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## Supplemental information

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# Upregulation of miR-520c-3p via Hepatitis B Virus Drives Hepatocellular Migration and Invasion by the PTEN/AKT/NF-κB axis

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Table S1. Primer sequences or siRNA sequences used in this study.

| Name | Sequences | Tm (°C) |
|---|---|---|
| MiR-520c promoter (-3887)-(+31)-FP | 5'-GTT**GCTAGC**GTACCATGCACTGATCACACCTTCG-3' | 65.1 |
| MiR-520c promoter (-3887)-(+31)-RP | 5'-AAC**CTCGAG**TGCTTCCCTCTAGAGGACGACAGC-3' | 65.8 |
| MiR-520c promoter (-3887)-(-1999)-FP | 5'-GTT**GCTAGC**GTACCATGCACTGATCACACCTTCG-3' | 65.1 |
| MiR-520c promoter (-3887)-( -1999)-RP | 5'-AAC**CTCGAG**GATCACGTCACTACACTCCAGCA-3' | 63.6 |
| MiR-520c promoter (-1999)-(+31)-FP | 5'-GAC**GCTAGC**TCAGCTCACTGCAACCTCCACCTCC-3' | 68.7 |
| MiR-520c promoter (-1999)-(+31)-RP | 5'-AAC**CTCGAG**TGCTTCCCTCTAGAGGACGACAGC-3' | 65.8 |
| MiR-520c promoter (-3887)-(-3110)-FP | 5'-GTT**GCTAGC**GTACCATGCACTGATCACACCTTCG-3' | 65.1 |
| MiR-520c promoter (-3887)-(-3110)-RP | 5'-AAC**CTCGAG**GCCACAGCCAACATTTCACAGC-3' | 70.9 |
| MiR-520c promoter (-3110)-(-1999)-FP | 5'-GTT**GCTAGC**TAATATGAGAAACAAACTATGGTAGACAAGATG-3' | 55.9 |
| MiR-520c promoter (-3110)-( -1999)-RP | 5'-AAC**CTCGAG**GATCACGTCACTACACTCCAGCA-3' | 63.6 |
| ChIP-Primer-1-FP | 5'-GGTGTGGTGGCGTGAACCTAGGA-3' | 67.9 |
| ChIP-Primer-1-RP | 5'-TAAGATTGGATAGACTTGAAGGCTTACTTACC-3' | 58.5 |
| ChIP-Primer-2-FP | 5'-GTCCTTGACACTGAAATTTTTTTCTCT-3' | 58.6 |
| ChIP-Primer-2-RP | 5'-ACCCGAAGAAAATAGCATAATCAGGG-3' | 62.6 |
| GAPDH-FP | 5'-GAGAAGGCTGGGGCTCATTT-3' | 60.7 |
| GAPDH-RP | 5'-TAAGCAGTTGGTGGTGCAGG-3' | 59.3 |
| PTEN-FP | 5'-GCGGAACTTGCAATCCTCAG-3' | 60.6 |
| PTEN-RP | 5'-AGGTTTCCTCTGGTCCTGGT-3' | 57.3 |
| CREB1-FP | 5'-GCC**GCTAGC**ATGACCATGGAATCTGGAGCC-3' | 70.7 |
| CREB1-RP | 5'-AAT**GCGGCCGC**TTAATCTGATTTGTGGCAGTAAAGG-3' | 69.1 |
| PTEN-3'UTR-FP | 5'-GG**ACTAGT**CTGTGGATGCTTCATGTGCTG-3' | 61.6 |
| PTEN-3'UTR-RP | 5'-AGCTTT**GTTTAAAC**CGTATGCAGTCTGGGCATATCA-3' | 61.6 |
| PTEN-3'UTR-M-FP | 5'-TATGATGTGTATATTCAATAGCTGTC-3' | 51.9 |
| PTEN-3'UTR-M-RP | 5'-ATTGAATATACACATCATAAATCTTC-3' | 50.5 |
| si-h-PTEN-1 | 5'-GGATAAAACACCATGAAAA-3' | |
| si-h-PTEN-2 | 5'-GAGCGTGCAGATAATGACA-3' | |
| si-h-PTEN-3 | 5'-GGCGCTATGTGTATTATTA-3' | |
| si-h-CREB1 | 5'-GUCUCCACA AGUCCA AACATT-3' | |
| hsa-miR-520c-3p-mimic | 5'-AAAGUGCUUCCUUUUAGAGGGU-3';<br>3'-UUUCACGAAGGAAAAUCUCCCA-5' | |
| hsa-miR-520c-3p-inhibitor/antagomir | 5'-ACCCUCUAAAAGGAAGCACUUU-3' | |
| HBx-FP | 5'-ACTTCGCTTCACCTCTGCAC-3' | 57.6 |
| HBx-RP | 5'-AGTATGCCTCAAGGTCGGTC-3' | 56.5 |
| MDH1-PGK-GFP_2.0-miR520c-FP | 5'-AATAT**CTCGAG**CCCTGCCCCACCTATACTCGTCT-3' | 70.1 |
| MDH1-PGK-GFP_2.0-miR520c-RP | 5'-CCG**GAATTC**AATTAATTTTTTCATCATCCATCTCTGATGATAAGC-3' | 61.0 |

GCTAGC: *Nhe* I; CTCGAG: *Xho* I; GCGGCCGC: *Not* I; ACTAGT: *Spe* I; GTTTAAAC: *Pme* I; GAATTC: *EcoR* I

**Figure S1**

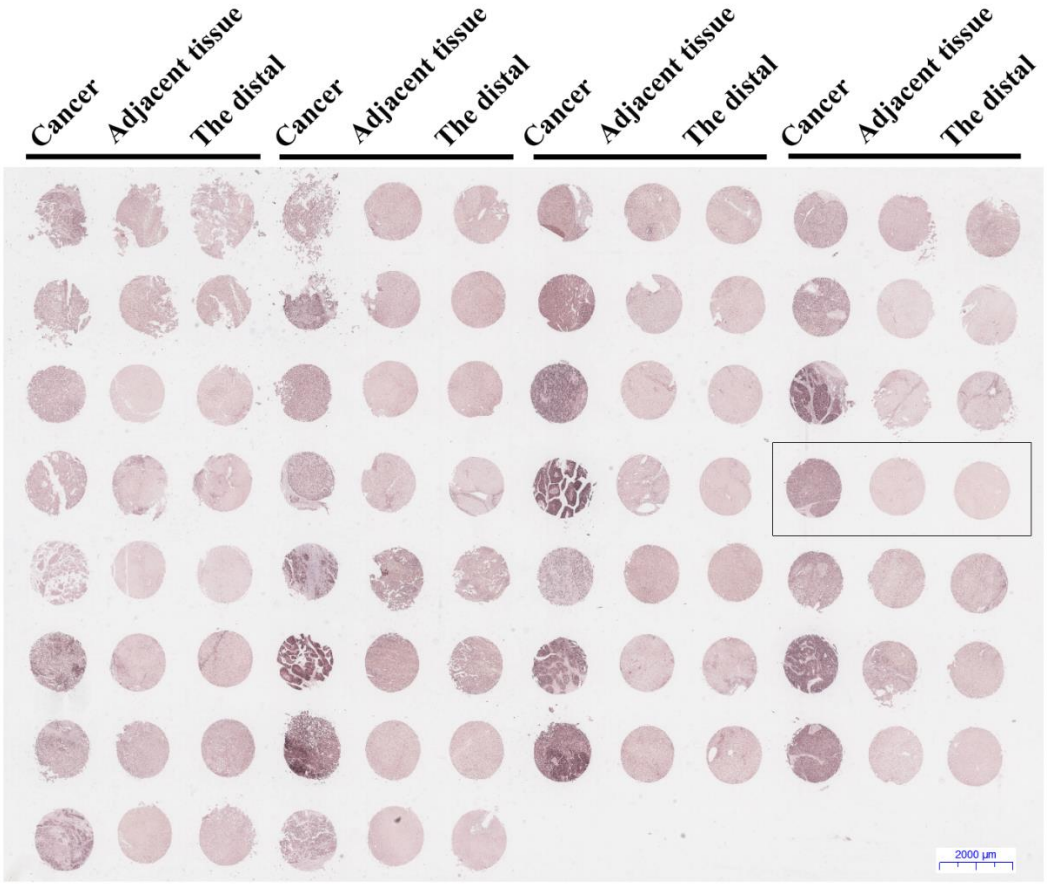


**Figure S1.** Analysis of miR-520c-3p level in tissue samples by in situ hybridization (ISH).

**Figure S2**

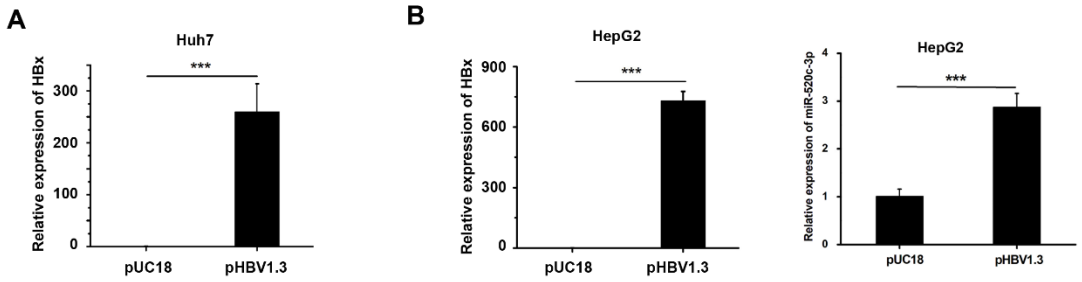


**Figure S2.** HBV upregulates the expression of miR-520c-3p. The success of the transfection of pHBV1.3 in different cells was demonstrated by the mRNA expression level of HBx (A and B). Increased expression of miR-520c-3p in HepG2 cells transfected with pHBV1.3 compared to the control plasmid (B, right panel).

**Figure S3**

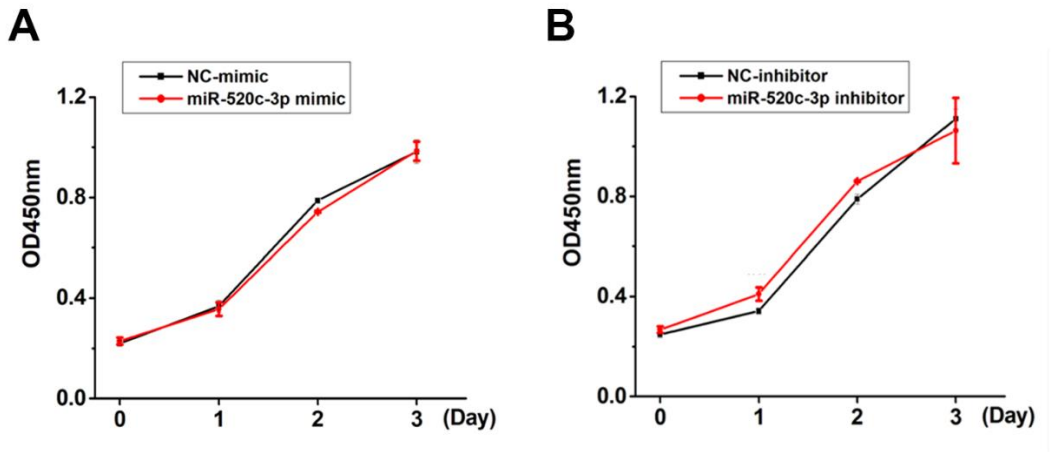


**Figure S3.** miR-520c-3p has little effect on the proliferation of HepG2.2.15 cells. miR520c-3p mimic (A) and miR520c-3p inhibitor (B) were transfected and the proliferation rate was detected.

**Figure S4**

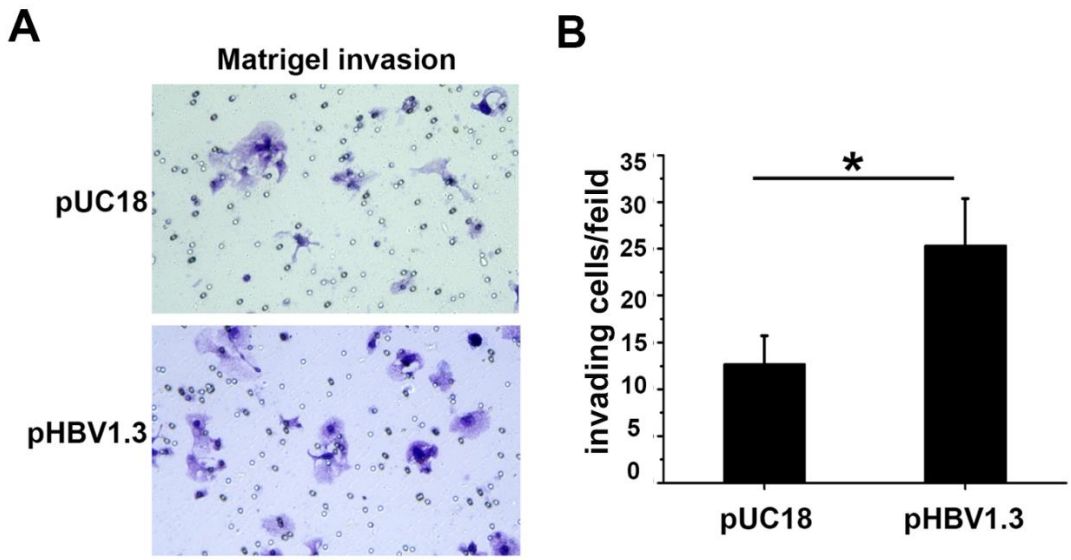


**Figure S4.** HBV promotes hepatomas cells invasion.

**Figure S5**

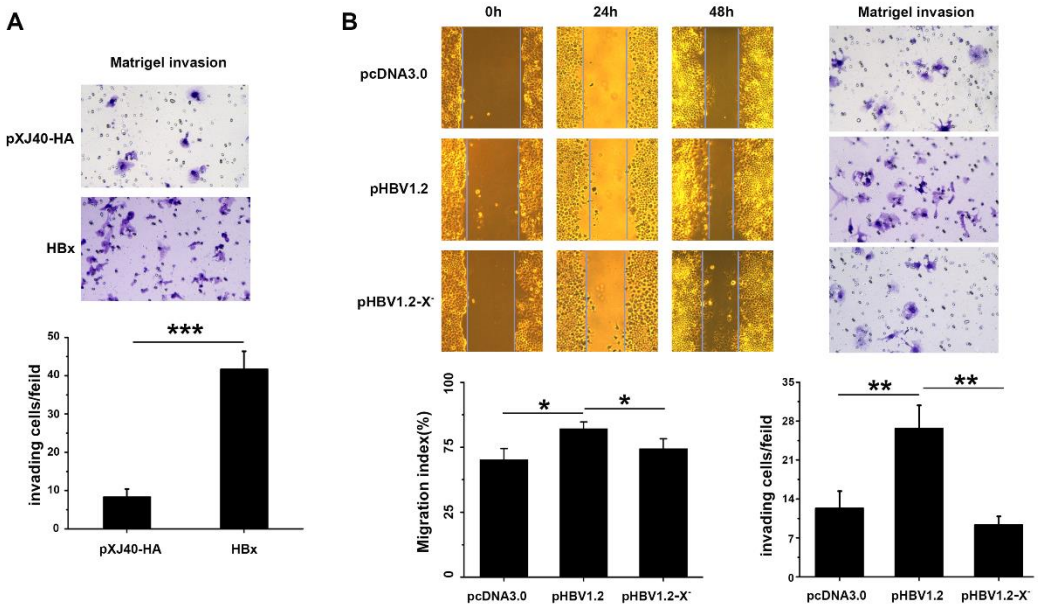


**Figure S5.** HBx promotes hepatomas cells invasion. (A) HBx-transfected Huh7 cells acquires greater invasive capabilities than the control transfection with pXJ40-HA. (B) Removing HBx from the HBV plasmid pHBV1.2 abolishes the enhanced migration (left panel) and invasion (right panel) of Huh7 caused by the transfection of the pHBV1.2. Note that pHBV1.2-$X^-$ is the same as pHBV1.2 except the removal of the HBx.

**Figure S6**

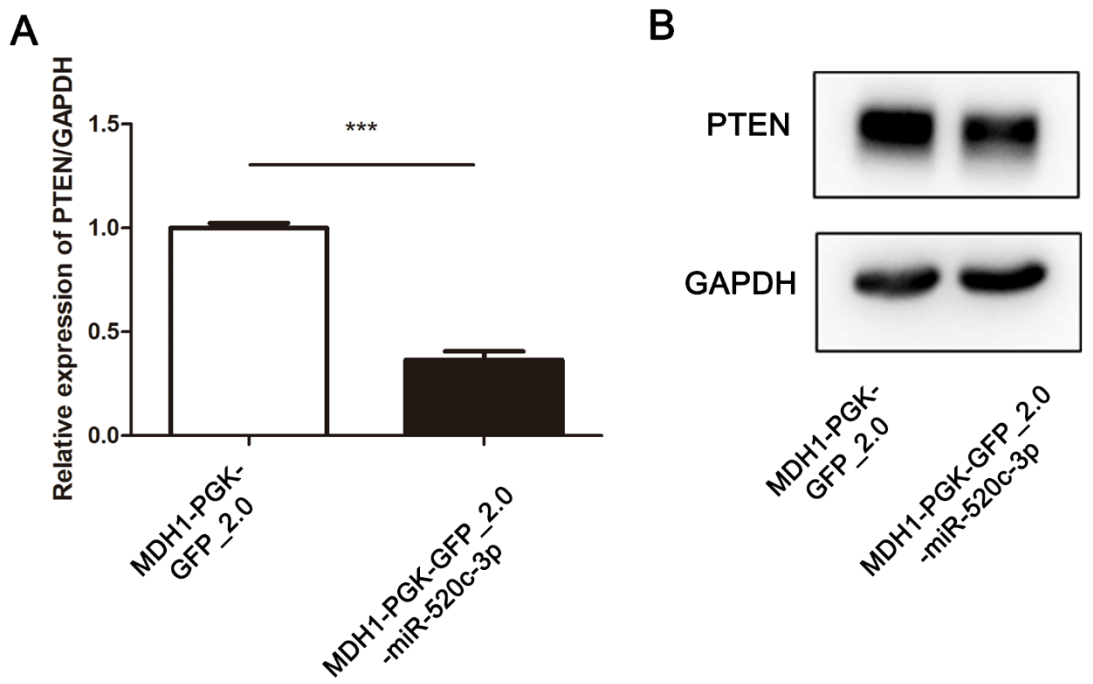


**Figure S6.** PTEN mRNA (A) and protein (B) levels in LM3-miR-520c stably transfected cells or control cells as detected by qPCR and western blot.

**Figure S7**

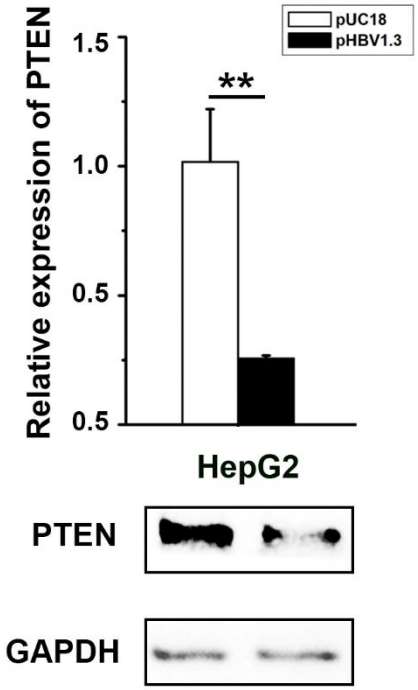


**Figure S7.** PTEN mRNA (up panel) and protein (right panel) levels are reduced by HBV in HepG2 cells.

**Figure S8**

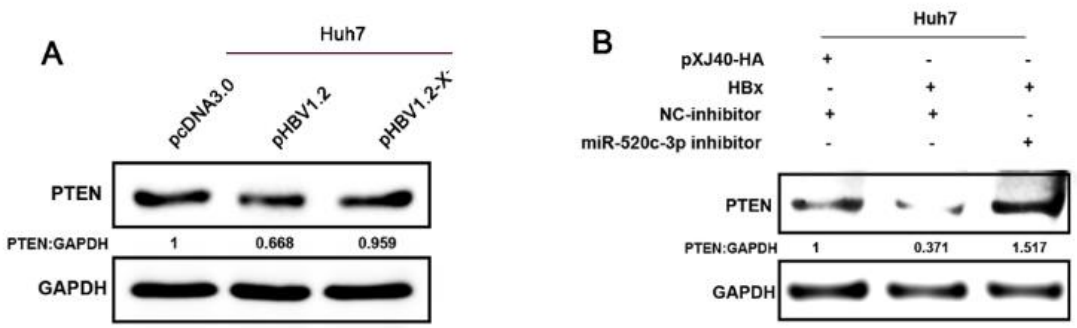


**Figure S8.** Anti-520c-3p reverses HBx-mediated PTEN repression. (A) HBx reduces PTEN level. Huh7 cells were transfected with pcDNA3.0, pcDNA3.0-HBV1.2 or pcDNA3.0-HBV1.2-$X^-$ (a mutant pHBV vector without HBx) plasmids and the level of PTEN was analyzed. (B) The western blot analysis shows that the repression of PTEN protein level by HBx requires miR-520c-3p. Huh7 cells were transfected with indicated plasmids and/or inhibitors and PTEN level was analyzed.

**Figure S9**

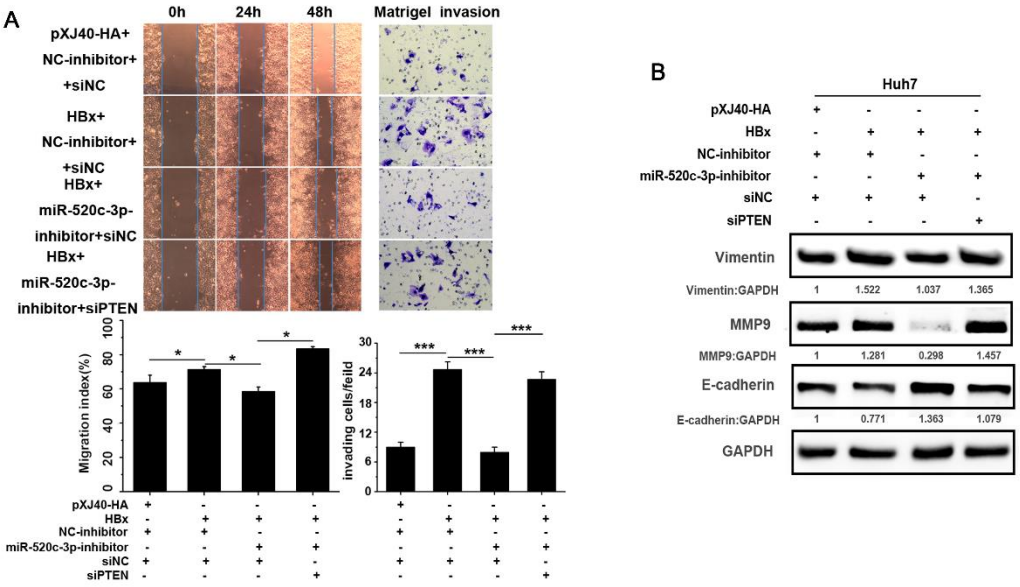


**Figure S9.** HBx-induced cell migration and invasion, and EMT require miR-520c-3p-mediated repression of PTEN. (A-B) After co-transfection of Huh7 cells with pxj40-HA or pxj40-HBx-HA, NC-inhibitor or miR-520c-3p inhibitor, and siNC or siPTEN, wound healing and invasion assay were used to measure the migration and invasive capacity of Huh7 as in Fig. 3F (A), and the levels of EMT-related proteins Vimentin, E-cadherin and MMP9 were examined by western blot analysis (B).

**Figure S10**

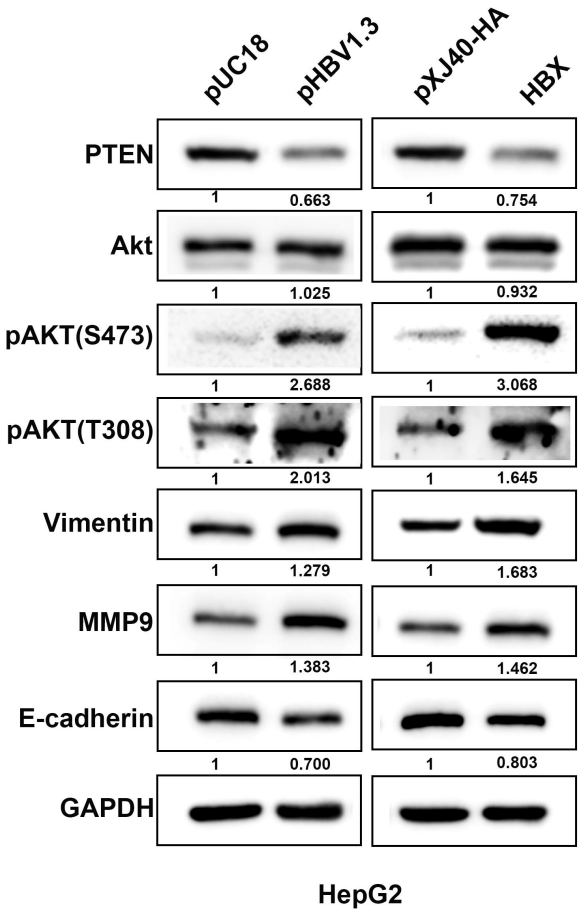


**Figure S10.** HBV or HBx reduces PTEN and activates AKT signaling. HepG2 cells were transfected with HBV or HBx or their corresponding control pUC18 or pXJ40-HA, respectively, 48 h after transfection, the cells were analyzed for the levels of AKT, p-AKT, Vimentin, E- cadherin, MMP9, and PTEN.

**Figure S11**

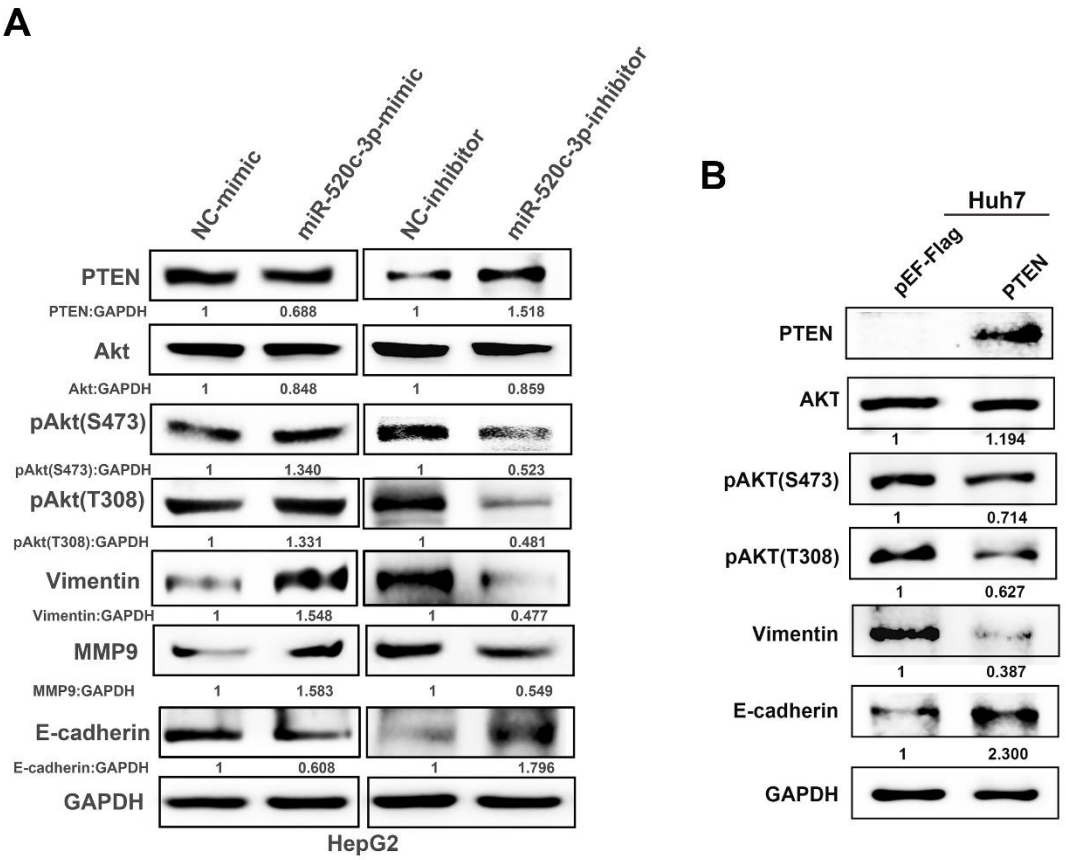


**Figure S11.** miR-520c-3p activates AKT and PTEN suppresses AKT activity. (A). HepG2 cells were transfected with miR-520c-3p. 48h after transfection, the cells were analyzed for the levels of AKT, p-AKT, Vimentin, E-cadherin, MMP9, and PTEN. (B) Huh7 cells were transfected with PTEN expression plasmid or control (pEF-Flag). 48h after transfection, the cells were analyzed for the levels of AKT, p-AKT, Vimentin, E-cadherin and PTEN.

**Figure S12**

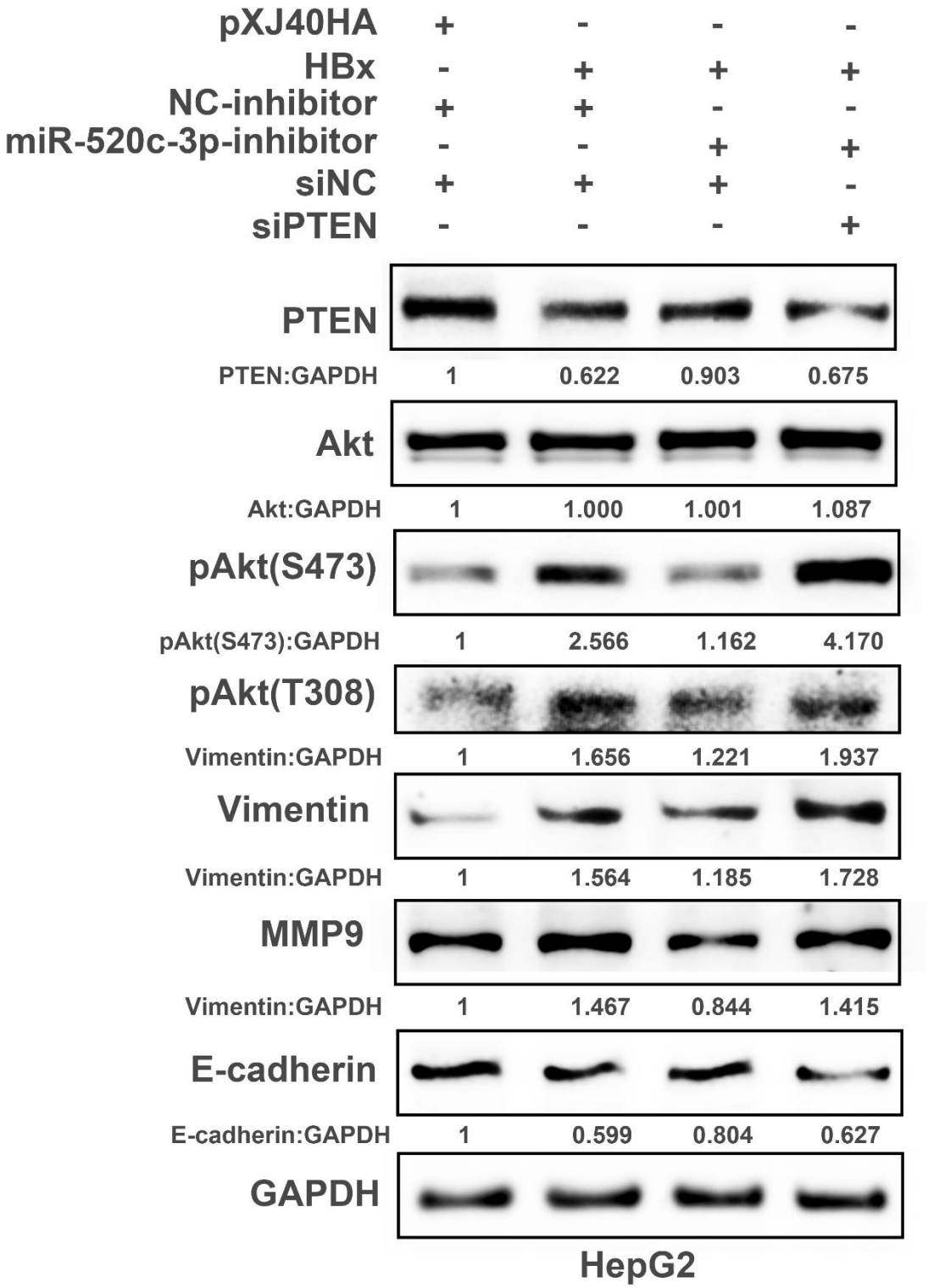


**Figure S12.** Inhibiting miR-520c-3p prevents the activation of AKT by HBx while knocking down PTEN reverses this effect. HepG2 cells were co-transfected with HBx, miR-520c-3p inhibitor and siPTEN and western blot analyses were done for indicated proteins.

**Figure S13**

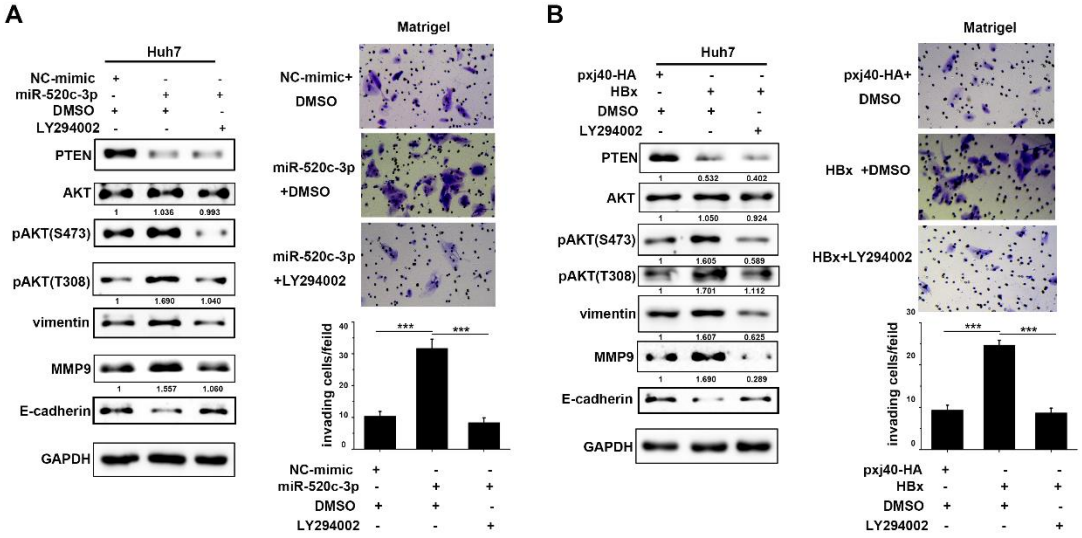


**Figure S13.** AKT activation is required for miR-520c-3p- or HBx-induced cell invasion. (A) Huh7 cells were transfected with miR-520c-3p mimic or NC-mimic for 36h, and treated with LY294002, a specific inhibitor of AKT phosphorylation, or DMSO for 12h. The cells were then subjected to western blot analyses of indicated proteins (Left) or cell invasion assay as in Fig. 2A (Right). (B) Huh7 cells at 36 hours after transfection with pxj40-HBx-HA or empty vector were treated with LY294002 or DMSO for 12 hours. The cells were analyzed as above. Three independent experiments were performed, and representative data are shown. $^{**}p< 0.01$, $^{***}p< 0.001$ in panels A-B.

## Figure S14

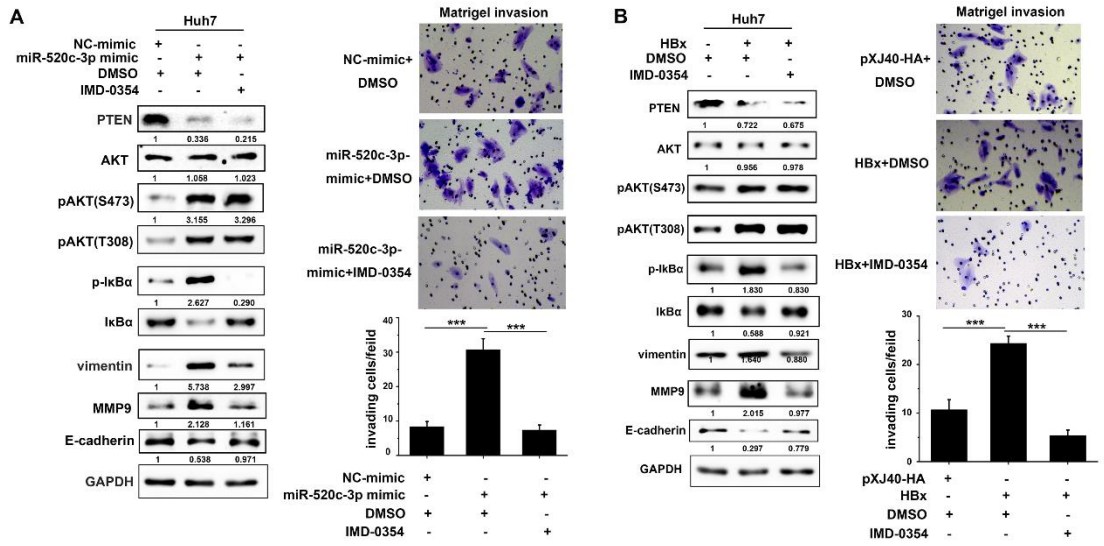


**Figure S14.** miR-520c-3p or HBx induces cell migration and invasion via activating NF-κB (p-IκBα). (A) Huh7 cells were pretreated with 5 mM NF-κB inhibitor IMD-0354 or DMSO for 4 hours followed by transfection with NC or miR-520c-3p mimic. 48 hours later, the levels of indicated proteins were examined by western blot analyses. (B) Huh7 cells were pretreated with IMD-0354 followed by transfection with pxj40-HA-HBx or empty vector. They were then subjected to western blot analyses of indicated proteins (Left) or cell migration and invasion assays as in Fig. 2A (Right).

**Figure S15**

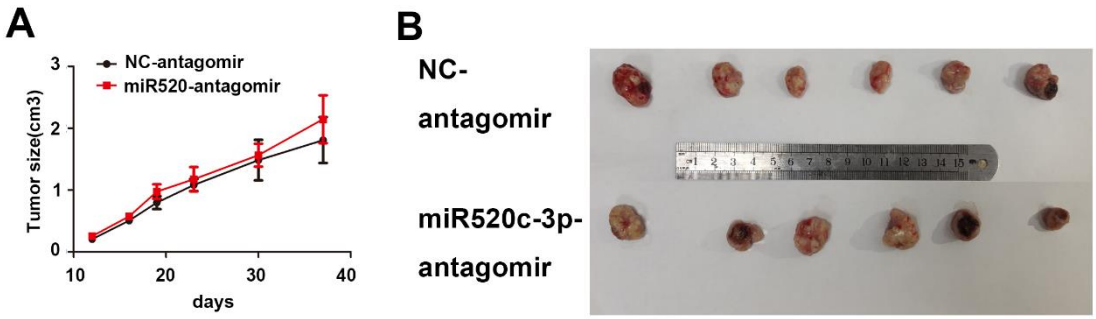


**Figure S15.** miR-520c-3p had no effect on tumor growth. (A) Tumor size was measured 2 weeks after subcutaneous implantation of tumor cells. The tumor growth curve was calculated. (B) After the death of the nude mice, the tumor size was compared.