in HBx-CRISPR-transfected cells: The HBx knockdown-HepG2.2.15 cells showed a marked reduction in cell proliferation in comparison to cells transfected with the control vector (2-fold $p < 0.05$ Fig. 2a-b) in the scratch assays. Colony formation assays showed that HBx knockdown clearly inhibited the cell proliferation in HBx-CRISPR transfected HepG2.2.15 as compared to cells transfected with the control vector (1.72-fold, $p < 0.05$ Fig. 2c-d). We observed a significant decrease in chemotactic migration and invasion in the cells treated with HBx-CRISPR as compared to cells treated with control vector (migration: 1.43-fold, $p < 0.05$ Fig. 2e-f and invasion: 1.75-fold, $p < 0.05$ Fig. 2g-h) indicating a reduced tumorigenic properties in HBx knockdown cells.

3.4 Knockdown of HBx inhibits genes related to epithelial to mesenchymal transition and cancer stemness in HBV-integrated-hepatoma cells: We next studied the effects of HBx-CRISPR plasmid on the tumor attributes of hepatoma cells. We evaluated the expression of E-cadherin (CDH1) and Vimentin (VIM), both of which have been associated with EMT. The HBx knockdown in the HepG2.2.15 cells induced CDH1 protein expression in HBx-CRISPR transfected cells (Fig. 3a-b) and reduced VIM protein expression in HBx-CRISPR transfected cells compared to control-transfected HepG2.2.15 cells (Fig. 3d-e) as seen by the flow cytometry assays. Gene expression studies further validated the above observations i.e, significant reduction in VIM gene (2.14-fold $p < 0.05$, Fig. 3f) and an upregulation in CDH1 gene (1.6-fold $p < 0.01$, Fig. 3c) in the cells treated with HBx-CRISPR as compared to the control plasmid transfected cells. The expression of stemness gene, CD133 was also significantly reduced at protein ($p < 0.01$) as well as gene level (3.22-fold, $p < 0.001$) in HBx-CRISPR transfected cells compared to control-transfected HepG2.2.15 cells (Fig. 3g-i). EMT in hepatoma cells was further validated by studying the gene expression of mesenchymal genes. RT-PCR studies showed that after HBx knockdown, there was a significant decrease in the expression of mesenchymal genes, Thy1, CDH2, Fibronectin and alpha-SMA in HBx-CRISPR treated cells compared to the cells transfected with the control vector (CDH2; 6.5-fold $p < 0.001$, Thy-1; 1.84-fold $p < 0.01$, alpha-SMA; 1.49-fold $p < 0.05$ and fibronectin; 1.25-fold Fig. 3j-m). Furthermore, HBx knockdown conditions led to a significant decrease in the expression of β-catenin and TGF-β genes in HBx-CRISPR treated cells as compared to the cells transfected with the control vector (β-catenin; 5.07-fold $p < 0.01$ and TGF-β; 7.75-fold $p < 0.001$ Fig. 3n-o). Given a close relation between EMT with matrix metalloproteases (MMPs) and CSC properties of the tumor cells, we also evaluated the gene expression of some of the MMPs and CSC markers (CD24 and CD44) in HepG2.2.15 cells. When HepG2.2.15 cells were transfected with HBx-CRISPR, expression of CD24 and CD44 was significantly downregulated as compared to that observed in the control vector (CD24; 2.03-fold $p < 0.05$ and CD44; 2.05-fold $p < 0.01$ Fig. 3p-q). Knockdown of HBx also led to decrease in expression of MMPs (MMP2, MMP9 and MMP14) in HepG2.2.15 cells, which were transfected with HBx-CRISPR compared to the control vector (MMP2; 2.6-fold $p < 0.01$, MMP9; 4.35-fold $p < 0.05$ and MMP14; 1.8-fold $p < 0.05$ Fig. 3r-t).

3.5 HBx knockdown resulted in downregulation of genes significantly associated with cell proliferation and stemness: To identify the global effect of HBx knock-down on HBV-hepatoma cell transcriptomics, the RNA profile of HBx-CRISPR treated cells were compared with control-transfected cells. Out of 1159 differentially expressed genes (DEGs), 70 genes were upregulated while 1089 genes were downregulated in HBx-CRISPR transfected cells ($p < 0.05$) (Fig. 4a). Four DEGs; C1orf54, HLA-DQB1, RAC2, and PLAAT4 that were significantly downregulated in HBx-CRISPR have been earlier shown to be induced in the liver during hepatitis B (HBV) infection in chimpanzees (GSEA; M11620). ELK1, AKT3, and EGR2 that are known to be highly expressed in human HBV infection (Wikipathway; WP4666), were also significantly downregulated after HBx knockdown (Fig. 4b). Next, we performed a functional enrichment analysis of the whole genome repertoire of the significant DEGs. The downregulated genes were associated with the biological processes including cell population proliferation, regulation of cell differentiation, while less than 3 upregulated genes with FDR < 0.1 were linked to metabolic associated pathways regulation of amine transport and glutamate secretion (Fig. 4c). Pathway enrichment using Wikipathway (Fig. 4d) and Reactome (Fig S6A) showed that while the upregulated genes were not significantly involved in any pathway, the downregulated genes were enriched for cell proliferation- and stemness-related pathways including EGF, PI3K-AKT, NOTCH, WNT, Ras, and Hippo signaling pathways. In addition, genes associated with signaling by nucleus receptors and EMT were downregulated. (Further details of various genes, their log FC and p-value is in supplementary data as excel file.

3.6 Knockdown of HBx decreased HBV replication in Three-dimensional HBV-integrated-hepatoma models: Three dimensional (3D) models more closely resemble the *in vivo* tumor microenvironment by exhibiting complex phenotypic heterogeneity. We thus also analysed the functional effects of HBx-CRISPR on the HepG2.2.15 cells in 3D HBV-integrated-hepatoma tumor models. The expression of HBx was significantly reduced (2.57-fold $p < 0.01$) in HBx-CRISPR transfected cells compared to control-transfected HepG2.2.15 cells (Fig. 5a) in 3D cultures. In 3D tumor cultures, the expression of HBV cccDNA reduced significantly in HBx-CRISPR transfected HepG2.2.15 cells as compared to the control cells (1.6-fold, $p < 0.01$ Fig. 5b).

3.7 Knockdown of HBx reduced metastasis in 3D HBV-integrated-hepatoma in vitro **models**: We next probed, if transfection with HBx-CRISPR reduced the tumorigenicity in HBV-HCC 3D model. HBx-CRISPR-HepG2.2.15 and control hepatoma cells (8000 cells/9.5cm²) formed distinct spheroids within 14 days of suspension cultures (Fig. 5c). Cells transfected with HBx-CRISPR formed less number ($p < 0.05$ Fig. 5c and 5d) and smaller sizes (165.355μm w.r.t 251.866μm $p < 0.001$, Fig. 5c and 5e) of spheroids in culture as compared to cells transfected with control vector.

In 3D models, to assess the tumorigenic property, we also used non-adherent hanging drop cultures for spheroid formation where cellular aggregation is promoted based on gravity and there is complete absence of a substratum [15]. At day3, cell to cell adhesion and spheroid compaction were significantly reduced in HBx knockdown cell spheroids as compared to spheroids derived from control cells (Fig. 5f and 5g). Results showed that at day 3, the percentage of spheroid migration in HBx knockdown cells was

47.25% as compared to control vector cells which showed a percentage migration of 65.8% (Fig. 5f and 5h, $p < 0.01$).

3.8 Knockdown of HBx inhibit epithelial to mesenchymal transitions and reduce stemness potential in 3D HBV-integrated-hepatoma spheroids model: There was a significant increase in CDH1 expression in HBx-CRISPR transfected cells compared to control vector cells (CDH1; 8.28-fold $p < 0.05$ Fig. 6a) and significant decrease in the expression of mesenchymal genes, VIM, CDH2, Thy1 in HBx-CRISPR treated cells compared to the cells transfected with the control vector (VIM; 2.04-fold $p < 0.05$, CDH2; 2.72-fold $p < 0.05$, Thy-1; 2.34-fold $p < 0.01$ Fig. 6b-d) Next, we also evaluated the expression of MMP9 and CSC marker, CD133 in HepG2.2.15 cells spheroids. The expression of CD133 was significantly downregulated in HBx-CRISPR treated spheroid cells as compared to that observed in the control vector (CD133; 4.33-fold $p < 0.01$ Fig. 6e). In 3D cultures, we also observed that knockdown of HBx led to a decrease in expression of MMPs in HepG2.2.15 cells, which were transfected with HBx-CRISPR compared to the control vector (MMP9; 1.87-fold $p < 0.05$ Fig. 6f). Furthermore, HBx knockdown conditions led to a significant decrease in the expression of TGF-β and β-catenin genes in 3D HBx-CRISPR treated cells compared to the cells transfected with the control vector (TGF-β; 1.83-fold $p < 0.05$ and β-catenin; 5.12-fold $p < 0.001$ Fig. 6g-h).

Discussion

The study reports a successful knock down of HBx expression and tumorigenic properties of hepatoma cells by our sgRNA designed against HBx. Since HBx plays a central role in HBV infection and is most probably appropriate for virus-induced liver disease, it represents the most suitable target for developing novel therapies to treat chronic HBV infection. Some studies have reported that it is possible to eliminate the majority of HBV cccDNA within 6 days in vitro by CRISPR/Cas9 mediated post-transfection [17]. Our data clearly indicated that HBx knockdown by the CRISPR/Cas9 system led to a marked downregulation of various signaling pathways associated with tumor progression. Li et al 2016 have reported that HBx promotes tumor invasion and metastasis in HCC via induction of c-Myc expression by activation of Ras/Raf/ERK1/2 pathway, which in turn results in tumor metastasis [18]. Other pathways that were attenuated in CRISPR-treated cells were Wnt, MAPK, Notch and Hedgehog. Notch, Hedgehog and Wnt pathways (evolutionarily conserved embryonic pathways) are most crucial to the tumorigenicity of CSCs and their association with MAPK and PI3K pathways play roles in induction of tumor aggressiveness through proliferation, anti-apoptosis, invasion, angiogenesis, metastasis and chemoresistance [19].

Many earlier studies have reported that HBx disrupts intercellular adhesion, and induces EMT, invasion, migration and metastasis in HCC. In our own previous study, we reported an upregulation of EMT and stemness markers including CDH2 (N-cadherin), Thy-1 (CD90), VIM (Vimentin), PROM1 (CD133) and TGFβR1 in HBx transfected hepatoma cells [10]. In the current study, we observed that CDH2, Thy-1, VIM, CD133 were significantly reduced after HBx knockdown in both 2D and 3D cultures. Transcriptomics data also confirmed a significant downregulation of VIM. Expression of MMPs (MMP2, MMP9 and MMP14), which are key regulators of EMT was also downregulated after HBx knockdown in both 2D as well as in 3D cultures. Previous studies have showed that HBx facilitates tumor cell invasion by upregulation of

various MMPs and subsequent destruction of the ECM. Liu et al. 2010 have reported that HBx increases the expression of MMP2 and MMP9 by activating the nuclear factor kB (NF-kB) pathway and upregulation of these MMPs promotes HCC cell invasion and metastasis [20]. Among biological processes, the HBx-CRISPR treated cells demonstrated a significant downregulation of cell proliferation, migration and angiogenesis. This was also observed on the functional level as HBx-CRISPR cells had decreased proliferation, invasion and tumorigenicity *in vitro.* HBx knockdown also resulted in reduced adhesion and metastatic properties in 3D HBV spheroid models. We also observed a downregulated expression of CSC markers, CD24, CD44 and CD133 in 2D as well as in 3D cultures. Overall, most of components of the EMT pathway were clearly downregulated both in HBx-CRISPR treated hepatoma cells in terms of gene/protein expression and functions.

Most noteworthy was the downregulation of several transcription factors in the HBx knock down cells. In our study, transcription factors like (HEY2, DACH1, TCF4, SOX2 and SP9) may help HBx to activate the HBV viral machinery, were downregulated in HBx-CRISPR group. The decrease in their expression by HBx-CRISPR clearly validated the targeted effects of our strategy.

Lucifora et al 2011 have reported that HBx does not determine the ability of HBV to enter the host cell but is essential for viral transcription from its natural transcription template, the nuclear HBV cccDNA [21]. We observed two-folds reduction of cccDNA gene expression in HBx knockdown HepG2.2.15 cells while in HepG2-HBV cells, reduction was about five-folds. This reduction in cccDNA was also observed in 3D cultures in HBx-CRISPR transfected HepG2.2.15 cells compared to the control cells. HBx is recruited to the cccDNA minichromosome and may be involved in epigenetic control of HBV replication [22]. Indeed, some of the enriched genes (SALL3, HDAC9, ELF5, PBX1) that are associated with chromatin dynamics and cancer, were also found to be deregulated in HBx-CRISPR group. Interestingly, in our study, we found that CRISPR/Cas9-mediated disruption of HBx decreased HBsAg secretion in supernatant of HBV-HCC cells in almost similar pattern as seen in cccDNA expression in HCC cells after HBx knockdown. RNA-Seq data also showed the downregulation of expression of several genes associated with HBV infection in human and chimapanzee liver (AKT3, ELK1 and EGR2, C1orf54, HLA-DQB1, RAC2 and PLAAT4) [23]. These data indicate that HBx might be impacting several components of HBV replication and a knockdown of its expression might be leading to a decreased viral replication.

To summarize, our study shows that targeting HBx by novel tailored CRISPR/Cas9 system effectively reduces HBx gene expression, HBV cccDNA, HBsAg levels as well as the stemness and mesenchymal characteristics of the HBV-integrated-hepatoma cells. The study highlights the use of HBx gene specific CRISPR/Cas9 technique as an effective targeted and non-viral therapeutic approach to control HBV-induced tumor progression and also reduce HBV replication. The study proposes CRISPR/Cas9 as an innovative and targeted strategy for the prevention of HBV associated HCC progression. For translation to humans, the safety and efficacy of the CRISPR/Cas9 approach further warrants a thorough in vivo evaluation.

Declarations

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Declaration of Conflict of Interest

The authors declare that they have no competing interests.

Authors' Contributions

PR performed all cell culture experiments, migration, chemotaxis, invasion, immunophenotyping, RT-PCR and 3D culture assays, collected and analysed all data and drafted the manuscript. HH helped in transcriptomics study. JK helped in performing the HBV cccDNA experiments. DMT helped PR in the editing of the manuscript. VN and SK got the funding for the study, designed the study, performed data analysis along with PR and finalized the manuscript.

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Figures

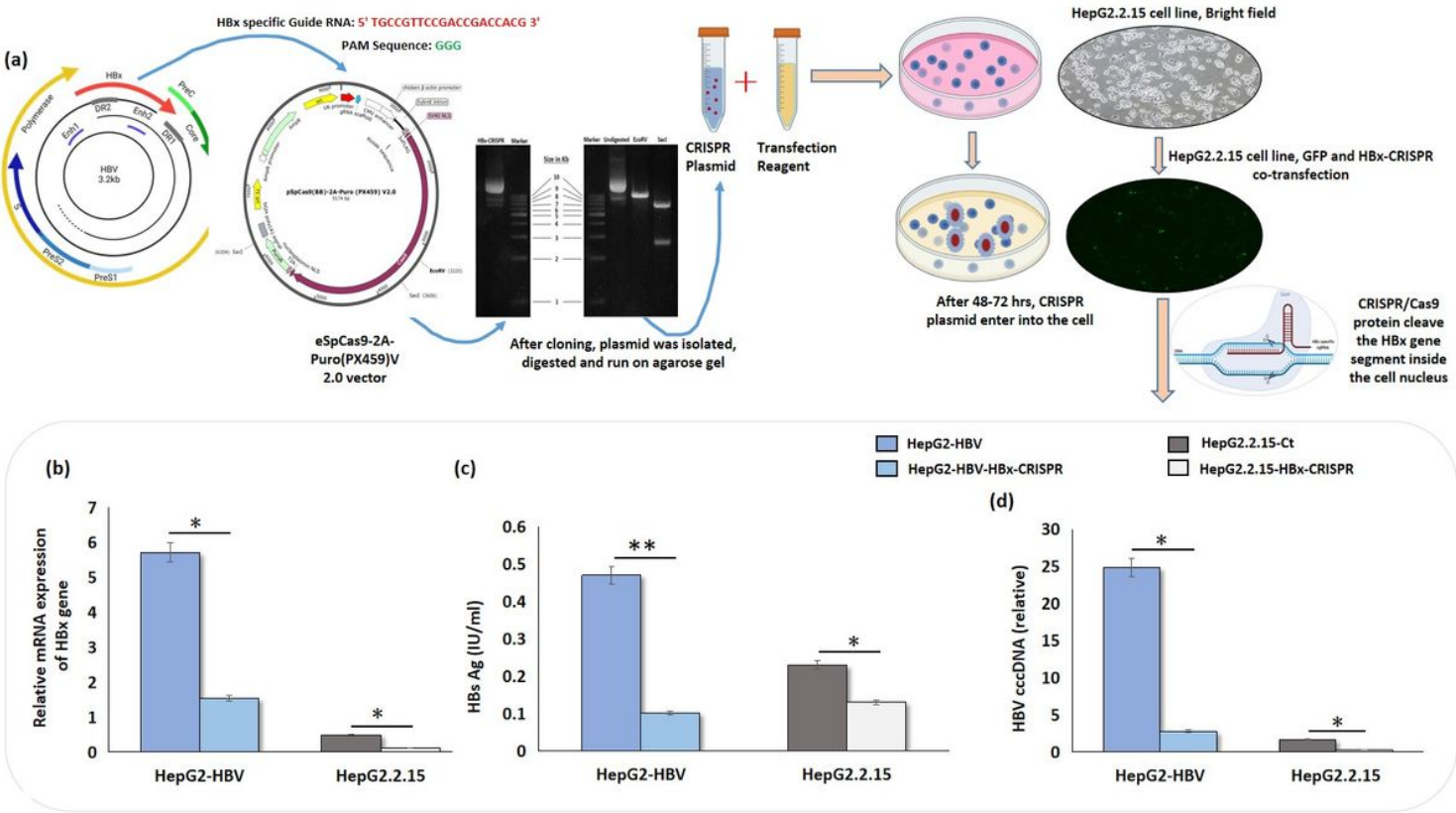

Figure 1

Knockdown of HBx by HBx-CRISPR plasmid and CRISPR/Cas9-mediated disruption of HBx decreased HBV replication in HBV-HCC model. (a) Schematic representation of the experimental set up for knockdown of HBx by CRISPR/Cas9 editing tool (b) Relative expression of HBx gene in HepG2 transfected with HBV plasmid and cotransfected with HBx-CRISPR (HepG2-HBV and HepG2-HBV-HBx-CRISPR) and HepG2.2.15 cells transfected with HBx-CRISPR with respect to HepG2.2.15 cells transfected with control vector (c) Levels of HBsAg (IU/ml) in HepG2-HBV, HepG2-HBV-HBx-CRISPR, HepG2.2.15-HBx-CRISPR and HepG2.2.15-Ct cells in HBV-HCC model (d) Relative expression of HBV cccDNA gene in HepG2-HBV, HepG2-HBV-HBx-CRISPR cells and HepG2.2.15-HBx-CRISPR, HepG2.2.15-Ct cells in HBV-HCC model. Data is represented as mean ± SD (n=3). '*'represents p< 0.05 and '**' represents p< 0.01.

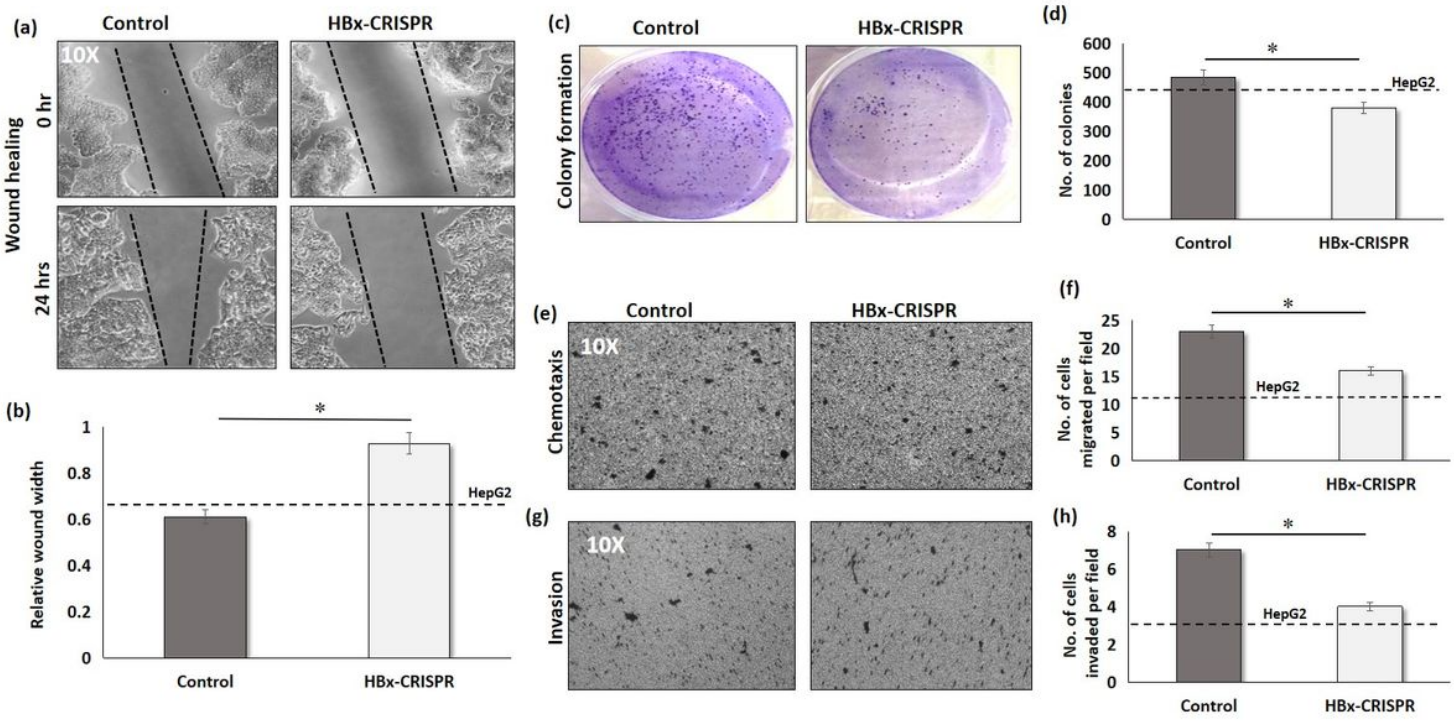

Figure 2

Functional properties in HBV-HCC cells after HBx knockdown. (a) Representative phase contrast images of wound closure assay in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR (b) Bar diagram showing relative wound width in the different experimental conditions (c-d) Representative images and bar diagram of colonies formed in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR (e-f) Phase contrast images and bar diagram of chemotactic migration in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR (g-h) Phase contrast images and bar diagram of invasion in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR. HepG2 cells without any treatment were used as respective controls (Dotted line). Data is represented as mean ± SD (n=3). '*'represents p< 0.05 and '**' represents p< 0.01.

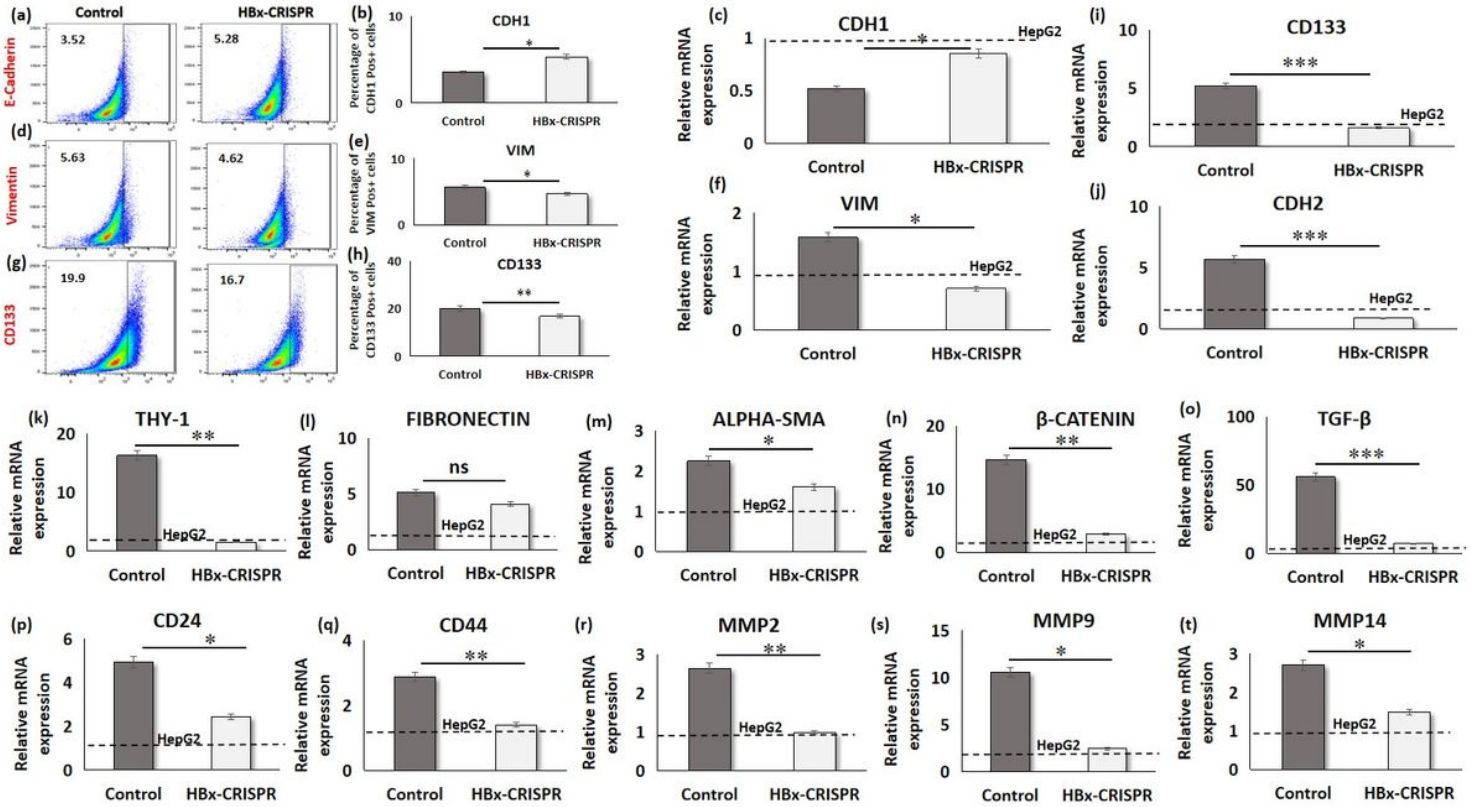

Figure 3

EMT, CSCs and proteases genes expression in HBV-HCC cells after HBx knockdown by CRISPR/Cas9. (a-b, d-e and g-h) Dot-plots and bar diagram of flow cytometry data depicting percentage of E-cadherin, Vimentin and CD133 positive cell population respectively (c, f and i). Relative gene expression of E-cadherin (CDH1), Vimentin (VIM) and CD133 respectively in HepG2.2.15-Ct and HepG2.2.15-HBx-CRISPR cells (j-m) Relative mRNA expression of CDH2, Thy-1, Fibronectin, Alpha-SMA in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR (n-o) Relative mRNA expression of β-catenin and TGF-β in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR. (p-q) Relative mRNA expression of CD24 and CD44 in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR (r-t) Relative mRNA expression of MMP2, MMP9 and MMP14 in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR. HepG2 cells without any treatment were used as respective controls (Dotted line). Data is represented as mean ± SD (n=3). '*'represents $p < 0.05$, '**' represents $p < 0.01$ and '***' represents $p < 0.001$.

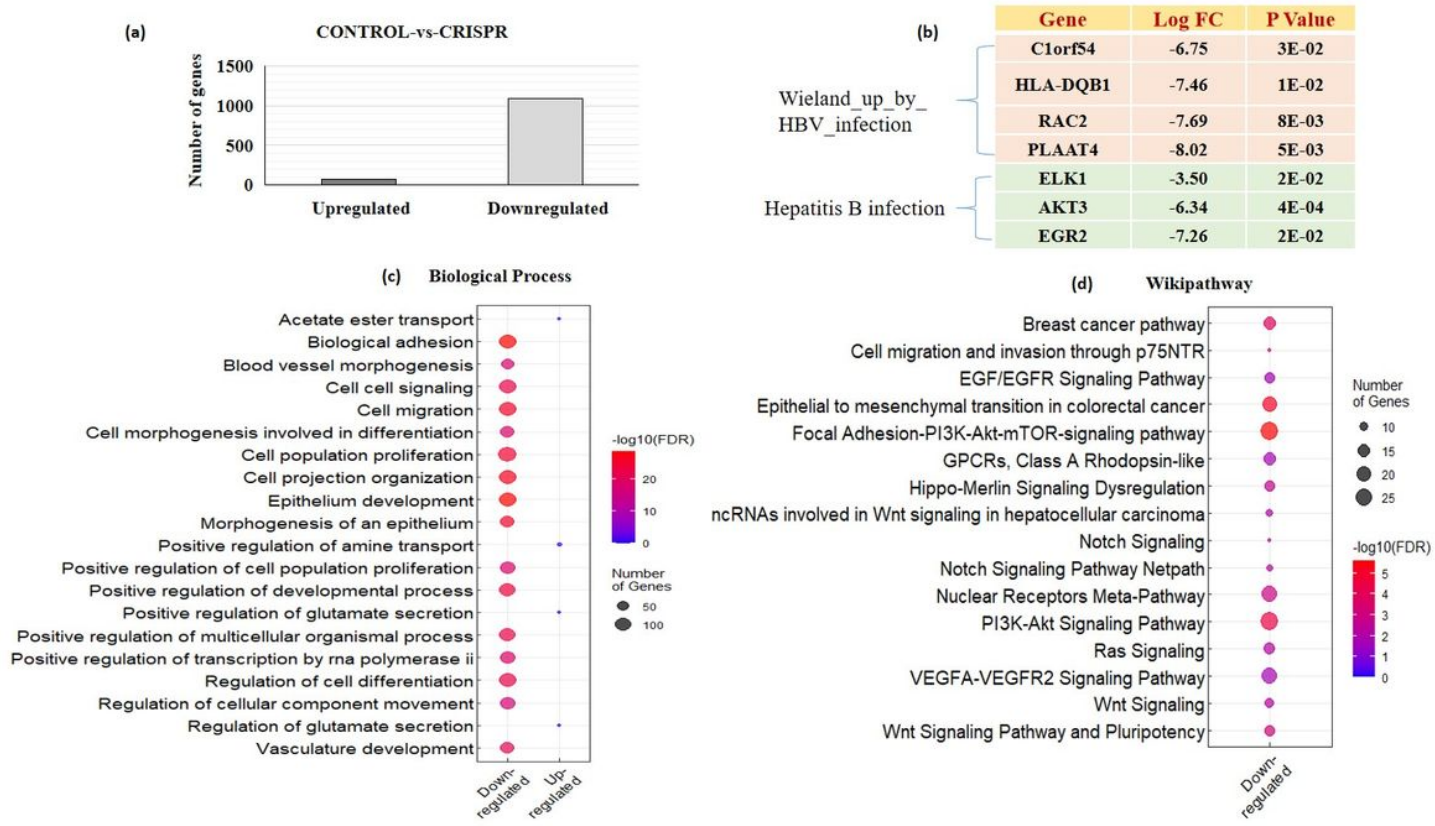

Figure 4

Knockdown of HBx resulted in downregulation of cell proliferation and stemness-associated genes. (a) Out of 1159 DEGs in HBx-CRISPR transfected cells, 70 genes were upregulated while 1089 genes were downregulated ($p<0.05$) (b) Significantly downregulated genes were associated with hepatitis B (HBV) infection the liver during. (c-d) The differentially expressed genes in HepG2.2.15 samples after HBx knockdown were analyzed for gene ontology (biological process) and pathway enrichment. The upregulated genes were not significantly enriched for any pathway even with FDR <0.1.

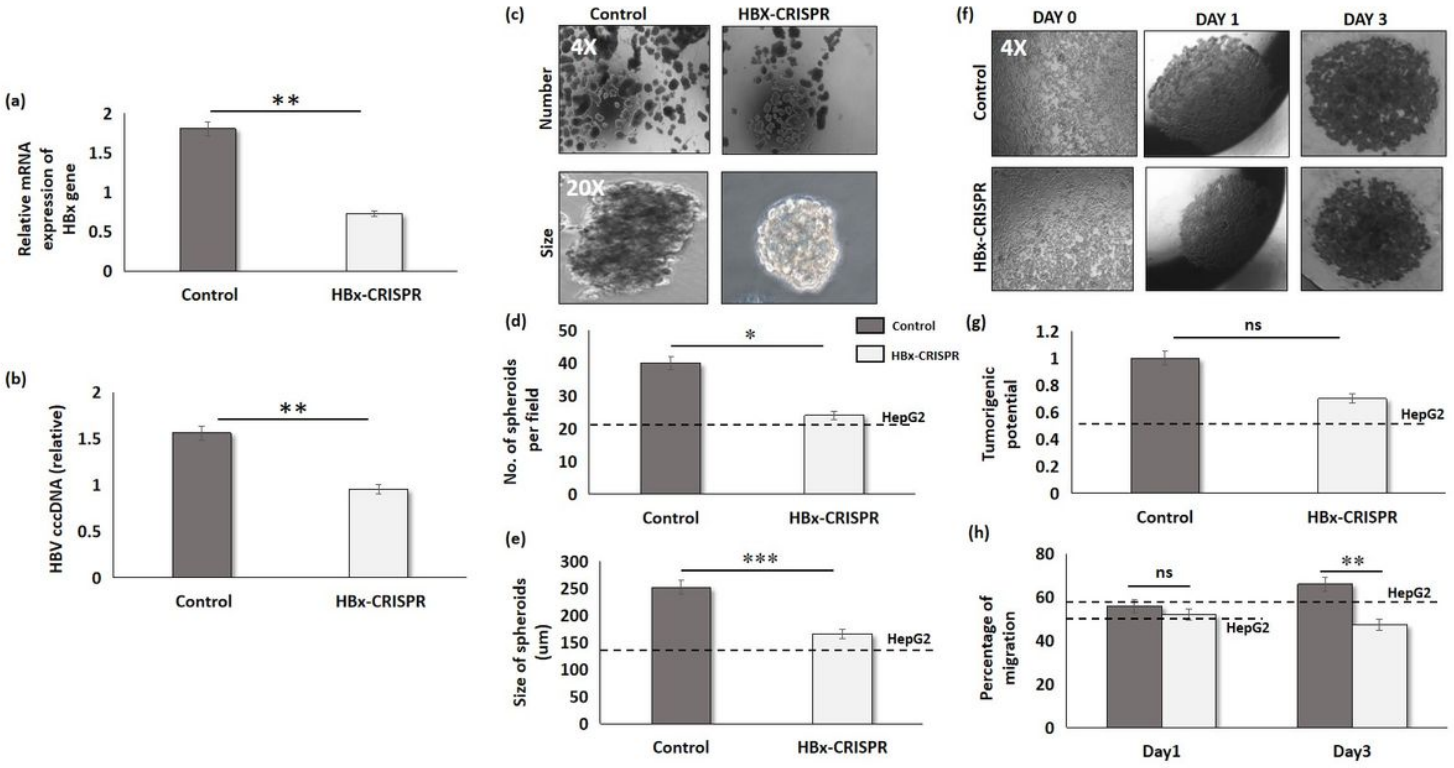

Figure 5

Knockdown of HBx reduced metastasis and decreased HBV replication in 3D HBV-HCC *in vitro* model. (a) Relative HBx gene expression in HepG2.2.15-HBx-CRISPR and HepG2.2.15-Ct cells in 3D-HBV-HCC model (b) Relative expression of HBV cccDNA gene in HepG2.2.15-HBx-CRISPR and HepG2.2.15-Ct cells in 3D-HBV-HCC model. (c) Representative phase contrast images of number (magnification 4x) and size (20x) of spheroids formed by various experimental models (HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR) per field respectively (d) Bar diagram showing the number of spheroids and (e) size of spheroids per field (in µm). (f) Phase contrast images of HCC cells spheroid formation in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR cells in day1-day3 time point to evaluate tumorigenic potential of cells (magnification at 4x) (g) Bar diagram showing the tumorigenic potential and (h) Relative migration percentage of various experimental HCC cells. HepG2 cells without any treatment were used as respective controls (Dotted line). Data is represented as mean ± SD (n=3). '*'represents $p < 0.05$, '**' represents $p < 0.01$ and '***' represents $p < 0.001$.

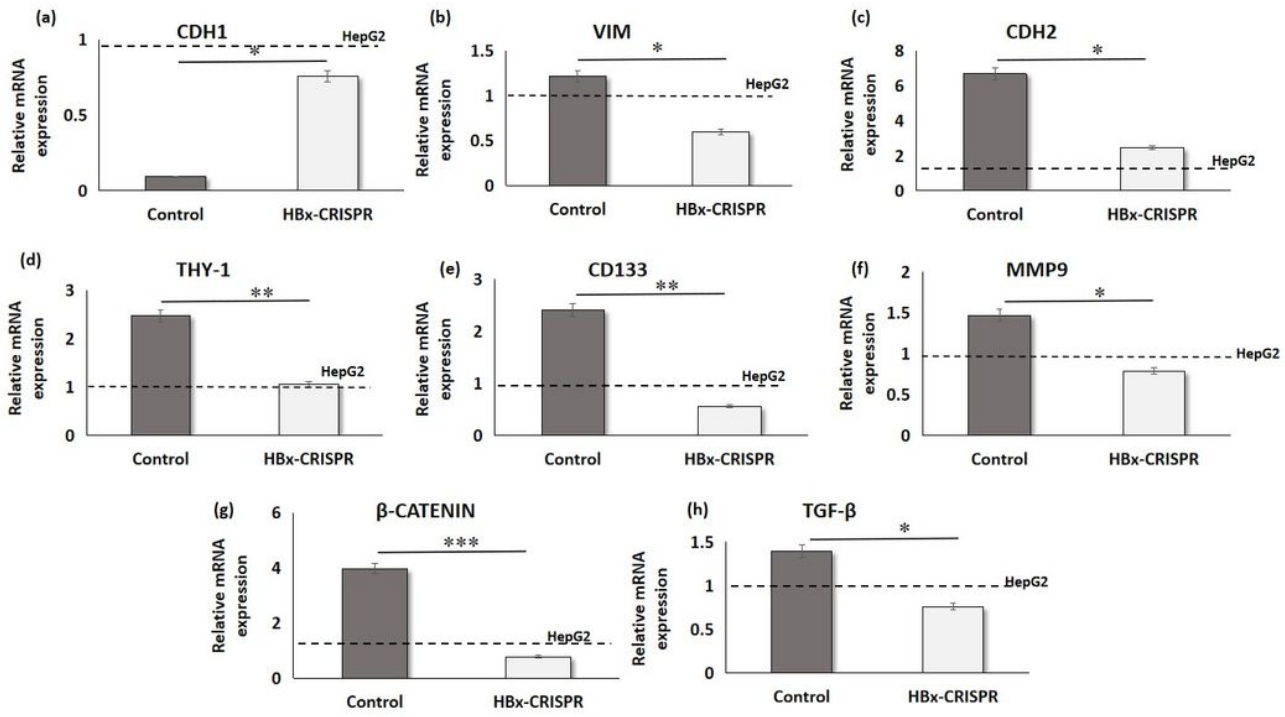

Figure 6

EMT, CSCs and proteases genes expression in 3D HBV-HCC *in vitro* model after HBx knockdown. (a-d) Relative mRNA expression of CDH1, CDH2, Vimentin, Thy-1 in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR (e) Relative mRNA expression of cancer stemness gene CD133, (f) protease gene MMP9 and (g-h) Relative mRNA expression of β-catenin and TGF-β in HepG2-2.15-Ct and HepG2-2.15-HBx-CRISPR. HepG2 cells without any treatment were used as respective controls (Dotted line). Data is represented as mean ± SD (n=3). '*'represents p< 0.05, '**' represents p< 0.01 and '***' represents p< 0.001.

Supplementary Files

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