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Hepatitis B virus X (HBX) protein upregulates β-catenin in a human hepatic cell line by sequestering SIRT1 deacetylase

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Abstract. Hepatitis B virus X (HBX) protein has been reported to induce upregulation of β-catenin, a known proto-oncogene, in p53-knockout and p53-mutant hepatic cell lines both in a GSK-3β-dependent manner and via interaction with adenomatous polyposis coli, which results in protection from β-catenin degradation. In this study, we describe a novel mechanism for HBX-mediated upregulation of β-catenin. We observed that HBX interacts with SIRT1, a class III histone deacetylase. Furthermore, the presence of HBX attenuated the interaction between SIRT1 and β-catenin, leading to protection of β-catenin from the inhibitory action of SIRT1. Reduction of SIRT1 with siRNA or suppression of SIRT1 activity with nicotinamide upregulated β-catenin protein levels. In contrast, enhancement of SIRT1 activity with resveratrol reduced β-catenin protein levels. Furthermore, in Hep3B cells stably expressing HBX, overexpression of SIRT1 or treatment with resveratrol enhanced sensitivity to doxorubicin-induced apoptosis, indicating that upregulation of SIRT1 could be a therapeutic strategy for HBV-related hepatocellular carcinoma. Based on these results, we propose that HBX upregulates β-catenin by sequestering SIRT1, which leads to anticancer drug treatment resistance.

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Introduction

The human hepatitis B virus (HBV) induces acute and chronic hepatitis and is closely associated with liver cancer (1,2). Among the 4 proteins derived from the HBV genome, the hepatitis B virus X (HBX) protein is involved in multiple signaling pathways associated with cell survival and proliferation. The cell signal transduction pathways that are activated by HBX include Jak1/Stat3, the PI-3 kinase pathways (3-6) and the Ras/Raf/MAPK signaling cascade which leads to NF-κB activation (7,8). HBX expression also increases reactive oxygen species (ROS) via calcium signaling and cellular kinases, which results in activation of the transcription factors NF-κB and Stat3 (3). In addition, HBX enhances Stat1 activity, which results in Type I IFN production (9). However, HBX-mediated downregulation of Type I IFN receptor inhibits extracellular IFN signal transduction (10). Other studies have shown that HBV-induced oxidative stress also stimulates the translocation of Raf-1 and a dominant negative PAK mutant and Src inhibitors abolish HBX-mediated Raf-1 mitochondrial translocation (11). We have previously reported that HBX induces upregulation of Foxo4, leading to the alleviation of oxidative stress through the JNK signaling pathway, which may provide a favorable environment for the survival of HBV-infected hepatic cells (12).

SIRT1, a member of the class III histone deacetylases, has been conserved throughout evolution from yeast to human (13) and is the key molecule responsible for extending life span in response to caloric restriction (14-16). SIRT1 can deacetylate not only histones (17) but also non-histone proteins including NF-κB p65 (18), p53 (19), Ku70 (20) and Foxo3a (21). It has been reported that SIRT1 expression is regulated at multiple levels and SIRT1 activity is regulated by cellular factors. Interaction of deleted in breast cancer (DBC)1 with SIRT1 inhibits SIRT1 activity, leading to a reduction of p53 deacetylation, which

indicates that DBC1 acts as a negative SIRT1 regulator (22,23). The active regulator of SIRT1 (AROS), identified as a SIRT1 interacting partner in a yeast two-hybrid screen, increases the enzymatic efficiency of SIRT1 through a conformational change (24). It has been also been demonstrated that interaction of the human immunodeficiency virus (HIV) Tat protein with SIRT1 blocks SIRT1 activity, similarly to the action of DBC1 (25).

The Wnt/β-catenin signaling pathway plays an important role in cell proliferation, differentiation, and oncogenesis (26,27). In particular, abnormal upregulation of Wnt/β-catenin activity has frequently been detected as an early event in many cancers (28). The β-catenin protein levels are normally regulated in part through the protein's targeted decay, which is mediated through a glycogen synthase kinase-3β (GSK-3β)-dependent or -independent pathway (29). In the absence of Wnt signaling, cytoplasmic β-catenin is sequentially phosphorylated by casein kinase and GSK-3β, which form a complex with adenomatous polyposis coli (APC) and axin, resulting in the degradation of β-catenin via a ubiquitination-dependent mechanism. Activation of the receptor by its Wnt ligands negatively regulates GSK-3β, leading to the stabilization of β-catenin in the cytoplasm (30). During Wnt/β-catenin signaling, β-catenin is translocated into the nucleus. There, it forms a complex with members of the T cell factor/lymphocyte enhancer factor(TCF/LEF) family of transcription factors, and thereby activates the expression of β-catenin responsive genes, such as *cyclin-D1, c-Jun, c-Myc*, and peroxisome proliferator-activated receptor-δ (*PPAR-δ*) (31-34), which play important roles in oncogenesis.

This study was undertaken to determine the novel oncogenic mechanism by which HBX functions in the proliferation and survival of hepatocellular carcinoma cells. We herein report that HBX binds to SIRT1. The interaction of HBX with SIRT1 protects β-catenin from the inhibitory action of SIRT1, leading to upregulation of β-catenin protein and activity. Therefore, we propose that HBX upregulates β-catenin by sequestering SIRT1 in a Wnt signaling-independent manner.

Materials and methods

Cell cultures and transient transfection. HEK293 T cells, parental Hep3B, and Hep3B cells stably expressing vector (Hep3B-Vec) or HBX (Hep3B-HBX) were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were plated at $5x10^5$ cells/well of a 6-well plate 24 h before transfection and the cells were transfected with 3-5 μg of DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Protein expression was examined by immunoblotting 48 h after transfection.

Reagent, antibodies, and plasmids. Nicotinamide, resveratrol, and wortmannin were purchased from Calbiochem (San Diego, CA, USA). LiCl was obtained from Sigma-Aldrich (St. Louis, MO). Anti-SIRT1, anti-Myc, anti-histone H3 and anti-actin antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-catenin antibody was purchased from BD Biosciences (San Jose, CA). Anti-Akt, anti-phospho-Akt (Ser-473), anti-GSK-3β, and anti-phospho-GSK-3β (Ser-9) antibodies were obtained from Cell Signaling Technology (Danvers, MA). For transient expression of proteins, pCMV-Myc-HBX (12), pEGFP-SIRT1 (35), pCDNA3.1-Myc-SIRT1 (36), and pCMV-Flag-β-catenin (37) were transfected into cells.

siRNA transfection. Cells were trypsinized and incubated overnight to reach 60-70% confluence before siRNA transfection. SIRT1 siRNA (100 nM; sense, 5'-ACUUUGCU GUAACCCUGUA (dTdT)-3' and antisense, 5'-UACAGGGU UACAGCAAAGU (dTdT)-3') (Bioneer Corporation, Daejeon, Korea), which has been described elsewhere (24) or negative control siRNA (Bioneer Corporation) was mixed with Lipofectamine 2000 (Invitrogen). The cells were incubated with the transfection mixture for 6 h, and then rinsed with DMEM containing 10% serum. The cells were incubated for 48 h before harvest.

Immunoprecipitation and immunoblotting. For immunoblotting, cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% NP-40, and 50 mM Tris-HCl, pH 7.5) containing 0.1 mM Na_2VO_3, 1 mM NaF and protease inhibitors (Sigma-Aldrich). Proteins from whole cell lysates were resolved on 10% or 12% SDS-PAGE gels, and then transferred to nitrocellulose membranes. Primary antibodies were used at 1:1,000 or 1:2,000 dilutions, and horseradish peroxidase-conjugated secondary antibodies were used at a 1:2,000 dilution in 5% nonfat dry milk. For immunoprecipitation, cells were harvested after 48 h of transfection, and the cell debris was removed by centrifugation at 10,000 g for 10 min at 4˚C. Cell lysates were pre-cleared with 25 μl of protein A/G agarose and incubated with the appropriate primary antibody and protein A/G agarose for 1 h at 4˚C. After 3 washes with lysis buffer, the precipitates were loaded on SDS-PAGE gels and analyzed by immunoblotting with the appropriate antibodies. After a final washing, nitrocellulose membranes were exposed to enhanced chemiluminescence using the LAS 3000 (Fujifilm, Tokyo, Japan).

Luciferase reporter assay. HEK293T and Hep3B cells were transfected with pCMV-Myc-HBX, pEGFP-SIRT1, or pCDNA3.1-Myc-SIRT1 plasmid in the presence of the TOPFlash-, or FOPFlash- luciferase reporter vector. To normalize transfection efficiency, a pGK-βgal vector that constitutively expresses β-galactosidase from a phosphoglucokinase promoter was included in the transfection mixture. At 48 h post-transfection, cells were washed with cold PBS and lysed in lysis solution [25 mM Tris (pH 7.8), 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton X-100]. Luciferase activity was measured using a luminometer with a Luciferase kit (Promega Corporation, Madison, WI).

MTT assay. In accordance with the manufacturer's instructions (Promega), dye solution containing tetrazolium was added to the cells and incubated for 4 h. The absorbance of the formazan produced by living cells was measured at 570 nm.

Results

HBX upregulates β-catenin in a human $p53^{-/-}$ hepatic cell line. The frequent observation of abnormal accumulations of

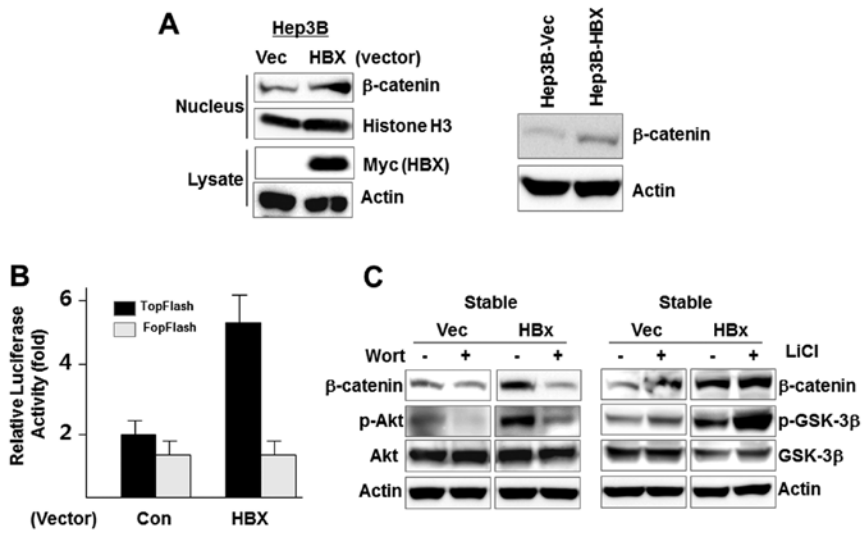

Figure 1. HBX increases β-catenin protein levels and transcriptional activity in Hep3B cells. (A) Nuclear extracts were prepared as previously described (52) from Hep3B cells transiently transfected with a control vector or a HBX-expressing vector (pCMV-Myc-HBX). Cell lysates from Hep3B-Vec and Hep3B-HBX cells were prepared and separated by a 10% SDS PAGE gel. The expression of β-catenin was detected by immunoblotting with anti-β-catenin antibody. (B) Hep3B cells were transfected with pCMV-HBX or pCMV-Myc plasmid in the presence of the pTopFlash or pFopFash luciferase reporter vector. At 48 h post-transfection, luciferase activity was measured and the transfection efficiency was normalized with β-galactosidase vector (pGK-β-gal). The results shown are the average of triplicate wells. Error bars indicate standard deviation. (C) Hep3B-Vec and Hep3B-HBX cells were treated with wortmannin (Wort; 30 μM) or LiCl (20 mM) for 24 h. Akt or GSK-3β activation was determined by its phosphorylation status and the expression of β-catenin was detected by immunoblotting using anti-β-catenin antibody.

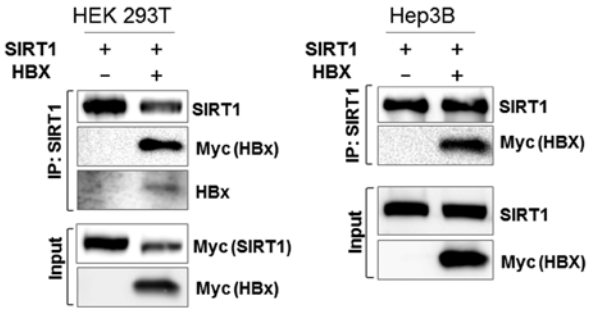

Figure 2. HBX binds to SIRT1 *in vivo*. HEK293T and Hep3B cells were transfected with pCMV-Myc-HBX (2 μg) and pCDNA3.1-Myc-SIRT1 (2 μg) plasmids. For immunoprecipitation, the cells were harvested 48 h post-infection and anti-SIRT1 antibody (1 μg) was added after pre-clearing the cell lysate. The cell lysates were then incubated for 1 h under agitation and ProteinA/G-coupled agarose beads (20 μl) were added. After extensive washing, the immunocomplexes were separated by a 12% SDS-PAGE gel and the HBX binding was detected by immunoblotting with anti-Myc or anti-HBX antibodies.

β-catenin in hepatocellular carcinoma suggests that the upregulated β-catenin may be attributed to the oncogenic activity of HBX protein in HBV-associated hepatocellular carcinoma (38). To address this possibility, we transiently introduced an HBX expression vector into Hep3B cells, which lack the p53 gene and separated the cytoplasmic and nucleic fractions. As shown in Fig. 1A, more β-catenin protein was detected in the nucleus of Hep3B cells that were transiently transfected with pCMV-Myc-HBX vector compared to Hep3B cells transiently transfected with a control vector. When we examined β-catenin protein levels in Hep3B cells stably expressing HBX (Hep3B-HBX) and in Hep3B cells with a control vector (Hep3B-Vec), we also found that the presence of HBX increased β-catenin protein levels in the cell lysates (Fig. 1A). In addition, to examine whether the HBX-mediated increase in β-catenin protein levels leads to functional activation and binding to the β-catenin/TCF binding element in the nucleus, we introduced a TopFlash luciferase plasmid controlled by the β-catenin/TCF-4 complex. As shown in Fig. 1B, the presence of HBX significantly enhanced β-catenin-mediated transcriptional activity in Hep3B cells while the introduction of HBX with FopFlash luciferase vector carrying a mutant TCF binding site failed to enhance β-catenin-mediated transcriptional activity. Next, to examine whether HBX utilizes Akt or GSK-3β protein as an upstream molecule in β-catenin upregulation, we treated Hep3B-Vec and Hep3B-HBX cells with wortmannin, an Akt inhibitor, or LiCl, a GSK-3β inhibitor. Wortmannin treatment inhibited Akt activity and reduced β-catenin protein levels in both cell lines (Fig. 1C). LiCl treatment inhibited GSK-3β as expected and increased β-catenin levels in both cell lines (Fig. 1C). This result indicates that HBX-mediated upregulation of β-catenin utilizes Akt and GSK-3β, which are upstream of Wnt signaling.

HBX can interact with SIRT1 in vivo. Although crucial pleitropic functions of SIRT1 in many biological process including stress response, apoptosis, and cellular metabolism have been well documented (39), SIRT1, a Class III histone deacetylase, has recently been implicated in tumor formation and suppression to a greater extent. For this reason, many studies have illustrated the regulation of SIRT1 expression and activity. Reports have shown that the enzymatic activity of SIRT1 may be modulated by the interactions of cellular proteins, such as DCB1 (22,23) and AROS (24). In addition, a viral regulatory protein has been shown to hijack and control SIRT1 enzyme activity for immune modulation. The HIV Tat protein has been reported to directly interact with the histone deacetylase (HDAC) domain of SIRT1, resulting in hyperactivation of

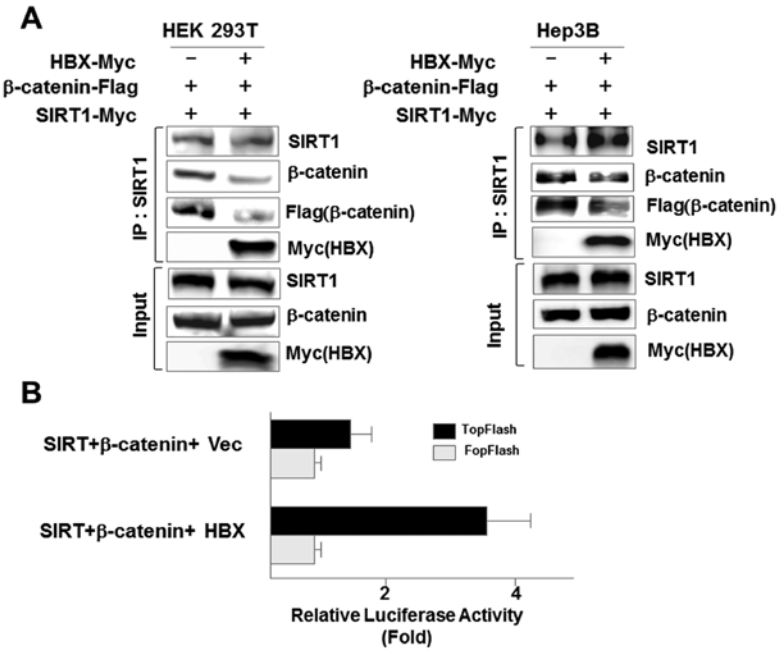

Figure 3. HBX weakens the interaction between SIRT1 and β-catenin. (A) HEK293T and Hep3B cells transfected with pCMV-Myc-HBX (2 μg), pCMV-Flag-β-catenin (2 μg) and pCDNA3.1-Myc-SIRT1 (2 μg) plasmids were harvested at 48 h post-infection for immunoprecipitation. The cell lysates were added with an anti-SIRT1 antibody (1 μg) and incubated for 1 h under agitation followed by the addition of Protein A/G-coupled agarose (20 μl). After extensive washing, the immunocomplexes were separated by 12% SDS-PAGE, and the levels of β-catenin, SIRT1 and HBX protein were detected by immunoblotting with the corresponding antibodies. (B) Hep3B cells were transfected with pCMV-Myc-HBX, pCMV-Flag-β-catenin and pCDNA3.1-Myc-SIRT1 plasmids in the presence of the pTopFlash or pFopFlash luciferase reporter vector. At 48 h post-transfection, luciferase activity was measured and transfection efficiency was normalized to a constitutive β-galactosidase expression plasmid (pGK-β-gal). The results shown are the average of triplicate wells. Error bars indicate standard deviation.

NF-κB responsive genes and promotion of immune-activating and pro-inflammatory cytokines by blocking the ability of SIRT1 to deacetylate the NF-κB p65 subunit at lysine 310 (25). Based on this line of evidence, we examined whether HBX interacts with SIRT1 and modulates SIRT1 activity as was observed with the Tat protein. After HEK293T cells were transiently co-transfected with SIRT1 and HBX expression vectors, the cell lysates were immunoprecipitated with anti-SIRT1 antibody and immunoblotted with anti-HBX antibodies (an anti-Myc antibody or polyclonal anti-HBX antibodies). As shown in Fig. 2, HBX protein was detected in the immune complex, indicating that HBX interacts with SIRT1 *in vivo*. Furthermore, to examine whether the interaction between SIRT1 and HBX can be reproduced in Hep3B cells, we co-expressed SIRT1 and HBX in Hep3B cells. After the SIRT1 protein was immunoprecipitated with an anti-SIRT1 antibody, HBX was detected in the immune complex, confirming the interaction between HBX and SIRT1 in Hep3B cells.

HBX attenuates the interaction between SIRT1 and β-catenin. It has been reported that SIRT1 binds to β-catenin both *in vitro* and *in vivo*, and results in inhibition of β-catenin transcriptional activity in a mouse colon cancer model (40). Our results in Fig. 2 display that HBX interacts with SIRT1 *in vivo*. Based on this evidence, we hypothesize that the presence of HBX weakens the interaction between SIRT1 and β-catenin, leading to the release of β-catenin from SIRT1 inhibition. To test our hypothesis, we introduced HBX, β-catenin, and SIRT1 plasmids in HEK293T and Hep3B cells. At 48 h post-transfection, the cell lysates were immunoprecipitated with anti-SIRT1 antibody and the level of β-catenin that co-precipitated with the immunocomplex was examined in the absence or presence of HBX. As shown in Fig. 3A, in both Hep3B cells and HEK293T cells, lower levels of β-catenin protein were detected in the immunocomplexes in the presence of HBX than in the absence of HBX. Moreover, when we compared β-catenin transcriptional activity in Hep3B cells transiently transfected with HBX, β-catenin and SIRT1 plasmids to the activity of Hep3B cells transiently transfected with β-catenin and SIRT1 plasmids, we discovered that the presence of HBX enhanced β-catenin transcriptional activity compared to the activity in the absence of HBX (Fig. 3B). The results support our hypothesis that HBX attenuates the interaction between SIRT1 and β-catenin, leading to the protection of β-catenin from the inhibitory action of SIRT1.

SIRT1 regulates β-catenin protein levels and activity in Hep3B-HBX cells. Since several studies have reported that ectopic induction of SIRT1 significantly reduced tumor formation, proliferation (41), and mouse morbidity in a β-catenin-driven mouse model of colon cancer (40), we speculated whether SIRT1 also functions as a suppressor of tumorigenesis through repression of β-catenin in our HBX-expressing hepatic cell line. Therefore, to examine whether SIRT1 expression or activity affects β-catenin protein levels in Hep3B-Vec and Hep3B-HBx cells, we transfected the cells with SIRT1 siRNA, nicotinamide (an inhibitor of SIRT1 activity) or resveratrol (an activator of SIRT1 activity). As shown in Fig. 4A, suppression of SIRT1 with siRNA resulted in upregulation of β-catenin protein levels. In addition, nico-

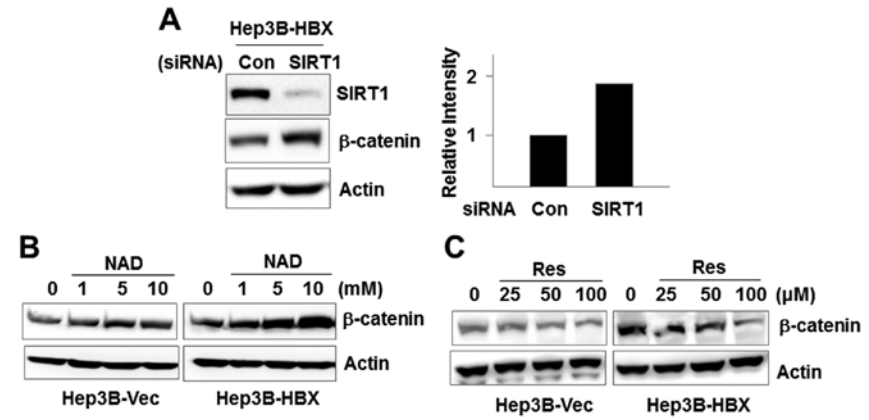

Figure 4. SIRT1 protein levels and activity modulate β-catenin expression. (A) SIRT1 and β-catenin protein levels were detected in cell lysates prepared from Hep3B-HBX cells transfected with SIRT1 siRNA (100 nM) 48 h post-transfection. Expression levels were determined by immunoblotting with anti-SIRT1 and anti-β-catenin antibodies. (B,C) Hep3B-HBX cells were treated with different concentrations of nitocinamide (NAD; 1, 5 and 10 mM) or resveratrol (Res; 25, 50 and 100 μM) for 24 h and the expression of β-catenin was detected by immunoblotting.

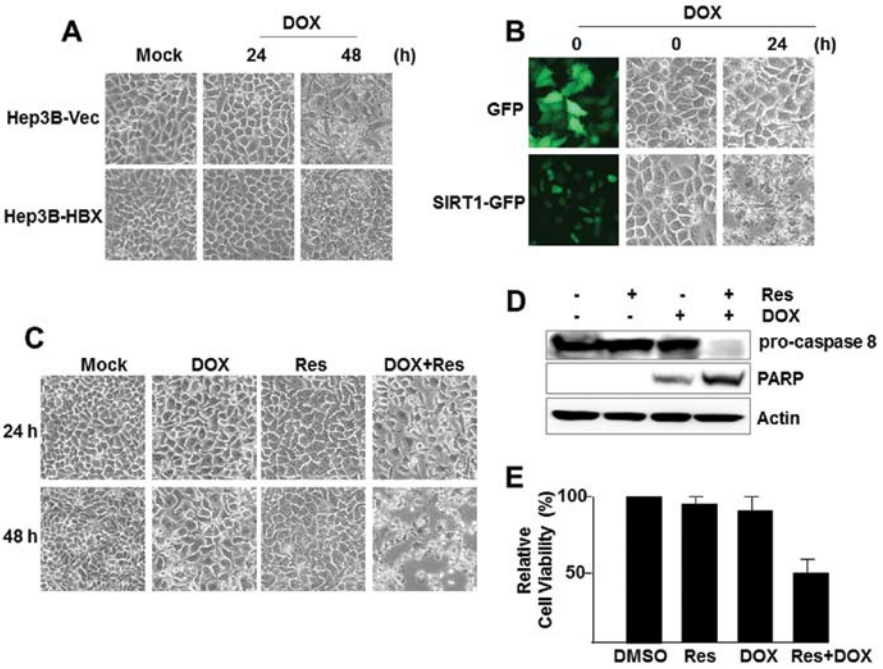

Figure 5. Upregulation of SIRT1 sensitizes Hep3B-HBX cells to doxorubicin-induced apoptosis. (A) Hep3B-Vec and Hep3B-HBX cells were treated with doxorubicin (DOX; 500 μg/ml) and cell viability was observed under microscopy for 48 h. (B) Hep3B-HBX cells were transfected with pEGFP-SIRT or a control vector and then treated with doxorubicin (500 μg/ml) for 48 h. Transfection efficiency was measured by GFP expression under a fluorescence microscopy. Cell viability was observed under light microscopy for 48 h. (C-E) Hep3B-HBX cells were treated with resveratrol and doxorubicin or doxorubicin alone for 48 h. Cell viability was measured by light microscopy or the MTT assay.

tinamide treatment increased the levels of β-catenin proteins while resveratrol treatment decreased the levels of β-catenin proteins in a dose-dependent manner (Fig. 4B and C). The results indicate that SIRT1 negatively regulates β-catenin protein levels in human hepatic Hep3B cells.

Upregulation of SIRT1 enhances the sensitivity of Hep3B-HBX cells to doxorubicin-induced apoptosis. To examine how modulation of SIRT1 affects the viability of hepatic cells stably expressing HBX under stress conditions such as DNA damage, we first optimized the concentration of doxorubicin, an anticancer drug using Hep3B-Vec and Hep3B-HBX cells. As shown in Fig. 5A, Hep3B-HBX cells survived in up to 500 μg/ml doxorubicin for 48 h while Hep3B-Vec cells were sensitive to 500 μg/ml doxorubicin. This result indicates that HBX confers resistance to doxorubicin. Using the Hep3B-HBX cells that exhibited resistance to doxorubicin, we next examined whether upregulation of SIRT1 enhances sensitivity to apoptosis during doxorubicin treatment. Hep3B-HBX cells were transfected with SIRT1-GFP and then incubated with doxorubicin 24 h after transfection. As shown in Fig. 5B, although introduction of GFP did not induce cell death of Hep3B-HBX cells during doxorubicin treatment, introduction of SIRT1-GFP enhanced doxorubicin-induced cell death. Furthermore, when Hep3B-HBX cells were treated with resveratrol in the presence of doxorubicin, the cells exhibited

greater sensitivity to apoptosis than Hep3B-HBX cells treated with doxorubicin alone (Fig. 5C and D). Consistent with these results, combined treatment with resveratrol and doxorubicin reduced the number of surviving cells compared to doxorubicin treatment alone (Fig. 5E). The results indicate that upregulation of SIRT1 protein and activity enhances doxorubicin-induced apoptosis in Hep3B-HBX cells.

Discussion

The pleiotropic effects of HBX can be attributed to its promiscuous interaction with many host cellular factors including HIF1-α to adapt the HBV-infected hepatic cells to a hypoxic environment (42); Jab1 to activate AP1 transcription activity (43); and DNA methyltransferease to facilitate cellular epigenetic modification (44). Additionally, it has been shown that HBX binds to APC, a signaling molecule of the complex involved in Wnt-/β-catenin signal transduction, which is involved in cell proliferation and tumor formation. HBX interacts with APC through a consensus domain necessary for APC binding to β-catenin, suggesting that HBX blocks β-catenin degradation by competitively displacing β-catenin from APC (45). Herein, we present a novel mechanism by which HBX sequesters SIRT1, thereby blocking SIRT1 inhibition of β-catenin in a Wnt signaling-independent manner. Therefore, this study provides evidence that β-catenin is superactivated, which could explain the oncogenic activity of HBX. We are investigating whether action of HBX toward SIRT1 is similar to the inhibitory action of HIV Tat protein toward SIRT1.

The roles of SIRT1 in tumor formation are controversial. Some studies have reported that SIRT1 can promote cancer cell growth by blocking cellular senescence through the direct deacetylation of p53 (19), Foxo (21), and E2F1 (46) and that overexpression of SIRT1 has been frequently detected in chemoresistant tumor cells and clinical tissues (47). In contrast, other groups have reported that increasing SIRT1 protein levels as a result of ectopic expression leads to reduced cell proliferation and tumor formation in a colon cancer model (40) and activation of SIRT1 activity by resveratrol also limits cell growth and reduces tumor formation in a breast cancer model (48). Subsequently, we explored the potential biological consequences of upregulating SIRT1 protein levels and activity in the presence of HBX. We discovered that upregulation of SIRT1 protein or activity enhances sensitivity of Hep3B-HBX cells to doxorubicin-induced apoptosis. In our HBX-induced hepatic cancer cell model, SIRT1 appears to act as a tumor suppressor, which is the opposite of its role in other studies which have shown that high SIRT1 activity promotes cell survival similar to an oncogene (49-51). Therefore, this result indicates that upregulation of SIRT1 proteins or activity could be a therapeutic strategy in HBX-induced hepatocellular carcinoma.

Acknowledgements

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