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RESEARCH REPORT

Construction of a *Bombyx mori* cell line that stably express the susceptible gene +$^{nsd-2}$ of *Bombyx mori* bidensovirus

Y Zhang, P Lü, Q Yu, R Li, D Miao, Z Hu, Y Yang, L Chen, K Chen*, Q Yao*

Institute of Life Sciences, Jiangsu University, Zhenjiang, China

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Abstract

Bombyx mori bidensovirus (BmBDV) is the only type species established in the new genus *Bidensovirus* within the new family *Bidnaviridae*. The BmBDV infected silkworm midgut shows symptoms such as the nucleus of midgut columnar cells expanded. It is confirmed the BmBDV-sensitive gene +$^{nsd-2}$, which encoding an amino acid transporter protein might be the receptor of BmBDV. The deletion of this gene causes the silkworm resistance to BmBDV infection. There is no permissive cell line for BmBDV infection has been established yet. The cell lines (BmN, etc.) are used broadly for silkworm viruses study are mainly resistant to BmBDV infection. Therefore, the establishment of BmBDV permissive cell line is particularly important for studying BmBDV in vitro. In this study, we successfully constructed a cell line BmN (+$^{nsd-2}$), which expressed +$^{nsd-2}$ gene stably. The NSD-2 expression and localization could be detected in BmN (+$^{nsd-2}$) cells via RT-PCR, western blot and immunofluorescence. The interaction between NSD-2 and the major structural protein VP of BmBDV was determined by Co-IP. However, we failed to detect the replication of BmBDV. This cell lines constructed has the potential for identifying the protein binding to the NSD-2 and provide a basis for study of BmBDV co-receptors and invasion mechanism.

Key Words: silkworm; bidensovirus; sensitive gene; cell lines; NSD-2

Introduction

Bombyx mori bidensovirus (BmBDV) is the only virus type species of the *Bidnaviridae* family established by the International Committee on Taxonomy of Viruses (ICTV) (Adams *et al*., 2012). BmBDV is a pathogen that can infect columnar cells of the silkworm midgut and cause the densonucleosis of *B. mori* larvae. BmBDV contains two single linear DNA (VD1 and VD2), which are spherical virus particles with non-envelope and a diameter of about 22-24 nm (Wang *et al*., 2007; Zhang *et al*., 2016). VD1-ORF3 encodes the major structural protein VP with β barrel structure similar to parvovirus and glycine rich sequence at the N-terminal region (Krupovic *et al*., 2014). The proteins are produced through a leaky scanning mechanism (Pan *et al*., 2014; Lü *et al*., 2017). VD2-ORF2 encodes a minor structural protein P133 with 1161 amino acids and a molecular weight of133 kDa, which is homologous to the C-terminus of the capsid protein of reovirus. P133 homologs are usually found in the outer coat of the capsid, which are closely related to host recognition and viral entry (Kong *et al*., 2011; Lv *et al*., 2011). The resistance of *B. mori* against the like-parvovirus strain BmDNV-2 is controlled by a recessive gene *nsd-2* (Abe *et al*., 2000; Murthy *et al*., 2014). The *nsd-2* linkage map was obtained by restriction fragment length polymorphism linkage method, and finally the non-sensitive gene *nsd-2* was determined to be an amino acid transporter (Ogoyi *et al*., 2003; Ito *et al*., 2008), which is expressed only in the midgut and encodes transmembrane proteins with 12-transmembrane domains, and lack of the 9-transmembrane domains of the C-terminal of the amino acid transporter, will cause the silkworm to be non-sensitive to BmBDV. Our group analyzed the transcription of +$^{nsd-2}$ gene in different developmental stages of silkworm and sensitivity in different tissues by RT-PCR, the results showed that the +$^{nsd-2}$ gene is only expressed in midgut of 1th-5th instar larvae *nsd-2*' and +$^{nsd-2}$' genes of BmBDV Zhenjiang strain (China) were cloned and sequenced. The results showed that the nucleotide sequences of *nsd-2* 'and +$^{nsd-2}$' genes were identical with *nsd-2* and +$^{nsd-2}$, suggesting that the silkworm had the same resistance mechanism to two BmBDV strains infection (Li *et al*., 2010; Qin *et al*., 1996; Ponnuvel

Corresponding authors:
Qin Yao and Keping Chen
Institute of Life Sciences
Jiangsu University
301 Xuefu Road, Zhenjiang 212013, PR China
E-mail: yaoqin@ujs.edu.cn, kpchen@ujs.edu.cn

et al., 2010; Eguchi *et al*., 2010). Ito K *et al* detected the expression level of $+^{nsd-2}$ and transcription of BmBDV from the anterior, middle, and posterior parts of the midgut of the sensitive silkworm by qRT-PCR, the results showed BmBDV-derived transcripts were clearly detected in all parts of the midgut and $+^{nsd-2}$ strongly expressed in the posterior part of the midgut. These results suggest that the infectivity of BmBDV depends mainly on the expression level of $+^{nsd-2}$ in the midgut and the viral infection is supported even by very faint expression of $+^{nsd-2}$ (Ito *et al*., 2016). However, the mechanism of $+^{nsd-2}$ mediates the entry of BmBDV into the cell is still unknown.

As a model insect, *B. mori* cell lines plays an important role in the study of immunology, insect pathology and insect virology (Taniai *et al*., 2006; Pan *et al*., 2010). Up to now, more than 30 silkworm cell lines have been established all over the world (Iwanaga *et al*., 2009). However, there is no report about the sensitive cell lines to BmBDV (Hu *et al*., 2010; Van *et al*., 2011; Zheng *et al*., 2014; Feng *et al*., 2017; Ming *et al*., 2017; Qi *et al*., 2017). At present, the widely used silkworm cell lines, *B. mori* embryo cell lines (BmE) and *B. mori* ovary cell lines (BmN) are non-sensitive to BmBDV. As we know, it is hard to promote the virus study without the proper sensitive cell lines, so we tried to clone and express the $+^{nsd-2}$ gene in BmN cells, and established a BmN ($+^{nsd-2}$) cell line that can stably express NSD-2 protein. We hope that this cell lines can be used for subsequent identification of receptor binding proteins and the mechanism of BmBDV invasion into the host cells.

Materials and Methods

Plasmid construction

According to the full lengh of gene sequence of National Center for Biotechnology Information Search database (NCBI), the specific primers of $+^{nsd-2}$ gene (NCBI Reference Sequence: NM_001130871.1) were designed, NSD-F:5 '-CGGGATCCATGGATTCAAATGGGATAAAT-3' (Underline means *Bam*H Ⅰ restriction endonuclease sites); NSD-R:5'-CCCTCGAGCCGCTTCCTGCGATACC-3' (Underline means *Xho* Ⅰ restriction endonuclease sites). PCR was amplified by high fidelity enzyme Pfu DNA polymerase (Takara, Dalian, China) with cDNA extracted from the sensitive silkworm strain 306 midgut. The PCR program consisted of 5 min pre-denaturation at 94 °C, 35 cycles (94 °C, 30 s; 56 °C, 30 s; 72 °C, 2'30s), and 10 min at 72 °C. PCR products are purified by Omega Gel Extraction kit (Omega Bio-tek), and the digested fragment was subcloned into the vector pIZ-V5/His also digested by *Bam*H I and *Xho* I (Invitrogen, Shanghai, China). The recombinant plasmid pIZ- $+^{nsd-2}$ was confirmed by sequencing.

Cell transfection and cell culturing

Plasmid Extraction Kit (Omega Bio) was used to extract plasmid pIZ- $+^{nsd-2}$ and pIB-GFP (laboratory constructed) was used for subsequent transfection.

BmN cells were cultured with TC-100 medium supplemented with 10% fetal bovine serum (FBS, Gibco, in Grand Island, NY, USA) and 1% antibiotics (penicillin-streptomycin; HyClone Gibco-BRL (Life) Technologies, Carlsbad, CA, USA) in T-25 flask. According to the manufacturer's instructions, the transfection was carried out with Cellfectin II (Invitrogen, Carlsbad, CA, USA). 2 μg pIZ- $+^{nsd-2}$ plasmid was mixed with 100 μL serum free TC-100 medium for 30 minutes; 5 μL Cellfectin II Resgent liposome reagent was added and mixed with 100 μL serum free TC-100 medium for 30 minutes. Finally, the DNA/Cellfectin II Resgent liposome reagent was mixed and incubated for 15 minutes at room temperature. At the same time, BmN cells were washed three times with serum-free TC-100 medium. Then, the DNA/Cellfectin II Resgent liposome reagent mixture was added to the culture flask in a 27 °C incubator. After 6 h, the DNA/Cellfectin II Resgent liposome reagent mixture was removed and 2 ml fresh TC-100 complete medium (containing 1% chloramphenicol and streptomycin +10% fetal bovine serum) was added. 48 h post-transfection, Zeocin (Invitrogen, Carlsbad, CA, USA) antibiotics with final concentration of 200 μg/ mL were added to screen stable cell lines. A new culture medium containing 200 μg/ mL Zeocin antibiotics was replaced every 3 days. The morphological characteristics of the cells were observed and recorded. The passage was cryopreserved after 30 passages.

PCR assay

RNA of BmN ($+^{nsd-2}$) cells, BmN cells and the midgut from the susceptible silkworm (Jing Song) were extracted by TRIzol reagent (Invitrogen); Total RNA (500 ng/μL) by RNase-free DNase (Vazyme Biotech, Nanjing, China) to remove DNA pollution, cDNA Synthesis Kit according to instructions (Vazyme Biotech) synthesis cDNA. 100-fold dilution of cDNA was used as template for PCR amplification. PCR amplification procedure and primers are as same as the section of plasmid construction.

Table 1 Primers for qRT-PCR

Gene	Sense primer 5'-3'	Anti-sense primer 5'-3'	Product size (bp)	Genbank Accession Number
Bm-*rpl3*	CAAAGTGAAATGGGCCAGAG	AGCACGAGCTACAGTGAACGA	235	NM-001126254.1
BmBDV-*ns1*	GTTGGTGGTGAAGGGTTTG	GGGAGATAGTTTACACTTTGGAG	198	DQ017268.1
BmBDV-*vp*	AGAAATGCTGGAGCCGAT	CCTCCACTGCCTGTAACTTC	209	DQ017268.1

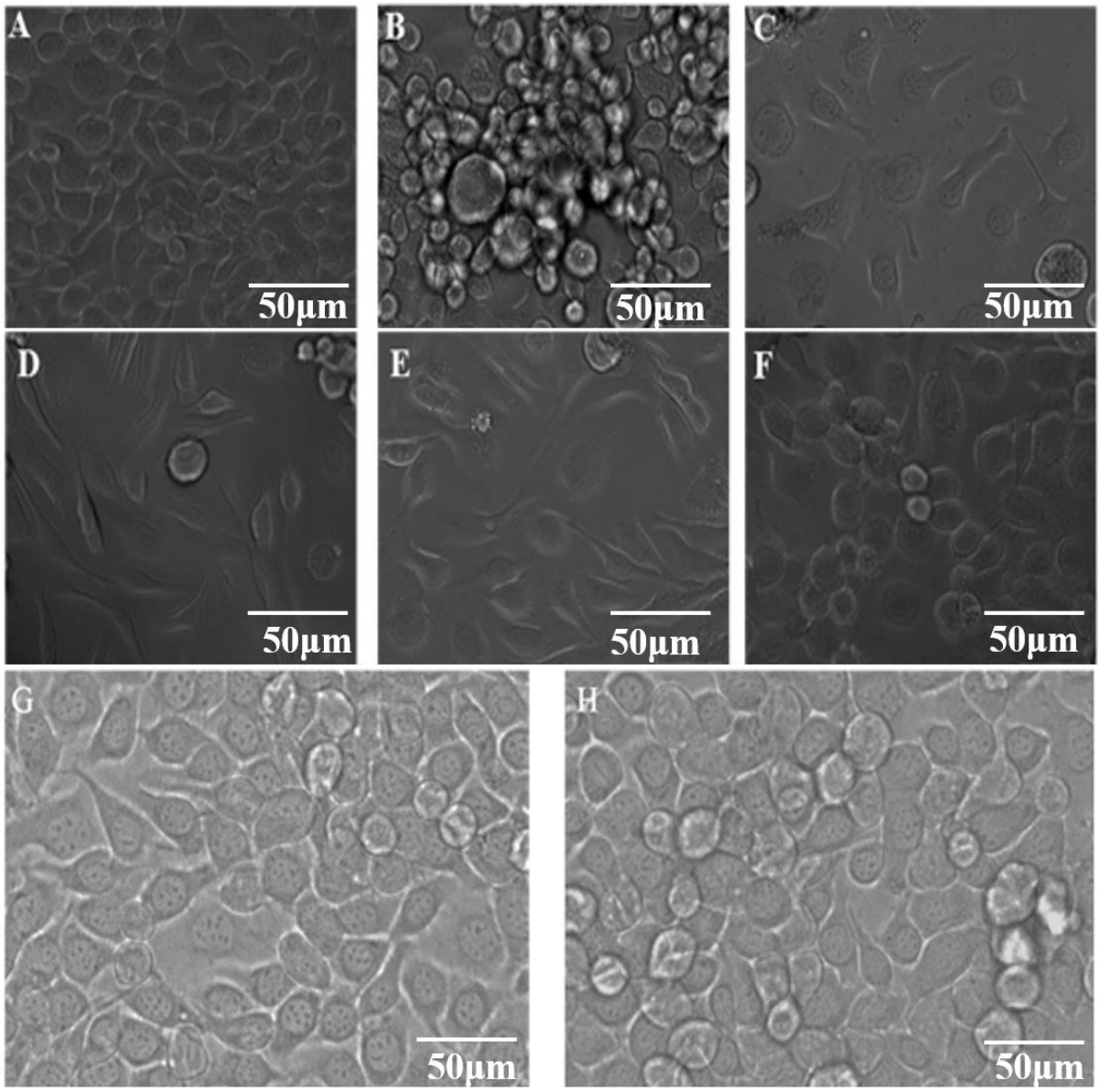

Fig. 1 BmN (+$^{nsd-2}$) cells morphological character of the zeocin selection. (A) BmN cells. (B) Zeocin selected cells, the transfected BmN cells started agglomerate from 4th days and (C) formed a few cell colones within 7-13 days. (D) The size of the cell colones appeared to increase over the following 5-8 days. (E) The number of the cell clones increased on 24 days. (F) The cells had nearly grown to confluent cell bottle after approximately 41 days in culture. (G) The morphology of BmN cells. (H) The morphology of BmN (+$^{nsd-2}$) cells after 30 passages

Western blot

BmN (+$^{nsd-2}$) cells were lysated by weak RIPA lysis buffer (Beyotime Biotechnology) including 50mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate and 1% PMSF (Cell Signaling Technology, USA). The lysates were collected and incubated on ice for 10 min. Lysates were cleared by centrifugation at 12,000 g for 5 min at 4 °C. The Total protein was separated by 12% SDS-PAGE and transferred onto 0.2 μm PVDF membranes (GE Healthcare Life Sciences, Germany). Membranes were blocked with 5% (w/v) non-fat milk (Sangon Biotech) for 4 h at 37 °C, and then incubated overnight at 4 °C with a specific polyclonal antibody anti-V5 (1:2000; Genscript, NJ). After three rinses with TBST, the membranes were incubated at room temperature for 2 h with the Goat anti-rabbit (1:3000) IgG-HRP (BBI Life Sciences) secondary antibody diluted in TBST. Membranes were washed three times with TBST, the results were detected by ECL Kit (Wanleibio).

Immunofluorescence assay

BmN (+$^{nsd-2}$) and BmN cells (5×10^5 cells/ml) were seeded into 35 mm glass bottom dish. Cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 min, washed with PBS again; 0.5% Triton X-100/PBS permeabilization for

10 min, PBS washed 3 times; 3% PBS/BSA was used to blocked at 37 °C for 30 min; V5-tag (Genscript, NJ) polyclonal antibody (1: 1000) as the first antibody, secondary antibody with CyTM5-labeled Antibody to Rabbit IgG (H+L) (KPL, USA). After three washes with PBS, the cells were observed under in Leica TCS SP5 confocal laser microscopy.

Co-immunoprecipitation (Co-IP)

BmN (+$^{nsd-2}$) cells were grown to approximately 80-90 % confluence in T-25 flasks (2), and pIB-VP recombinant plasmid (laboratory construction) was transfected into BmN (+$^{nsd-2}$) cells. 48 h post-transfection, the cells were washed twice with PBS and harvested by scraping. Cells were pelleted by centrifugation at 1000 g for 10 min at 4 °C and resuspended in RIPA lysis buffer. The sample were pre-cleared by centrifugation and then the immunoprecipitation antibody was added and the samples rocked at 4 °C for a minimum of 1 h. Protein A/G PLUS-Agarose (Bioworld Technology) was added to the samples and rocked overnight at 4°C. Immune complexes were washed three times with PBS; 5× sample buffer was added to the beads and the immunoprecipitated material was separated by 12% SDS-PAGE, transferred onto PVDF membranes and analyzed by western blotting using the anti-vp and anti-v5 antibodies.

Virus infection experiments

5 x 10^5 BmN (+$^{nsd-2}$) cells were seeded into 6-well plates and each well was added with BmBDV virus suspension (20 mg/mL) 100 μL, respectively. Cells were harvested at 0 h, 2 h, 4 h, 8 h, 12 h and 24 h post-infection. Each total RNA was extracted using TRIzol reagent (Invitrogen). 20-fold dilution cDNA was used as template for qRT-PCR (RPL3 as a reference gene). The transcription of BmBDV genes *vp* and *ns1* were detected. The list of primers is as follows (Table 1).

Results

Construction of NSD-2 stable expressing cell line

NSD-2 protein encoded by sensitive gene +$^{nsd-2}$ is one of the important factors of virus infect cells. We transfected the recombinant plasmid pIZ-+$^{nsd-2}$ into BmN cells and selected with 200 μg/ mL Zeocin antibiotics. Finally, we obtained cell lines with stable expression of +$^{nsd-2}$ gene and named the BmN (+$^{nsd-2}$) cells after 30 times. In our culture conditions, the cells began to bunch up after 4 days (Fig. 1B) with the normal morphology of BmN cells as control group (Fig. 1A), and formed clonal cells in 7-13 days (Fig. 1C); clonal cells began to grow in the following 5-20 days (Fig. 1D, E). The cells overspreaded the T-25 flask (Fig. 1F) at about 41 days after the following culture. When the cell growth gradually stabilized, the cells adapted quickly to the new medium with 200 μg/ mL Zeocin antibiotics, exhibited a good growth condition and could be stably passaged. From the final cellular morphology after 30 passages, there is no significant difference between the BmN cells (Fig. 1G) and the BmN (+$^{nsd-2}$) cells morphology (Fig. 1H).

The transcription of +$^{nsd-2}$ gene in BmN (+$^{nsd-2}$) cells

The total RNA of BmN (+$^{nsd-2}$) and BmN cells were extracted and reverse transcripted to cDNA as the template of PCR amplification. Theoretically, the target size of sensitive and non-sensitive genes is 1878 bp and 591 bp, respectively. In fact, NSD-F and NSD-R primers used in the process of plasmid constructing for PCR amplification and the size of the amplified products from BmN (+$^{nsd-2}$) cells is near 2000 bp (Fig. 2A), and the size of the amplified products from BmN cells and BmBDV susceptive strains of silkworm (Jingsong) midgut is near 600 bp and 2000 bp, respectively (Fig. 2B), consistent with the the theoretical size. These experimental results indicate that the +$^{nsd-2}$ gene fragment could stably express in BmN (+$^{nsd-2}$) cells after the 30 passages.

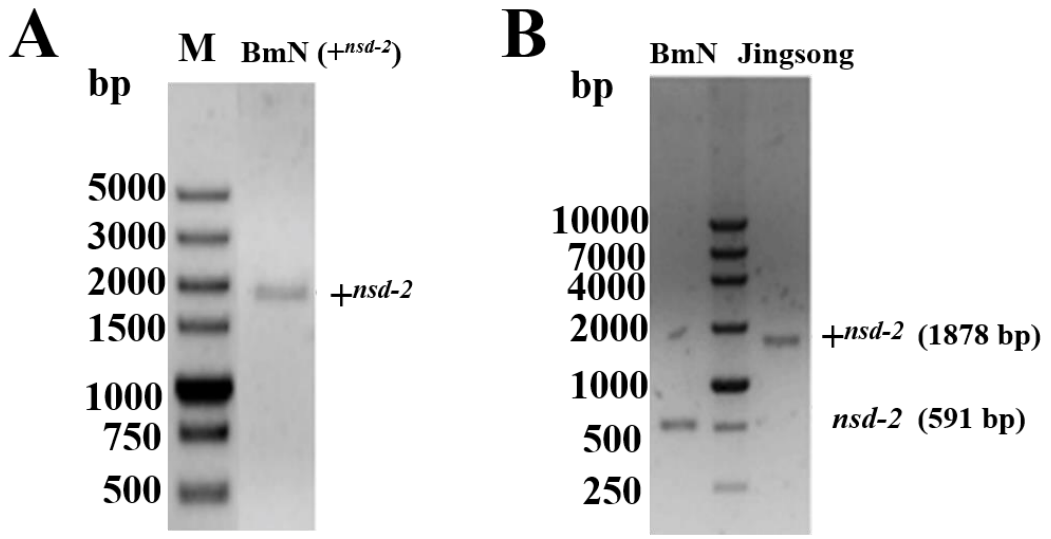

Fig. 2 PCR amplification of the + $^{nsd-2}$ gene in BmN (+ $^{nsd-2}$) cells and Jingsong strain of silkworm midgut, BmN cells extracted total RNA. (A) M, DNA marker. BmN (+$^{nsd-2}$): BmN (+$^{nsd-2}$) cells well collected and extracted total RNA, and amplification of +$^{nsd-2}$ gene by RT-PCR; (B) BmN: The amplification of cDNA reverse transcription from BmN cells RNA; Jingsong: The amplification of cDNA reverse transcription from Jingsong midgut RNA

Western blot analysis of NSD-2 protein expression in BmN (+$^{nsd-2}$) cells

After 30 passages of BmN (+$^{nsd-2}$) cells, the expression of NSD-2 protein was detected by western blot. After lysis, BmN (+$^{nsd-2}$) cells and BmN cells were analyzed by western blot with V5 polyclonal antibody as the first antibody, and the theoretical size of protein was about 68 kDa. In this experiment, a specific protein of about 68 kDa was detected in BmN (+$^{nsd-2}$) cells while it wasn't found in BmN cells (Fig. 3). These results demonstrate that NSD-2 protein could stably express in the passages of BmN (+$^{nsd-2}$) cells.

Immunofluorescence analysis of NSD-2 expression in BmN ($^{nsd-2}$) cells

NSD-2 is a transmembrane protein. Therefore, we want to ascertain wether it is specific located on BmN (+$^{nsd-2}$) cell lines membranes. Treated BmN (+$^{nsd-2}$) cells with V5-tag polyclonal antibody (1: 1000) as the first antibody, the Cy^{TM}5-labeled antibody to rabbit IgG (H+L) was used as the secondary antibody, and the BmN cells were used as negative controls. Red fluorescence (Fig. 4A) was detected in BmN (+$^{nsd-2}$) cells while no red fluorescence (Fig. 4B) was observed in the cell membrane of BmN cells. The results show that NSD-2 protein was mainly expressed in the cell membrane of BmN (+$^{nsd-2}$) cells.

Interaction between VP protein and NSD-2 protein in BmN (+$^{nsd-2}$) cells

VP protein is the main structural protein of BmBDV, and NSD-2 protein is a viral receptor candidate. However, the interaction between VP protein and NSD-2 protein is still unknown. The BmN (+$^{nsd-2}$) cells transfected with pIB-VP recombinant plasmid (laboratory construction) were collected for

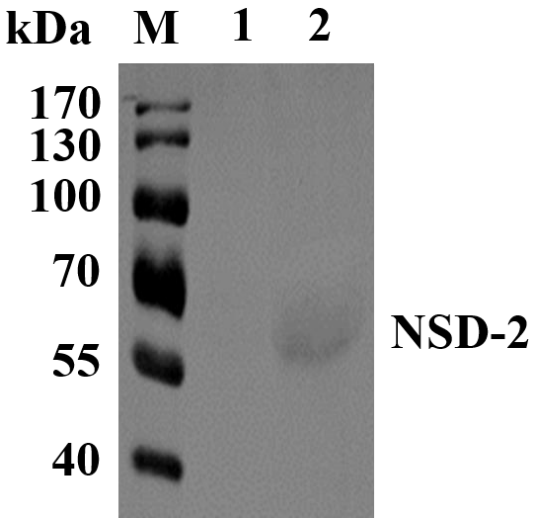

Fig. 3 Expression analysis of NSD-2 by Western blotting in BmN (+$^{nsd-2}$) cells and BmN cells. M, DNA marker. (1) BmN Cells; (2) BmN (+$^{nsd-2}$) cells well collected and lysised，and subjectedc to Western blot analysis with anti-V5 antibody

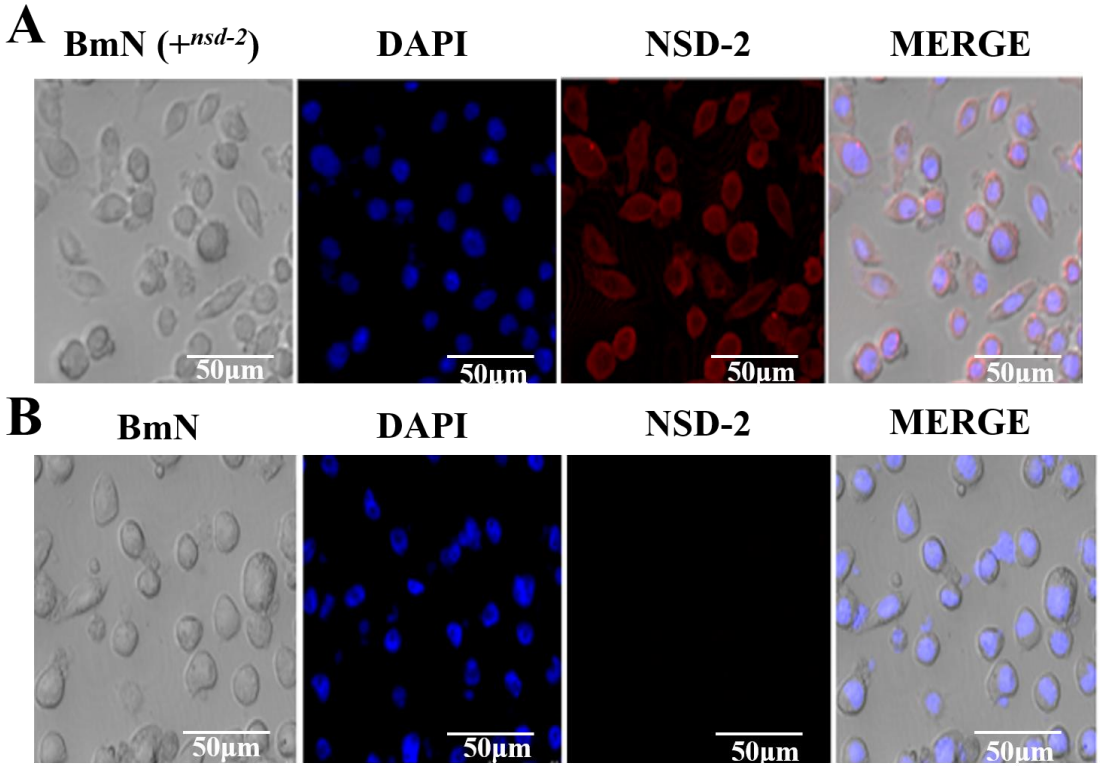

Fig. 4 Detection of NSD-2 protein in BmN (+$^{nsd-2}$) cells by Immunofluorescence assay. (A) Red fluorescence was observed in BmN (+$^{nsd-2}$) cells. (B) Red fluorescence wasn't observed in BmN cells, which as a negative controls. DAPI staining shows unclear DNA (blue).

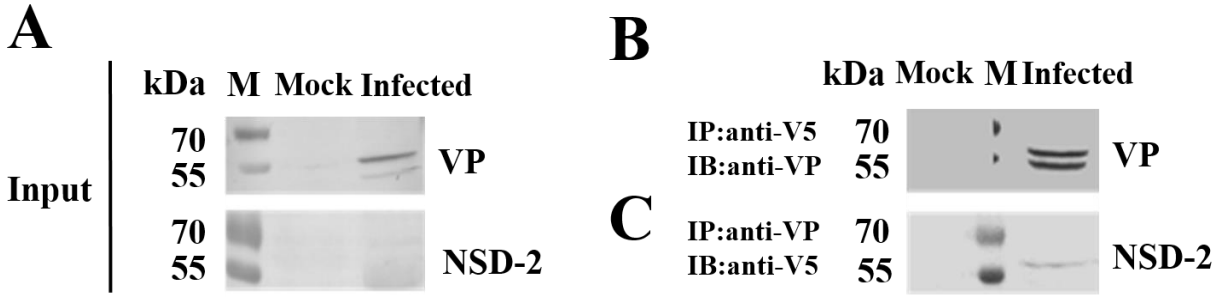

Fig. 5 In vitro interaction between VP and NSD-2. (A) The presence of viral structural protein VP and host protein NSD-2 in lysate of infected BmN (+$^{nsd-2}$) cells by IB assay. (B) Co-immunoprecipitation of VP with NSD-2 by anti-V5. (C) Co-immunoprecipitation of NSD-2 with VP by anti-VP

the Co-IP experiments. VP protein and NSD-2 protein were detected in BmN (+$^{nsd-2}$) cells (Fig. 5A), the use of anti-V5 polyclonal antibody immunoprecipitation pulled down VP (Fig. 5 B) and using anti-VP monoclonal antibody (laboratory preparation) immunoprecipitation pull down NSD-2 (Fig. 5 C); while the corresponding protein band were not detected in the control group.

Virus infection in BmN (+$^{nsd-2}$) cells

NSD-2 protein plays an important role in BmBDV infection, whether BmN (+$^{nsd-2}$) cells could mediate BmBDV into the cell is still unclear. qRT-PCR could quantitatively detect BmBDV genes *vp* and *ns1* in BmN(+$^{nsd-2}$) cells infected by BmBDV. In addition, we make the *Bombyx mori* gene *rpl3* as the internal reference standard. Cultured BmN(+$^{nsd-2}$) cells with BmBDV for 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, the RNA were extracted and reverse transcribed into cDNA (RPL3 as the control of qRT-PCR). The theory sizes of *vp* and *ns1* genes were 209 bp and 198 bp, respectively. As a result, the corresponding segments (Fig. 6 A, B) were not detected in BmN (+$^{nsd-2}$) cells. The results showed that BmBDV could not infect BmN (+$^{nsd-2}$) cells only in the presence of NSD-2, indicates that there may exist co-receptors to mediate virus infection.

Discussion

As an important economic insect and a model insect of Lepidoptera, the silkworm has made important contributions to the development of society and the progress of science (Mita *et al*., 2008). BmBDV is one of the most dangerous viruses, which causes decrease of cocoon yield in summer and autumn in China. Therefore, the research of virus invasion mechanism has a positive meaning for preventing of the silkworm diseases.

Host tissues and specific receptors on cell surface are the main factors that determine the pathway of virus invasion, the way of diffusion and the disease characteristics of the host. The presence of virus receptors on the cell surface directly affects cells susceptibility to viruses. Ito transfected the +$^{nsd-2}$ gene into the resistant strain silkworm through Germ-Line transport system to restore the BmBDV-sensitive silkworm strain, indicated that NSD-2 protein was the essential factor for BmBDV infection. We cloned the +$^{nsd-2}$ gene and constructed the recombinant plasmid pIZ-+$^{nsd-2}$ successfully. The recombinant plasmid was transfected into BmN cells and selected by Zeocin antibiotics. BmN (+$^{nsd-2}$) cell lines, which stably expressed BmBDV sensitive gene +$^{nsd-2}$, were successfully constructed. RT-PCR technology was used to detect the transcription of +$^{nsd-2}$ gene in BmN (+$^{nsd-2}$) cells, and the expression of NSD-2 protein was tested by immunofluorescence analysis and western blot. The results showed that BmN (+$^{nsd-2}$) cell lines were able to express the +$^{nsd-2}$ gene stably. The immunofluorescence experiment revealed that the NSD-2 protein of the C-terminal with V5-tag was mainly located in the cell membrane area of BmN (+$^{nsd-2}$) cells. Co-IP results showed that the interaction between NSD-2 and VP protein really existed. However, the results of qRT-PCR, routine PCR and immunofluorescence experiments showed that the virus genes was not detected in BmN (+$^{nsd-2}$) cells.

Therefore, we speculate that NSD-2 may mediate virus entry with other co-receptors and suitable ambient conditions, such as temperature and pH. It is well known that the HIV receptor has CD4 molecules as well as the co-receptor CCR5 or CXCR4 (Lee *et al*., 2017; Liu *et al*., 2017). HIV-1 forms a trimer complex to invade target cells, namely HIV-1 gp120 envelope protein, CD4 molecule and a co-receptor. The majority of its co-receptors are chemotactic cytokine receptor. Hepatocytes are the main target cells for HCV infection, and in the initial stage of HCV infection, some liver specific factors / molecules are required to be involved except for HCV receptor in vitro (EA *et al*., 2011). Moreover, virus enters the cell has two ways: pH is independent on the fusion of the plasma membrane directly, and the receptor mediated pH dependent endocytosis. For example, cell invasion of HCVpp is pH dependent: cytoplasmic acidification inhibitor Bafilomycin A1 can reduce the infectivity of HCVpp to Huh-7 cells, and has dose response relationship. Similar results are also observed with the treatment of chloroquine, a weakly alkaline drug. In addition, the temperature and ions also have great influence on the virus invading cells (Zhao *et al*., 2009).

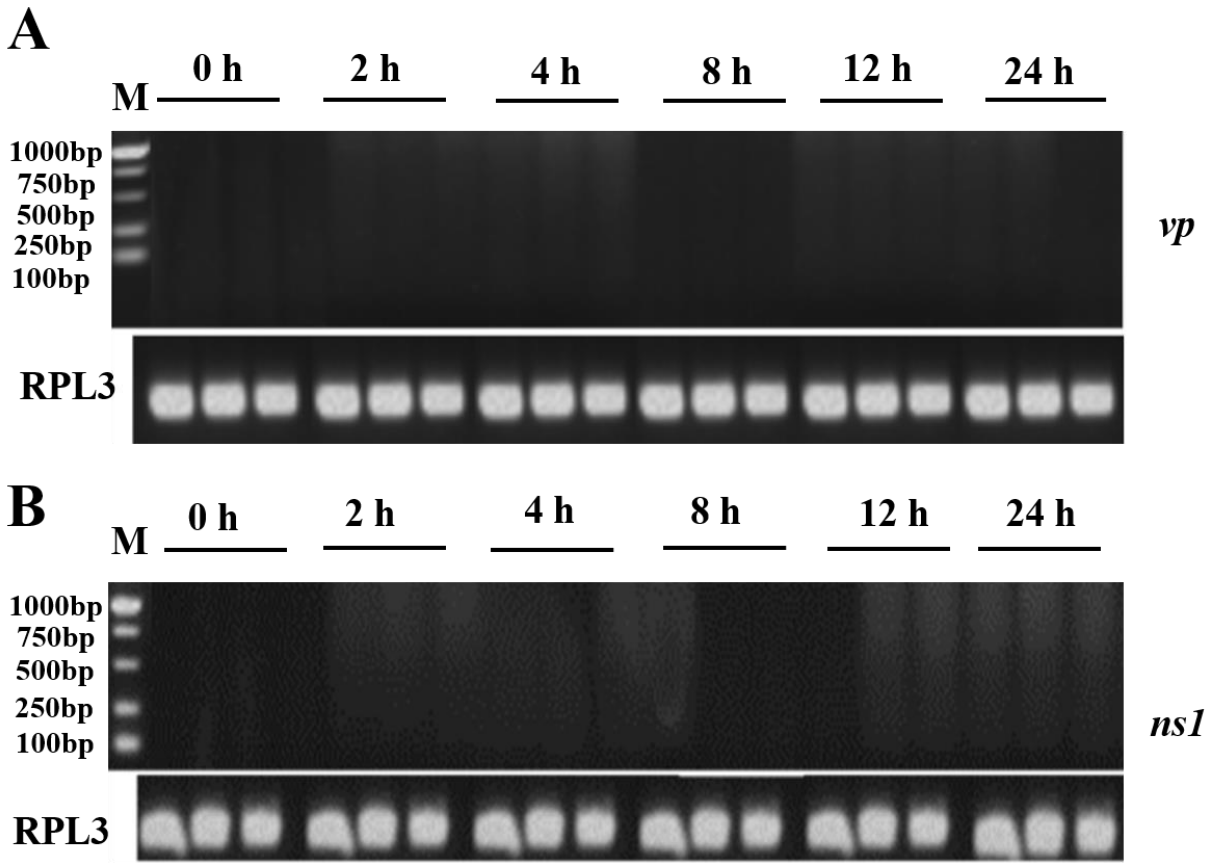

Fig. 6 The virus infection experiment results. (A) qRT-PCR amplification of the *vp* gene in BmN (+$^{nsd-2}$) cells from six different times; (B) qRT-PCR amplification of the *ns1* gene in BmN (+$^{nsd-2}$) cells from six different times; Data are performed in triplicate

In this study, a BmN cell line is established which stably expresses the potential BmBDV receptor protein NSD-2 of the silkworm. Although the results show that BmBDV cannot invade the cell, the stable recombinant cell lines could still be applied to the study of NSD-2, a protein closely related to viral invasion and the authentication of the receptor binding proteins. The subsequent task may focus on the analysis and identification of other components of the co-receptor by means of Co-IP and mass spectrometry. And the identified co-receptors will be added into BmN (+$^{nsd-2}$) cells to find whether the virus can invade cells. Moreover, the virus dose, pH and so on factors will be considered to be related to virus infection. The study of the viral receptor could deeply reveal the mechanism of the interaction between virus and host cells, which provides a new idea for developing effective virus prevention measures.

Acknowledgments

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