

## Original Article

# HBx transfection limits proliferative capacity of podocytes through cell cycle regulation

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**Our previous studies have shown that podocyte number is significantly decreased in glomeruli of children with hepatitis B virus (HBV)-associated glomerulonephritis. In this study, we aimed to explore whether exogenous expression of HBx protein could directly inhibit podocyte proliferation *in vitro*, and to investigate its role in cell cycle regulation. HBx gene was delivered into cultured mouse podocytes through an adenovirus-based vector. Cell morphology was evaluated with Wright-Giemsa staining. Cell growth and proliferation were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5,6-carboxy-fluorescein diacetate, succinimidyl ester (CFSE)-based proliferation assays. Cell cycle phase was analyzed by flow cytometry, and the expression of cell cycle regulatory proteins was examined by western blot analysis. It was found that the aberrant nuclear changes like double and multiple micronuclei, which reflect mitotic catastrophe, accumulated in podocytes after 5 days post-infection. MTT assay showed that Ad.HBx-infected podocytes grew much more slowly than controls at day 4 post-infection and thereafter. Furthermore, CFSE-based proliferation assay also showed that the proliferation of HBx-expressing podocytes was significantly inhibited than that of controls at 3-day post-infection, and that the difference became much more obvious at day 5 post-infection. Cell cycle analysis showed that the transfection of HBx resulted in significant up-regulation of both cyclin B1 and CDK-inhibitor p21 expression and G<sub>2</sub>/M phase arrest, and slight down-regulation of cyclin A expression. These results demonstrated that exogenous expression of HBx might limit the proliferative capacity of podocytes through cell cycle regulation, thus suggesting that HBx may play a role in podocyte injuries in HBV-associated glomerulonephritis.**

**Keywords** hepatitis B virus X protein; podocyte; cell cycle; HBV-associated glomerulonephritis

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## Introduction

Hepatitis B virus-associated glomerulonephritis (HBV-GN) was initially reported by Combes *et al.* in 1971 [1]. The relationship between chronic HBV infection and glomerulonephritis is confirmed, which draws an increasing attention due to widespread infection of HBV in Chinese children. Most patients with HBV-GN present with nephrotic syndrome, and some of them show refractory proteinuria and eventually progress to end-stage renal failure [2,3]. Membranous nephropathy is the predominant pathological type of HBV-GN in children. The pathogenesis of HBV-associated membranous nephropathy remains to be elucidated. Immune-mediated damage of podocytes is considered to be one of the most important mechanisms in HBV-associated membranous nephropathy. Podocytes are terminally differentiated quiescent cells lining the exterior of glomerular capillaries and function as a critical component of the glomerular filtration barrier [4]. Injury of these podocytes leads to the development of proteinuria, while loss of podocytes is considered to be an important risk factor for glomerulosclerosis and progressive renal impairment [5–7]. Our previous studies have shown that podocytes are injured and the cell number is significantly decreased in glomeruli of children with HBV-associated membranous nephropathy [8]. We hypothesize that, in addition to immune complex-mediated mechanism, HBV may directly infect glomerular podocytes and cause the damage to the infected podocytes through HBV proteins, which will be a new explanation of podocytopathy in HBV-associated membranous nephropathy.

HBV encodes a small regulatory protein, termed HBx which plays an important role in virus replication and cellular transcription and signaling. HBx can localize within different subcellular compartments like the nucleus, cytoplasm, and mitochondria, where it can not only activate many signaling pathways such as mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and Src kinases,

but also work as a transcriptional transactivator to regulate the expression of a wide variety of genes, thus it can influence HBV replication, cell cycle progression, apoptosis, DNA repair, protein degradation, transformation, cell-cell interactions, etc. [9,10]. As a result, HBx may be associated with some cellular events like apoptosis, proliferation, or carcinogenesis after HBV infection. It has been confirmed that HBx plays an important role in the development of hepatocellular carcinoma in chronic hepatitis B patients. Using *in situ* hybridization, *HBx* mRNA has been identified within glomeruli in 30% of HBV-GN patients [11]. But the role of HBx in podocyte damage has not been studied. In this study, we showed that exogenous expression of HBx in podocytes could limit cell proliferative capability probably through the up-regulation of both cyclin B1 and CDK-inhibitor p21 expression and the induction of G<sub>2</sub>/M phase arrest. To our knowledge, this is the first report to demonstrate that HBx may play a role in podocyte injuries in HBV-associated glomerulonephritis.

## Materials and Methods

### Immunohistochemistry assay

Renal tissues were obtained through biopsy on 14 children aged 5–14 years and hospitalized in Pediatric Department of Tongji Hospital (Wuhan, China). The diagnosis of HBV-GN was confirmed by pathological examination. Normal renal tissues obtained from five patients who received operation due to kidney tumor served as the control in this study. The study was approved by our hospital's institutional review board, and all subjects gave prior informed consent.

For immunohistochemical assay, the 4 mm paraffin-embedded sections of renal tissues were deparaffinized in xylene and rehydrated in a descending ethanol series. Endogenous peroxidase was treated with 3% hydrogen peroxide for 20 min. And then the sections were treated with 5% skim milk in phosphate buffered saline (PBS) for 30 min. After that, the sections were incubated with the primary rabbit antibody against HBx (1:100 dilution; BioVendor Laboratory Medicine Inc., Modrice, Czech Republic) overnight at 4°C, then stained with a sensitive streptavidin-peroxidase technique using DAB Detection Kit (Dako, Glostrup, Denmark).

### Cell culture

Studies were performed on the conditionally immortalized mouse podocyte cell line MPC5 (provided by Prof. Peter Mundel, Albert Einstein College of Medicine, New York, USA), which was maintained in type I collagen-coated flasks at 33°C (permissive conditions) in RPMI 1640 media (Gibco, Carlsbad, USA) containing 10% fetal bovine serum (FBS; Gibco), and 10 U/ml interferon- $\gamma$  (PeproTech, Rocky Hill, USA). To evaluate the effect of HBx on podocyte

proliferation, MPC5 cells were divided into blank group, Ad.Null group, and Ad.HBx group according to whether infected with vectors or not and whether the vectors were inserted with HBx-expressing sequence or not.

### Construction of recombinant adenovirus and infection

A type 5 E1–E3-deletion recombinant adenovirus expressing HBx under the control of the cytomegalovirus promoter was constructed as described previously and named Ad.HBx [12]. The presence of Ad.HBx was verified by polymerase chain reaction (PCR) and sequence analysis. The adenoviral vector pAdxsi without HBx insert was served as a control named Ad.Null (Sinogenomax, Beijing, China). Virus stock titers were determined by plaque formation assay using HEK293 cells.

Stock virus preparations were diluted in RPMI 1640 without FBS, then inoculated onto cell monolayer at the indicated multiplicity of infection (MOI). After incubation at 33°C for 2 h, the virus inoculum was removed. RPMI 1640 containing 10% FBS was added to cover the cells and culture at 33°C for the indicated time.

### Morphological analysis of podocytes

At 5 day post-infection, podocytes were stained with Wright-Giemsa staining solution (Sigma-Aldrich, St Louis, USA) and observed under an optical microscope. At least 200 interphase cells were observed to evaluate the accumulation of aberrant nuclear changes in grouped podocyte.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The number of cells present at a given time point was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MPC5 cells were seeded in 96-well plates ( $4 \times 10^3$  cells per well), cultured for 12 h and then infected with the recombinant adenovirus or control. At the indicated time points, MTT assay was performed according to manufacturer's instruction (Promega, Madison, USA). Absorbance (*A*) was measured at a wavelength of 490 nm using a Microplate Reader (BIO-TEK, Winooski, USA).

### 5,6-Carboxyfluorescein diacetate, succinimidyl ester labeling and proliferation analysis

The amine-reactive dye 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, USA) is incorporated into cells and divided equally into daughter cells during proliferation. Thus, cell proliferation can be determined by measuring the mean fluorescence intensity by flow cytometry on a FACScan flow cytometer [13,14]. Briefly, when MPC5 cells reached the confluence as monolayers, cells were synchronized with IFN-free and serum-free medium for 24 h. These cells were harvested and incubated with 10  $\mu$ M CFSE for 15 min at 33°C. After being washed, the labeled cells were divided into two parts. One was plated

onto six-well culture plates, cultured for 12 h and then infected with adenovirus vectors for the indicated time. The other, representing the parent cells, was immediately fixed in cold PBS containing 4% paraformaldehyde, and stored at 4°C. The treated cells were harvested and immediately analyzed by flow cytometry. Data were analyzed using CellQuest and ModFitLT software (Becton Dickinson, Franklin Lakes, USA). Cell proliferation was calculated using the Proliferation Wizard Model. The proliferation index is the sum of the cells in all generations divided by the number of original parent cells.

### Bivariate flow cytometric analysis

Multiparameter flow cytometry (cellular DNA content versus cyclin expression) was performed according to Gong *et al.* [15]. In brief, cells were fixed in 70% ice-cold ethanol, permeabilized with 0.25% Triton X-100 on ice, and incubated with the mouse primary antibody against cyclin B1 (1 : 100; Santa Cruz, Santa Cruz, USA) containing 1% BSA overnight at 4°C. After incubation with FITC-conjugated goat anti-mouse IgG (Santa Cruz), 100 µg/ml of DNase-free RNase (Sigma-Aldrich) and 50 µg/ml of propidium iodide (Sigma-Aldrich), the cells were analyzed by flow cytometry. A control group was specially set with isotype control antibody to confirm the specificity of primary antibody binding.

### Western blot analysis

Podocytes were lysed in sodium dodecyl sulfate (SDS) sample buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM PMSF, and 0.1 mM sodium orthovanadate] at 4°C. Protein concentrations of the resulting lysates were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The protein extracts (50 µg) were electrophoresed on an 8% SDS-polyacrylamide gel at 60 V for 4–5 h and then electroblotted onto a polyvinylidene difluoride membrane, followed by blocking in 5% milk/TBS-T [25 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20] or 3% bovine serum

albumin/TBS-T at room temperature for 1–3 h. The membrane was primarily incubated overnight at 4°C with antibodies against HBx (BioVendor Laboratory Medicine Inc.), p21<sup>WAF1/CIP1</sup> (Santa Cruz), cyclin A (Santa Cruz), and β-actin (Sigma), respectively, and then subject to incubation with peroxidase-conjugated anti-mouse immunoglobulin G (1 : 5000 in 3% bovine serum albumin/TBS-T). The activity of membrane-bound peroxidase was detected by enhanced chemiluminescent detection kit (ECL system; Pierce, Rockford, USA). Thereafter, the densitometric quantitation of protein bands was performed using NIH image software. Protein expression was quantified as the ratio of a specific band to the corresponding β-actin value in the same lane.

### Statistical analysis

Data were presented as mean ± standard deviation (SD) from at least three experiments. One-way analysis of variance was used to evaluate the significance of differences. A value of  $P < 0.05$  was considered statistically significant.

## Results

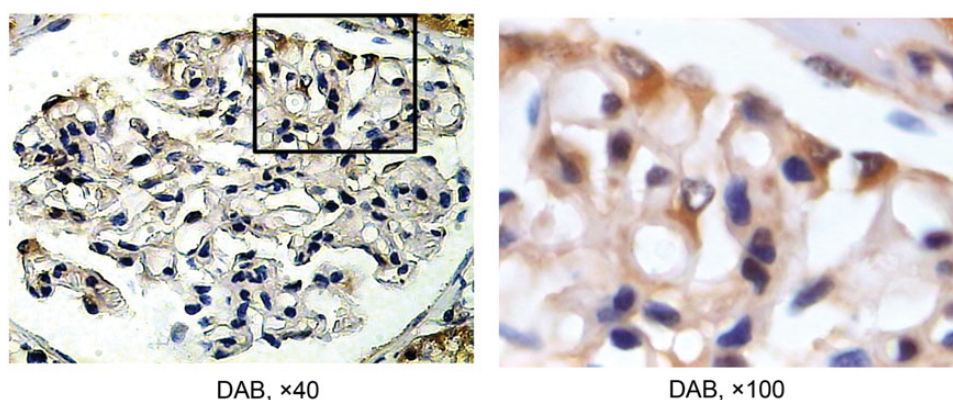
### Visualization of HBx proteins in the glomeruli of children with HBV-GN

HBx protein was visualized within the glomerulus in 10 of 14 (71%) children with HBV-GN by immunohistochemical staining. The expression of HBx protein was localized mainly in the cytoplasm of podocytes, with somewhat weak expression in the nuclei of podocytes (**Fig. 1**).

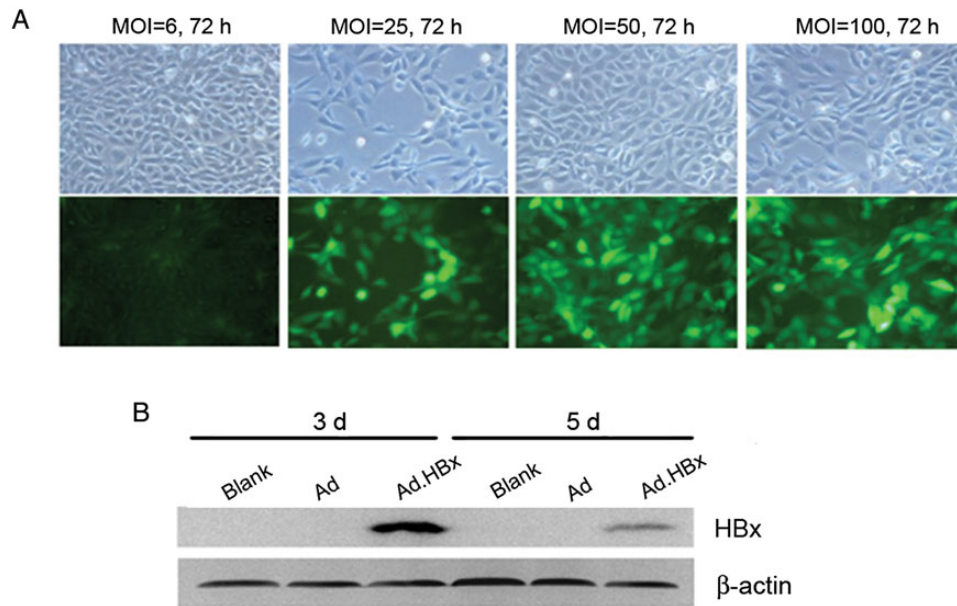
### Construction of recombinant adenovirus and HBx expression in podocytes

The HBx-inserted recombinant adenovirus, named Ad.HBx, was verified by PCR and sequencing (data not shown), purified and titrated to  $2 \times 10^{10}$  plaque formation units (pfu)/ml.

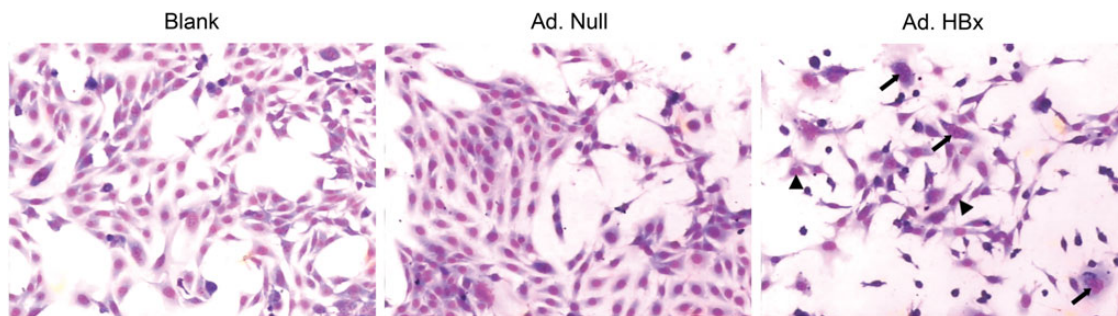
Podocytes were first infected with Ad.GFP at MOIs of 6, 25, 50, and 100 to find an appropriate concentration with efficient expression (**Fig. 2A**). At day 3 post-infection, the green fluorescence was shown in nearly all podocytes



**Figure 1.** HBx expression in the kidney tissue of pediatric patients with HBV-GN



**Figure 2. HBx was expressed in podocytes** (A) Podocyte transfection with Ad.GFP at MOIs of 6, 25, 50, and 100. (B) Detection of the HBx protein in Ad.HBx-transfected podocytes.



**Figure 3. Podocyte morphology** The cellular and nuclear morphology of HBx-transfected podocytes was analyzed by Wright-Giemsa staining. Double nuclei (arrowhead) and multiple nuclei (arrow) were significantly accumulated in podocytes of Ad.HBx group, and a few shrunken bodies were seen.

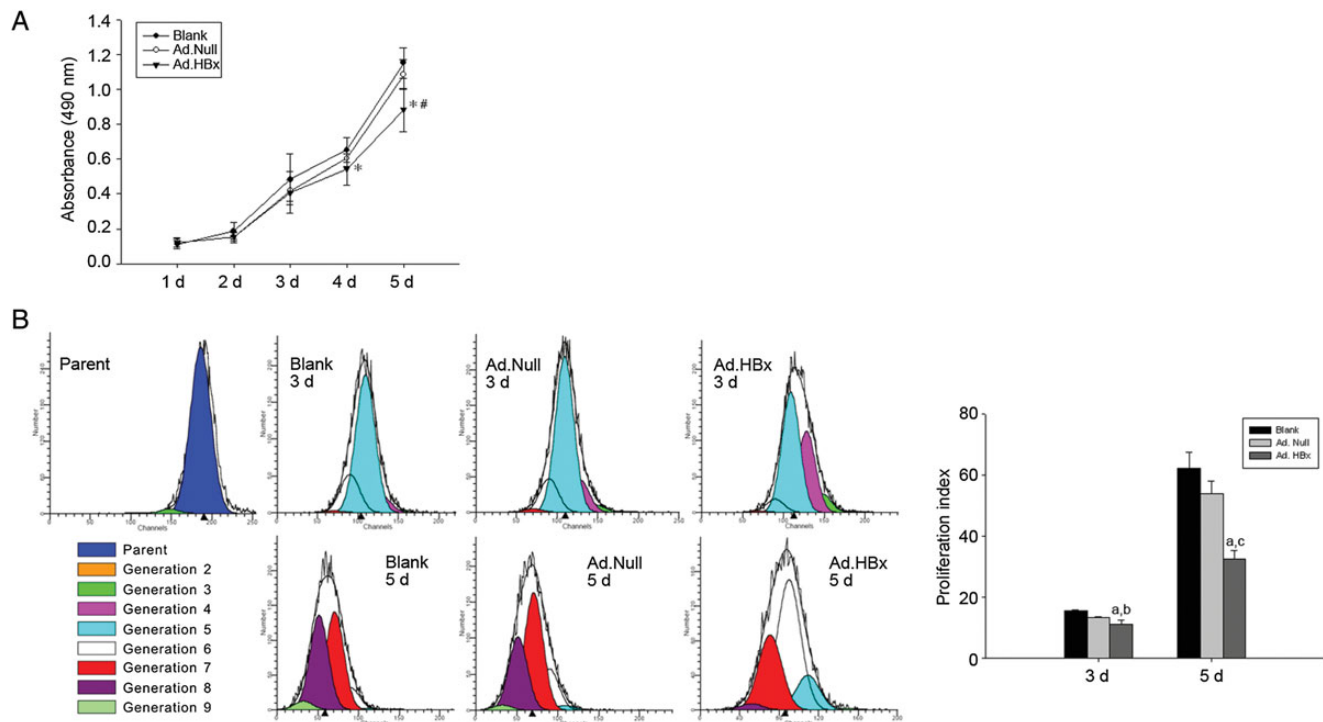
infected with Ad.GFP at MOI of 100, and the podocytes remained in excellent condition. Therefore, MOI of 100 was considered as the optimal concentration and used in all subsequent experiments. As shown in **Fig. 2B**, HBx was detected as a single protein band at 17 kDa in Ad.HBx group.

#### HBx induced aberrant nuclear changes of podocytes

As shown in **Fig. 3**, most of the blank control cells and Ad.Null-infected podocytes remained single nucleus at day 5 post-treatment, while aberrant nuclear changes like double nuclei and multiple nuclei were significantly accumulated in the Ad.HBx-infected podocytes. These results demonstrated that exogenous expression of HBx alone could induce mitotic catastrophe in podocytes. The aberrant nuclear changes in podocytes were lagged behind the expression of HBx which was strong at day 3 post-infection.

#### Exogenous expression of HBx in MPC5 cells induced proliferative incapacity of podocytes

We further detected the proliferative incapacity of podocytes under the different infections by MTT proliferation assay. As expected, the numbers of podocyte in blank and Ad.Null control groups increased similarly with the extension of culture time. In contrast, podocytes in Ad.HBx group grew significantly more slowly than controls at day 4 and even more obvious at day 5 post-infection (**Fig. 4A**). The results of CFSE-based proliferation assay also showed that the predominant podocytes in both blank and Ad.Null control groups at day 3 post-infection were already in the fifth generation, but in the Ad.HBx group, the predominant podocytes were in both the fourth and fifth generations (**Fig. 4B**). At day 5 post-infection, most podocytes were in the seventh and eighth generations in both blank group and Ad.Null group; however, the cells were just in the sixth and seventh



**Figure 4. HBx expression in podocytes inhibited cell proliferation** (A) Podocytes were treated with medium alone or infected with either Ad.Null or Ad.HBx, and cell viability was determined by MTT assay at the indicated time points from three independent experiments. \*  $P < 0.01$  vs. control cells; #  $P < 0.01$  vs. Ad.Null-infected cells. (B) Proliferation profiles of CFSE-labeled podocytes. The proliferation index is the sum of the cells in all generations divided by the number of original parent cells present at the start of the experiment. a,  $P < 0.01$  vs. control cells; b,  $P < 0.05$  vs. Ad.Null group; c,  $P < 0.01$  vs. Ad.Null group.

**Table 1. The percentage of cells in different cell cycle phases**

Cell cycle phases	3 days			5 days		
	Blank	Ad.Null	Ad.HBx	Blank	Ad.Null	Ad.HBx
G <sub>0</sub> /G <sub>1</sub>	64.5% ± 4.0%	63.5% ± 1.1%	53.2% ± 6.0%** <sup>#</sup>	61.2% ± 4.0%	64.4% ± 7.3%	47.9% ± 3.4%* <sup>##</sup>
S	19.2% ± 1.8%	21.4% ± 1.7%	25.3% ± 3.8%*	26.8% ± 1.8%	25.6% ± 3.8%	30.6% ± 2.1%
G <sub>2</sub> /M	16.3% ± 2.5%	15.1% ± 0.9%	21.5% ± 2.5%* <sup>##</sup>	12.0% ± 2.1%	10.0% ± 3.5%	21.5% ± 1.3%** <sup>##</sup>

\*\*<sup>##</sup>Data from three independent experiments were expressed as mean ± SD. \* $P < 0.05$  vs. control cells; \*\* $P < 0.01$  vs. control cells; # $P < 0.05$  vs. Ad.Null-transfected cells; ## $P < 0.01$  vs. Ad.Null-transfected cells.

generations in Ad.HBx group. The proliferation indexes were 15.4, 13.3, 11.2 at day 3, and were 61.7, 54.0, 32.5 at day 5 for the blank group, Ad.Null group, and Ad.HBx group, respectively. The delayed podocyte division and lower proliferation index after Ad.HBx transfection demonstrate that HBx has an inhibitory effect on podocyte proliferation.

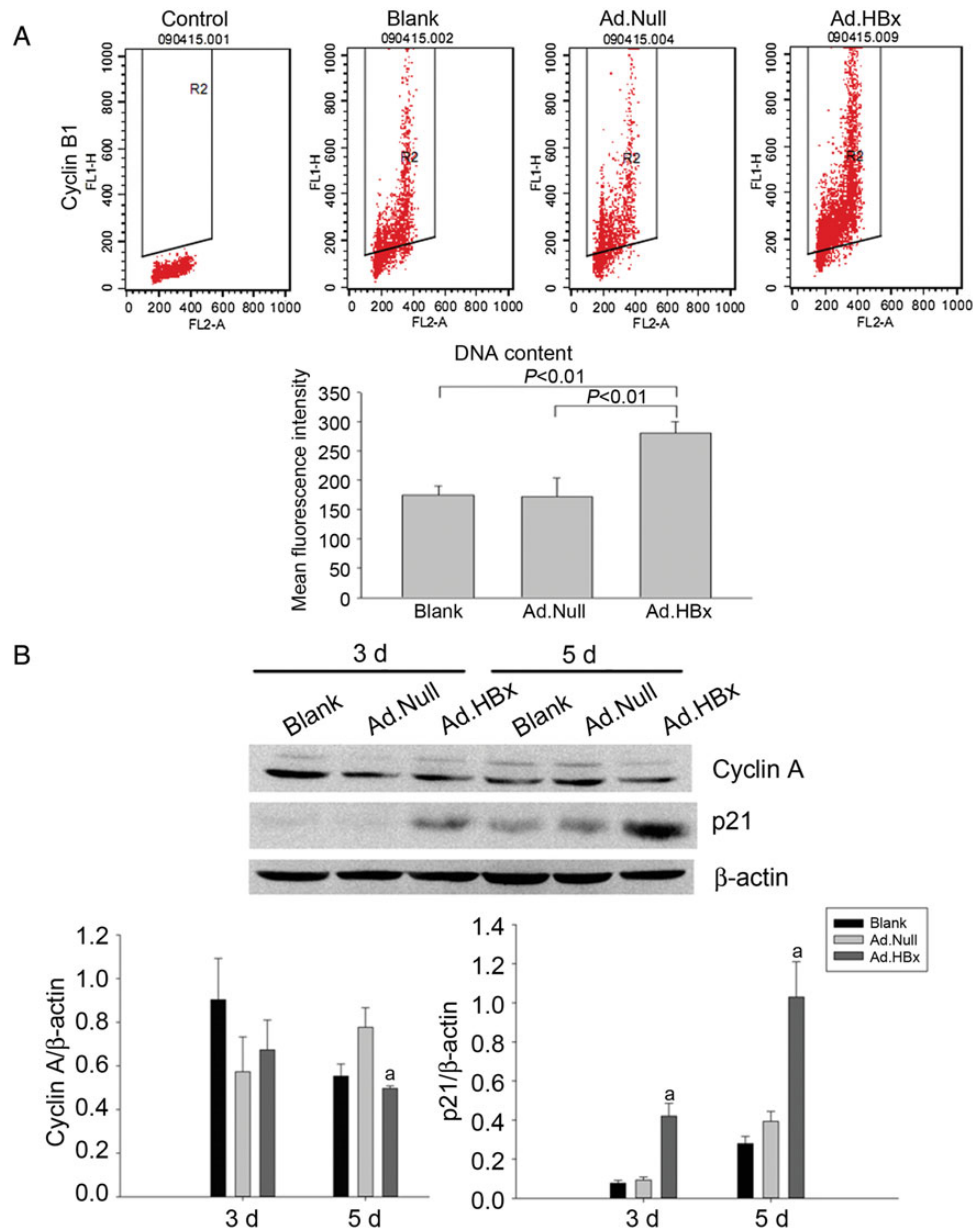
### Exogenous expression of HBx-induced G<sub>2</sub>/M cell cycle arrest in podocytes

As shown in **Table 1**, the percentage of cells in the G<sub>2</sub>/M phase was significantly increased in Ad.HBx group than that in the both controls at days 3 and 5 post-treatment, while the percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phase was significantly decreased in Ad.HBx group than that in both controls at

days 3 and 5 post-treatment. These results suggest cell cycle arrest at the G<sub>2</sub>/M phase.

### Exogenous expression of HBx in podocytes increased both cyclin B1 and p21 expression, but decreased cyclin A expression

The expression of cyclin B1 was significantly up-regulated in Ad.HBx group than that in control groups at day 5 post-infection (**Fig. 5A**). A more dramatic increase of CDK-inhibitor p21 expression was found in Ad.HBx group at day 5 post-infection (**Fig. 5B**). But the changes in cyclin A expression were not significantly different between the Ad.HBx group and controls at day 3 post-infection, only a



**Figure 5. Changes of cyclin B, cyclin A, and p21 levels following exogenous HBx expression** (A) Effect of HBx on cyclin B1 expression. The cells were subjected to flow cytometry for analysis of the DNA content vs. cyclin B1 level. Representative results of three independent experiments are shown. (B) Effect of HBx on cyclin A expression and p21 expression. a,  $P < 0.01$  vs. Ad.Null group.

slight decrease was found in Ad.HBx group at day 5 post-infection compared with that in the controls (Fig. 5B).

## Discussion

HBx has been shown to be associated with either proliferation or apoptosis in hepatoma cells and hepatocytes. Thus, it could be involved in either the proliferation or apoptosis of HBV-infected podocytes in the similar ways. In this study, we observed the effects of HBx alone on podocyte cell survival, and found that at day 5 post-infection, HBx-expressing podocytes exhibited the characteristics of mitotic suspension. To further confirm the inhibition of podocyte proliferation by

HBx, we measured cell proliferation using both the MTT assay and CFSE-based proliferation assay. MTT assay showed that Ad.HBx-infected podocytes grew much more slowly than controls at day 4 post-infection and thereafter. Furthermore, CFSE-based proliferation assay also showed that the proliferation of HBx-expressing podocytes was significantly inhibited when compared with that of controls at 3 day post-infection, and that the difference became much more obvious at day 5 post-infection. Taken together, these results showed that exogenous expression of HBx alone could significantly decrease podocyte proliferative capability.

In response to injury, podocytes can undergo several kinds of cell fates, including detachment, de-differentiation,

hypertrophy, proliferation, incapacity of proliferation, apoptosis, or even necrosis. Podocytes are terminally differentiated quiescent cells. The number of podocyte in glomeruli is delicately maintained and ultimately determined by cell cycle regulation [16]. Incapacity of proliferation can be a consequence of either limited DNA synthesis secondary to G<sub>1</sub>/S checkpoint arrest, or mitotic restriction secondary to G<sub>2</sub>/M checkpoint arrest [16]. This study clearly showed that exogenous expression of HBx resulted in cell cycle arrest of podocyte at G<sub>2</sub>/M phase. This finding, together with the abnormal morphologic changes in nuclei seen in HBx-expressing podocytes provided strong evidence that HBx may limit podocyte proliferation through inducing G<sub>2</sub>/M phase arrest.

It is well known that the main phase-specific cell cycle regulatory proteins involved in the regulation of G<sub>2</sub>/M transition are cyclin A, cyclin B, cdc2, and the CDK inhibitor p21 [17]. The amount of cyclin B1, a mitotic cyclin, gradually rises through the cell cycle until mitosis, where it falls abruptly due to degradation. If cyclin B1 is not degraded in a timely manner, the cell will remain frozen in mid-metaphase [18]. In this study, we found that concomitant with the HBx-induced G<sub>2</sub>/M phase arrest, the cyclin B1 level was significantly increased at day 5 post-infection in HBx-expressing podocytes as compared with control podocytes. Thus, we believe that the persistently high level of cyclin B1 may be involved in the HBx-induced G<sub>2</sub>/M phase arrest. Cyclin A participates in the regulation of both S phase and G<sub>2</sub>/M phase progression by interaction with distinct kinases. The decrease in cyclin A level can result in G<sub>2</sub>/M phase arrest [19,20] or premature entry into mitosis [21]. Our results also showed that along with the HBx-induced G<sub>2</sub>/M phase arrest, the levels of cyclin A were lower, although slightly, in HBx-expressing podocytes than in control podocytes, at day 5 post-infection. Furthermore, it is known that ubiquitin-dependent proteolysis makes a major contribution to the degradation of p27 as well as p21, and it has been reported that cyclin A-cdk2 plays an ancillary noncatalytic role in the ubiquitination of p27, by way of the SCFskp2 complex [22]. Thus, we speculate that HBx-induced downregulation of cyclin A may contribute to a steady expression of p21. The CDK inhibitor p21, a 21 kDa protein, is a member of the Cip/Kip family. In our conditionally immortalized transgenic mouse podocytes, the expression of p21 is undetectable under normal growth-permissive conditions, and increases gradually under non-permissive conditions, until it is maximal in fully differentiated podocytes [23]. In this study, we found that the level of p21 protein was significantly up-regulated in the HBx-expressing podocytes. At day 3 post-infection, p21 was just slightly detected in the Ad.Null and blank control podocytes, but was highly expressed in HBx-expressing podocytes. Moreover, the expression of p21 remained significantly high at day 5 post-infection. Likewise, several other reports have shown that in podocytes, the up-regulation of p21 expression is

closely related to G<sub>2</sub>/M phase arrest and the subsequent inhibition of proliferation [24,25].

In summary, our results showed that exogenous expression of HBx in podocytes could significantly limit the proliferation of podocyte through induction of cell cycle arrest at the G<sub>2</sub>/M phase. This inhibitory effect was associated with an increased expression of both cyclin B1 and CDK-inhibitor p21, and a decreased expression of cyclin A. All the results indicate that HBx may play a role in podocyte injuries in HBV-associated glomerulonephritis. This study may be the first step in revealing a new pathogenesis of HBV-GN.

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