

HBx co-localizes with COXIII in HL-7702 cells to upregulate mitochondrial function and ROS generation

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Received November 16, 2014; Accepted February 19, 2015

DOI: 10.3892/or.2015.3852

Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignant diseases, and HBx leads to the development of HBV-associated HCC. Mitochondria are key organelles that regulate apoptosis, cellular energetics and signal transduction pathways, and are the source of HBx-induced reactive oxygen species (ROS). Recent findings have shown that HBx interacts with the inner mitochondrial membrane protein, COXIII, via the yeast two-hybrid system, mating experiment and coimmunoprecipitation. The aim of the present study was to examine the co-localization of HBx and COXIII in HL-7702 cells and to investigate ensuing alterations of mitochondrial function. An HL-7702 cell line stably expressing the *HBx* gene by lentivirus vectors was constructed. Confocal microscopy was utilized to assess the interaction between HBx protein and COXIII. Expression of COXIII, activities of cytochrome *c* oxidase (COX) and the mitochondrial membrane potential, which were functionally relevant to the HBx protein-COXIII interaction, were investigated in cell cultures. Moreover, the intracellular ROS levels were detected by flow cytometry. The results demonstrated that HBx co-localized with the inner mitochondrial protein, COXIII, in HL-7702 cells, causing the upregulation of COXIII protein expression as well as COX activity. However, HBx did not alter the mitochondrial membrane potential and mitochondria exhibited only slight swelling in HL-7702-HBx cells. Moreover, HBx elevated the generation of mitochondrial ROS in HL-7702-HBx cells. The main finding of the present study was that the co-localization of HBx and COXIII leads to upregulation of the mitochondrial function and ROS generation, which are associated with the oncogenesis of HBV-associated HCC.

Introduction

Hepatocellular carcinoma (HCC) remains one of the most prevalent malignant diseases and has the fourth highest mortality rate worldwide (1). Chronic HBV infection remains the major etiological factor of HCC worldwide with >50% of HCC patients being chronic carriers (2). The activities of HBx are known to play major roles in the onset and progression of HBV-associated HCC. HBx, encoded by the smallest open reading frame of mammalian hepadnaviruses, is 154 amino acids in size, with a molecular mass of ~17.5 kDa (3). HBx protein interacts with different host factors to modulate cell signal transduction pathways, transcriptional regulations, cell cycle progression, DNA repair, apoptosis and genetic stability (1).

HBx is predominately localized in the cytoplasm with a low level of nuclear distribution (4). Mounting evidence from immunofluorescence microscopy and subcellular fractionation techniques have demonstrated that a fraction of cytosolic HBx co-localizes with mitochondria in primary rat hepatocytes (5), in the human WRL68 liver cell line and human HepG2 hepatocarcinoma cell line (6), in Huh7 (7) and in HBx-expressing COS cells, a monkey kidney cell line (8), and in HBx-transgenic mouse hepatocytes (9), suggesting a key role of HBx in affecting mitochondrial physiology, metabolism and other relevant functions. The abovementioned studies explain using different cell lines that HBx localizes to the outer mitochondrial membrane (10). However, our recent studies have shown that the inner mitochondrial membrane protein, COXIII, also interacts with hepatitis B virus X protein *in vivo* by the yeast two-hybrid system, further verified by mating experiment, coimmunoprecipitation and confocal laser scanning microscopy (11-13).

Mitochondria are key organelles that regulate apoptosis, cellular energetics and signal transduction pathways (14), and are the source of HBx-induced ROS (15). Accumulating evidence has suggested that inflammation contributes to HCC development due to the adverse effects of inflammatory mediators such as proinflammatory cytokine and reactive oxygen species (ROS). They play a key role on DNA repair, DNA methylation, DNA oxidation and lipid peroxidation (16,17). There are five enzyme complexes (complexes I-V) involved in oxidative phosphorylation in mitochondria. Cytochrome *c*

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Key words: hepatitis B virus X protein, cytochrome *c* oxidase subunit III, mitochondrial function, reactive oxygen species, hepatocellular carcinoma

oxidase (COX; or complex IV), composed of the COXI, COXII and COXIII mtDNA encoded subunits and 10 nuclear DNA encoded subunits, is an essential component of the respiratory chain that catalyzes the reduction of molecular oxygen by reduced cytochrome *c* (18). COX is the terminal enzyme of the ETC and plays a pivotal role in the generation of ATP and maintenance of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Increased expression of COX subunits is associated with restoration of COX activity, increased cellular ATP levels, and a delayed restoration of $\Delta\Psi_m$ (19). In human colorectal carcinoma cell lines metastasis is closely associated with COXIII, which functions in the process of growth and differentiation, transcription, apoptosis and signal transduction (20). Furthermore, upregulation of mitochondrial energy production-associated genes, *COXIII* and *COXVb*, are capable of enhancing cell growth by supporting higher energy requirements for cells (21,22). The abovementioned findings indicate that changes of mitochondrial function may be important in the HBx-associated HCC.

In the present study, we used HL-7702 cells which expressed HBx stably as a biologically relevant system to explore the physiological effect of HBx. We found that HBx located to the inner mitochondrial membrane protein COXIII, leading to alterations of mitochondrial function and the subsequent upregulation of inflammatory mediator, ROS.

Materials and methods

Antibodies and reagents. Anti-DYKDDDDK-Tag (anti-flag) antibody was purchased from Abmart (Shanghai, China). Anti-cytochrome *c* oxidase subunit III (anti-COXIII) (N-20) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti- β -actin antibody was purchased from ZSGB-BIO (Beijing, China). CFTM488-conjugated donkey anti-mouse IgG (H+L), CFTM350-conjugated donkey anti-goat IgG (H+L), and puromycin were obtained from Sigma (St. Louis, MO, USA). MitoTracker Red was obtained from Molecular Probes (Eugene, OR, USA). Primers were produced by Biosune (Shanghai, China).

Plasmids. The HBx expression plasmid PcDNA3.1-x (HBV subtype ayw) was a gift from Professor Michael J. Bouchard (Drexel University College of Medicine, Philadelphia, PA, USA). The lentivirus packaging system: pLOV.CMV.eGFP.2A.EF1a.PuroR (pLOV), psPAX2 and pMD2.G were provided by Neuron Biotech Co., Ltd. (Shanghai, China). The lentivirus vector pLOV contains the gene coding for green fluorescent protein (GFP), puromycin and the peptide DYKDDDDK (flag).

Cells cultures. The human 293T embryonic kidney cell line was provided by ATCC (UK). The human HL-7702 hepatocytes cell line was purchased from the Shanghai Cell Bank (Shanghai, China). The 293T and HL-7702 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution and maintained at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂.

Generation of the recombinant lentivirus and establishment of the stably transfected HL-7702 cell line. Full-length HBx

(465 bp) was amplified from PcDNA3.1-x by polymerase chain reaction (PCR) and then infused with flag epitope at the N-terminus and then cloned into pLOV. The plasmid was digested with *Xba*I to construct the recombinant lentiviral vector pLOV.flag-HBx. The recombinant lentiviral particles were generated by transient cotransfection with 293T cells by the three-plasmid expression system, and harvested by filtration through a 0.45 μ m filter and ultracentrifugation at 100,000 x g for 2 h at 4°C. The recombinant lentiviral particles titer was calculated by qPCR and used to transduce HL-7702 cells. The cell clones were then treated with 0.2 μ g/ml puromycin for 10 days. Expression of GFP in the objective clones was examined directly by fluorescence microscopy. Positive clones expressing GFP and resistant to puromycin were screened and designated as HL-7702-HBx and HL-7702-mock, respectively. HBx, mock and control were considered to represent HL-7702-HBx, HL-7702-mock and HL-7702 cells, respectively. The expression of flag-HBx was detected by qPCR and western blot analysis.

Indirect immunofluorescence. To eliminate the impact of green fluorescence caused by GFP in HL-7702-HBx cells, we used 100% methanol as a fixative (23). As a result, green fluorescence disappeared (data not shown). The distributions of HBx and COXIII proteins were measured by confocal microscopy. The cells were seeded directly onto coverslips and incubated for 48 h. When the density of cells achieved 60-70%, the culture medium was removed by repeated washes with phosphate-buffered saline (PBS), and the cells were stained for 30 min with 150 nM MitoTracker Red. The cells were first fixed with a 100% ice methanol solution, pre-incubated in blocking solution (5% donkey serum albumin in PBS) and incubated with the appropriate primary antibodies (anti-flag and anti-COXIII) at 4°C overnight. After overnight incubation, the cells were washed. The fluorescence-labeled secondary antibodies (CFTM488-conjugated donkey anti-mouse, CFTM350-conjugated donkey anti-goat) were added and incubated for 60 min at 37°C in the dark, and the sections were mounted using glycerol. Fluorescence images were captured by confocal laser scanning microscopy (Leica, Germany).

Isolation of mitochondria and measurement of COX activity. Mitochondria were isolated using the mitochondrial isolation kit for mammalian cells (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, following lysis of $\sim 2 \times 10^7$ cells, cell debris and nuclei were pelleted at 700 x g for 10 min at 4°C, followed by centrifugation at 3,000 x g for 15 min at 4°C to pellet a mitochondrially enriched fraction, and then 12,000 x g for 5 min at 4°C to pellet the isolated mitochondria. The isolated mitochondria were vortexed with 30 μ l of 1X enzyme dilution buffer (10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose). Protein concentrations were measured using the bicinchoninic acid (BCA) assay and then adjusted to 1 μ g/ μ l. COX activity was determined using the Cytochrome *c* Oxidase Assay kit (GenMed, Shanghai, China) according to the manufacturer's instructions. COX activity was based on a colorimetric assay that quantifies the oxidation of ferrocytochrome *c* to ferricytochrome *c* via cytochrome *c* oxidase, a reaction that results in a decrease in absorbance at 550 nm.

This decrease was monitored by an enzyme mark instrument (Bio-Tek, Winooski, VT, USA) calibrated to zero. Isolated mitochondria (10 μ l) were combined with 10 μ l of lysis buffer, then mixed with 205 μ l of buffer solution in 96-well plates. The reaction was initiated by the addition of 25 μ l of reaction liquid, and the decrease in absorbance at 550 nm was measured for 1 min. Activity was calculated based on the equation: U/ml = $[(\Delta\text{Abs}_{550}/\text{min for the sample} - \Delta\text{Abs}_{550}/\text{min for the blank}) \times \text{dilution factor} \times \text{total reaction volume}] / [\text{mitochondria isolate volume} \times \text{the difference in extinction coefficients between ferro- and ferri-cytochrome } c \text{ at 550 nm (21.84)}]$. One unit was considered the amount that oxidizes 1 μ mol reduced cytochrome *c*/min at pH 7.0 and 25°C.

Flow cytometric analysis. Cells in the three groups were harvested with trypsin and resuspended in PBS (1x10⁶ cells/ml) to analyze $\Delta\Psi_m$, ROS and cell cycles by flow cytometry. The $\Delta\Psi_m$ was assessed using the cationic fluorescent dye, TMRM. When cells reached 1x10⁶ cells/ml, they were incubated in 100 nM TMRM solution at 37°C in the dark for 20 min. The cells were washed twice with PBS and resuspended in PBS for analysis by a flow cytometer. Intracellular ROS levels were subsequently detected by staining cells with 50 μ M dihydroethidium (DHE) fluorescence probe (Vigorous Biotechnology, Beijing, China) for 30 min in the dark. The cells were collected by trypsinization and the mean fluorescence intensity was quantified by flow cytometry.

Transmission electron microscopy (TEM). The morphological changes of cell and mitochondria were observed by TEM. Cells (1x10⁶) were collected and subjected to fixation with 3% fresh glutaraldehyde and 1.5% paraformaldehyde solution at 4°C for 1 h, followed by post-fixation with 1% osmium tetroxide and 1.5% potassium ferrocyanide solution at 4°C for 1.5 h. The cells were dehydrated with a graded series of ethanol solution and embedded in Epon-618. Ultrathin sections were cut to stain with uranyl acetate and lead citrate, and observed under TEM (Phillips, Madison, WI, USA).

qPCR detects gene expression. Total RNA was extracted using TRIzol reagent (Life Technologies, Rockville, MD, USA). The primer sequences of each gene are listed in Table I. The first-strand cDNA was generated using MMLV transcriptase [New England Biolabs (NEB), Ipswich, MA, USA], and qPCR was performed with FastStar Universal SYBR-Green Master (Roche, Foster City, CA, USA) in triplicate in an Applied Biosystems StepOnePlus Real-Time PCR system (Life Technologies). Endogenous mRNA values were normalized to the level of β -actin mRNA.

Western blot analysis. The cells were prepared by washing with ice-cold PBS and lysed. Protein concentrations were measured using the bicinchoninic acid (BCA) assay. Equal amounts of protein were loaded and separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk in TBST [0.1% Tween-20, 20 mM Tris (pH 7.4) and 150 mM NaCl] for 2 h at room temperature, followed by overnight incubation with the primary antibody at 4°C. The membrane was washed and incubated with a horseradish peroxidase-conjugated secondary antibody for

Table I. Primer sequences.

Gene name	Primer sequence (5' to 3')
HBV X	F: ACTCTCTCGTCCCCTTCTCC R: GGTTCGTTGACATTGCTGAGA
COXIII	F: CCCGCTAAATCCCCTA'AAG R: GGAAGCCTGTGGCT'CAAAA
β -actin	F: CTCCATCCTGGCCTCGCTGT R: GCTGTACCTTCACCGTTCC

F, forward; R, reverse.

1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) kit (ZSGB-BIO Co. Ltd., Beijing, China). Images of the blots were captured using the Image Scanner (Epson, Nagano, Japan). The band intensity was quantified using ImageJ software.

Statistical analysis. Each set of experiments was repeated at least three times with similar results. Results were presented as means \pm standard deviations (SD). Statistical evaluation was carried out by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant result.

Results

Construction and identification of an HL-7702 cell line stably transduced with a lentivirus expressing the HBx gene. Lentiviral vectors lead to prolonged transgene expression (24). In the present study, we used the vectors to establish long-term HBx expressing HL-7702 cells *in vitro*. The sequences of the recombinant vector PCMV.flag-HBx examined by the sequencing process showed that the full-length HBx gene had been successfully subcloned into the lentiviral vector (data not shown). The titre of the recombinant lentivirus was 8.61x10⁷ TU/ml. GFP expression of HL-7702-HBx and HL-7702-mock were observed by fluorescence microscopy. The GFP-positive cells were 95-98% (Fig. 1A). Expression of HBx mRNA and protein were detected in HL-7702-HBx cells (Fig. 1B and C). This showed that the HL-7702 cell line stably expressing HBx was established.

HBx co-localizes with inner mitochondrial protein COXIII in HL-7702 cells. Our recent studies have verified that COXIII interacts with HBx (11-13). To confirm this finding, we subjected HL-7702-HBx and control cells to fluorescent staining and examination by confocal microscopy. The co-localization of HBx protein (green color) with COXIII protein (blue color) in mitochondria (red color) was in white in HL-7702-HBx cells, while in HL-7702 cells the co-localization of COXIII protein (blue color) and mitochondria (red color) was in purple (Fig. 2). In addition, HBx localized in the nucleus and cytoplasm, with a fraction of cytosolic HBx co-localized with mitochondria as identified in previous studies (4-9). Thus, our study demonstrated that HBx co-localized with inner mitochondrial protein COXIII in HL-7702 cells.

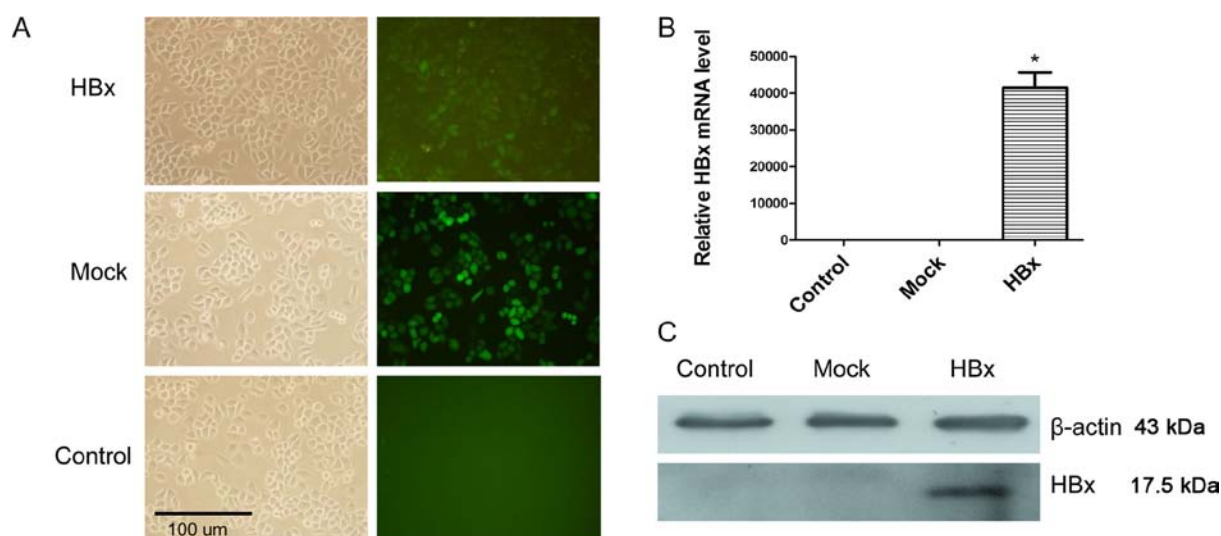


Figure 1. Construction of an HL-7702 cell line stably expressing *HBx* gene. HBx, mock and control represented HL-7702-HBx, HL-7702-mock and HL-7702 cells, respectively. (A) Fluorescence microscope analysis of HL-7702 cells transduced with lentiviral vectors expressing green fluorescent protein (GFP). Cells were observed by visible light (left) and fluorescence microscope (right), respectively (original magnification, $\times 200$). (B) qPCR detection of HBx mRNA expression. (C) Western blot detection of HBx protein expression by the antibody specific against flag protein and β -actin.

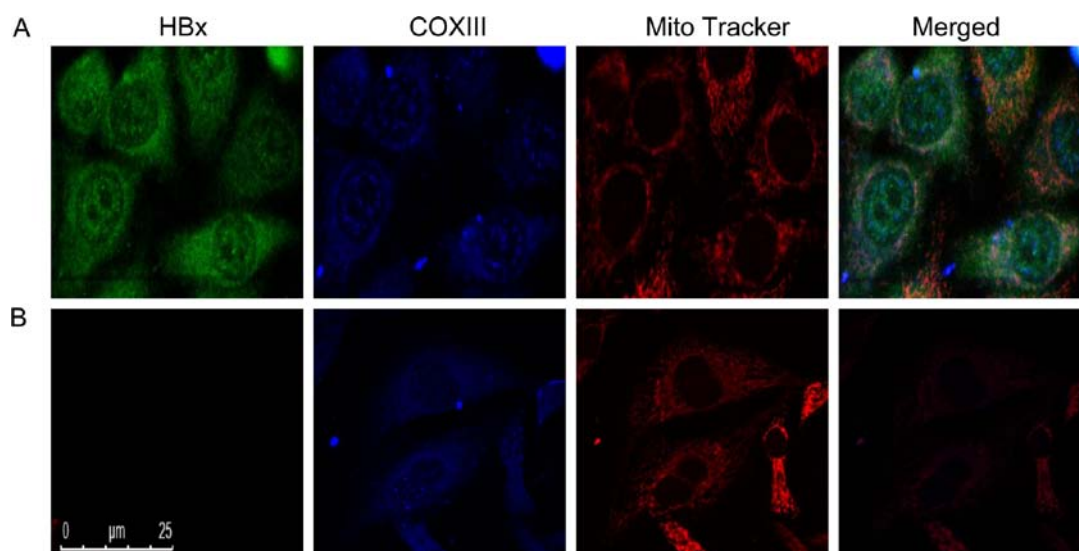


Figure 2. Co-localization of HBx with COXIII in mitochondria by confocal microscopy. Subcellular localization of HBx and COXIII were visualized by immunofluorescence staining with anti-flag antibody (green) and anti-COXIII (blue) antibodies. Mitochondria were stained with MitoTracker (red). The merged images are shown in the right panel. (A) HL-7702-HBx cells, (B) HL-7702 cells (original magnification, $\times 800$).

Upregulation of the mitochondrial function in HL-7702-HBx cells. COXIII is an essential component of the mitochondrial respiratory chain. Thus, the co-localization of HBx and COXIII was expected to induce an alteration in the normal mitochondrial electron flow. To determine mitochondrial function alteration in HL-7702-HBx cells, we compared the expression of mitochondrial protein subunits of complexes IV (COXIII subunit). The expression of COXIII protein was increased in HL-7702-HBx cells, while the RNA level did not change (Fig. 3A and B). The result indicated that HBx regulated *COXIII* gene function at the post-transcription level. Then we measured the activities of COX (Fig. 3C). As expected, COX activity of HL-7702-HBx cells was significantly increased, correlating with their upregulated protein activities. However, there was no difference in the $\Delta\Psi_m$ of any of the cells (Fig. 3D).

Changes of mitochondrial ultrastructure in HBx steadily expressed cells. Abnormal aggregation of mitochondria has been found in HBx-expressing cells (9,25). To assess the mitochondrial morphological consequences in HL-7702-HBx cells, we observed the mitochondria, nuclei and other intracellular membrane structures by TEM. As shown in Fig. 4, mitochondria had only slight swelling in HL-7702-HBx cells. Mitochondria in HL-7702-mock and HL-7702 cells were round or oval, and their membrane was integral, crest was dense and arranged in order. Our results indicated that cell and nuclear morphology were not significantly changed and there was no mitochondrial aggregation in any of the three groups.

Upregulation of ROS production in HL-7702-HBx cells. Since we demonstrated that HBx upregulates genes are involved

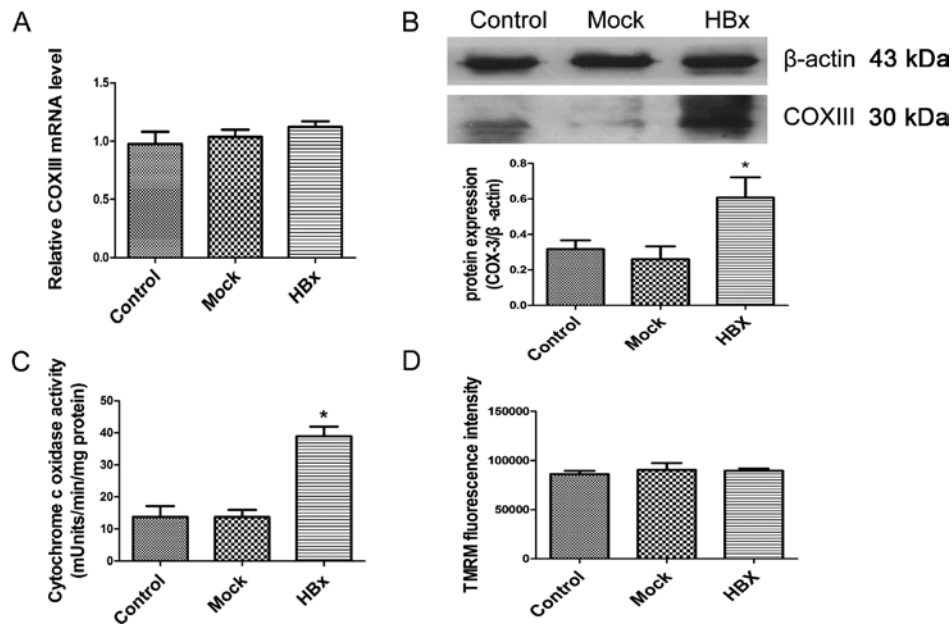


Figure 3. HBx leads to the upregulation of mitochondrial function in HL-7702-HBx cells. HBx, mock and control represented HL-7702-HBx, HL-7702-mock and HL-7702 cells, respectively. (A) Expression of COXIII mRNA was detected using qPCR. Levels of β -actin mRNA were used as an internal control. (B) Levels of COXIII protein were detected in lysates of HL-7702, HL-7702-mock and HL-7702-HBx cells using western blotting by antibody specific against COXIII protein and β -actin. (C) Changes of cytochrome *c* oxidase (COX) activity were determined using the Cytochrome *c* Oxidase Assay kit. (D) Mitochondrial membrane potential was measured using fluorescence dye, TMRM, followed by flow cytometry. Values are the means \pm SD for at least three independent experiments. * $P < 0.05$.

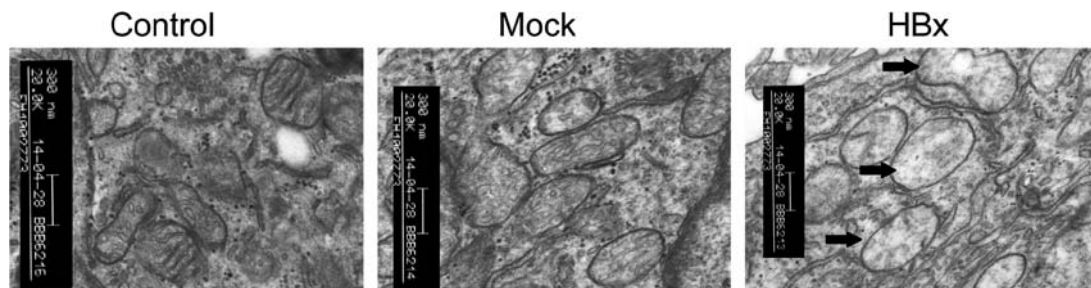


Figure 4. Changes of mitochondrial ultrastructure in HL-7702-HBx cells. Morphological changes of mitochondria were examined by electronic microscopy (original magnification, $\times 16,000$). Slight swelling in mitochondria was found in the HL-7702-HBx cells (shown by the arrow).

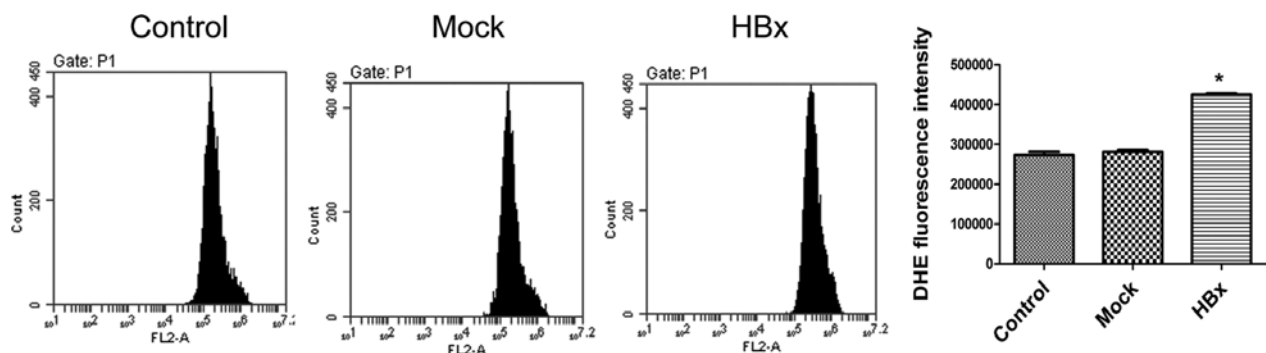


Figure 5. HBx induces ROS generation in HL-7702 cells. HBx, mock and control represented HL-7702-HBx, HL-7702-mock and HL-7702 cells, respectively. Intracellular ROS levels were determined using fluorescence dye, DHE, followed by flow cytometry. Values are the means \pm SD for at least three independent experiments. * $P < 0.05$ compared with HL-7702 and HL-7702-mock cells.

in oxidative phosphorylation at mitochondria, a major ROS generator, we examined whether HBx induces ROS production in HBx-transfected cells. Therefore, we examined

ROS levels by flow cytometry using the DHE probe. Fig. 5 shows that DHE fluorescence revealed an increase in the HL-7702-HBx cells.

Discussion

HBx plays a key role in the development of HBV-associated HCC (3). Using a variety of methods such as liposome preparation, electrical transduction and adenovirus can deliver HBx. However, lentiviral vectors are more suitable for long-term expression in non-dividing or infrequently dividing cells due to advantages of low toxicity and lack of immunogenicity (26,27). In the present study, we successfully established HL-7702 cells that stably expressed HBx *in vitro* using lentiviral vectors. They served as a biologically relevant system for exploring the effect of HBx in human normal hepatocytes in physiology.

HBx protein is located in the cytoplasm and partly in the nucleus (4). Accumulating evidence has demonstrated that a fraction of cytosolic HBx co-localizes with the mitochondria in different cell lines (5-10) in addition to the outer mitochondrial membrane protein, VDAC3 (8,10). HBx has been found to localize to mitochondria, leading to mtDNA damage, which encodes COXIII subunit (18,28). Recent findings have shown that COXIII also interacts with HBx (11-13). These findings strongly predict the interaction between HBx and COXIII. However, the localization of HBx within the inner mitochondrial membrane and the effect of HBx on mitochondrial physiology in HL-7702 cells have not been previously addressed. To the best of our knowledge, our results were the first to suggest the inner mitochondrial membrane localization of HBx in HL-7702 cells (Fig. 2). These results provide insight into the mitochondrial localization of HBx.

COX is the terminal complex of the electron transport chain and its biogenesis is a critical part of mitochondrial biogenesis (29,30). Mounting evidence suggests that the upregulation of COX has been found in human prostate (31) and breast carcinoma (32), and human colon cancers (22). In the present study, we characterized the upregulation of COX biogenesis in HL-7702-HBx cells, including the increasing protein expression of COXIII and upregulation of the activities of COX, which may provide more energy for cells to grow. These results were consistent with recent studies showing HBx (HBV serotype ayw) upregulated the protein expression of COXIII and activities of COX in HepG2 cells which stably expressed HBx using lentiviral vectors (13). By contrast, HBx (HBV genotype B) downregulated COXIII expression and inhibited COX activity in HL-7702 cells that expressed HBx using eukaryotic vector (33). The reasons for this discrepancy include that HBx plays pro-apoptotic and anti-apoptotic roles in different situations (34), and different genotypes of HBV may play different roles. On the other hand, HBx in long-term transfected cells is different from that in transiently transfected cells. COX accepts electrons from cytochrome *c* and transfers them to molecular oxygen. At the same time protons are pumped across the inner mitochondrial membrane leading to the generation of $\Delta\Psi_m$ (35). It has been reported that HBx located in mitochondria may cause loss of $\Delta\Psi_m$ in hepatoma cells (8,36). By contrast, in our system, the co-localization of HBx with COXIII protein did not induce changes in $\Delta\Psi_m$. We consider that long-term HBx-transfected cells in our system were more stable than the abovementioned HBx transiently transfected cells. Thus, the upregulation of COX biogenesis did not result in $\Delta\Psi_m$ alteration. Additionally, the role of HBx in human normal hepatocytes (HL-7702 cells) may be

different from that in hepatoma cells. We then showed the slight swelling of mitochondria in HL-7702-HBx cells accompanied by increased respiratory enzyme activities and protein levels in mitochondria, as indicated by electron microscopy. This is an adaptive state for mitochondria, which adapt to the change of microenvironment. These results suggest that the co-localization of HBx with COXIII led to changes of mitochondrial function and morphology.

Mitochondria are the source of ROS (15). COX encoded gene expression changes are associated with the development of tumors mainly through an increase in ROS in mitochondria oxidative phosphorylation (37-40). In the present study, we observed that HBx expression elevated the generation of ROS (Fig. 5). HBx increases the level of mitochondrial ROS, which is associated with hepatocellular carcinogenesis (15,41), although the mechanism involved remains controversial.

In summary, we have demonstrated that the HBx protein co-localizes with the inner mitochondrial membrane protein COXIII in HL-7702 cells, leading to changes of mitochondrial function, morphology and increasing the generation of mitochondrial ROS. These results provide insight into the molecular mechanism of HBV-associated HCC.

Acknowledgements

The present study was supported by the National Natural Science (grant no. 81300321) from the Foundation of China, the Key Clinical Specialty Discipline Construction Program of Fujian Province (2012-149), and Young and Middle-Aged Personnel Training Project of Fujian Province Health Department (2014-ZQN-ZD-9). We thank Professor Bouchard for the pCDNA3.1-X plasmid.

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