



Original Article

HBx Facilitates Drug Resistance in Hepatocellular Carcinoma via CD133-regulated Self-renewal of Liver Cancer Stem Cells



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Abstract

Background and Aims: Hepatitis B virus (HBV) infection contributes to hepatocellular carcinoma (HCC) tumorigenesis, drug resistance, and recurrence, although the underlying molecular mechanisms remain unclear. Recent studies suggest that HBV infection may be associated with liver cancer stem cells (LCSCs), but the exact mechanisms are yet to be resolved. In this study, we aimed to analyze the role of HBV infection in regulating the stemness of HCCs, which is closely linked to drug resistance. **Methods:** Sphere formation assay and real-time Polymerase Chain Reaction quantification were used to isolate and confirm liver cancer stem cells. The inhibitory concentration values of sorafenib and regorafenib were calculated and compared using the Cell Counting Kit-8 assay. HBV infection was used to assess the effect of HBV replication on LCSC markers. Co-immunoprecipitation assay was performed to detect the interaction between CD133 and SRC. Furthermore, we utilized the CRISPR-Cas9 system to knock-out CD133 expression in HepG2.2.15 cells. **Results:** LCSCs derived from HCCs exhibited high expression of stem cell markers and demonstrated reduced sensitivity to sorafenib and regorafenib. HBV replication promoted both drug resistance and stemness in hepatoma cells and clinical samples. Overexpression of HBx protein in HepG2 cells upregulated the expression of CD133, EpCAM, and CD24, enhancing resistance to sorafenib and regorafenib. Knockout of CD133 expression using the CRISPR-Cas9 system significantly inhibited drug resistance to both sorafenib and regorafenib in HepG2.2.15 cells. Mechanistically, HBV replication promoted CD133 expression, which in turn interacted with the SRC/STAT3 signaling pathway. **Conclusions:** Our data suggest that HBV replication enhances the stemness and drug resistance of HCC, providing a strong theoretical foundation for the development of targeted and efficient treatments for HBV-infected HCCs.

Keywords: Hepatocellular carcinoma; Liver cancer stem cells; Hepatitis B virus; CD133; Drug resistance; Sorafenib; Regorafenib.

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Introduction

Liver cancer is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide.^{1,2} Primary liver cancers include hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and combined carcinoma (HCC-ICC).³ Over 90% of liver cancer cases are HCC, and only 5–15% of early-stage liver cancer patients are eligible for surgical resection.⁴ Hepatitis B virus (HBV) infection is a major pathogenic factor of HCC, accounting for 50–80% of HCC cases.⁵ The HBV gene mainly encodes four antigens (HBsAg, HBcAg, HBeAg, and HBxAg), which have been linked to HCC by activating various cancer-related signaling pathways and regulating cell metabolism, drug resistance, stemness, and other mechanisms.⁶ Currently, sorafenib, an oral multi-kinase inhibitor, is recommended worldwide as the first-line therapy for advanced HCC.⁷ However, only approximately 30% of patients benefit from the treatment, with drug resistance developing within six months of initiating the regimen.⁸ Regorafenib is recommended as a second-line multi-kinase inhibitor for patients with HCC who experience disease progression during first-line treatment with sorafenib.⁹ It is postulated that cancer stem cells (CSCs) are more resistant than non-CSCs within the same tumors to elimination by conventional chemotherapy and radiotherapy.¹⁰ The success of anti-cancer therapy for HCC is limited by both primary and acquired drug resistance, which is related to the presence of liver cancer stem cells (LCSCs).

HCC is an aggressive tumor with a poor prognosis. Accumulating evidence supports the notion that LCSCs are responsible for poor outcomes due to their highly stem-cell-like abilities and resistance traits. Several LCSC markers have been identified, including CD133,¹¹ EpCAM,¹² CD13,¹³ CD24,¹⁴ CD44,¹⁵ CD90,¹⁶ and OV6.¹⁷ In our previous study, we indicated that HBV might contribute to LCSC formation.¹⁸ HBV PreS1 facili-

tates HCC development by promoting the appearance and self-renewal of liver cancer stem cells.¹⁹ These findings suggest a correlation between HBV replication and the stemness of HCC. Targeting MYH9 blocked HBx-induced GSK3 β ubiquitination to activate the β -catenin destruction complex, thus suppressing cancer stemness.²⁰ Therefore, targeting LCSCs has been identified as a potential strategy to improve the outcomes of HCC patients by reducing drug resistance.

In the current study, we provide evidence that HBV infection is involved in sorafenib and regorafenib resistance in HCC by modulating expressions of CD133, EpCAM, and CD24, which are critical for maintaining the stemness and self-renewal of LCSCs.

Methods

Patient samples

Forty HCC tissues (T) and matched liver tissues [adjacent non-tumor (NT)] were obtained from patients who underwent surgery at the First Medical Center of Chinese PLA General Hospital, Beijing, China. Of these, twenty patients were HBsAg negative and twenty were HBsAg positive. We collected frozen samples of HCC tissues and adjacent non-tumor tissues immediately after tumor resection, along with relevant clinical information. The patients were enrolled in this Institutional Review Board-approved study (S2018-111-01). Informed consent was obtained regarding the use of their samples for further studies. All patients were negative for serological markers of hepatitis C virus, hepatitis D virus, and human immunodeficiency virus infections.

Cell culture

The liver cancer cell lines Huh7, HepG2,²¹ HepG2.2.15 (HBV viral genome-integrated cells), HepAD38, and HepG2-NTCP (HBV receptor-expressing cells), which are derived from HCC and hepatoblastoma, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C in a humidified 5% CO₂ atmosphere.

Reagents

Cells were treated with various concentrations and durations of sorafenib (MCE, HY-10201) and regorafenib (MCE, HY-10331).

Sphere formation assay

Sphere formation was performed according to a previously published protocol with some modifications.²² Briefly, cells were seeded at a density of 100 cells per well in ultra-low adherent 96-well plates and cultured in DMEM/F12 supplemented with B27 (1 \times), EGF (10 ng/mL), and Basic-FGF (10 ng/mL) for seven to fourteen days. Tumor spheres were then observed under a microscope.

Real-time PCR quantification (RT-qPCR)

Total RNA was collected using TRIzol reagent (Invitrogen) from cultured cells and liver tissues, after homogenizing the frozen tissues with Precellys beads, and quantified by qPCR as previously described.²³ RT-qPCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and detected with the Applied Biosystem 7500 PCR instrument. GAPDH was used as an endogenous control. The primers for qPCR are shown in Table 1. The qPCR was performed in a 5 μ L reaction volume following the manufacturer's protocol. The relative quantification PCR protocol for

Table 1. RT-qPCR primer sequences

Gene name	Sequence 5'→3'
<i>CD133</i>	
Sense	TGACAAGCCCATCACAAACATT
Antisense	CGCCTGAGTCACTACGTTGC
<i>EpCAM</i>	
Sense	AGCAGTTGTTGCTGGAATTGT
Antisense	AGTTCCCTATGCATCTCACCC
<i>CD24</i>	
Sense	CTCCTACCCACGCAGATTTATTC
Antisense	AGAGTGAGACCACGAAGAGAC
<i>CD13</i>	
Sense	TTCAACATCACGCTTATCCACC
Antisense	AGTCGAACTCACTGACAATGAAG
<i>CD44</i>	
Sense	TCCGAATTAGCTGGACACTC
Antisense	CCACACCTTCTCCTACTATTGAC
<i>Sox2</i>	
Sense	TGG ACA GTT ACG CGC ACA T
Antisense	CGA GTA GGA CAT GCT GTA GGT
<i>AFP</i>	
Sense	AGA CTG AAA ACC CTC TTG AAT GC
Antisense	GTC CTC ACT GAG TTG GCA ACA
<i>Oct4</i>	
Sense	GATGGCGTACTGTGGGCC
Antisense	TGGGACTCCTCCGGGTTTTG
<i>GAPDH</i>	
Sense	TGTGAACGGATTTGGCCGTAT
Antisense	ACAAGCTTCCCATTCTCGGC

RT-qPCR, real-time polymerase chain reaction.

all markers was as follows: 50°C for 2 m, 95°C for 2 m, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

Chemiluminescent immunoassay (CLIA)

After HBV infection, HBsAg and HBeAg levels in cell culture supernatants were detected using commercial CLIA kits (Aurobio Diagnostic Co., China), according to the manufacturer's instructions.

Cytotoxicity assay

Cells were seeded at a density of 5,000 cells per well in 96-well plates and incubated overnight. The medium was replaced with fresh medium containing varying concentrations of sorafenib and regorafenib, and cells were incubated for an additional 48 h. Cells were then washed three times with PBS and 10 μ L of CCK8 solution was added. After a 2-h incubation, the absorbance of each well was measured at 450 nm using an EnSpire Multimode Reader (PerkinElmer, Norwalk, CT, USA). The following formula was used to calculate cell viability:

$$\frac{AE - AB}{AC - AB} \times 100\%$$

AE, AB, and AC represent the absorbance of experimental samples, blank controls, and untreated samples, respectively.

Western blot

Western blot analysis was conducted following a previously described protocol.²⁴ Briefly, cells were lysed using RIPA lysis buffer containing phenylmethanesulfonyl fluoride and phosphatase-protease inhibitor cocktails. Protein concentrations were determined using a BCA Kit according to the manufacturer's instructions. The following antibodies were used: CD133 (Abcam, ab19898), EpCAM (Abcam, ab223582), CD24 (Abcam, ab179821), β -actin (TransGen, HC201-01), HA (TransGen, HT301-01), HBc (Prof. Guangxiang Luo, University of Alabama Birmingham), SRC (CST, #2109), p-STAT3 (CST, #9145), and STAT3 (CST, #9139).

HBV infection

HepG2-NTCP cells were seeded in six-well collagen-coated plates and cultured in DMEM supplemented with 3% FBS and 2% DMSO. Once confluent, the cells were infected with HBV (500 geq/cell) and cultured in DMEM supplemented with 3% FBS, 4% PEG8000, and 2% DMSO. After 24 h incubation, the cells were washed five times with PBS to remove residual HBV and HBsAg, and fresh medium was added for another seven days of culture in DMEM with 3% FBS, 2% DMSO, and IFN α . The supernatant was collected for HBsAg and HBeAg quantification using CLIA kits (Autobio Diagnostics Co., Zhengzhou, China), and the cells were harvested for quantification of HBV pgRNA and total RNA by RT-qPCR.^{25,26}

Co-immunoprecipitation

Cells were lysed in IP Lysis/Wash Buffer supplemented with phenylmethanesulfonyl fluoride and a protease inhibitor cocktail at 4°C for 30 m and then centrifuged at 13,000 g for 20 m at 4°C. The lysate was immunoprecipitated with the appropriate antibodies and Protein A/G Magnetic beads at 4°C for 6 h. The beads were washed five times with IP Lysis/Wash Buffer, and the immunocomplexes were eluted for 5 m and analyzed by Western blot. The antibodies used were CD133 (Abcam, ab216323) and SRC (CST, #2109).

Knockout of CD133 by CRISPR-Cas9

HepG2.2.15 cells were seeded on collagen-coated 24-well plates and infected with CD133-KO lentivirus (purchased from Tsingke Biotechnology Co.) in the presence of 5 μ g/mL polybrene. After 24 h infection, the virus-containing medium was discarded and replaced with fresh medium for another 72 h of culture. Cells were then treated with 10 μ g/mL of puromycin and 500 μ g/mL of hygromycin for five days. CD133 expression was validated by RT-qPCR and Western blot.

Statistical analysis

Differences between the means of independent groups were tested using an unpaired Student's t-test. Analyses were carried out using GraphPad Prism 7 (GraphPad software). A *p*-value of less than 0.05 was considered statistically significant. Each experiment was repeated at least three times.

Results

Hepatocellular carcinoma stem cells enhance sorafenib and regorafenib resistance

Drug resistance in HCCs may be linked to LCSCs. LCSCs are often in a quiescent state, which contributes to their resistance to chemotherapy and radiotherapy. Tumor sphere for-

mation is considered a promising method for isolating various types of cancer stem cells.²⁷ Thus, we constructed LCSCs using the sphere formation assay. We successfully obtained tumor spheres enriched with LCSCs (Fig. 1A). We verified the expression of CD133, EpCAM, CD24, CD13, and CD44 in HepG2 and Huh7 cells by RT-qPCR (Fig. 1B, C). CD133, EpCAM, CD24, and CD13 expressions were upregulated in HepG2S1 cells compared to parental HepG2 cells, while CD44 expression was not significantly altered. Meanwhile, CD133, EpCAM, CD24, CD13, and CD44 expressions were significantly upregulated in Huh7S1 cells compared to parental Huh7 cells. The cytotoxicity of sorafenib and regorafenib was assessed in parental HCCs and LCSCs. The half-maximal inhibitory concentrations (IC₅₀) of sorafenib and regorafenib were calculated and compared between parental HCCs and LCSCs. The IC₅₀ values for sorafenib were 6.616, 14.69, 8.61, and 42.58 μ M in HepG2, HepG2S1, Huh7, and Huh7S1, respectively (Fig. 1D, E). The IC₅₀ values for regorafenib were 5.39, 8.213, 10.19, and 13.69 μ M in HepG2, HepG2S1, Huh7, and Huh7S1, respectively (Fig. 1F, G). The IC₅₀ values of sorafenib and regorafenib were significantly higher in HepG2S1 and Huh7S1 cells compared to their parental counterparts. These data indicate that the selected LCSCs of HepG2S1 and Huh7S1 cells were more resistant to sorafenib and regorafenib than the parental cells.

HBV replication promotes stemness of HCCs in human hepatoma cells and clinical cancer tissues

To investigate the role of HBV in HCC resistance to sorafenib, we performed HBV infection in HepG2-NTCP cells (Fig. 2A). Secreted HBsAg and HBeAg levels increased during HBV infection but decreased upon treatment with IFN α at a final concentration of 100 IU/mL (Fig. 2B). Similarly, intracellular HBV pgRNA and total RNA were both elevated following HBV infection (Fig. 2C). We then performed RNA sequencing in HBV-infected (HBV group) and HBV-infected cells treated with IFN α (HBV + IFN α group), showing that LCSC markers (CD133, EpCAM, CD24, CD13, and CD90) were significantly more highly expressed in the HBV group than in the HBV + IFN α group (Supplementary Fig. 1). We verified the expressions of CD133, EpCAM, and CD24 in these cells using RT-qPCR and Western blot (Fig. 2D, E). These results demonstrated that HBV infection upregulated the expression of CD133, EpCAM, and CD24, which are associated with the stemness of HCCs.

To further evaluate the role of HBV replication in the stemness of HCCs, we assessed the expression of CD133, EpCAM, and CD24 in HepG2.2.15 cells, which harbor the HBV viral genome, using RT-qPCR and Western blot (Fig. 3A, B). The levels of CD133, EpCAM, and CD24 were higher in HepG2.2.15 cells compared to parental HepG2 cells. Tumor sphere formation was also significantly increased in HepG2.2.15 cells compared to parental HepG2 cells (Fig. 3C). Additionally, the IC₅₀ values of sorafenib and regorafenib were elevated in HepG2.2.15 cells compared to HepG2 cells (Fig. 3D, E), indicating that HBV replication promotes the stemness of HCCs by upregulating CD133, EpCAM, and CD24 expressions and contributing to drug resistance to sorafenib and regorafenib.

We also investigated the effect of HBV on the stemness of clinical samples from HCC patients. As shown in Figure 3F, CD133 and CD24 expressions were significantly higher in T and NT of HBV-positive patients compared to those from HBV-negative patients. However, no differences were observed in the expression of EpCAM, AFP, OCT4, or Sox2 in these tissues. These data suggest that HBV replication correlates with the stemness of HCC by upregulating markers

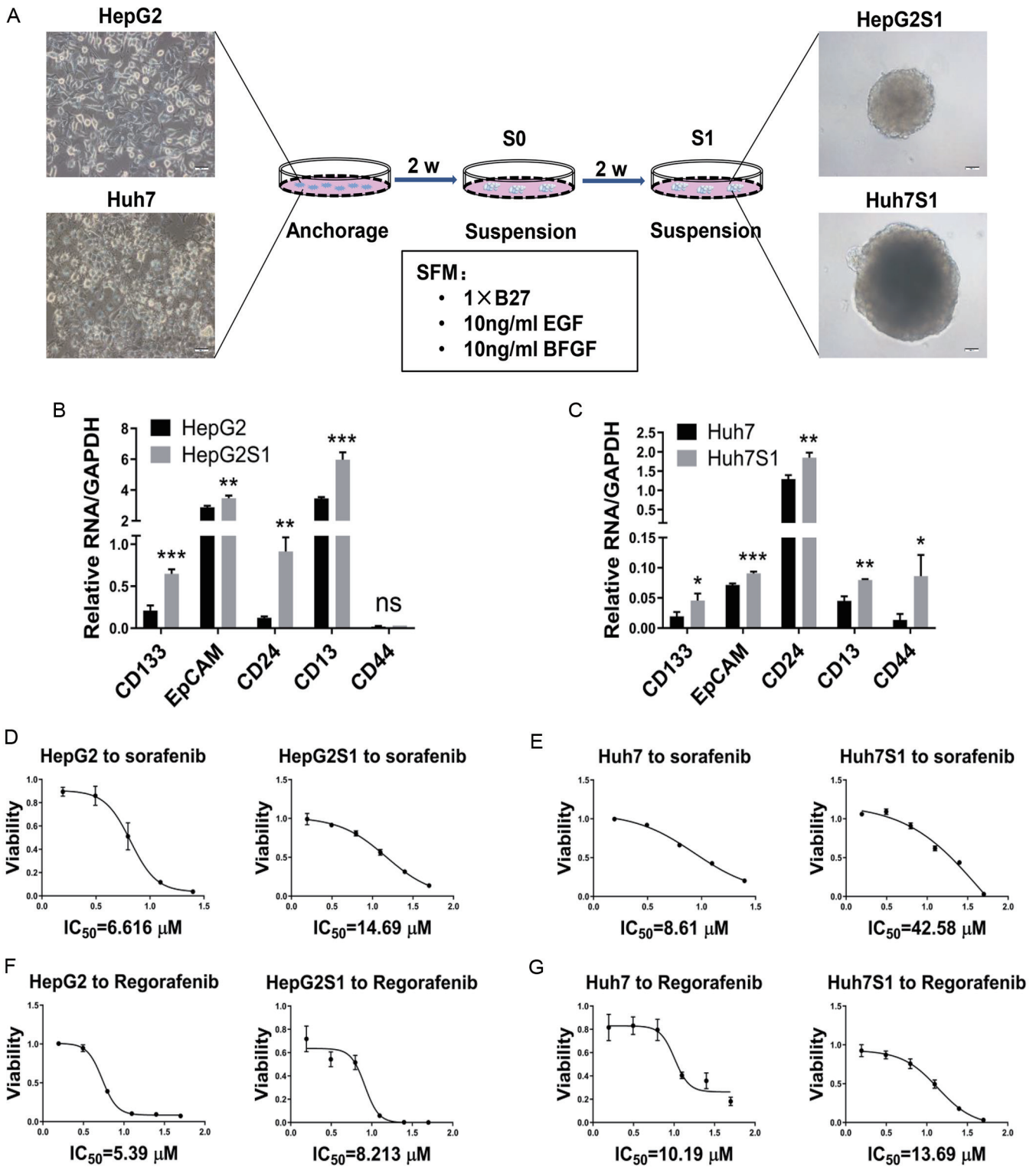


Fig. 1. Hepatocellular carcinoma stem cells enhance sorafenib and regorafenib resistance. (A) Schematic diagram of experimental design for tumor sphere formation. (B) LCSC markers CD133, EpcAM, CD24, CD13, and CD44 were detected in parental HepG2 and tumor spheroids from HepG2 cells (HepG2S1) by RT-qPCR. (C) LCSC markers CD133, EpcAM, CD24, CD13, and CD44 were detected in parental Huh7 and tumor spheroids from Huh7 cells (Huh7S1) by RT-qPCR. (D) IC_{50} values of sorafenib were determined in HepG2 and HepG2S1 cells by cytotoxicity assay. (E) IC_{50} values of sorafenib were determined in Huh7 and Huh7S1 cells by cytotoxicity assay. (F) IC_{50} values of regorafenib were determined in HepG2 and HepG2S1 cells by cytotoxicity assay. (G) IC_{50} values of regorafenib were determined in Huh7 and Huh7S1 cells by cytotoxicity assay. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, no significance. LCSC, liver cancer stem cell; RT-qPCR, real-time polymerase chain reaction.

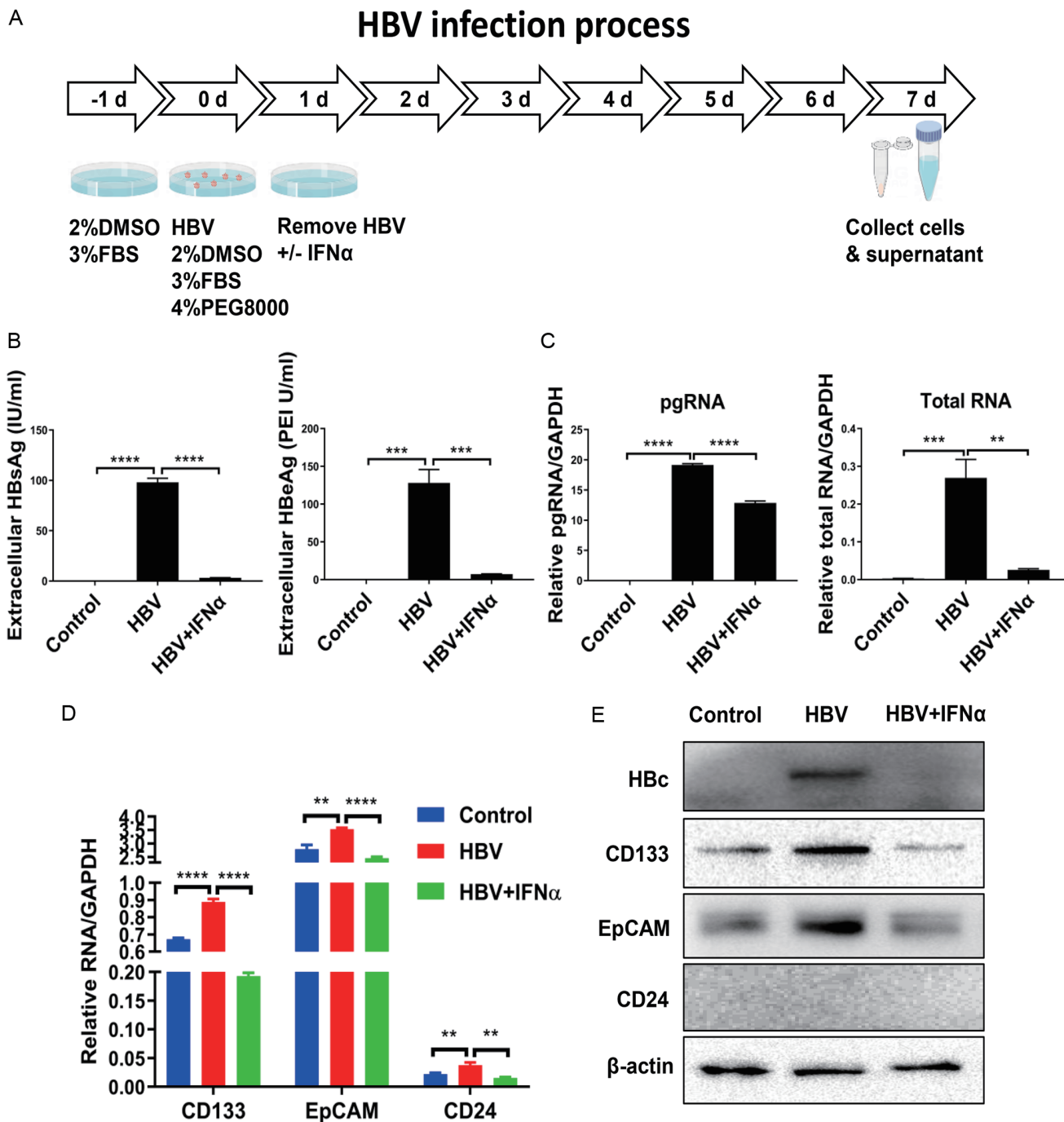


Fig. 2. HBV replication promotes stemness of HCCs in HepG2-NTCP cells. (A) Schematic diagram of experimental design for HBV infection. (B) Secreted HBsAg and HBeAg were measured by CLIA. At 7 dpi of HBV infection, HBV-related markers were detected. (C) HBV total RNA and pgRNA were evaluated by RT-qPCR. (D) CD133, EpCAM, and CD24 expressions were determined by RT-qPCR. (E) HbC, CD133, EpCAM, and CD24 expressions were determined by Western blot. Full-length blots are presented in Supplementary Figure 2. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RT-qPCR, real-time polymerase chain reaction.

such as CD133 and CD24. Taken together, these results reveal that HBV facilitates the generation of CSCs in human hepatoma and clinical HCC tissues.

HBx enhances the stemness of HCC

HBx was considered an oncogenic factor in liver cancer ini-

tiation and progression. Therefore, we speculated that the HBx protein might be involved in regulating the stemness of HCC. To verify the effects of HBx on the stemness of HCC, we established stable HBx-overexpressing HepG2 cells. The results showed that HBx was successfully overexpressed in HepG2 cells (Fig. 4A) and that tumor sphere formation was

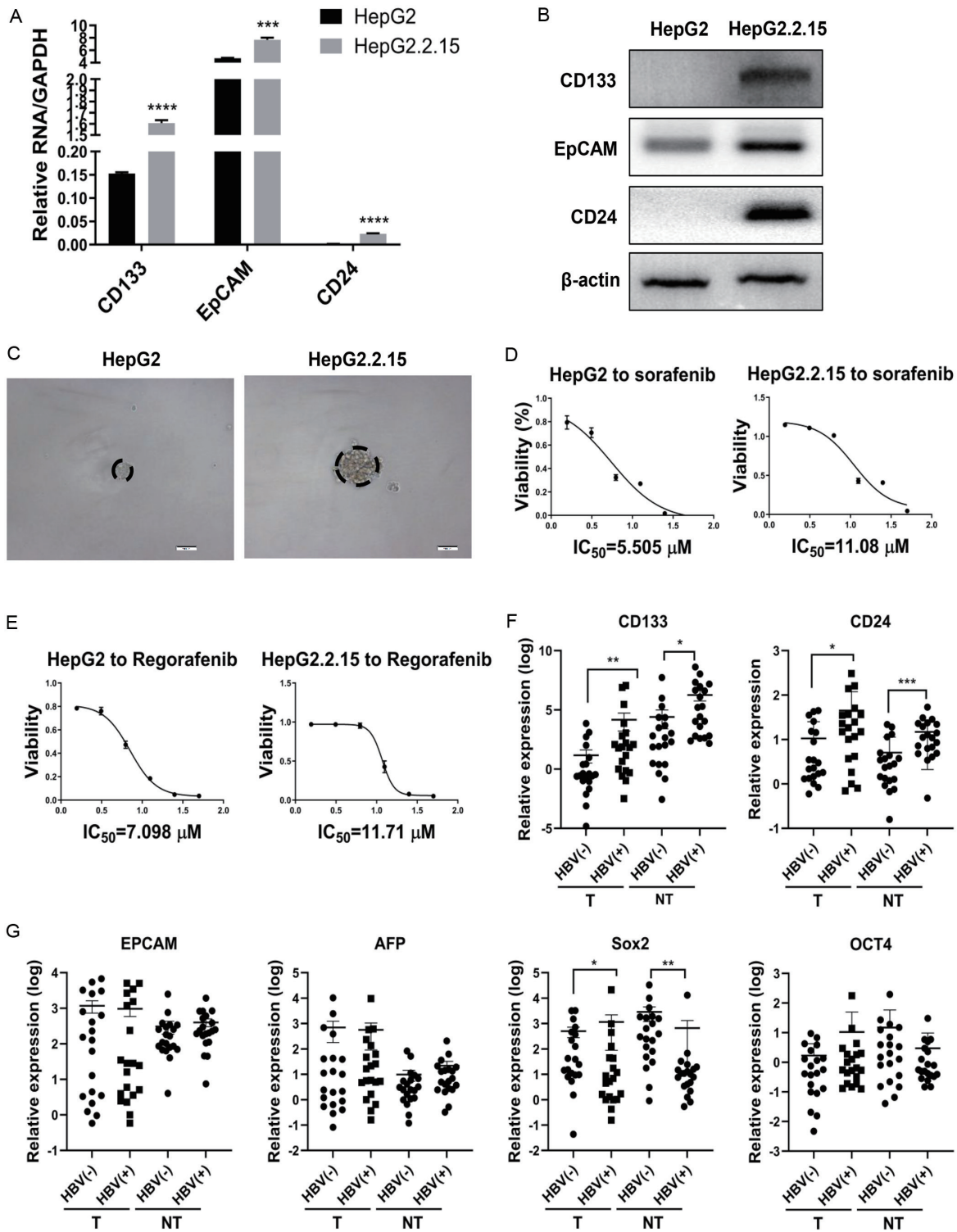


Fig. 3. HBV replication affects stemness of HCCs in HepG2.2.15 cells (A-E) and clinical cancer tissues (F-G). (A) The expression of CD133, EpCAM, and CD24 was determined in HepG2 and HepG2.2.15 cells using RT-qPCR. (B) The levels of CD133, EpCAM, and CD24 were determined in HepG2 and HepG2.2.15 cells by Western blot. Full-length blots are presented in Supplementary Figure 3. (C) Tumor spheroids of HepG2 and HepG2.2.15 cells were formed after seven days by sphere formation assay using ×40 magnification. (D) IC₅₀ values of sorafenib were determined in HepG2 and HepG2.2.15 cells by cytotoxicity assay. (E) IC₅₀ values of regorafenib were determined in HepG2 and HepG2.2.15 cells by cytotoxicity assay. (F-G) The mRNA expressions of CD133 and CD24 (F) and EPCAM, AFP, OCT4, and Sox2 (G) in T and NT of HBV-positive HCC patients compared to HBV-negative HCC patients were detected by RT-qPCR. Dots represent individual values, and horizontal lines represent the median value with an interquartile range. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001; T, tumor tissues; NT, adjacent non-tumor tissues; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RT-qPCR, real-time polymerase chain reaction.

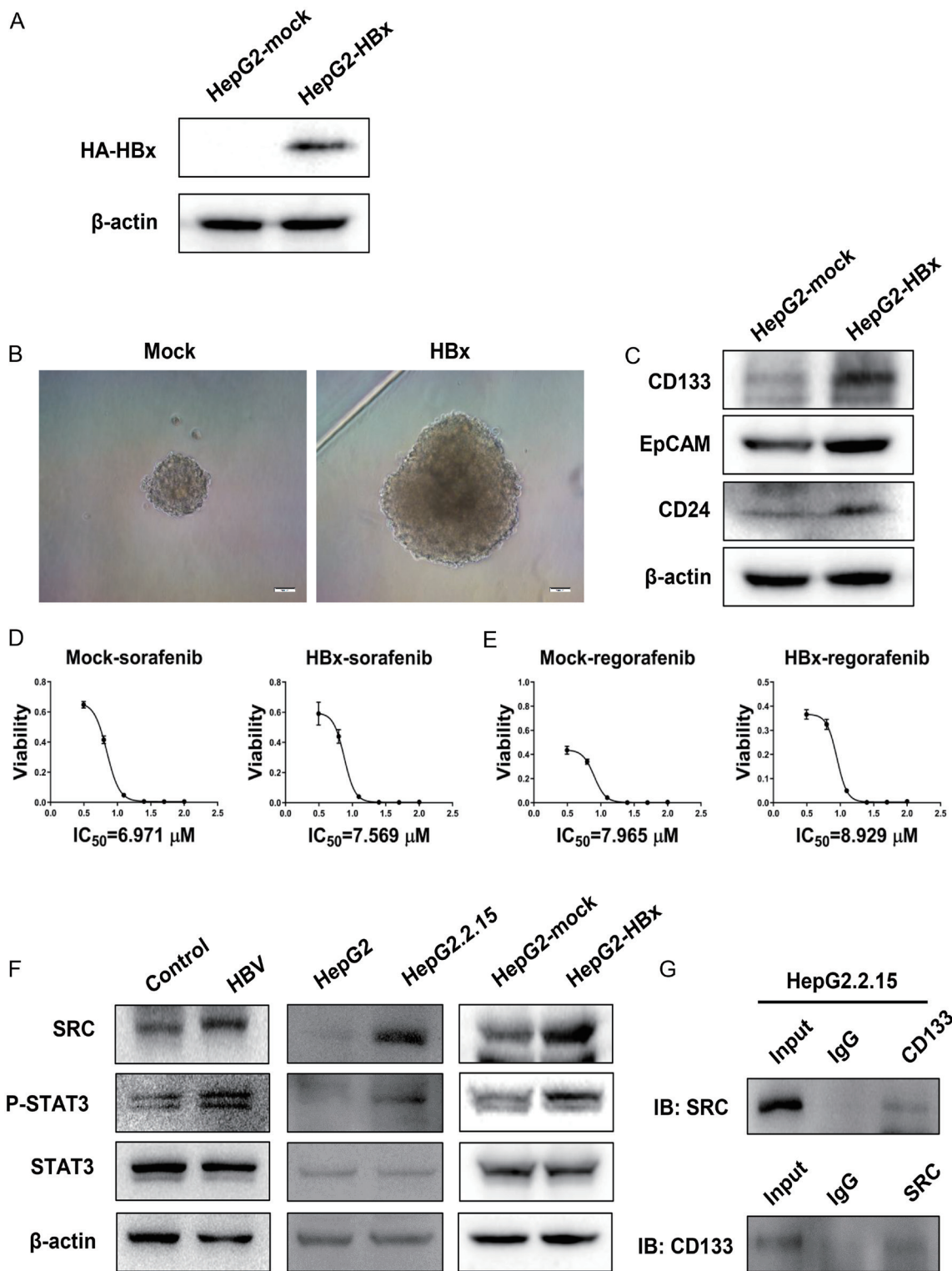


Fig. 4. HBx enhances the stemness of HCC, leading to drug resistance. (A) HBx with HA tag was overexpressed in HepG2 cells, and HBx expression was detected using the HA antibody by Western blot. Full-length blots are presented in Supplementary Figure 4. (B) Tumor spheroids of HepG2-mock and HepG2-HBx cells were formed after 14 days by sphere formation assay using ×40 magnification. (C) CD133, EpCAM, and CD24 expressions were determined in HepG2-mock and HepG2-HBx cells by Western blot. Full-length blots are presented in Supplementary Figure 4. (D) IC₅₀ values of sorafenib were determined in HepG2-mock and HepG2-HBx cells by cytotoxicity assay. (E) IC₅₀ values of regorafenib were determined in HepG2-mock and HepG2-HBx cells by cytotoxicity assay. (F) SRC, p-STAT3, STAT3, and β-actin expressions were detected by Western blot. Full-length blots are presented in Supplementary Figure 5. (G) The interaction of CD133 and SRC was determined by co-immunoprecipitation (CoIP) in HepG2.2.15 cells. Full-length blots are presented in Supplementary Figure 6. HCC, hepatocellular carcinoma.

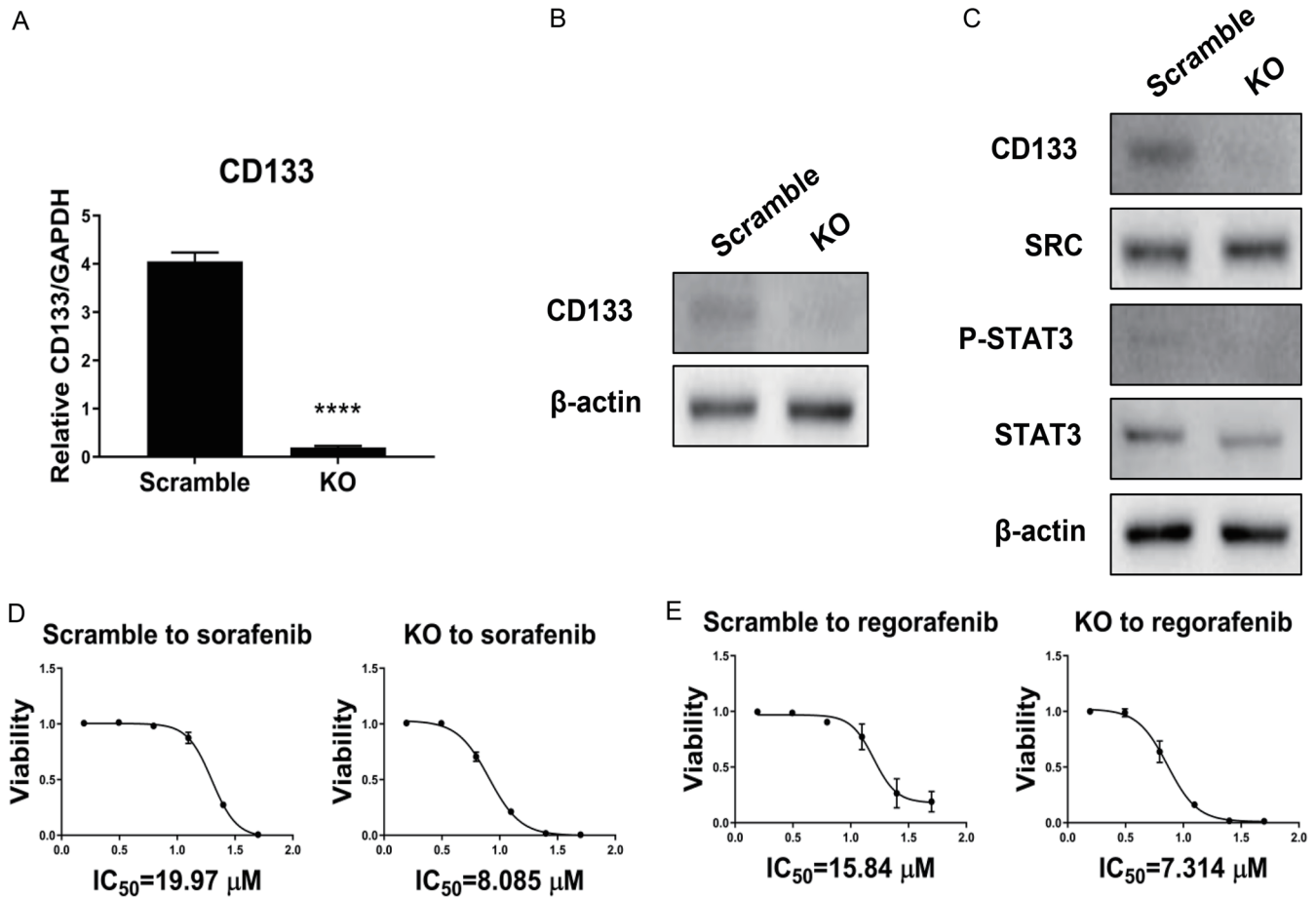


Fig. 5. Knockout of CD133 decreases the stemness of HCC. CRISPR-Cas9 system was applied to knockout CD133 in HepG2.2.15 cells. (A) The efficiency of CD133 knockout was detected by RT-qPCR. (B) The efficiency of CD133 knockout was detected by Western blot. (C) CD133, SRC, p-STAT3, STAT3, and β-actin expressions were detected by Western blot. Full-length blots are presented in Supplementary Figure 7. (D) IC₅₀ values of sorafenib were determined in HepG2.2.15-scramble and HepG2.2.15-CD133-KO cells by cytotoxicity assay. (E) IC₅₀ values of regorafenib were determined in HepG2.2.15-scramble and HepG2.2.15-CD133-KO cells by cytotoxicity assay. *****p* < 0.0001. HCC, hepatocellular carcinoma.

increased (Fig. 4B). Furthermore, HBx upregulated the expression of CD133, EpCAM, and CD24 in HepG2 cells (Fig. 4C). We then determined the IC₅₀ values of sorafenib and regorafenib, which showed that HBx reduced the sensitivity of HepG2 cells to these drugs (Fig. 4D, E). These findings collectively demonstrate that HBx promotes cancer cell stemness in HCCs and enhances resistance to sorafenib and regorafenib.

A recent study has shown that the C-terminal amino acid region of CD133 (845–857) can interact with SRC.²⁸ SRC has been identified as a non-canonical kinase for STAT3, phosphorylating it at the Tyr705 residue, which enhances stemness-related properties.²⁹ To determine if HBV replication impairs CD133-regulated SRC expression, we performed the following experiments. As shown in Figure 4, HBx upregulated SRC expression and activated the STAT3 signaling pathway (Fig. 4F). We also confirmed that CD133 interacts with SRC in HepG2.2.15 cells (Fig. 4G). These findings suggest that HBV may regulate the stemness of HCC via the SRC/STAT3 signaling pathway.

Knockout of CD133 enhances sorafenib and regorafenib sensitivity in HCCs

To investigate the role of CD133 in drug resistance in HCCs,

we used the CRISPR-Cas9 system to knockout CD133 expression in HepG2.2.15 cells. PCR and Western blot confirmed the successful knockout of CD133 in HepG2.2.15 cells (Fig. 5A, B). We further assessed the expression of CD133, SRC, p-STAT3, and STAT3 by Western blot. Knockout of CD133 downregulated the expression of p-STAT3 and STAT3, but did not affect SRC expression, indicating that CD133 regulates the STAT3 signaling pathway (Fig. 5C). Subsequently, we determined the IC₅₀ values of sorafenib and regorafenib, which showed that CD133 knockout enhanced the sensitivity of HepG2.2.15 cells to these drugs (Fig. 5D, E). These results demonstrate that CD133 contributes to sorafenib and regorafenib resistance in HCCs.

Discussion

The majority of HBV infections ultimately lead to HCC tumorigenesis and progression. HCC is characterized by a poor prognosis and a low five-year survival rate, which are closely related to drug resistance. Drug resistance is commonly observed in advanced HCC patients, and LCSCs are a major contributor to this resistance. In this study, our analysis of human HCC data revealed that the HBx protein promotes HCC stemness and drug resistance by upregulating the ex-

pression of CD133, EpCAM, and CD24, and by activating the SRC/STAT3 signaling pathway in HCCs. Furthermore, the knockout of CD133 expression by CRISPR-Cas9 enhanced drug sensitivity. Thus, we demonstrate that HBV promotes the stemness of HCCs, resulting in drug resistance, including to sorafenib and regorafenib.

Moreover, HBV promotes chemotherapy resistance in HCC by regulating Wnt, MAPK, STAT, p53, Akt, and Notch signaling pathways.^{30–33} Studies have found that sorafenib has a poor prognosis in HCC patients with HBV infection. However, the mechanism by which HBV regulates drug resistance in HCCs remains unclear. Our findings indicate that HBx enhances sorafenib and regorafenib resistance in HCCs by up-regulating the expression of LCSC markers such as CD133, EpCAM, and CD24. Additionally, overexpression of HBx activates the SRC/STAT3 signaling pathway in HepG2 cells.

CD133 expression in cancers correlates with tumorigenesis and progression and affects cancer cell stemness. Stemness in cancer cells is often a major cause of chemoresistance, leading to tumor recurrence and progression.³⁴ Liver cancer cells expressing CD133 have been reported to be involved in chemotherapy resistance by activating AKT/PKB and Bcl-2 survival response signals.³⁵ In addition, CD133 inhibits apoptosis and upregulates FLIP expression, resulting in chemotherapy resistance.³⁶ Our previous study showed differential gene expression between the HBV and HBV + IFN α groups by RNA sequencing. CD133 expression was significantly lower in the HBV + IFN α group than in the HBV group.³⁷ Moreover, HBV replication markers, such as pgRNA levels, were positively correlated with CD133 expression in distal non-tumor tissues of HCC patients.¹⁸ Therefore, we speculate that HBV may regulate liver cancer stem cells through CD133, which, in turn, results in drug resistance. A recent study demonstrated that the C-terminal amino acid region of CD133 (845–857) can interact with SRC.²⁸ The non-receptor tyrosine kinase SRC directly activates the STAT3 signal, and phosphorylated STAT3 leads to homodimerization and translocation to the nucleus, where it regulates downstream target gene expression.³⁸

In this study, we analyzed how HBV infection can regulate the stemness of HCCs, and CD133 may act as a key regulator of HCC chemoresistance. Our data propose an innovative mechanism of drug resistance in HBV-infected liver cancer patients, which could help design clinical strategies to improve drug response in liver cancers. Targeting CD133 could increase the drug sensitivity to sorafenib in HCC patients who are infected with HBV. Additionally, combination therapy using sorafenib alongside SRC/STAT3 signaling pathway inhibitors could potentially delay disease progression by suppressing drug resistance in HCC patients. However, these conclusions have not yet been validated in clinical studies. Currently, the molecular mechanisms by which HBV-regulated LCSC markers contribute to drug resistance require further investigation. This study identifies the CD133-SRC/STAT3 pathway as a key mechanism, but further mechanistic insights into how HBx upregulates CD133 and how this interaction enhances stemness and drug resistance need to be explored. Additionally, the roles of other HBV proteins in drug resistance, as well as the underlying mechanisms of HBx-mediated regulation of CD133 in HCCs, need to be determined.

Conclusions

This study found that HBV increases sorafenib and regorafenib resistance in HCCs through upregulation of CD133 and activation of the SRC/STAT3 signaling pathway. Our data provide a potentially key molecular target for drug resistance

in HBV-infected HCCs, offering a strong theoretical basis for the development of specific and efficient treatments for HBV-infected HCCs.

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Study concept and design (KX, TL), acquisition of data (XJ, JW), analysis and interpretation of data (XJ, HD, XL, GO, LW, XT), drafting of the manuscript (XJ, JW), critical revision of the manuscript for important intellectual content (KX), administrative, technical, or material support (KX), and study supervision (KX, HZ, TL). All authors have made significant contributions to this study and have approved the final version and publication of the manuscript.

Ethical statement

The patients were enrolled in this Institutional Review Board-approved study (S2018-111-01). Informed consent was obtained regarding the use of their samples for further studies.

Data sharing statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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