Human bocavirus induces apoptosis and autophagy in human bronchial epithelial cells

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Abstract. Human bocavirus (HBoV) is classified in the *Bocavirus* genus within the Parvoviridae family, first identified from children with respiratory diseases. Previous studies have investigated the stimulating effect of HBoV on cell apoptosis and autophagy. In the present study, human bronchial epithelial cells (HBECs) were utilized to examine the mechanism of HBoV recombination expressing vector (pWHL-1) on the promotion of cell apoptosis and autophagy. The results from the present study indicated that pWHL-1 inhibited the proliferation of HBECs in a time-dependent manner. Additionally, pWHL-1induced apoptosis, as substantiated by an increased apoptotic rate and presence of autophagosomes. Following pWHL-1 transfection, proliferating cell nuclear antigen, caspase-3 and B cell lymphoma 2 (Bcl-2) protein expression levels were decreased, with the exception of Bcl-2 associated x (Bax) protein, which increased. mRNA and protein expression levels of microtubule-associated protein 1A/1B-light chain 3 (LC3) II and autophagy protein 5 were increased in pWHL-1-transfected HBECs, whereas, the mRNA and protein levels of LC3I and sequestosome 1 were decreased. Notably, pWHL-1 also enhanced the activation of p53 and inhibited AKT activation in HBECs. Results from the present study suggest that pWHL-1 induces apoptosis and autophagy, thus providing a novel insight into the effect of HBoV and its uses in respiratory diseases.

Introduction

Human bocavirus (HBoV) is classified in the *Bocavirus* genus within the Parvoviridae family, and was first identified in children with respiratory diseases (1). HBoV infections have been observed worldwide not only in respiratory tract secretions but also in urine, fecal and serum samples (2,3). Newly identified HBoV including HBoV2, HBoV3 and HBoV4 were identified in human stool samples. Previous findings suggest that HBoV is associated with human diseases and notable pathogenesis; however, there is an inadequate amount of evidence to support this due to the limited establishment of *in vitro* HBoV culture systems and animal models (4). HBoV encodes three nonstructural proteins including: NP1, NS1 and NS1-70, in addition to two structural proteins, VP1 and VP2. HBoV NP1 is a nuclear protein, which has an important role in the DNA replication process of HBoV within the nuclei of infected cells (5,6). It has been reported that NP1 is able to cause cell cycle arrest at G2/M phase followed by apoptosis, via the mitochondrion pathway in HeLa cells (7). Infection with Bocavirus minute virus of canines (MVC) has also been demonstrated to induce apoptosis, dependent on the replication of the viral genome and arrest G2/M phase in Walter Reed/3873D (WRD) canine cells (8).

The multiple integration of biological and pathological progresses, such as proliferation, differentiation, apoptosis and metabolism, is able to affect the pathogenesis of human diseases. Advanced understanding of the cellular and molecular mechanism of these progresses is important for developing novel diagnostic and therapeutic targets. Previous studies have indicated the cellular bases and roles of autophagy in human health and disease world-wide (9,10). Autophagy is responsible in the *in vivo* protection against human pathogens that are degraded *in vitro* by bacteria, viruses and parasites (11,12). Autophagy-related proteins (ATGs), are conserved in mammalian cells and have been indicated to be essential components of the autophagic progress (13). The ubiquitin-like conjugation of ATG5-ATG12 contributes to autophagosome formation and induces LC3 lipidation (LC3I) (10,14). The conversion of cytosolic LC3I to phosphatidylethanolamine-conjugated form (LC3II), a key marker for the autophagosome, indicates the formation of autophagosome (15). SQSTM1, also referred to as p62 protein, is decreased in the presence of autophagy and accumulated in the absent of autophagy, suggesting SQSTM1 may be a marker for autophagy (16).

The molecular mechanism by which HBoV induces apoptosis and autophagy is not yet well understood. Previous studies have demonstrated that celecoxib and caffeine induced autophagy through inhibition of the PI3K/ATK signaling pathway (17,18). The PI3K/AKT signaling pathway is widely utilized in normal and abnormally activated cells, such as in cancer cells (19). Activation

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of AKT triggers phosphorylation of downstream targets which impacts cell proliferation, apoptosis, migration and autophagy progresses (20). In the present study, human bronchial epithelial cells (HBECs) were used to examine the effect of HBoV on cell proliferation, apoptosis and autophagy, as well as the mechanism involved. We propose a model of positive regulation of autophagy as part of the host response to HBoV infection in HBEC.

Materials and methods

Cell culture. Human bronchial epithelial cells (HBECs; Institute of Biochemistry and Cell Biology, Shanghai, China) at $1x10^5$ cells/well were grown on rat-tail collagen I-coated dishes and incubated at 37°C in a humidified chamber containing 5% CO_2 for 12 h. Subsequently, HBEC were changed to grow in bronchial epithelial cell basal medium (Clonetics, Co., San Diego, CA, USA) supplemented with 100 mg/ml penicillin G and 50 μg/ml streptomycin, 0.5 μg/l human epidermal growth factor and 50 mg/l bovine pituitary extract (all obtained from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in tissue culture flasks and expanded in the same growth medium. All HBECs were cultured using an incubator and maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Lentiviral production and transduction. The HBoV coding sequence was cloned into a pBluescript SKII vector donated from Yi Li from the Wuhan Engineering Institute (Wuhan, China). The HBoV recombination expression vector was referred to as pWHL-1. Constructs were subsequently transfected into HEK 293T cells using Lipofectamine 2000 according to the manufacturer's instructions. Viruses were collected following 48 h transfections and HBECs were infected. HBECs without transfection were used as a control and HBEC with the empty PLKO.1-EGFP vector transfection was used as the negative control (NC).

Cell proliferation assay. HBECs ($3x10^3$ cells/well) transfected with or without 50 nM pWHL-1 were harvested and plated in 96-well plates. A total of 10 μl Cell Counting assay kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol, was added to each well and the absorbance was measured at 450 nm using a microplate reader.

Apoptosis assay. HBECs ($3x10^3$ cells/well) transfected with or without 50 nM pWHL-1 were harvested and plated in 96-well plates. A cell fixation and permeabilization kit (cat. no. ab185917; Abcam, Cambridge, UK) was used to fix the HBECs in suspension and then permeabilizing the cell membranes, according to the manufacturer's protocol. Subsequently, cells were washed with PBS for 5 min at 25°C, harvested and stained with 195 μl Annexin V-FITC and 5 μl propidium iodide (PI; BD Biosciences, San Jose, CA, USA) for 15 min in the dark at room temperature followed by flow cytometry analysis using BD Accuri™ C6, version 1.0.264.21 software (BD Biosciences, San Jose, CA, USA).

Immunohistochemistry. HBECs transfected with or without 50 nM pWHL-1 were fixed with 10% formaldehyde for 48 h at 25°C and blocking of endogenous peroxidases was completed

Table I. Primer sequences used in the present study.

Gene	Primer sequences
LC3I-forward	5'-TCCGACCGGCCTTTCAAGCAG-3'
LC3I-reverse	5'-GAGAACCTGACCAGAACTCCC AG-3'
LC3II-forward	5'-GGAAAGCAGCAGTGTACC-3'
LC3II-reverse	5'-CTTTAAGCCGGAAGGCAG-3'
ATG5-forward	5'-GGCTGAGTGAACATCTGAG-3'
ATG5-reverse	5'-CCCAGTTGCCTTATCTGAC-3'
SQSTM1-forward	5'-GGAGTCGGATAACTGTTC-3'
SQSTM1-reverse	5'-GATTCTGGCATCTGTAGG-3'
GAPDH-forward	5'-CACCCACTCCTCCACCTTTG-3'
GAPDH-reverse	5'-CCACCACCCTGTTGCTGTAG-3'

LC3, microtubule-associated protein 1A/1B-light chain 3; ATG5, autophagy protein 5; SQSTM1, sequestosome 1.

by soaking slides in a solution of 90% methanol/3% H_2O_2 for 10 min at 37°C. For antigen retrieval, the HBECs were microwaved in 10 mM citrate buffer (pH 6.0) at 95°C for 10 min. HBECs were then incubated with proliferating cell nuclear antigen (PCNA) rabbit monoclonal antibody (cat. no. ab18197; Abcam; 1:1,000) for 1 h at room temperature after blocking non-specific binding with 10% normal goat serum (cat. no. 005-000-121; Qcbio Science & Technologies Co., Ltd., Shanghai, China) in PBS at 37°C for 30 min, followed by incubation with goat anti-rabbit biotin-conjugated IgG (cat. no. ab181744; Abcam; 1:1,000) at 25°C for 30 min. HBECs were stained with 3,3'-diaminobenzidine (Shanghai Long Island Biotec., Co., Ltd., Shanghai, China) and hematoxylin staining (Sigma-Aldrich; Merck KGaA). Immunohistochemical signals were calculated with the positive staining of cells using a light microscope (CX41RF; Olympus Corporation, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from HBECs transfected with or without pWHL-1 using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific Inc.). A total of 1 μg RNA was reverse transcribed to synthesize cDNA using Primescript RT Reagent (Takara Biotechnology Co., Ltd., Dalian, China). DNaseI treatment was used to remove genomic DNA. RNA-Primer Mix (12 μl), 5xRT Reaction Buffer (5 μl), 25 mM dNTPs (1 μl), 25 U/μl RNase Inhibitor (1 μl), 200 U/μl M-MLV Rtase (1 μl), Oligo(dt)$_{18}$ (1 μl) and ddH$_2$O (DNase-free; 4 μl). SYBR-Green qPCR Master Mix (X2; Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) was used to Real-time PCR performed on an ABI 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). SYBRGreen Mix (12.5 μl), forward primer (0.5 μl), reverse primer (0.5 μl), ddH$_2$O (9.5 μl), cDNA (2 μl). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. The primers are listed in Table I. GAPDH mRNA was used as internal control. mRNA expression levels were calculated by the comparative $\Delta\Delta$Cq method
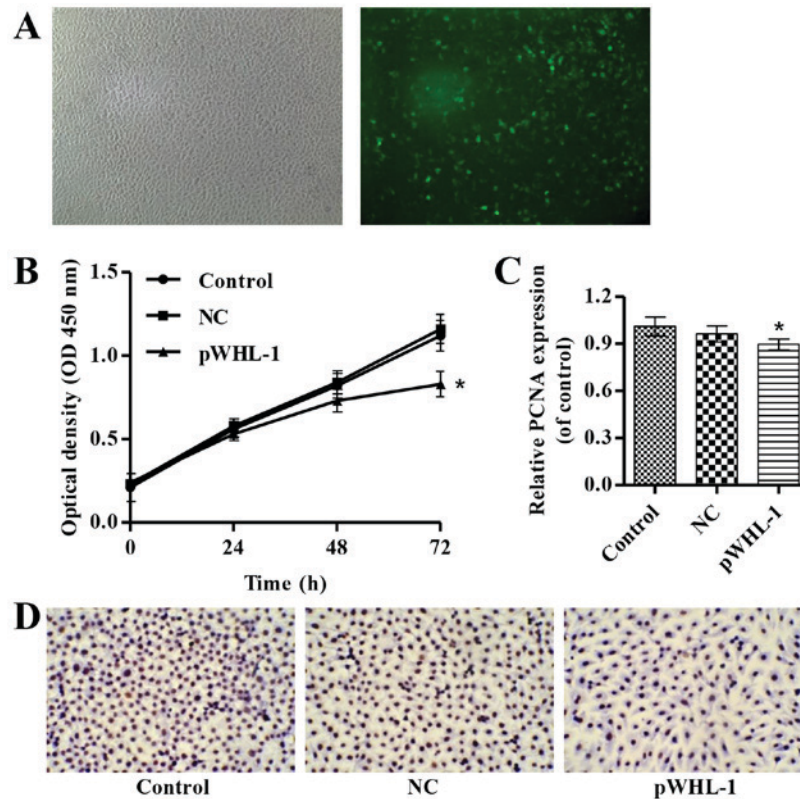
Figure 1. HBoV transfection inhibited HBEC proliferation. HBoV recombination expressing vector was transfected into HBEC for 48 h. (A) Transfection efficiency was evaluated by fluorescence microscopy (left: white light, right: green fluorescence). (B) Proliferation ability was measured using Cell Counting kit-8 analysis. (C) Quantification and (D) representative images of PCNA expression analyzed by immunohistochemistry. The HBoV recombination expression vector was referred to as pWHL-1. HBEC without transfection was used as a control and HBEC with the empty PLKO.1-EGFP vector transfection was used as the NC. *$P<0.05$ vs. NC. Data are presented as the mean ± standard deviation. HBoV, human bocavirus; HBEC, human bronchial epithelial cells; PCNA, proliferating cell nuclear antigen; NC, negative control. Magnification, x200.

and the fold changes were analyzed by $2^{-\Delta\Delta Cq}$ (21). The experiment was repeated three times.

Western blot analysis. Total proteins were extracted from HBEC transfected with or without pWHL-1 using RIPA buffer containing 50 mM Tris-HCl, (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, phosphatase and protease inhibitor cocktail (CalbioChem; Merck KGaA) at 4°C for 20 min, followed by centrifugation at 12,000 x g for 1 min at 25°C. A total of 30 µl protein was separated using 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in fat-free milk overnight at 4°C following three washes with Tris-buffered saline with Tween-20 (Amresco, LLC, Solon, OH, USA) for 5 min at 25°C and subsequently incubated with primary antibodies at 4°C overnight. Antibodies used in western blot analysis were as follows: Rabbit monoclonal antibodies for LC3 I/II (cat. no. 4108; 1:1,000; CST Biological Reagents Company Limited, Shanghai, China), SQSTM (cat. no. ab109012; 1:10,000), ATG5 (cat. no. ab109490; 1:1,000), caspase-3 (cat. no. ab32042; 1:1,000), Bcl-2 (cat. no. ab32124; 1:1,000), Bax (cat. no. ab320503; 1:500), p-p53 (cat. no. ab1431; 1:1,000), p-AKT (cat. no. ab38449; 1:1,000), AKT (cat. no. ab8805; 1:500; all purchased from Abcam), GAPDH (cat. no.5174; CST Biological Reagents Company Limited; 1:1,500), and mouse monoclonal antibody for p53 (cat. no. ab1101; Abcam; 1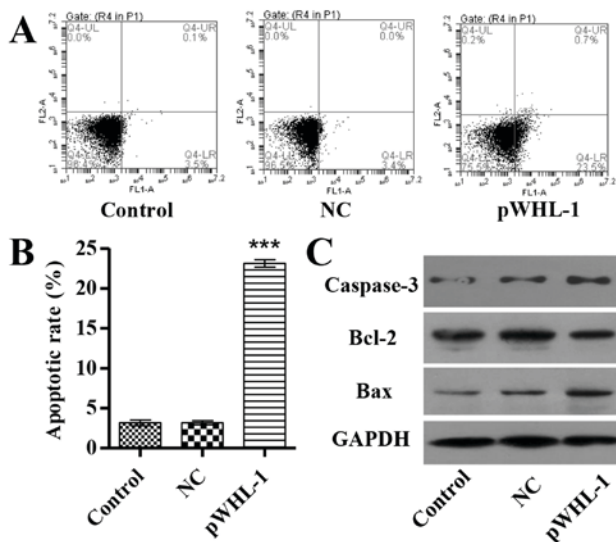:1,000). Membranes were subsequently washed three times with Tris-buffered saline with Tween-20 (Amresco, LLC), and the peroxidase-conjugated goat anti-rabbit/mouse secondary antibody (1:1,000; cat. no. A0208 and A0216; Beyotime Institute of Biotechnology, Haimen, China) was incubated at 37°C for 1 h and washed three times with Tris-buffered saline with Tween-20 (Amresco, LLC). Immunoreactivity was detected with enhanced chemiluminescence (Merck Millipore; Merck KGaA) and signals were quantified by densitometry (Quantity One software version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH mRNA was used as internal control and the experiment was repeated in triplicate.

Statistical analysis. Data are presented as the mean ± standard deviation. The paired, two-tailed Student's t-test was used to analyze the significance of difference between groups. Each experiment was performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HBoV suppresses HBEC proliferation. To investigate the biological significance of HBoV in HBEC, HBoV stably expressing cell lines of HBEC were established by pWHL-1 transfection. As indicated in Fig. 1A, fluorescence microscopy evaluation demonstrated a transfection efficiency in HBECs following transfection with pWHL-1. Furthermore, pWHL-1 transfection significantly reduced the proliferation of HBEC
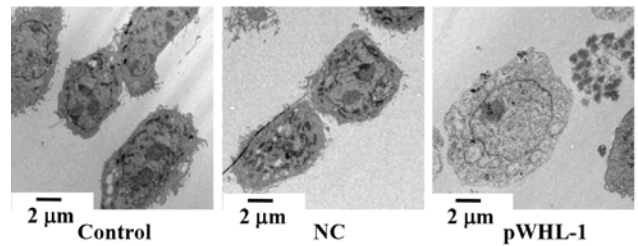
Figure 3. HBoV transfection induced HBEC autophagy. HBoV recombination expressing vector transfected into HBEC for 48 h. The HBoV recombination expression vector was referred to as pWHL-1. HBEC without transfection was used as a control and HBEC with the empty PLKO.1-EGFP vector transfection was used as the NC. In comparison to the control and NC groups, pWHL-1 transfected HBEC exhibited disordered nuclei, damage of the cellular membrane in organelles such as mitochondria, and a large number of autophagic vacuoles and autophagosomes were present in the cytoplasm. HBoV, human bocavirus; HBEC, human bronchial epithelial cells; NC, negative control.

Figure 2. HBoV transfection induced HBEC apoptosis. HBoV recombination expressing vector was transfected into HBEC for 48 h. The HBoV recombination expression vector was referred to as pWHL-1. HBEC without transfection was used as a control and HBEC with the empty PLKO.1-EGFP vector transfection was used as the NC. (A) Representative plot and (B) quantification of apoptotic rate as assessed by flow cytometry. (C) Protein expression levels of caspase-3, Bcl-2 and Bax were detected by western blot analysis. ***P<0.001 vs. NC. Data are presented as the mean ± standard deviation. HBoV, human bocavirus; HBEC, human bronchial epithelial cells; NC, negative control; Bcl-2, B cell lymphoma 2; Bax, Bcl-2 associated protein X.

when compared with the control and NC groups (P<0.05; Fig. 1B). Immunohistochemistry analysis of PCNA, a marker of cell proliferation, indicated that PCNA exhibits significantly decreased levels in pWHL-1 transfected HBEC when compared with the NC (P<0.05; Fig. 1C and D).

HBoV promotes HBEC apoptosis. A total of 48 h after transfection, apoptosis of HBEC was investigated by Annexin V-FITC/PI staining and flow cytometry analysis. The results revealed that pWHL-1 transfection significantly induced HBEC apoptosis by 6.3-fold when compared with the NC groups (P<0.001; Fig. 2A and B). To further investigate the mechanism of HBoV associated with apoptosis of HBEC, three apoptosis associated proteins were also detected in HBEC. Furthermore, the protein expression level of B cell lymphoma (Bcl-2) was significantly decreased (P<0.01), whereas the protein expression levels of Bcl-2 associated X (Bax, P<0.01) and caspase-3 were significantly increased in HBEC with pWHL-1 transfection, whereas in pWHL-1 transfected HBEC (P<0.01; Fig. 2C).

HBoV induces the autophagy progress in HBEC. To investigate the effect of HBoV on autophagy of HBEC, a transmission electron microscope was used to observe the cellular morphology of HBEC. The results indicated that pWHL-1 transfected HBEC possessed disordered nuclei, damage of the cellular membrane of organelles, including the mitochondria, and a large number of autophagic vacuoles and autophagosomes in cytoplasm. In comparison with the pWHL-1 transfected HBEC, the control and NC groups showed normally ordered nuclei, abundant and complete cellular membrane of organelles, such as mitochondria, and a fewer number of autophagic vacuoles and autophagosome in cytoplasm (Fig. 3).

Subsequently it considered whether HBoV functionally regulates the autophagy-associated protein in HBEC. To address this question, RT-qPCR and western blot analysis was performed to detect the mRNA and protein expression levels of core proteins involved in the autophagy progress, respectively. These results showed that the mRNA expression levels of LC3I and SQSTM1 were significantly decreased in pWHL-1 transfected HBEC when compared with the control and NC groups, whereas the mRNA expression levels of LC3II and ATG5 were significantly increased in pWHL-1 transfected HBEC when compared with the control and NC groups (P<0.01; Fig. 4A). Similarly, the protein expression levels of LC3I and SQSTM1 were significantly decreased in pWHL-1 transfected HBEC when compared with the control and NC groups, whereas the protein expression levels of LC3II and ATG5 were significantly increased in pWHL-1 transfected HBEC when compared with the control and NC groups (P<0.01; Fig. 4B and C).

HBoV inhibits activation of p53 and AKT. It has been widely recognized that signaling pathways such as p53 and PI3K/AKT pathways are often activated in tumor cells and promote cell proliferation and repress apoptosis and autophagy (19,20). To examine the role of HBoV on p53 and AKT in HBECs, the activation of these proteins was assessed by western blot analysis. In NC HBECs, the P-53/p53 and p-AKT/AKT ratios were unchanged compared with the control cells. In HBECs transfected with pWHL-1, p53 phosphorylation was significantly increased, while AKT phosphorylation was significantly decreased, compared with the NC cells (P<0.01; Fig. 5A and B).

Discussion

Human bocavirus (HBoV) has been widely regarded to induce apoptosis and autophagy, which is associated with viral pathogenesis (22). In a previous report, human airway epithelia infected with HBoV were revealed to result in cilia loss and airway epithelial cell hypertrophy; however this was not associated with apoptotic or necrotic cell death (23). Similarly, a previous study demonstrated that HBoV nonstructural
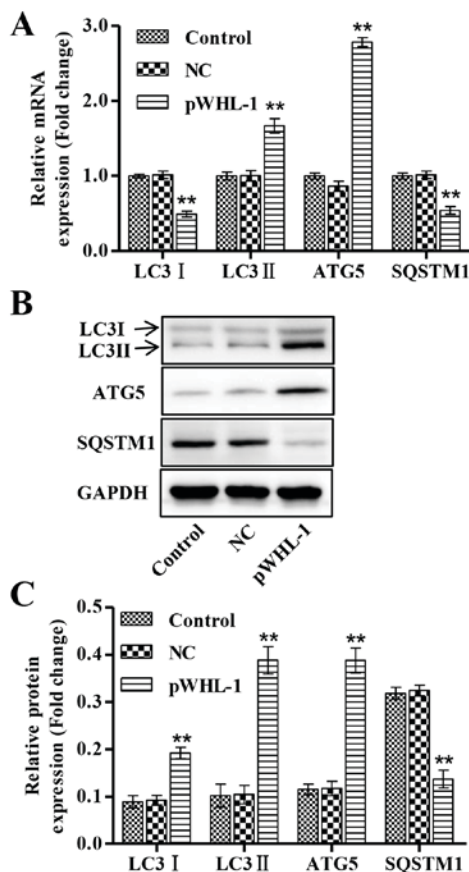
Figure 4. HBoV infection regulated autophagic core proteins expression. HBoV recombination expressing vector was transfected into HBEC for 48 h. The HBoV recombination expression vector was referred to as pWHL-1. HBEC without transfection was used as a control and HBEC with the empty PLKO.1-EGFP vector transfection was used as the NC. mRNA and protein expression levels of LC3I, LC3II, ATG5 and SQSTM1 were measured by (A) reverse transcription-quantitative polymerase chain reaction and (B and C) western blot analysis. **P<0.01 vs. NC. Data are presented as the mean ± standard deviation. HBoV, human bocavirus; HBEC, human bronchial epithelial cells; NC, negative control; LC3, microtubule-associated protein 1A/1B-light chain 3; ATG5, autophagy protein 5; SQSTM1, sequestosome 1.
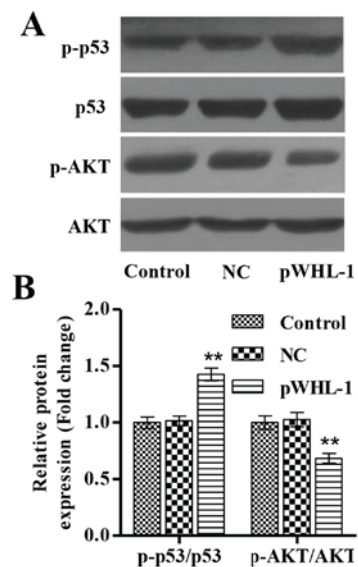

Figure 5. HBoV transfection inhibited the activation of p53 and AKT. HBoV recombination expressing vector was transfected into HBEC for 48 h. The HBoV recombination expression vector was referred to as pWHL-1. HBEC without transfection was used as a control and HBEC with the empty PLKO.1-EGFP vector transfection was used as the NC. (A and B) Protein expression levels of p-p53, p53, p-AKT and AKT were measured by western blot analysis. **P<0.01 vs. NC. Data are presented as the mean ± standard deviation. HBoV, human bocavirus; HBEC, human bronchial epithelial cells; NC, negative control; p53, tumor protein p53; AKT, protein kinase B.

protein failed to cause cell death (5). However, in the present study, we demonstrated that HBoV recombination expressing vector (pWHL-1) induced proliferation inhibition, apoptosis and autophagy in HBECs.

To investigate the effect of HBoV recombination expressing vector transfection of HBEC, the proliferation of HBEC was measured using aCCK-8 assay. The results showed that pWHL-1 transfection significantly inhibited the proliferation of HBEC when compared with the control and NC groups. It has been reported that HBoV is able to arrest the cell cycle at S phase; however, cell cycle arrest occurs predominantly at the G2/M phase, followed by cell apoptosis and proliferation suppression (8). The expression of PCNA was detected in pWHL-1 transfected HBEC by immunohistochemistry assay. The expression of PCNA was significantly decreased in pWHL-1-transfected HBECs when compared with the control and NC groups, which is consisted with the decreased proliferation observed following pWHL-1 transfection in HBECs.

Previous studies have demonstrated that HBoV induced apoptosis in several cell types, including HeLa, Walter

Reed/3873D canine and various types of epithelial cells (7,8). Therefore, the predominant focus of the present study was on the effect of pWHL-1 on HBEC apoptosis. The present findings identified that pWHL-1 significantly induced apoptosis of HBECs and regulated the protein expression of caspase-3, Bax and Bcl-2. Western blot analysis demonstrated that the protein expression levels of caspase-3 and Bax were increased, whereas the protein expression levels of Bcl-2 were decreased in pWHL-1-transfected HBECs compared with control and NC groups, suggesting that HBoV may induce apoptosis through a mitochondrion-mediated pathway and increase the ratio of Bax/Bcl-2. In agreement with our findings, a previous study reported that HBoV also induced cell apoptosis through the activation of caspase-3 and caspase-9 and an increase of Bax/Bcl-2 ratio was observed (7).

Autophagy is a crucial component of the cellular stress adaptation response that maintains mammalian homeostasis (24). Autophagosome formation proceeds through a series of stages and has various roles in cancer development and progression, and is involved in the proliferation of normal cells (25). In the present study, pWHL-1 infection was indicated to induce autophagy, as evidenced by the presence of disordered nuclei, damage of the cellular membrane of organelles, such as mitochondria, and large numbers of autophagic vacuoles and autophagosome observed in cytoplasm. In addition, specific autophagy associated proteins were also detected in HBEC. RT-qPCR and western blot analysis demonstrated that the mRNA and protein expression of LC3II and ATG5 were significantly increased, whereas LC3I and SQSTM1 mRNA and protein expression levels were significantly decreased in pWHL-1-transfected HBECs. An increased ratio of LC3II/LC3I is a marker of an enhancement in

autophagosomes formation, whereas SQSTM1 expression is negatively correlated with autophagy (26). ATG5 is involved in the early stage of autophagosome formation and has multiple functions in various physiological contexts (27).

The molecular mechanism by which HBoV induces autophagy in HBEC is not yet fully understood. Autophagy and apoptosis may coadjust through p53 and PI3K/AKT signaling (28,29). The p53 and PI3K/AKT signaling pathway are two well-known pathways involved in the regulation of autophagy (30). Both pathways are associated with tumorigenesis and activated in a number of cancers (19). *Yersinia pestis* infection of HBECs has been associated with the negative regulation of autophagy via the observed decrease exhibited of p53 cytoplasmic localization and PI3K/AKT activation (31). Furthermore, plumbagin has been demonstrated to induce autophagy via the inhibition of the PI3K/AKT pathway in human non-small cell lung cancer cells (32) In the present study, the activation of p53 was significantly increased; however, AKT protein activation via phosphorylation was significantly decreased in pWHL-1-transfected HBECs when compared with the control and NC groups, indicating that HBoV induced HBEC autophagy predominantly through enhancing p53 activation and blocking AKT activation.

In conclusion, the present study demonstrated that HBoV promoted the inhibition of proliferation, apoptosis and autophagy in HBEC and the apoptosis and autophagy were associated with the regulation of p53 and AKT. The present study may be useful to address the precise effect of HBoV in HBEC proliferation, apoptosis and autophagy and to delineate the molecular mechanism of HBoV in respiratory diseases.

Acknowledgements

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