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ABSTRACT

Hepatocellular carcinoma (HCC), a primary type of liver cancer, has one of the highest mortality rates in cancer related death worldwide. HCC patients have poor prognosis due to intrahepatic and extrahepatic metastasis. Hepatitis B virus (HBV) infection is one of the major causes of various liver diseases including HCC. Among HBV gene products, HBV X protein (HBx) plays important roles in the development and metastasis of HCC. However, the mechanisms of HCC metastasis induced by HBx has not been elucidated yet.

In this study, we first report that HBx interacts with regulates suppressor of cytokine signaling 1 (SOCS1) which negatively controls NF-κB by degrading p65, a subunit of NF-κB. NF-κB activates the transcription of factors associated with epithelial-mesenchymal transition (EMT) which is a crucial cellular process associated with invasiveness and migration of cancer cells. Here, we report that HBx physically binds to SOCS1, subsequently prevents the ubiquitination of p65, activates the transcription of EMT transcription factors, and enhances cell migration and invasiveness suggesting a new mechanism of HBV-associated HCC metastasis.

INTRODUCTION

Hepatocellular carcinoma (HCC), a primary liver cancer, is the sixth most common cancer and the third leading cause of death from cancer worldwide (1). Especially HCC is a very critical and important public health problem in Asia and Sub-Saharan Africa (2). The major risk factors for HCC are hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholism, and non-alcoholic fat liver diseases. Approximately, over 50% of HCC patients are related to chronic HBV infection (3). Especially HBV-induced HCC has poor prognosis because of its highly metastatic recurrence. However, the exact mechanisms underlying cancer development and metastasis of HBV-induced liver cancer remain unclear. Among HBV

proteins, HBV X protein (HBx) plays a major role in the development and pathogenesis of HCC (4, 5). HBx is a multifunctional regulatory protein essential for viral replication that requires various host cellular components (6, 7). HBx is also a promiscuous protein involved in cell cycle regulation, transactivation of genes related to cell growth, oncogenic transformation, and metastasis. In addition, HBx has been reported to activate a variety of signal transduction pathways including NF-κB signaling (8, 9).

It has been suggested that epithelial-mesenchymal transition (EMT) is associated with HBV-induced HCC metastasis (10, 11). During EMT, tumor cells lose their epithelial features and acquire mesenchymal features leading to the gain of invasive and migratory characteristics. There are changes in gene expressions that contribute to the suppression of the epithelial phenotype and augmentation of the mesenchymal phenotype. EMT-associated changes in gene expression are triggered by EMT regulators including Snail, Slug, and Twist1 which have central roles in the processes of development, fibrosis, and carcinogenesis (12, 13).

EMT regulatory factors are tightly associated with NF-κB activity (14). The NF-κB family has been described as a critical regulator of a large number of biological processes including cell proliferation, differentiation, immune responses, and inflammation (15, 16). This family consists of five subunits: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel (Rel), and RelB, which associate to form functional homo- and heterodimers. It has been reported that NF-κB is the upstream regulator of MMPs and EMT transcription factors which promote cell migration and invasion of hepatocellular carcinoma (17).

Interestingly, SOCS1 is down-regulated in tissues of HCC associated with HBV infection (18). SOCS1 is a major regulator responsible for suppressing the cell signals and cell motility. SOCS1 plays critical roles in several kinds of signaling pathways (19, 20) and SOCS1 regulates NF-κB activity by degrading p65 (21).

SOCS1 is negatively associated with the prognoses of HBV-induced liver failure (22). Liver

fibrosis has strong correlation with the methylation of SOCS1 gene. Carcinogen-induced HCC development was also enhanced by heterozygous deletion of SOCS1 gene. These findings suggest that SOCS1 contributes to resistance against hepatic injury, fibrosis, and hepatocellular carcinogenesis (23). It was found that the gene loss and epigenetic silencing of SOCS1 are strongly associated with HBV-related HCC (18). In addition, SOCS1 also represses the metastases of certain cancers. SOCS1 suppresses the gene expression of EMT transcriptional factors, leading to the repression of EMT associated cell migration and metastasis of HCC in mice models (24).

Here we report that HBx binds to SOCS1 in liver cancers cells. HBx also represses p65 degradation induced by SOCS1 and stabilizes p65 to enhance NF-κB activity. In addition, HBx promotes the expression of EMT transcription factors and elevates cell migration and metastasis of HCC by regulating SOCS1. Now, it is evident that SOCS1 repression by HBx may be a bona fide regulatory way how HBx is involved in the metastasis of HBV-induced HCC.

RESULTS

HBx increases the level of p65

Since NF-κB is a pivotal regulator of various biological processes including cell proliferation, differentiation, and inflammation, NF-κB is a major signaling factor in HBx mediated-pathogenesis (25, 26). In order to elucidate how HBx regulates the NF-κB signaling pathway in HBV-infected HCC cells, we tested the effect of shRNA against HBx in HepG2.2.15 cells which produce infectious HBV virus particles. Since HBV infection activates NF-κB activity, as expected, when the level of HBx was suppressed by HBx shRNA, the phosphorylation of IKK was decreased and the level of IκBα was increased (Fig. 1A). In addition, the level of

p65 was also decreased, in the presence of HBx shRNA. We also tested HBx shRNA in Hep3B cells which produce HBV particles (Fig. 1B). Again, when HBx was knocked-down by HBx shRNA in Hep3B cells, the level of p65 was deceased, the level of phosphorylated IKK was decreased, and the level of IκBα was increased. However, since IKK-IκBα pathway can not account for the remarkable decreased level of p65, we attempted to test whether HBx affects the level of p65. We transfected HepG2 cells with various amounts of HBx expressing plasmid to analyze the level of p65 in the presence of HBx. Interestingly, HBx increases the level of p65 in a dose dependent manner (Fig. 1C) suggesting that HBV infection enhances NF-κB activity by elevating p65 level along with activating IKK-IκBα pathway.

HBx interferes with SOCS1-mediated suppression of p65 and physically binds to SOCS1

It has been reported that suppressor of cytokine signaling 1 (SOCS1) affects several signaling pathways, in addition to the classical JAK/STAT pathway. SOCS1 can also promote p65 degradation by its ubiquitin ligase activity (21). Several studies suggest that SOCS1 is associated with HCC, because of methylation-mediated silencing of SOCS1 gene in HBV-related HCC (27, 28). Since SOCS1 degrades NF-κB p65 subunit, whereas HBx enhances the level of p65, it is very possible that SOCS1 and HBx conversely control NF-κB which plays important roles in HBV-associated HCC development and metastasis.

To elucidate the relationship between SOCS1 and HBx on the regulation of NF-κB, we tested the effects of SOCS1 and HBx on NF-κB transcriptional activity by luciferase assay using NF-κB reporter plasmid. As expected, SOCS1 repressed NF-κB transcriptional activity in a dose dependent manner and HBx restored NF-κB transcriptional activity which was reduced by SOCS1 (Fig. 2A). We also confirmed the level of p65 in the presence of SOCS1 and HBx in HepG2 cells. SOCS1 reduced the level of p65, whereas HBx augmented the level of p65

reduced by SOCS1 (Fig. 2B). Furthermore, we tested the levels of p65 in the presence of shRNA against SOCS1 in HepG2 cells and Hep3B cells. When the levels of SOCS1 were knocked-down by SOCS1 shRNA, the levels of p65 was increased in both cell lines (Fig. 2C). To further dissect the reciprocal effects of SOCS1 and HBx on the level of p65, we tested whether HBx interacts with SOCS1 physically or not. The cell extracts of Hep3B that is producing HBV particles were prepared and immuno-precipitated by HBx antibody and analyzed by SOCS1 antibody (Fig. 2 D). Surprisingly, it clearly shows that two proteins, HBx and SOCS1, physically interact with each other. In addition, we also confirmed the interaction between HBx and SOCS1 in 293T cells transfected with plasmids expressing HBx and SOCS1 (Fig. 2E). These data suggest that HBx may interfere with SOCS1-induced p65 degradation by binding to SOCS1 physically.

HBx stabilizes p65 by blocking p65 ubiquitination mediated by SOCS1

Since it has been suggested that SOCS1 regulates p65 by inducing proteosomal degradation (29), we tested whether HBx affects the stability of p65 by checking the half-life of p65 in the presence of cyclohexamide. As expected, while SOCS1 enhanced the degradation rate of p65, HBx retarded the degradation rate of p65 which was elevated by SOCS1 (Fig. 3A). In addition, when the expression of HBx was knocked-down by HBx shRNA in Hep3B cells, the effect of SOCS1 on p65 degradation rate was increased (Fig. 3B). However, in the presence of scramble shRNA in Hep3B cells, the effect of SOCS1 on p65 degradation rate was reduced compared with HBx shRNA suggesting that the amount of HBx in Hep3B might be high enough to mask the inhibitory effect of overexpressed SOCS1. Now, it is evident that HBx interferes with SOCS1-mediated degradation of p65.

To determine how HBx regulates SOCS1-mediated degradation of p65 post-translationally, we investigated whether HBx affects physical interaction between SOCS1 and p65. The

physical interaction between SOCS1 and p65 was repressed by HBx in a dose-dependent manner (Fig. 3C). In addition, HBx binded to SOCS1 competitively with p65 and released p65 from SOCS1 in a dose-dependent manner (Fig. 3D).

Since SOCS1 promotes the ubiquitination of p65 for proteasomal degradation, we next checked whether HBx is involved in the ubiquitination of p65 by SOCS1. While SOCS1 elevated the ubiquitination of p65, HBx decreased the level of p65 ubiquitination and interfered with the interaction between SOCS1 and p65 (Fig. 3E). These results suggest that HBx suppresses SOCS1-mediated ubiquitination/degradation of p65 by interfering with the interaction between p65 and SOCS1 through competing with p65.

HBx increases the levels of EMT factors and promotes cell migration

Previous reports have shown that NF-κB signaling has a major role in metastasis of HCC (30). To investigate whether HBx could affect EMT process by stabilizing p65 through SOCS1 regulation, we examined the effects of SOCS1 and HBx on the levels of EMT transcription factors and EMT markers. While SOCS1 reduced the levels of EMT transcription factors including Twist1 and Snail, HBx restored the levels of EMT transcriptional factors reduced by SOCS1 (Fig. 4A). In addition, while SOCS1 elevated the level of epithelial marker E-cadherin and decreased the level of mesenchymal marker vimentin, HBx repressed the level of E-cadherin elevated by SOCS1 and restored the level of vimentin reduced by SOCS1 (Fig. 4 B). In addition, we checked the morphology of HepG2 cells in the presence of SOCS1 and HBx. While HepG2 cells had epithelial-like morphology, SOCS1 kept the morphology of HepG2 cells as epithelial-like shape and HBx changed the cells to mesenchymal-like shape (Fig. 4 C).

We also examined the effects of SOCS1 and HBx on cellular mobility and invasiveness. By wound healing assay, it turned out that SOCS1 retarded the rate of cell motility and HBx

elevated cell motility rate decreased by SOCS1 (Fig. 4D). Cell invasion assay also showed that SOCS1 repressed the rate of invasiveness and HBx restored invasiveness rate reduced by SOCS1 (Fig. 4E).

These results suggest that HBx induces EMT through interaction with SOCS1 and subsequent up-regulation of Twist1 and Snail, resulting in the elevated migration and invasion of HCC.

DISCUSSION

Since the high rate of metastasis and recurrence of HCC is the main reason for the poor prognosis of HCC patients, the elucidation of biochemical mechanisms underlying HCC metastasis and invasion is important to provide possible new molecular therapeutic strategy. HBx protein, with a wide range of biological activities, plays important roles in the development and recurrence of HBV-related HCC. However, the mechanisms of HCC metastasis induced by HBx are largely unknown. Recently, EMT has been considered as a key process in multiple steps associated with the changes of cell movements including metastasis. HBx has been shown to be involved in the regulation of EMT which is controlled by several signal transduction pathways including NF-κB.

SOCS1 is associated with various HBV-induced liver diseases including liver fibrosis and HCC. SOCS1 also inhibits the invasion of HCC cells by regulating EMT signaling cascade (24).

SOCS1 has been previously implicated in controlling the levels of p65 and functioning in concert with the ECS complex independently of its role in regulating cytoplasmic signaling pathways (31). Canonical SOCS box-containing proteins interact with the Elongin BC complex to have ubiquitin ligase activity. Elongin B and C complex is used as an adaptor in the ECS complex (32). Recently the COMMD1 protein, which inhibits NF-κB, was shown to be interacting with the ECS complex by promoting the association between p65 and SOCS1

to facilitate the ubiquitination of p65 and subsequent proteasomal degradation (33).

In this study, we identified the molecular mechanism underlying HBx-mediated activation of NF-κB which leads to the acceleration of EMT in HCC cells. We found that HBx restores the half-life of p65 repressed by SOCS1. In addition, HBx inhibits the interaction between p65 and SOCS1. Moreover, HBx binds to SOCS1 directly to block the ubiquitination of p65 induced by SOCS1. Since NF-κB is the master regulator of inflammation and its constitutive activation promotes cancer development by increasing cell proliferation, angiogenesis, invasion, metastatic potential, and resistance to apoptosis, therefore SOCS1 is a new cellular regulatory protein which is involved in HBx-induced hepatocellular carcinogenesis and metastasis. Taken together, these results present the protein network consisting of HBx, SOCS1, and NF-κB in EMT of HCC associated with HBV infection.

MATERIALS AND METHODS

Plasmids

FLAG-HBx was constructed as previously described (34). shHBx plasmid was a kind gift from Dr. Irene Oi-lin Ng. Myc-SOCS1 plasmid was kindly provided by Dr. Akihiko Yoshimura. SOCS1 shRNA and scramble shRNAs were obtained from VectorBuilder.

Cell culture and transfection

HepG2, HepG2.2.15, Hep3B, and 293T cells were maintained in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2. Cells were transfected with PEI (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction.

Cycloheximide chase assay

Protein stability was determined by treating the cells with 400μM cycloheximide (Tocris Bioscience, Bristol, UK). Cell lysates were prepared at indicated time points and analyzed by Western blotting.

Immunoprecipitation and Western blot analysis

Cell lysates were prepared in modified RIPA buffer and incubated with specific antibodies overnight at 4°C. The immuno-complexes were isolated using protein A Sepharose (Incospharm), resolved by SDS-PAGE, and transferred to nitrocellulose membrane. Immunoblotting was performed with the indicated antibodies. Antibodies against HBx, SOCS1, p65, p-IKK, IκBα were purchased from Santa Cruz. Antibodies against E-cadherin and vimentin were provided by BD and Cell Signaling Technology respectively.

Wound healing assay

HepG2 cells were seeded in six-well culture plates at a density of $3x10^5$ cells/mL and cultured overnight. At 24 h after transfection, the cell monolayers were scratched to generate wounds. The area of covered by the migrating cells was measured using IMAGE J software at 0 and 24 h after scratching. The experiments were repeated three times.

Luciferase assay

At 24 h after transfection, cell lysates were prepared and the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Firefly luciferase activity was normalized to Renilla luciferase activity.

Ubiquitination assay

At 4 h before harvesting, cells were treated with 20μM MG-132 (AG scientific, San Diego, CA, USA), lysed with modified RIPA buffer containing MG-132, and pulled down with Glutathione-Sepharose 4B beads. The bead complexes were resolved and analyzed by SDS/PAGE followed by Western blotting with the indicated antibodies.

GST pull-down assay

Transiently transfected HepG2 cells were lysed in modified RIPA buffer for 30 min at 4°C. The cell lysates were incubated overnight at 4°C with Glutathione-Sepharose 4B beads (Incospharm). The bead complexes were washed three times with modified RIPA buffer and the bound proteins were analyzed by Western blotting.

Matrigel invasion assay

After overnight serum starvation, $0.5x10^5$ HepG2 cells were suspended in serum-free DMEM and seeded on Matrigel-coated Transwell filters (8-㎛ pore) in the upper chamber. The lower chamber was filled with the medium containing 10% serum. Then whole chamber was incubated for 24 h at 37 °C. After non-invading cells remaining on the upper surface of the filter were removed, the cells on the lower surface of the filter were fixed with 4% formaldehyde and permeabilized with 100% methanol for 20 min. Invaded cells were stained with Giemsa and counted under a microscope. Experiments were independently repeated three times.

Statistical analysis

Statistical comparisons of results were evaluated by one-way analysis of variance (ANOVA) and Student's t-test for unpaired values. Data are expressed as means ± standard deviation and

P < 0.05 was considered statistically significant. Levels of statistical significance are indicated by asterisks (*, P < 0.05, **, P < 0.01; ***, P < 0.001).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. HBx upregulates the level of p65. (A) HepG2.2.15 cells were transfected with shRNA against HBx. At 72 h after transfection, the levels of HBx, p65, IκBα, IKK, and phosphorylated IKK were determined by immunoblotting with anti-HBx, anti-p65, anti-IκBα, anti-IKK, and anti-phosphorylated IKK antibodies. (B) Hep3B cells were transfected with HBx shRNA and scramble shRNA. At 72 h transfection, the cell lysates were analyzed by Western blotting with anti-HBx, anti-p65, anti-IκBα, anti-IKK, and anti-phosphorylated IKK antibodies. (C) HepG2 cells were transiently transfected with various amounts of Flag-HBx plasmid. At 48 h after transfection, the cell lysates were prepared and analyzed by Western blotting with anti-Flag, anti-p65, and anti-ß-actin antibodies.

Figure 2. HBx inhibits SOCS1-mediated suppression of p65 and physically binds to SOCS1 (A) HepG2 cells were co-transfected with HA-p65, Flag-HBx, and Myc-SOCS1 plasmids. At 48 h after transfection, NF-κB activities were determined by a dual luciferase reporter assay. The results are denoted as the relative luciferase activities. The data shown are means ± SD of values from three independent experiments. (B) HepG2 cells were co-transfected with Flag-

HBx and Myc-SOCS1 plasmids. Cell lysates were subjected to Western blot analysis using antibodies against Flag, p65, Myc, and ß-actin. (C) HepG2 cells and Hep3B cells were transfected with SOCS1 shRNA and scramble shRNA. At 72 h transection, cell lysates were prepared and analyzed by Western blotting with anti-SOCS1, anti-p65, anti-IKK antibodies. (D) The lysates of Hep3B cell expressing HBx constitutively were prepared and immunoprecipitated with anti-HBx antibody. Immunoprecipitated complex was analyzed by Western blotting with anti-HBx and anti-SOCS1 antibodies. (E) 293T cells were transfected with Flag-HBx and Myc-SOCS1 plasmids. The cell lysates were immunoprecipitated with anti-Myc and analyzed by Western blotting with anti-Myc and anti-Flag antibodies.

Figure 3. HBx stabilizes p65 by blocking p65 ubiquitination induced by SOCS1.

(A) HepG2 cells were co-transfected with Flag-HBx and Myc-SOCS1 plasmids. At 48 h after transfection, cells were treated with 400μM cycloheximide for 0, 12, and 24 h. Cell extracts were analyzed by Western blotting with anti-Flag, anti-Myc, anti-p65, and anti-ß-actin antibodies. (B) Hep3B cells were transfected with Myc-SOCS1 plasmid and HBx-shRNA plasmid. Cell extracts were prepared and analyzed by Western blotting with anti-p65, anti-HBx, anti-Myc, and anti-ß-actin antibodies. (C) HepG2 cells were co-transfected with Flag-HBx, Myc-SOCS1, and GST-p65 plasmids. At 48 h after transfection, cells were treated with 20μM MG-132 for 4 h. Cell extracts were prepared, pulled down with Glutathione-Sepharose 4B, and analyzed by Western blotting with anti-Myc and anti-GST antibodies. (D) HepG2 cells were co-transfected with Flag-HBx, Myc-SOCS1, and His-p65 plasmids. At 48 h after transfection, cells were treated with 20μM MG-132 for 4 h. Cell extracts were immunoprecipitated with anti-Myc antibody and analyzed by Western blotting. (E) HepG2 cells were co-transfected with Flag-HBx, Myc-SOCS1, GST-p65, and HA-Ub plasmids. At 48 h after transfection, cells were treated with 20μM MG-132 for 4 h. Cell extracts were

pulled down with Glutathione-Sepharose 4B and analyzed by Western blotting.

Figure 4. HBx promotes cellular migration and invasion by restoring the levels of EMT factors suppressed by SOCS1.

(A) HepG2 cells were transfected with Flag-HBx and Myc-SOCS1 plasmids. At 48 h after transfection, cell lysates were analyzed by Western blotting with anti-Flag, anti-p65, anti-Twist1, anti-Snail, and anti-ß-actin antibodies. (B) HepG2 cells were transfected with Flag-HBx and Myc-SOCS1 plasmids. Cell lysates were prepared and analyzed by Western blotting with anti-Flag, anti-p65, anti-vimentin, anti-E-cadherin, and anti-ß-actin antibodies. (C) HepG2 cells were transfected with Flag-HBx and Myc-SOCS1 plasmids. At 48 h after transfection, the shapes of transfected HepG2 cells were analyzed by light microscopy. Scale bar = 50㎛ (D) Wound healing assay was carried out in HepG2 cells transfected with Flag-HBx and Myc-SOCS1 plasmids. Representative images were taken at 24 h after scratching. (E) HepG2 cells were transfected with Flag-HBx and Myc-SOCS1 plasmids. The invasion ability of cells was measured using Transwell cell culture chambers.

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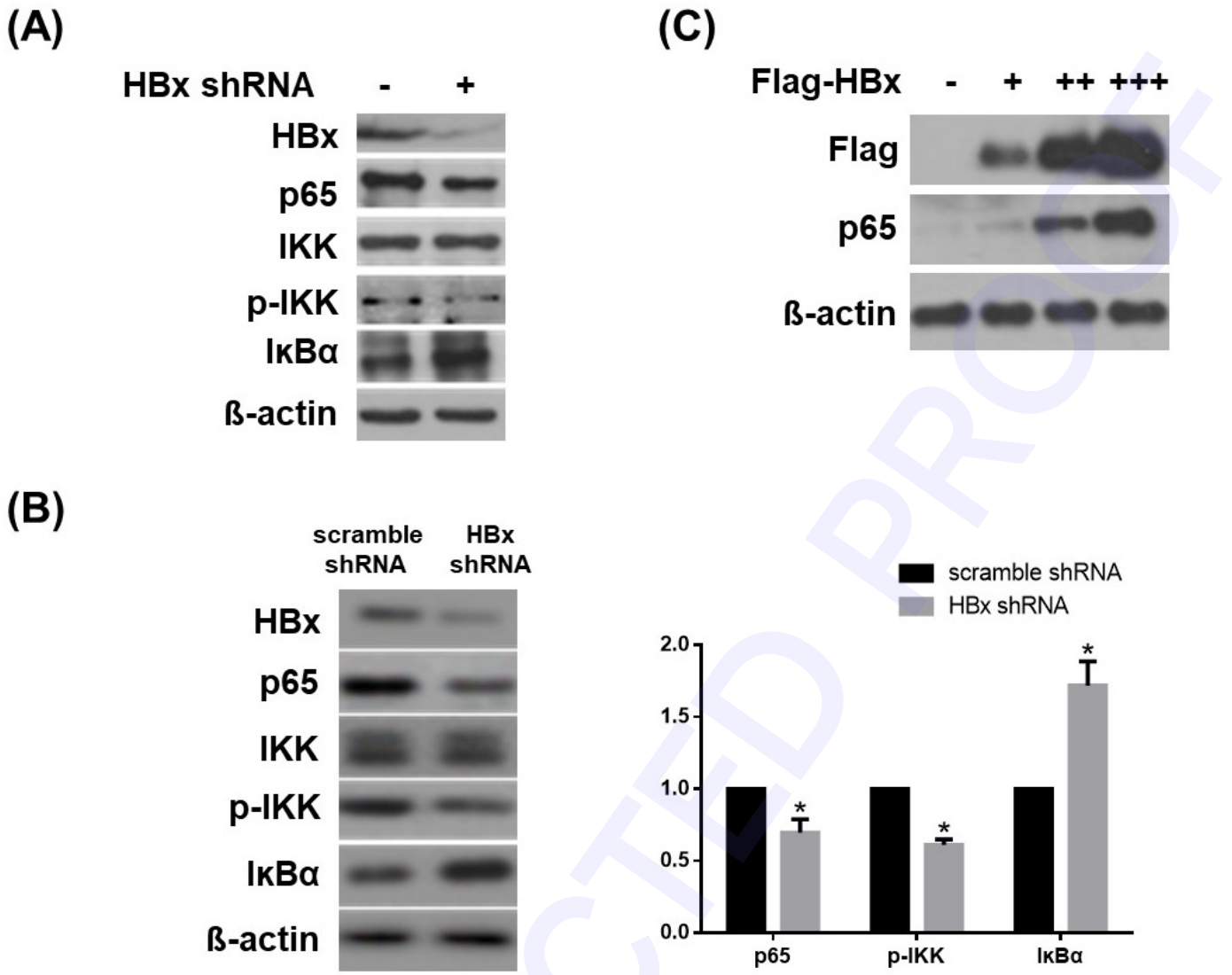

Fig. 1.

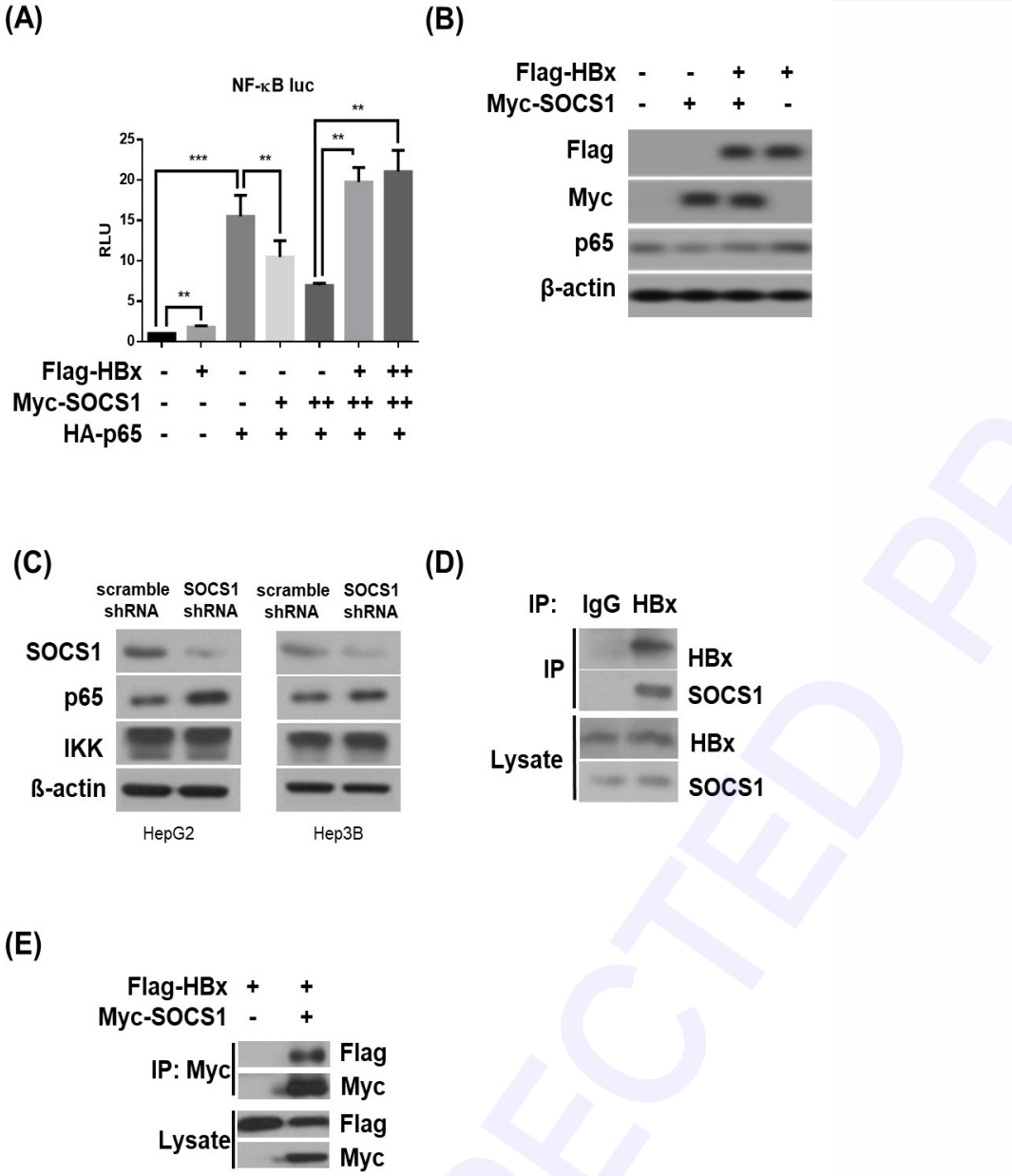

Fig. 2.

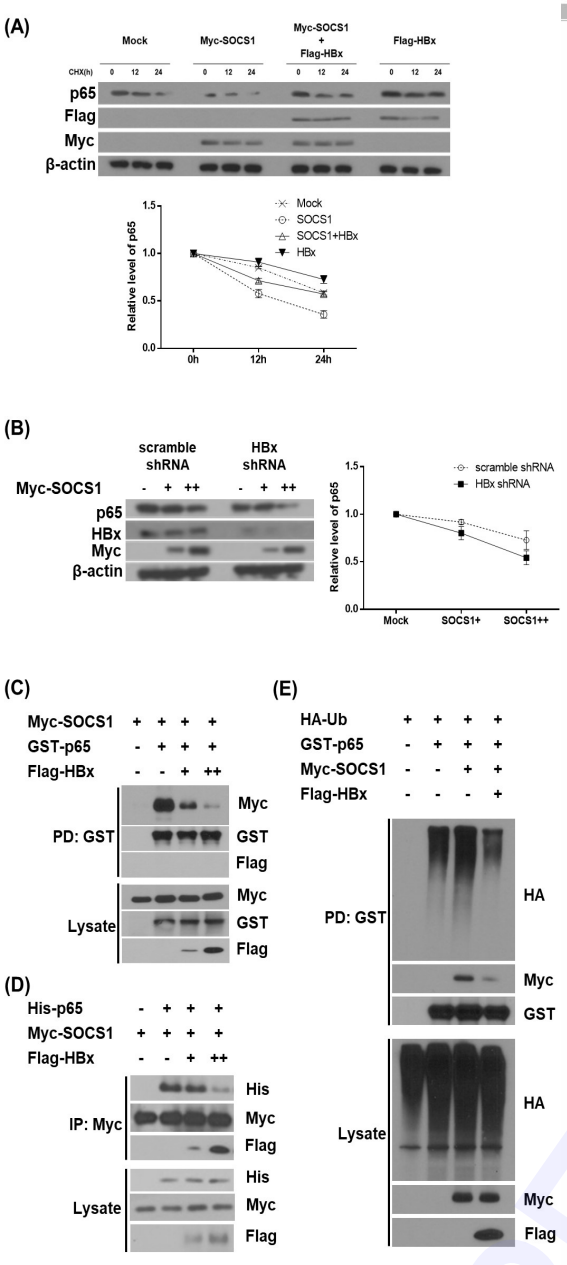

Fig. 3.

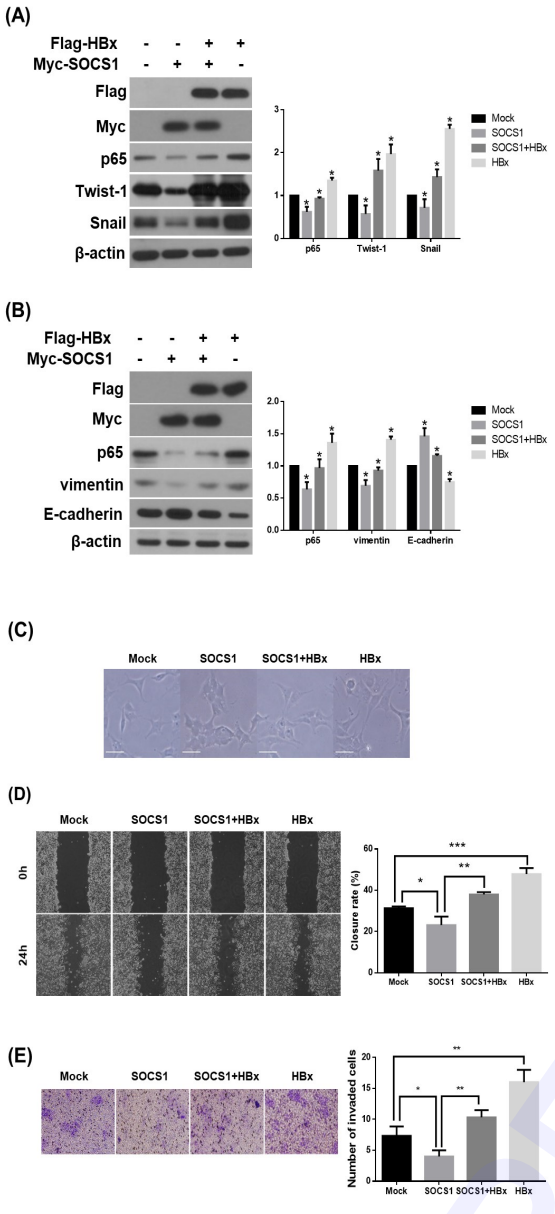

Fig. 4.