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# The signal peptide of BmNPV GP64 activates the ERAD pathway to regulate heterogeneous secretory protein expression

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**Abstract**

As a powerful eukaryotic expression vector, the baculovirus expression vector system (BEVS) is widely applied to the production of heterogeneous proteins for research and pharmaceutical purposes, while optimization of BEVS remains a work in progress for membrane or secreted protein expression. In this study, the impact of the signal peptide (SP) derived from Bombyx mori nucleopolyhedrovirus (BmNPV) GP64 protein on protein expression, secretion, and the endoplasmic reticulum-associated degradation (ERAD) pathway were investigated in BmN cells and BEVS. Transient expression studies in BmN cells revealed that SP alters the localization and expression levels of recombinant proteins, reducing intracellular accumulation while enhancing secretion efficiency. Quantitative analysis demonstrated that SP-mediated secretion was markedly higher compared to controls, albeit with lower total expression levels. Further exploration into SP-mediated ERAD pathway activation showed increased expression of BiP and other ERAD-associated genes (PDI, UFD1, S1P, and ASK1), correlating with higher SP-driven protein expression levels. RNA interference (RNAi) experiments elucidated that knockdown of ERAD-associated genes enhances both the secretion efficiency of SP-guided proteins and the infectivity of BmNPV. Particularly, interference with BiP demonstrated the most pronounced effect on protein secretion enhancement. Viral infection experiments further supported these findings, showing upregulated ERAD-associated genes during BmNPV infection, indicating their role in viral protein processing and infectivity. In conclusion, this study elucidates the complex interplay between SP-mediated protein secretion, ERAD pathway activation, and viral infectivity in BmNPV-infected cells. These insights suggest strategies for optimizing recombinant protein production and viral protein processing in baculovirus expression systems, with potential implications for biotechnological and biomedical applications. Further research could refine our understanding and manipulation of protein secretion pathways in insect cell-based expression systems.

**Keywords** Baculovirus expression vector system, BmNPV, Signal peptide, ERAD, Secretion

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## Introduction

Baculoviridae is a family of double-stranded DNA viruses that are pathogenic to insects and mainly infect Lepidoptera, Hymenoptera, and Diptera [1, 2]. In addition to their application as pesticides, the baculovirus expression vector system (BEVS) has become a powerful eukaryotic vector for the production of large quantities of heterogeneous proteins for research and biomedical purposes. BEVS has strong polyhedrin and p10 promoters for mass production of recombinant proteins [3] and unique advantages such as the strong modification ability of the target protein, the high expression level of foreign proteins, and a limited host range [4, 5]. To date, a number of BEVS products have been approved, including human vaccines and veterinary vaccines [6]. Baculovirus is also employed as a transduction vector capable of entering various mammalian cells to facilitate foreign gene expression under mammalian promoters, without replicating the viral genome [7], hereby offering a safer alternative to many mammalian viral vectors [8].

Membrane proteins play crucial roles in cellular functions such as responding to environmental changes, maintaining membrane stability, transporting nutrients, and defending the cells [9]. They are also prominent targets for drug development, making them subjects of intensive researches. BEVS has demonstrated notable success in expressing integral membrane proteins [10, 11]. Over the past decade, BEVS has contributed to obtaining approximately 31% of membrane protein structures [12]. However, a significant challenge of BEVS is achieving high expression yields of membrane proteins or secreted proteins; the levels of secreted proteins can be 10 to 100 times lower than those produced intracellularly [13]. Understanding the mechanisms of protein expression, translation, and secretion is crucial for overcoming this bottleneck.

Integral membrane proteins or secretory proteins are targeted to the appropriate membrane by their signal peptides (SPs) in a signal recognition particle-dependent manner [14–16]. The SPs mediate the synthesis of proteins in the endoplasmic reticulum (ER) and transport them to the extracellular space via the ER-Golgi secretory pathway [17]. These SPs are subsequently removed co- or posttranslationally by the cellular membrane-bound signal peptidase (SPase) complex [18]. Therefore, a wide variety of SPs from baculoviruses or host factors have been utilized to enhance the secretory efficiency of membrane proteins or secreted proteins [19–21], and optimization of amino acid composition within the SPs can enhance the secretion of recombinant proteins in BEVS [22]. Moreover, co-expression of SPase of *Bacillus subtilis* increased the expression in the insect cells [23].

Secreted proteins are intended to move from ER through the ER-Golgi apparatus to the cell exterior. However, some proteins fail to properly fold or assemble, leading to their identification and degradation via the ER-associated protein degradation (ERAD) pathway to maintain ER homeostasis [24]. Binding immunoglobulin protein (BiP), a major member of the heat shock protein 70 (hsp70) family, not only promotes protein folding to reduce the aggregation of protein in the ER [25] but also enhances protein transport and ER-related protein degradation [26, 27]. The ER oxidoreductases, such as protein disulfide isomerase (PDI) can oxidize cysteine residues of substrates to form disulfide bonds, thereby aiding in protein synthesis and folding [28]. Depletion of BiP or PDI has been shown to reduce the secretion and expression of recombinant proteins [29]. Conversely, overexpression of BiP significantly increases protein expression [13, 29]. Moreover, the ubiquitin-recognition protein (Ufd1) facilitates clearance of misfolded proteins through the ERAD pathway [30], and apoptosis signal-regulating kinases 1 (ASK1) and sphingosine-1 phosphate (S1P) are involved in unfolded protein response (UPR) [31–34]. However, their roles in the expression of membrane proteins remain largely unclear.

Bombyx mori nucleopolyhedrovirus (BmNPV) has been extensively used for heterogeneous protein expression in insect cells, larvae, and pupae due to the cost-effectiveness of this virus-insect expression system compared to cultured cells [13, 29, 35]. Various SPs derived from host factors, non-host factors, and viral factors have been employed for secreted protein expression, such as bombyxin, 30k, SP1, honeybee melittin, and GP64 [13, 22, 36, 37]. GP64, a type I integrated membrane protein, plays a crucial role in mediating fusion of budded virus (BV) with the host cell. The SP of GP64 is commonly used for expressing membrane proteins, secretory proteins, and protein surface display [13, 38]. Recently, we reported that GP64 in BmNPV retains its SP within the BV, and this uncleaved SP is essential for protein trans-plasma membrane secretion [39, 40]. Interestingly, GP64 and its SP guide foreign proteins to distribute within cells in a scattered dot pattern, as observed by immunofluorescence assays [41, 42]. Thus, further elucidation of the mechanisms governing SP-mediated protein secretion is warranted.

In this study, we investigated the expression of membrane proteins or secreted proteins guided by the SP of BmNPV GP64. Both eGFP and Luciferase fused with SP [43, 44] were expressed in BmN cells through transient expression and BEVS. We examined membrane protein expression and its influence on ERAD-associated proteins using quantitative assays and qPCR. Additionally, the impact of membrane protein expression on virus production was explored through RNA interference targeting ERAD-associated proteins. Our findings indicate that SP application enhances the secretory efficiency of

proteins; however, the overall expression levels were significantly reduced compared to non-secreted proteins. Furthermore, SP application effectively triggered the expression of ERAD-related proteins. Conversely, suppressing the expression of ERAD pathway proteins led to a notable increase in membrane protein expression. This study contributes to a deeper understanding of the BEVS and proposes a novel approach to enhance protein secretion levels by targeting ERAD proteins.

## Materials and methods

### Cells and plasmids

The BmN cells were cultured at 27 °C in TC-100 insect medium (Applichem, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) using standard techniques. Transient expression plasmids including pIZ/V5-SP-eGFP-TMD (SP-eGFP), pIZ/V5-eGFP-TMD (eGFP), pIZ/V5-SP-Luc (SP-Luc) and pIZ/V5-Luc (Luc) were constructed previously [42, 45]. In brief, *egfp* or *luciferase* gene was fused with the SP and the transmembrane domain (TMD) of BmNPV GP64 to generate transient expression plasmid SP-eGFP-TMD or SP-Luc. The vectors eGFP-TMD or Luc, lacking the SP, were used as controls.

### Stable cell line screening

BmN cells were initially seeded in a 6-well plate (Nest Biotechnology, Wuxi, China) and cultured over-night. Subsequently, the cells were transfected with either 4 μg SP-eGFP-TMD or eGFP-TMD plasmid using H-4000 (Engreen Biosystem, Beijing, China). To select stable cell lines, the cells were cultured in TC100 medium supplemented with 200 μg/mL Zeocin for screening purposes. After three months of screening, stable BmN cell lines were established, with over 90% of the cells exhibiting fluorescence. The expression of eGFP was evaluated using western blot analysis with an eGFP antibody (Sangon, Shanghai, China).

### Live-cell imaging

The stable cell lines were seeded onto confocal dishes (NEST Biotechnology, Wuxi, China), followed by staining with rhodamine B chloride (R18, Sigma–Aldrich, MS, USA) and Hoechst, as described previously [41]. Confocal microscopy (Leica SP8) was employed to image the fluorescence, and the intensity of eGFP fluorescence was compared using identical excitation and exposure times.

### Cells transfection

BmN cells were seeded into 6- or 24-well plates. After 24 h, the cells were transfected with the specified amount of plasmid DNA using H-4000 following the manufacturer's instructions. At 72 h post-transfection (p.t.) or at specified times, the cells were either harvested for measuring the relative activity of Luciferase or lysed with TRIzol (Thermo Fisher Scientific, MA, USA) for qPCR analysis.

### Luciferase activity assay

BmN cells in 24-well plates were transfected with 0.8 μg transient expression vector. Subsequently, both cells and media were collected directly at the indicated time points for the luciferase activity assay (Promega, MI, USA). For cell samples, 120 μl of 1× Cell Lysis Buffer per well was added, followed by shaking at moderate speed for approximately 15 min until complete lysis. The lysate was then transferred into a centrifuge tube after brief centrifugation. From the supernatant, 10 μl was used for the luciferase assay. The secretory efficiency, used to assess the impact of SP application on protein secretion, was calculated as the ratio of the total relative Luciferase Unit (RLU) in the media divided by the sum of RLU in both media and cells. Three independent biological replicates were performed and significance was determined using one-way ANOVA.

### Separation of secretory protein from supernatant

BmN cells were transfected with transient expression vectors, and cells were harvested for SDS-PAGE separation at 72 h p.t. In parallel, the supernatant from 24-well plates was collected and mixed with four times the volume of acetone, followed by overnight precipitation at -20 °C to recover secretory proteins. The precipitated proteins were pelleted by centrifugation, dissolved in 100 μl of phosphate-buffered saline (PBS) at pH 7.0, and subjected to SDS-PAGE analysis. Luciferase expression was assessed by western blot using a Luciferase antibody (Santa Cruz, CA, USA).

### qPCR analysis

The concentration of RNA samples was determined using a NanoDrop spectrophotometer. Subsequently, 500 ng of total RNA was subjected to reverse transcription using a PrimeScript RT Reagent Kit (Takara, Dalian, China). For qPCR analysis, specific primers (Table 1) targeting genes were utilized with NovoStart SYBR Q-PCR SuperMix Plus (Novoprotein, Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control [46]. Relative gene expression was evaluated using $2^{-\Delta\Delta Ct}$ method, and all experiments were performed using three biological replicates and three technical replicates.

### Small interfering RNA (siRNA) transfection

The primers for target genes in RNAi experiments were designed as detailed in Table 1. Scrambled siRNA sequences served as controls (NC), and siRNAs were synthesized using the in vitro transcription T7 Kit (TaKaRa,

**Table 1** Target gene sequence of RNAi and the primers used in this study

| Gene name | Target gene sequence |
|---|---|
| BiP | CCTCATGTTCAAGTACAAA |
| BiP NC | CAATACATGCATAACGCTT |
| PDI | GCTGAAGAAGAATCTCCTA |
| PDI NC | TTTAGTACCACGGAAACGA |
| UFD1 | GCATTAGAACAACTCACAA |
| UFD1 NC | AGTACCGAAAAATTAACCC |
| S1P | GCACTCAGCCGCTGTATTA |
| S1P NC | GTCAGACGTTAGGTCGACA |
| ASK1 | GCCCTTGAAGAAGTTCGTT |
| ASK1 NC | AACTGGCTAGCTGCATTTG |
| BiPQF | TGGAGTTTGTTCGCGCTAGT |
| BiPQR | AGGCCACGTATGAAGGTGTG |
| PDIQF | TCAAGAACAGGATCTCGCCG |
| PDIQR | TCATCAGCTTGACGACCACC |
| UFD1QF | TGATGCCCGAATCTAGTGGC |
| UFD1QR | GTTCCCGGTTCGGAATCACT |
| S1PQF | GCCGTTGACGCTGGACTATC |
| S1PQR | CGCCACCTGATACATGCTGAG |
| ASK1QF | TGGCGGCTGAACTGGTTAATG |
| ASK1QR | AGGTGATACAGCAGCGAACTCT |

Dalian, China). BmN cells were pre-seeded at total of $3.5\times10^5$ per well in 24-well plates and cultured overnight. They were then transfected with 50 pmol of siRNA using R4000 according to the manufacturer's instructions. Parallel experiments included scrambled siRNA as a control. At 24 h p.t., cells were collected for detection of the interference effect using qPCR analysis.

#### Cell viability assay

BmN cells were transfected with siRNA, and cell viability was assessed at 24 h p.t. using a Cell Counting Kit-8 (CCK-8) (Byotime, Shanghai, China). The culture medium was aspirated, and each sample in a 96-well plate received 100 µL of PBS and 10 µL of CCK-8 solution. Following a 2-hour incubation in darkness, absorbance was measured at a wavelength of 450 nm using a Perkin-Elmer multimode plate reader (Perkin-Elmer, MA, USA).

#### Recombinant viruses construction

To generate BmNPV bacmids containing the *luciferase* gene with or without SP, the *luciferase* gene SP-Luc and Luc was amplified by PCR. For SP-Luc, the forward primer sequence was 5'-CGGGATCCATGCTACTAGTAAATCAGTCATACC-3' and the reverse primer sequence was 5'-TTGCGGCCGCTTACACGGCGATCTTTCCG-3'. For Luc without SP, the forward primer sequence was 5'-CGGGATCCATGGAAGACGCCAAAAACATAAAG-3' and the same reverse primer was used. These PCR products were then inserted downstream of the polyhedrin promoter in pFBD-egfp [47] using *Bam*H I and *Not* I restriction sites to generate pFBD-egfp-SP-Luc and pFBD-egfp-Luc constructs. Subsequently, these constructs were transposed into BmNPV bacmid [48] using the Bac-to-Bac system according to the manufacturer's instructions. This resulted in the creation of BmBac-egfp-SP-Luc and BmBac-egfp-Luc. These bacmids were transfected into BmN cells. Subsequently, recombinant viruses were harvested from the transfected cells, and the titers were measured by end-point dilution assay (EPDA) [49].

#### ERAD-associated genes expression in BmNPV infection

The BmN cells were seeded into a 6-well plate and cultured overnight. Subsequently, the cells were infected with BmBac-eGFP [47] at a multiplicity of infection (MOI) of 5. At 24 h post-infection (h p.i.), the cells were harvested for analysis of ERAD-related gene expression using q-PCR. Primers specific for these genes are detailed in Table 1.

#### Western blot

BmN cells in a 6-well plate were transfected with 200 pmol of siRNA targeting BiP and subsequently infected with BmBac-eGFP at an MOI of 5, 24 h p.t.. At 48 h p.i., cells were harvested for western blot analysis using anti-BiP (Thermo Fisher Scientific, MA, USA), anti-GP64 (Santa Cruz, CA, USA), and anti-Tubulin (Sangon, Shanghai, China) antibodies. The relative expression of BiP was determined by scanning the bands in the western blot images with ImageJ and quantifying BiP expression relative to Tubulin.

#### Immunofluorescence assay

BmN cells transfected with siRNA were fixed at 72 h p.t. using 4% paraformaldehyde (PFA, Sangon, Shanghai, China) for 15 min. After washing with PBS, cells were blocked with 2% BSA for 30 min, followed by incubation with a rabbit antibody specific for *Bombyx mori* Tetraspanin A (BmTPA) at a dilution of 1:500. Following a 2 h incubation at room temperature and subsequent PBS washes, bound antibodies were detected using Alexa 488-conjugated goat anti-rabbit antibody (Sangon, Shanghai, China) at a dilution of 1:3000. PBS washes were performed between each step. Imaging was performed using a fluorescence microscope (OLYMPUS IX83, Tokyo, Japan), and fluorescence intensity was quantified using a Perkin-Elmer multimode plate reader (Waltham, MA, USA).

#### Comparison of virus infectivity

After transfecting BmN cells with siRNA, cells were subsequently infected with BmBac-eGFP at 24 h p.t. at an MOI of 5. The BVs were collected for titration at 48 h p.i., and BV DNA was isolated for qPCR. To quantify the BmBacmid DNA, it was isolated according to the Bac-to-Bac manual and quantified by spectrophotometry at

OD260. A standard curve was generated using ten-fold dilutions of BmBamid DNA. The viral infectivity was assessed by calculating the titer normalized to the copy number of genomic DNA ($TCID_{50}$/copy).

### Comparison of virus production at different MOI

To investigate the impact of ERAD-associated genes on BV production, BmN cells were transfected with siRNA and subsequently infected with BmBac-eGFP at 24 h p.t. using MOIs of 0.1 and 1. After 48 h p.i., BVs present in the cell culture supernatants were collected, and their titers were determined using an EPDA. The significance of BV titers was statistically analyzed using one-way ANOVA in GraphPad Prism 9.

### Comparison of luciferase expression by BEVS

BmN cells were seeded in 24-well plates at a density of $1.5\times10^5$ cells per well and cultured overnight. They were then transfected with siRNA targeting ERAD-associated genes. After 24 h, the cells were infected with the recombinant virus BmBac-egfp-SP-Luc or BmBac-egfp-Luc at an MOI of 5 for 2 h. Non-adherent viruses were removed by washing the cells three times with PBS. Subsequently, the cells were incubated in TC100 insect medium with 10% FBS at 27 °C for Luciferase assay analysis or qPCR assay at 72 h p.i.

## Results

### The existence of SP decreased the total expression of secretory protein

To assess the impact of SP application on protein secretion, initial experiments involved transient expression in BmN cells. Specifically, BmN cells were transfected with SP-eGFP-TMD or eGFP-TMD construct, and the stable cell lines were selected with Zeocin. Subsequently, cell localization was examined using a laser confocal microscope under uniform excitation and exposure conditions. Image analysis using ImageJ-v1.8.0 revealed distinct patterns: eGFP-TMD localized in the cytoplasm, often accumulating into large bubble structures (Fig. 1a), whereas SP-eGFP-TMD was found both on the plasma membrane and in the cytoplasm, forming dispersed small dots in the cytoplasm. Particularly, the fluorescence of SP-eGFP-TMD transfected cells (8.23), was markedly lower than that of eGFP-TMD cells (73.99) (Fig. 1b), a statistically significant difference ($P=0.0004$). Furthermore, western

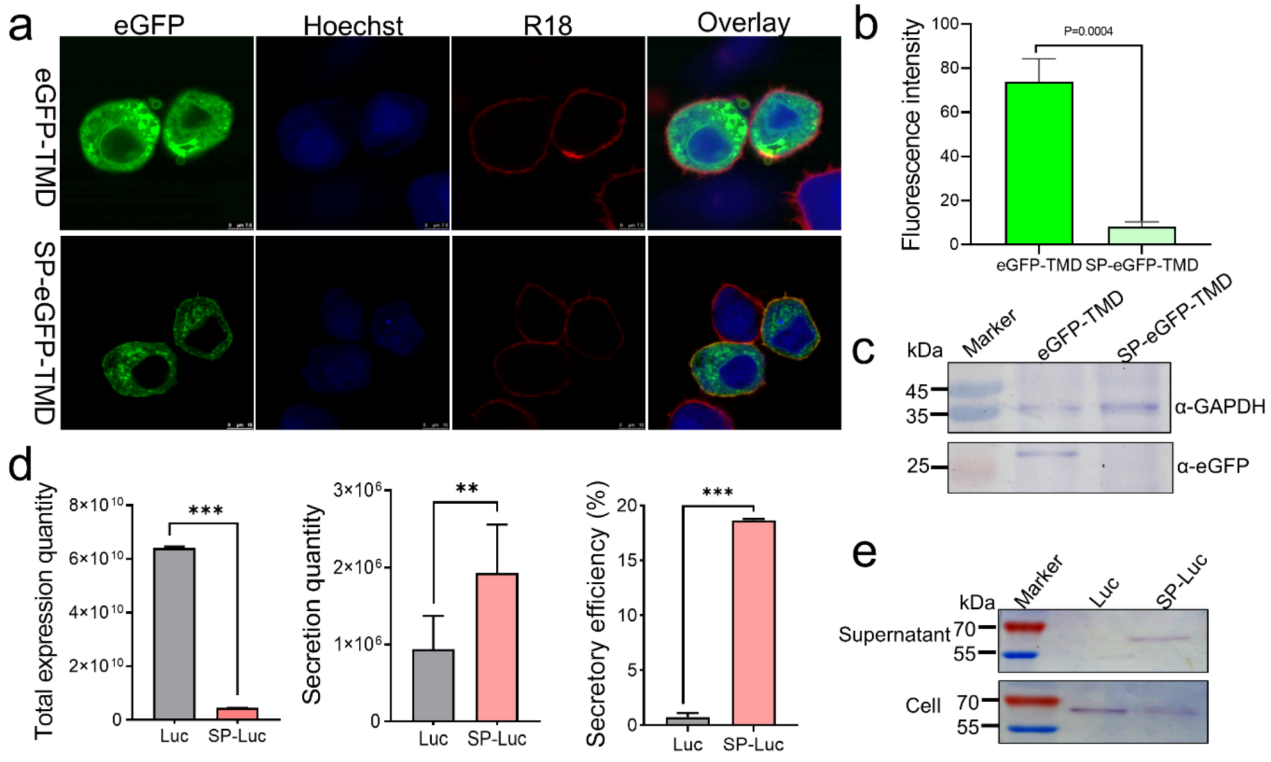


**Fig. 1** Impact of GP64 SP on protein expression and secretion. **(a)** Localization assay using confocal laser microscope of stable BmN cells transfected with pIZ/V5-SP-eGFP-TMD and pIZ/V5-eGFP-TMD. Nucleus was stained with Hoechst33258, and the plasma membrane was stained by R18. **(b)** Comparison of fluorescence intensity measured by ImageJ-v1.8.0 in transfected cell lines. **(c)** Western blot analysis comparing protein expression levels using an GFP antibody; GAPDH was used as an internal control. **(d)** Quantitative analysis of SP effect on Luciferase expression. BmN cells were transfected with SP-Luc or control Luc, and Luciferase activity was measured in both supernatant and cell lysates collected at 72 h p.t. **, $P<0.01$, ***, $P<0.001$. **(e)** Western blot analysis of SP influence on protein expression using a Luciferase antibody, with proteins precipitated from the supernatant and cell lysates of transfected cells

blot analysis (Fig. 1c) indicated similar GAPDH expression levels between samples; however, while eGFP-TMD was readily detectable, SP-eGFP-TMD was challenging to detect, suggesting a decrease in total protein expression with SP application.

Furthermore, to quantitatively analyze the effect of SP on protein expression, SP-Luc and Luc was transiently expressed in BmN cells by transfection. At 72 h p.t., supernatant and cell samples were collected to detect Luciferase activity. The results showed that the total expression level of SP-Luc was significantly lower than that of the Luc control (Fig. 1d), representing only about 7% of the total expression quantity of Luc. However, with the assistance of SP, both the quantity and secretory efficiency of SP-Luc were both higher compared to the control (Fig. 1d). Moreover, supernatant from transfected cells were collected, proteins were precipitated, and cells were lysed for western bolt. The result indicated that Luc exhibited higher expression than SP-Luc within the cells; however, Luc was not detected in the supernatant (Fig. 1e). Taken together, these findings suggested that the presence of SP increases the yield of secreted proteins while reducing the overall intracellular protein expression level.

### SP activated expression of BiP and genes related to -ERAD pathway

In transient expression, protein levels correlated with the dosage of the expression vector. We subsequently investigated the relationship between secretory protein expression and plasmid dosage. As depicted in Fig. 2a, within a certain range (16–80 ng), secretion protein quantity increased in a dose-dependent manner. However, a stable plateau was reached with a dosage of exceeding 80 ng

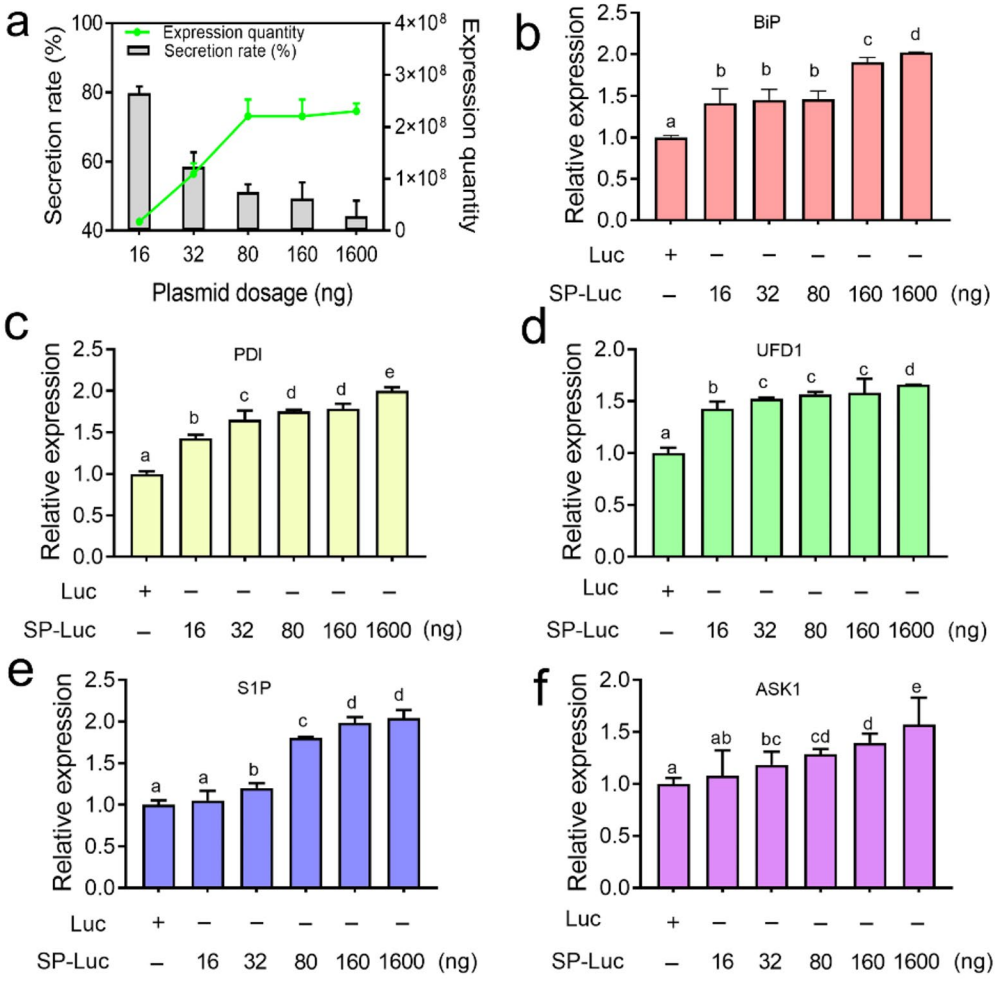


**Fig. 2** Secretory protein expression activates BiP and ERAD-associated gene expressions. **(a)** Correlation analysis between Luciferase expression and plasmid dosage in transfected cells. **(b-f)** ERAD-related gene expressions in transfected cells. BmN cells were transfected with increasing doses of the SP-Luc plasmid, with Luc serving as the control. Cells were collected at 72 h p.t., and total cellular RNA was extracted. The relative gene expression of BiP (b), PDI (c), UFD1 (d), S1P (e), and ASK1 (f) was measured by qPCR, with GAPDH used as the internal control. Significance levels are denoted by letters above the columns. All experiments were conducted in triplicate

DNA (Fig. 2a, green line), showing no significant increase in protein secretion. Consequently, the secretory efficiency declined with increasing plasmid dosage (Fig. 2a, histogram), indicating that higher plasmid dosage could produce more protein up to a limit. However, total secretion quantity was constrained in the transfected cells, suggesting a restriction in secreted protein capacity within cells.

Secretory protein folding and processing occur in the ER, necessitating precise cellular regulation. Protein accumulation in the ER triggers the ERAD process, which eliminates misfolded proteins [50]. Thus, we examined ERAD-related gene expression using qPCR. As shown in Fig. 2b, relative to Luc treatment (set as 1), SP-Luc transfection a significant increased BiP expression compared to Luc, with no difference observed between dosage of 16–80 ng. However, BiP expression significantly rose with dosages exceeding 80 ng plasmid, suggesting that excessive SP-Luc expression activated BiP. Furthermore, expression levels of ERAD-related genes (PDI, UFD1, S1P, and ASK1) increased with higher dosage (Fig. 2c-f), positively correlating with ERAD-related proteins expression levels according to Pearson correlation analysis. These finding indicate that excessive SP-Luc expression activats the expression of ERAD-associated genes.

#### ERAD pathway-related proteins influence protein secretion

ERAD is a crucial pathway for protein quality control in eukaryotic cells, responsible for identifying, classifying, and degrading misfolded proteins to prevent their accumulation in the cytoplasm [51, 52]. Our previous study demonstrated that BiP knockdown significantly increased the secretory efficiency of SP-Luc [42], suggesting BiP's role in the SP-activated ERAD pathway. We further investigated the involvement of ERAD-associated proteins in protein secretion. Initially, we used siRNA targeting BiP (SiBiP) to suppress BiP expression and measured protein secretion. As depicted in Fig. 3a, siBiP application markedly enhanced SP-Luc secretion. Concurrently, knockdown of BiP led to a significantly reduction (91.9%, 71.2%, 37.4%, and 82.1%, respectively) in the relative expression level of PDI, UFD1, S1P, and ASK1 compared with the NC group (Fig. 3b), indicating BiP's regulation of these proteins. Subsequently, BmN cells were transfected with siRNA targeting PDI, UFD1, S1P, and ASK1, or scrambled siRNA (NC), and knockdown efficiency was assessed by qPCR at 24 h p.t. Result showed significant reduction in intracellular mRNA levels of PDI, Ufd1, S1P, and ASK1 following siRNA transfection (Fig. 3c).

To evaluate the impact of these proteins knockdown on cells viability, BmN cells were transfected with either NC or siRNA targeting PDI, Ufd1, S1P, and ASK1. Cell viability was assessed using a Cell Counting Kit-8 at 24 h p.t. The results indicated that knockdown of these proteins minimally affected cell viability, with no significant difference observed (Fig. 3d). Additionally, membrane protein BmTPA expression was compared following knockdown of these proteins using a cell-based ELISA with an antibody against BmTPA. Knockdown of BiP and Ask increased BmTPA secretion, whereas interference with PDI, Ufd1 and S1P decreased BmTPA secretion (Fig. 3e). However, neither interference condition showed a significant inhibitory effect on host membrane protein secretion. In contrast, BmN cells were transfected with the plasmid SP-Luc or Luc 24 h after siRNA transfection, and supernatant and cell samples were collected at 72 h p.t. for Luciferase activity assay. Notably, Luc synthesis remained unchanged in NC or siRNA-transfected cells (Fig. 3f). In contrast, interference with PDI, UFD1, S1P, and ASK1 led to increasing of 3.39%, 1.39%, 2.97%, and 1.99%, respectively, in SP-Luc secretory efficiency (Fig. 3f), with significant difference observed for PDI, S1P, and ASK1, indicating their involvement in the protein secretion.

#### Down-regulated ERAD-related genes increased BmNPV infectivity

GP64, the viral membrane fusion protein facilitating BmNPV infection, is transported to the cytoplasmic membrane and integrated into virion during budding. Therefore, activation of ERAD-associated genes may influence BmNPV infectivity. Here, we investigate the relationship between virus infectivity and ERAD-related genes expression. BmN cells were infected with BVs at an MOI of 5, and at 24 h p.i., cells were collected for ERAD-related genes expression analysis. As shown in Fig. 4a, intracellular mRNA levels of BiP, PDI, UFD1, S1P, and ASK1 were significantly upregulated in virus-infected cells compared to healthy cells (CTRL group), with notable increases in BiP, PDI, and SIP by 13.3, 14.5, and 19.3 folds, respectively, indicating activation of ERAD-associated gene expression by BmNPV infection. Subsequently, we knocked down ERAD-related genes expression and accessed its impact on virus infectivity. To assess the impact of RNAi on these target genes, we evaluated BiP expression, a key protein in the ERAD pathway, using a western blot with an anti-BiP antibody during viral infection. siRNA targeting BiP significantly decreased BiP expression compared to the NC control, as shown by the relative expression analysis of BiP/Tubulin (Fig. 4b and c). Although viral infection in siRNA-transfected cells increased BiP expression, a significant difference remained, indicating that siRNA effectively reduced the target gene expression. The BV titers were determined by EPDA at 48 h p.t., revealing increased virus titers in BmN cells treated with siRNAs targeting BiP, PDI, UFD1, S1P, or ASK1 compare to NC cells (Fig. 4d).

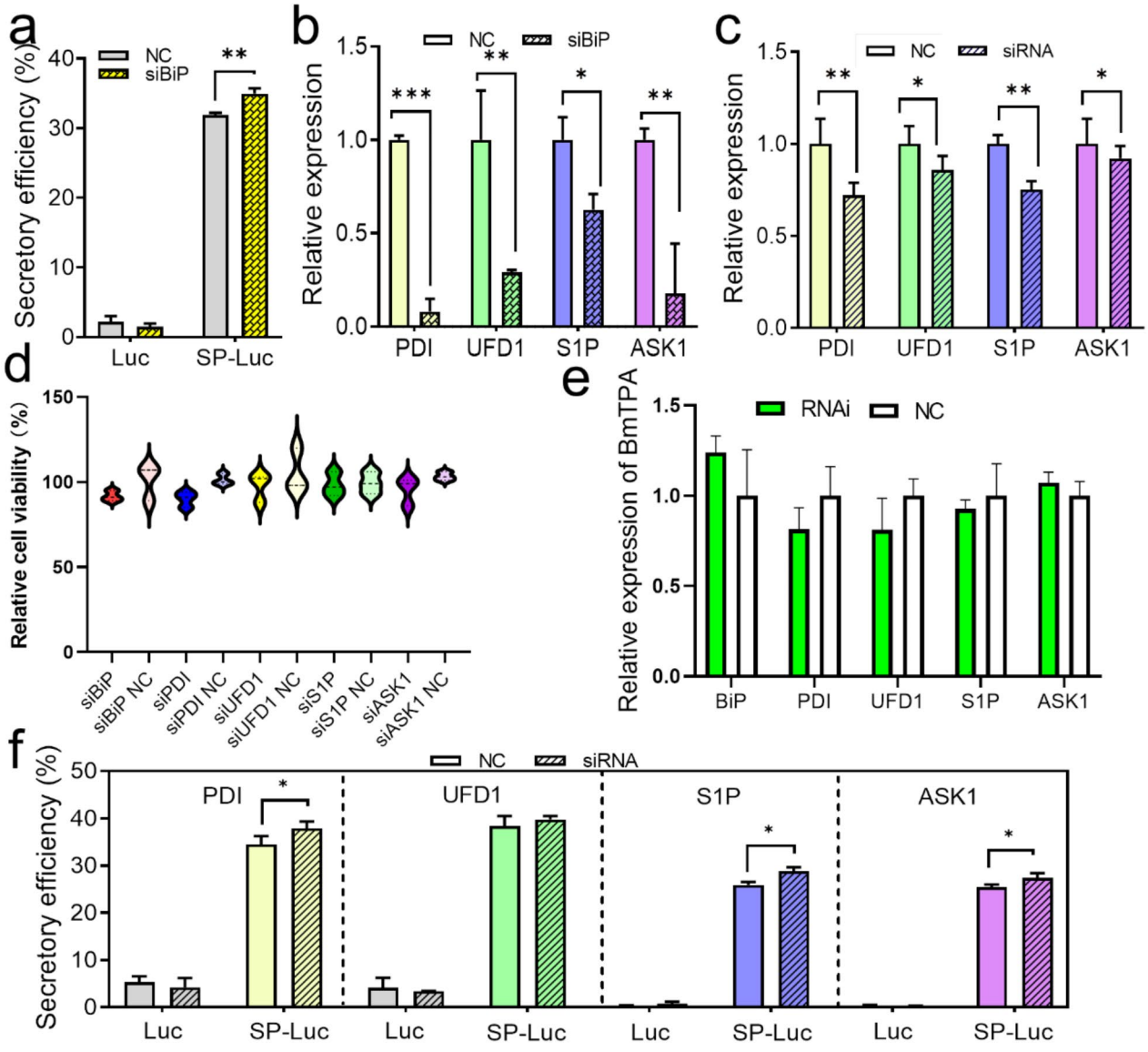


**Fig. 3** ERAD pathway-related genes involved in protein secretion. **(a)** SiBiP application increased protein secretion. BmN cells were transfected with siBiP and subsequently transfected with either SP-Luc or Luc plasmid. Supernatant and cell samples were collected at 72 h p.t. and subjected to a Luciferase activity assay. **(b)** Effects of BiP knocking down on the expression of PDI, UFD1, S1P, and ASK1. BmN cells were transfected with siBiP, and total cellular RNA was extracted at 24 h p.t. The relative gene expression of PDI, UFD1, S1P, and ASK1 was assessed by qPCR with GAPDH served as the internal control. Experiments were conducted at least three times. **(c)** Efficiency of siRNA knockdown of target genes. BmN cells were transfected with siRNA against PDI, UFD1, S1P, and ASK1 or scrambled siRNA (NC). Cells were collected at 24 h p.t., and RNA copies of target genes were quantified by qPCR to assess siRNA efficiency. (**d**) Cell viability assay of BmN cells transfected with siRNA. Cell viability was assessed at 24 h p.t. using a Cell Counting Kit-8 after transfection with siRNA and NC. (**e**) Effect of ERAD-associated gene knockdown on membrane protein expression. BmN cells were transfected siRNA targeting BiP, PDI, UFD1, S1P, and ASK1 for 24 h. Cells were fixed and subjected to cell-based ELISA with an antibody against BmTPA. Intensity of cells was recorded, with NC control set as 1. **(f)** Effects of PDI, UFD1, S1P, and ASK1 knockdown on SP-Luc secretion. After 24 h of siRNA transfection, cells were transfected with the plasmid SP-Luc or Luc. Supernatant and cell samples were collected at 72 h p.t. and subjected to a Luciferase activity assay. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

To explore the mechanism behind this increase, viral genomes were extracted and quantified using qPCR with gp64 specific primers. No significant difference was observed in genome numbers between the RNAi samples and untreated CTRL (Fig. 4e), suggesting that interference with ERAD-associated genes did not affect viral particle production. However, analysis of BV DNA relative infectivity indicated that knockdown of BiP, PDI, S1P, ASK1, and UFD1 significantly decreased genomic DNA copy numbers per TCID50, implying enhanced infectivity of BVs (Fig. 4f). Further evaluation of gene knockdown effects on virus production at lower MOIs

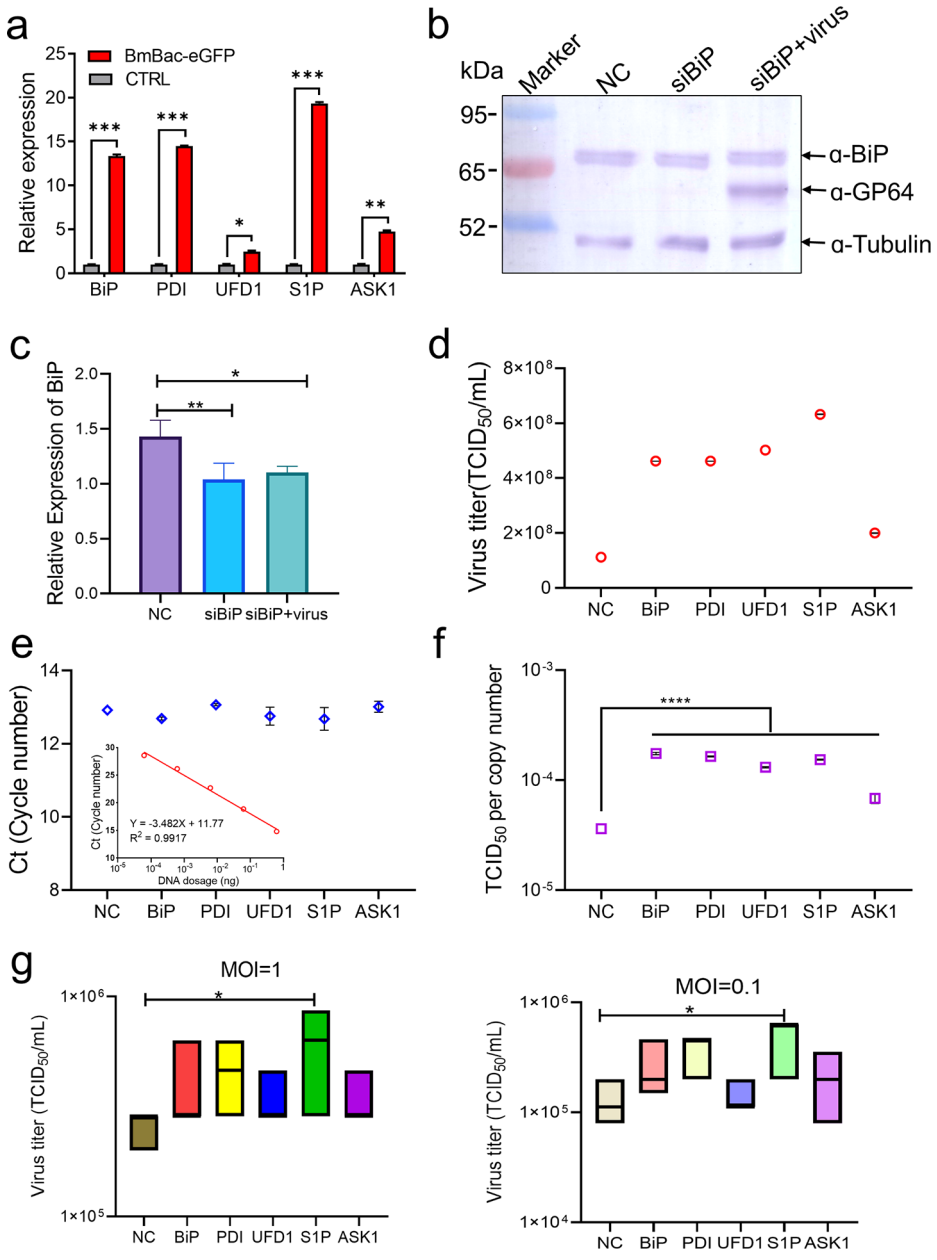


**Fig. 4** Knockdown effect of ERAD-related gene expression on viral infectivity. **(a)** Relative expression of ERAD-associated genes during BmNPV infection. BmN cells were infected by BmNPV at a MOI of 5, followed by qPCR analysis of ERAD-associated gene expression. **(b)** Western blot analysis of BiP expression. BmN cells were transfected with siRNA targeting BiP and subsequently infected with or without BmNPV at an MOI of 5, 24 h post-transfection. Cells were harvested for western blotting with anti-BiP, anti-GP64, and anti-Tubulin antibodies at 48 h p.i. **(c)** Relative expression of BiP. The relative expression of BiP was determined by scanning the bands in the western blot image with ImageJ, and quantifying BiP expression relative to Tubulin. **(d)** Impact of ERAD-associated gene interference on BV titers. BmN cells were transfected with siRNA targeting ERAD-related genes and subsequently infected with the virus. Viral titers in the supernatant were determined by EPDA. **(e)** Quantification of BV genomes by qPCR. Viral genomes were extracted from BVs, and their numbers were determined by qPCR. A standard curve was generated using a series of diluted bacmid DNAs. **(f)** Comparison of BV relative infectivity. The infectivity of BVs was assessed by normalizing BV titers to genomic DNA copies. **(g)** BV production from BmN cells transfected with siRNA and infected at lower MOIs. The impact of siRNA-mediated gene knockdown on BV titers was evaluated under reduced MOI conditions. *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$

showed increased BV titers compared to control, particularly notable with S1P interference at MOIs of 1 and 0.1 (Fig. 4g). These findings collectively demonstrate that down-regulation of ERAD-related genes enhances BV infectivity, highlighting their potential as targets for improving viral production.

### Interference with ERAD-related genes enhances protein secretion in BEVS

To explore the impact of ERAD-related genes on the recombinant proteins secretion in BEVS, we constructed recombinant viruses BmBac-egfp-SP-Luc and BmBac-egfp-Luc. The BmN cells were infected with these viruses at an MOI of 5, and both cells and supernatants were collected at 72 h p.i. for Luciferase activity analysis. As shown in Fig. 5a, the total Luciferase production from BmBac-egfp-SP-Luc was significantly lower compared to BmBac-egfp-Luc (Fig. 5a, upper panel), while the secretory efficiency was markedly increased by 3-fold (Fig. 5a, lower panel).

Next, we investigated the effect of knocking down ERAD-related genes on the expression and secretion of foreign proteins in BEVS. BmN cells were transfected with siRNA targeting BiP, PDI, UFD1, S1P, and ASK1, followed by infection with BmBac-egfp-SP-Luc at an MOI of 5 at 24 h p.t. Protein expression and infection progress were monitored at 72 h p.i. using fluorescence microscope and Luciferase assay. The results revealed increased eGFP expression when the expression of ERAD-related genes was effectively interfered with, particularly with BiP knockdown (Fig. 5b). Disturbance of BiP significantly enhanced the total expression quantity, secretion quantity, and secretory efficiency of Luciferase compared to the NC control (Fig. 5c). Similar enhancements were observed with knockdown of the other four ERAD-associated genes compared to the NC control, although the effectiveness was less pronounced than with BiP knockdown (Fig. 5c).

## Discussion

BEVS exhibits high expression level of cytoplasmic proteins but a relatively lower level of secreted and transmembrane proteins. This study systematically analyzed the impact of the SP from BmNPV GP64 on protein secretion, using both transient expression plasmid and BEVS. The presence of SP significantly increased the quantity of secreted protein while decreased total expression level. SP directs proteins into ER-Golgi secretory pathway, but also triggers ERAD pathway activation due to protein accumulation in the ER, leading to protein degradation. RNA interference targeting ERAD-associated genes—BiP, PDI, UFD1, S1P, and ASK1—markedly enhanced the quantity of secreted protein. Additionally, knockdown of ERAD-associated genes increased both BV infectivity and secreted protein levels, suggesting potential optimizations for membrane protein and secreted protein expression in BEVS.

GP64, the principal membrane fusion protein of alphabaculovirus, is crucial for BV entry into host cell [53]; GP64's SP is commonly used for membrane protein

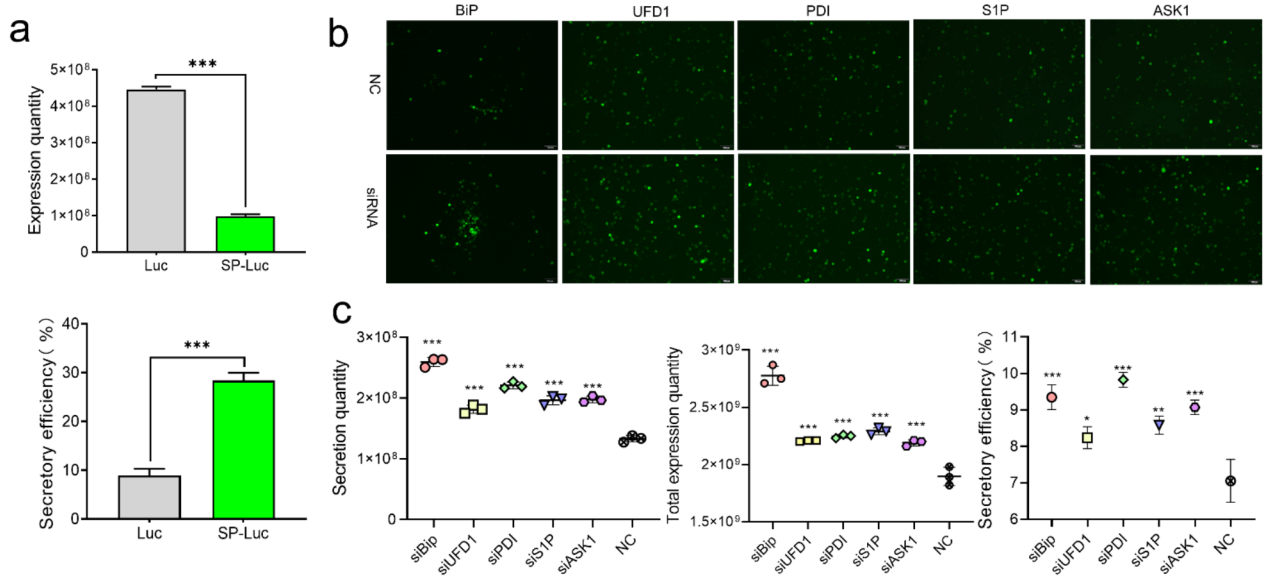


**Fig. 5** Effects of ERAD-related genes on protein secretion by BEVS. (**a**) Influence of SP application on protein secretion by BEVS. **(b)** Fluorescence microscopy of BmN cells following ERAD-related gene interference and infection with BmBac-egfp-SP-Luc. **(c)** Enhancement of secreted protein expression in BEVS by knocking down ERAD-associated gene expression. BmN cells were transfected with siRNA and subsequently infected with BmBac-egfp-SP-Luc; cells and supernatants were collected for a Luciferase activity assay at 72 h p.i. The experiments were performed in triplicate, and values represent means ± SD. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA)

expression and glycoprotein surface display [54, 55]. Unlike AcMNPV, where GP64 decreased significantly late in infection [56], BmNPV GP64 remains abundant throughout infection [57]. Recent finding indicate that BmNPV GP64 SP remains uncleaved from mature BV, essential for BV egress and infectivity [41, 58, 59], including its role in facilitating Ebola virus glycoprotein secretion in BmN cells [39]. Moreover, BmNPV GP64 SP led arrowhead proteinase inhibitor B and human epidermal growth factor secretion in cells and larvae, which generated fusion products (papers in Chinese). Thus, BmNPV GP64 SP mediated protein secretion was further explored in this study. However, the diverse SPs of baculovirus membrane proteins, beyond GP64 tested here, and more SPs of host membrane proteins were not explored, leaving room for further optimization of specific secreted protein expressions.

In BmNPV infection, we observed over a 10-fold in BiP, PDI, UFD1, S1P, and ASK1 expression compared to control (Fig. 4a), indicating their correlation with BmNPV infection. During viral infection, multiple viral membrane proteins are synthesized, with some accumulating in the ER and activating in the late infection stage [1]. As molecular chaperone, ERAD-related proteins likely play key role in these processes. BiP aids viral protein folding and ensure quality control of newly synthesized glycoproteins [42, 60, 61]. In contrast to other studies where depletion of BiP or PDI reduced the secretion and expression of recombinant proteins [29], overexpression of BiP has shown to increase protein secretion [13, 29, 62]. Our study demonstrated interference targeting BiP had a more pronounced enhancement on SP-mediated protein secretion. Furthermore, in combination with BiP expression was activated by GP64 SP [42]. We hypothesized that this activation mediated the translocation of proteins from the ER for degradation [63].

Virus infection involves complex interactions between the virus and host, potentially activating the ERAD pathway through various mechanism. To isolate these effects, transient expression assays were employed. We found that SP-Luc expression positively correlated with BiP and ERAD-associated gene expression, suggesting that overexpression of secreted proteins efficiently triggers the ERAD pathway (Fig. 2). Similarly, hyperexpression of the polyhedrin promoter in BEVS leads to large quantities of heterogeneous secreted protein. When guided into the ER by the SP, molecular chaperones such as BiP and PDI facilitate correct protein fold and transport to Golgi. However, ER capacity is finite; excessive protein entry triggers the ERAD degradation pathway, resulting in significant protein degradation. Importantly, RNAi targeting ERAD-associated genes did not increase BV particles (Fig. 4) but did enhance virus infectivity, suggesting that RNAi treatment could boost viral infectivity by enhancing the membrane protein GP64 on BVs.

## Conclusions

This study provides new insight into enhancing membrane proteins expression using molecular targets like BiP and other ERAD-associated genes by BmNPV-insect cells/larvae, and offer a basis for further research into GP64 SP mechanism in protein secretion. Additionally, the study uncovers the link between BmNPV infection and ERAD pathway activation, providing insights for understanding BmNPV pathogenesis with uncleaved SP.

### Abbreviations

| | |
|---|---|
| ABC | ATP binding cassette |
| ASK1 | apoptosis signal-regulating kinase 1 |
| BEVS | baculovirus expression vector system |
| BiP | Binding immunoglobulin protein |
| BmNPV | Bombyx mori nucleopolyhedrovirus |
| BmTPA | Bombyx mori Tetraspanin A |
| BV | budded virus |
| EPDA | end-point dilution assay |
| ER | endoplasmic reticulum |
| ERAD | endoplasmic reticulum-associated protein degradation |
| FBS | fetal bovine serum |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GPCRs | G protein-coupled receptors |
| h p.i. | hours post infection |
| h p.t. | hours post transfection |
| hsp70 | heat shock protein 70 |
| MOI | multiplicity of infection |
| PBS | phosphate buffer saline |
| PDI | protein disulfide isomerase |
| RLU | relative Luciferase unit |
| S1P | sphingosine-1 phosphate |
| siRNA | Small interfering RNA |
| SP | signal peptide |
| SPase | signal peptidase |
| $TCID_{50}$ | 50% tissue culture infective dose |
| Ufd1 | ubiquitin-recognition protein |
| UPR | unfolded protein response |

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### Author contributions

Conceptualization, B.H. and J.H.; methodology, B.H.; validation, N.L. Y.X., and L.S.; formal analysis, N.L., L.S., Y.X., and M.L.; investigation, N.L. and L.S.; data curation, B.H; writing—original draft preparation, B.H.; writing—review and editing, B.H. and J.H.; supervision, B.H.; project administration, B.H.; funding acquisition, B.H. All authors have read and agreed to the published version of the manuscript.

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### Data availability

No datasets were generated or analysed during the current study.

## Declarations

### Ethics approval and consent to participate

Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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