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Therapeutic effects of duck Tembusu virus capsid protein fused with staphylococcal nuclease protein to target Tembusu infection in vitro

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ABSTRACT

Tembusu virus (TMUV), a member of the genus *flavivirus*, primarily causes egg-drop syndrome in ducks and is associated with low disease mortality but high morbidity. The commercially available live vaccines for treating TMUV currently include the main WF100, HB, and FX2010-180P strains, and efficient treatment and/or preventative measures are still urgently needed. Capsid-targeted viral inactivation (CTVI) is a conceptually powerful new antiviral strategy that is based on two proteins from the capsid protein of a virus and a crucial effector molecule. The effector molecule can destroy the viral DNA/RNA or interfere with the proper folding of key viral proteins, while the capsid protein mainly plays a role in viral integration and assembly; the fusion proteins are incorporated into virions during packaging. This study aimed to explore the potential use of this strategy in duck TMUV. Our results revealed that these fusion proteins can be expressed in susceptible BHK21 cells without cytotoxicity and possess excellent Ca^{2+}-dependent nuclease activity, and their expression is also detectable in DF-1 cells. Compared to those in the negative controls (BHK21 and BHK21/pcDNA3.1(+) cells), the numbers of viral RNA copies in TMUV-infected BHK21/Cap-SNase and BHK21/Cap-Linker-SNase cells were reduced by 48 h, and the effect of Cap-Linker-SNase was superior to that of Cap-SNase. As anticipated, these results suggest that these fusion proteins contribute to viral resistance to treatment. Thus, CTVI might be applicable for TMUV inhibition as a novel antiviral therapeutic candidate during viral infection.

1. Introduction

More than 70 types of viruses have been confirmed as *flaviviruses*, most of which are arthropod-borne viruses, including West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DEV), yellow fever virus (YFV), tick-encephalitis virus (TBE), Ntaya virus (NTAV), Bagaza virus, and Zika virus (ZIKV), which are predominantly transmitted by arthropod vectors, such as mosquitos, midges, ticks, and sandflies (Esther et al., 2012). Many of these viruses cause significant human and animal diseases (Wolfe et al., 2001), such as encephalitis (German et al., 2006; Turtle et al., 2012), haemorrhagic fever, and shock syndrome (Mackenzie and Gubler DJPetersen, 2004).

Tembusu virus (TMUV) is a newly emerging *flavivirus* (Li et al., 2012; Yun et al., 2012) identified as an enveloped positive-stranded RNA virus that is approximately 55 nm in diameter (Vaidya et al., 2012). TMUV contains only a large open reading frame (ORF) that encodes a single polyprotein, which is processed by viral and host proteases into three structural proteins, a capsid protein (Cap), a precursor membrane protein (prM), and an envelope glycoprotein (E), as well as several nonstructural (NS) proteins (NS1, NS2A/B, NS3, NS4A/B, 2K, NS5) (Zhang et al., 2017). These NS proteins primarily comprise proteases and play important roles in viral replication, proteolysis and maturation (Mukhopadhyay et al., 2005). In egg-laying ducks, TMUV primarily causes hyperpyrexia and feed intake and egg production losses and leads to neurological symptoms such as ataxia and paralysis in commercial meat-type ducks.

Cap proteins of *Flaviviridae* are α-helical and multifunctional and are mostly responsible for genome packaging to ensure specific

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capsidation (Fajardo et al., 2016). Capsid-targeted viral inactivation (CTVI) can be applied to viruses that have relatively flexible cap structures, such as retroviruses, hepadnaviruses, caulimoviruses, flaviviruses and circoviruses. CTVI was first proposed by Natsoulis and Boeke (Natsoulis and Boeke, 1991) in 1991 after the discovery of "intracellular immunization". This tactic is based on a cap protein, which can be assembled into virions, targeting exogenous effector molecules, such as nucleases, lipases, proteases, and single-chain antibodies (scAbs) (Zhang et al., 2016). These effectors possess a specific functional ability to destroy the virion component (genomic DNA/RNA or key proteins) from within. Staphylococcus nuclease (SNase) is widely applied in CTVI and is a calcium (Ca^{2+}) dependent enzyme that becomes active in response to millimolar levels of Ca^{2+} (Schumann et al., 2001). Previous CTVI technology has primarily focused on retroviruses (e.g., human immunodeficiency virus-1, HIV-1 (Okui et al., 1998); murine leukaemia virus, MLV (Vanbrocklin and Federspiel, 2000); and classical swine fever virus, CSFV (Wang et al., 2010; Zhou et al., 2010)) or retrovirus-like viruses (e.g., hepatitis B virus, HBV (Beterams and Nassal, 2001)), which replicate by reverse transcription. However, the technological development of CTVI has been successfully expanded to other nonretroviruses, such as DENV2 (Qin and Qin, 2006), JEV (Pang et al., 2013), porcine circovirus 2 (PCV2), and reovirus. Herein, our research explored whether the CTVI strategy shows potential applicability against DTMUV infection.

2. Materials and methods

2.1. Cells

Baby hamster kidney 21 (BHK21) cells and DF-1 cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Gibco by Life Technologies) containing 10% foetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA). All cell cultures were incubated at 37℃ in a humidified 5% carbon dioxide incubator.

2.2. Virus, antibodies and reagents

DTMUV (CQW1 strain, GenBank accession no. KM233707.1) was isolated from Chongqing Wanzhou (Zhu et al., 2015). A mouse polyclonal antibody directed against SNase serum was prepared in our laboratory. Polyclonal horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-tagged goat anti-mouse secondary antibodies were purchased from Beijing Biodragon Immunotechnologies Co., Ltd. and Proteintech, respectively.

2.3. Construction of plasmids

To construct the recombinant plasmids, several pairs of specific primers were designed based on the cap protein of DTMUV CQW1 strains and the *Staphylococcus aureus* nuclease, which were provided by Wencan Jiang, Professor of Sichuan Agriculture University (ID: CP006630.1) (Table 1).

RNA was extracted from TMUV strain CQW1 allantoic fluid and reverse transcribed into cDNA using RNAiso plus (Takara, Dalian, China) and a RT-PCR Kit (purchased from Vazyme Biotech Co. Ltd). First, the Cap_1, $SNase_1$, Cap_2, and $SNase_2$ genes were amplified using the cDNA template, the pGMT-SNase plasmid, and the primers listed in Table 1. Then, the amplified $Cap_1/SNase_1$ and $Cap_2/SNase_2$ fragments were utilized to construct the fusion fragments Cap-SNase and Cap-Linker-SNase using overlapping PCR technology. The two fusion fragments and pcDNA3.1 (+) were then digested separately using *EcoR* I and *Xho* I, and digested fragments were cloned into the digested pcDNA3.1 (+) vector to obtain pcDNA3.1 (+)-Cap-SNase and pcDNA3.1 (+)-Cap-Linker-SNase. The integrity and fidelity of the inserted fragments were confirmed by sequencing (Qingke, Chengdu).

The cap protein acts as the nucleic chaperone, and the cell-penetrating peptides (CPPs) derived from some flavivirus capsid proteins serve as good delivery systems for some viruses. pepR and pepM are two novel CPPs; pepM mainly binds the membrane, while pepR binds RNA. The CPPs include 6–30 amino acid residues, mainly ranging from highly charged (rich in Arg and Lys) to hydrophobic or amphipathic sequences, and these two peptides of Cap protein regions are conserved among *Flaviviridae* family members. The C-terminus of the flavivirus capsid protein serves as the RNA-binding region (Roland and Jean-Luc, 2012). For better contact with viral RNA, SNase is fused to the C-terminus of the capsid protein. Glu43 and Arg87 are the main active sites of the SNase enzyme (Qin and Qin, 2006; Wang et al., 2010). A schematic diagram of the fusion parts is shown in Fig. 1A–B.

2.4. Transfection and sample handling

BHK21 and DF-1 cells were cultured in DMEM containing 10% FBS in six-well plates to 80% confluence. Transient transfection was performed using Lipofectamine™ 3000 (Invitrogen, Shanghai, China). Mock transfection (no plasmid) was performed as a negative control. Total cellular proteins were harvested after 48 h for transient expression analysis. Cells were lysed in 100 μL of native lysis buffer (Beyotime) containing 1% phenyl methanesulfonyl fluoride (PMSF). Then, the cell lysate was centrifuged, and the supernatant was collected and subjected to Western blot analysis.

2.5. Western blot analysis

Samples were collected as described above, and the protein concentration was determined using a BCA kit. Protein samples were subsequently separated by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes, which were blocked in 5% nonfat dry milk at room temperature for 1 h. The mouse anti-SNase polyclonal antibody (1:200 dilution) and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Shanghai, China) were used at a 1:200 dilution overnight at 4 ℃. Goat anti-mouse IgG polyclonal HRP-conjugated secondary antibodies (1:5000 dilution; Santa Cruz Biotechnology, Shanghai, China) were then applied. Proteins were visualized using enhanced chemiluminescence (ECL) and quantified using Quantity One (Bio-Rad, California, USA).

Table 1
Primers for amplification of the fusion genes.

Primer name	Primer sequence (5′–3′)
$Cap(F)_1$	CG***GAATTC***GCCACCATGGCTAACAAAAAACCAGGAAGACC
$Cap(R)_1$	GCACTATATACTGTTGGATCCCCAGCAACTATCGGGAGTAA
$Cap(R)_2$	TGGATCGGATCCTCCTCCTCCGGATCCTCCTCCTCCGGATCCTCCTCCTCCCCCAGCAACTATCGGGAGTAA
$SNase(F)_1$	TTACTCCCGATAGTTGCTGGGGATCCAACAGTATATAGTGC
$SNase(F)_2$	CTGGGGGAGGAGGAGGATCCGGAGGAGGAGGATCCGGAGGAGGAGGATCCGATCCAACAGTATATAGTGC
$SNase(R)_1$	CCG***CTCGAG***TTATTGACCTGAATCAG

The underlined bases represent protective bases, the bold and italic bases represent *Eco*R I and *Xho* I sites, respectively, and the underlined and italic bases represent Kozak sequences.

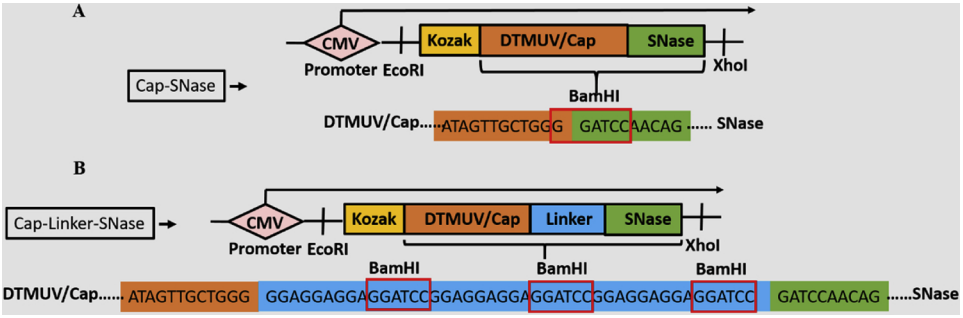

Fig. 1. Schematic of the recombinant plasmids. A and **B** show the junction between the TMUV Cap and SNase in detail. The orange frame represents Cap, and the green frame depicts SNase. The blue frame depicts a linker with 15 amino acids $(G_4S)_3$ in C, and the red frames show *BamH* I. The Kozak sequence is shown in yellow, and the CMV promoter is shown in pink.

2.6. Immunofluorescence assay (IFA)

The expression of fusion proteins was monitored by immunofluorescence detection. Transfected cells were plated on coverslips in 6-well plates 48 h post transfection, fixed with 4% paraformaldehyde, permeabilised with 0.25% Triton-100, and blocked with 5% skim milk. After blocking, cells were incubated in a mouse anti-SNase (1:200) primary antibody overnight at 4°C. The FITC-tagged secondary antibody (purchased from Proteintech) was first diluted 1:100 in 1% BSA and then applied to the cells. Finally, nuclear staining was performed in the dark using 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and the cells were washed with 1 × PBS three times after each incubation. Confocal microscopy was used to observe fluorescence signals.

2.7. Nuclease activity assay

To detect the nuclease activity of BHK21 cells transfected with the different constructs, we utilized plasmid DNA digestion assays in vitro. The samples were boiled in loading buffer for 10 min to eliminate residual cellular nuclease activity, but SNase possesses thermal stability. Different sample concentrations (obtained as previously described) were added to the nuclease reaction cocktail buffer (100 mM Tris-HCl [pH 8.8], 10 mM $CaCl_2$) containing 500 ng of the substrate plasmid pcDNA3.1(+). Each reaction system contained a total volume of 20 μL and was incubated at 37 °C for 30 min. Purified SNase protein served as the standard (positive control), and 0.5 M EDTA was used to chelate calcium ions. Reaction products were mixed with 1 μL of buffer and electrophoresed on 1% agarose gels to visualize DNA using gold view staining.

2.8. Cytotoxicity assay

BHK21 cells were seeded into 96-well plates and then subjected to microscopic observation to detect changes every day after transfection. To further analyse the effects of the fusion proteins Cap-SNase and Cap-Linker-SNase on BHK21 cell viability, the MTT assay was performed. Forty-eight hours after transfection, 20 μL of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 5 h. Next, media was removed with a pipette, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well and pipetted up and down to dissolve the precipitate. The plate was then placed in the incubator for 5 min to dissolve air bubbles. Finally, the absorbance was measured at 550 nm, and the percent viability was calculated according to the following formula: viability (%) = (mean absorbance of sample/mean absorbance of control) × 100%.

2.9. Real-time fluorescence quantitative reverse transcription PCR (qRT-PCR)

BHK21 cells grown in 12-well plates were infected with virus preparations at a multiplicity of infection (MOI) of 1 for 1 h at 37°C. Next, the plasmids were transfected into BHK21 cells using Lipofectamine™ 3000 (Life Technologies) according to the manufacturer's instructions. At various time points, culture supernatants (250 μL) were collected for total RNA extraction using TRIzol reagent. Ten microlitres of diethyl pyrocarbonate (DEPC) was added to dissolve the RNA, and 1 μL of RNA was subsequently reverse transcribed into cDNA using 10 μL of reverse transcriptase (Takara). Virion copies were tested at different times using real-time fluorescence quantitative PCR (qRT-PCR) (CFX96 Real-Time Detection System, Bio-Rad). Then, cDNA (0.2 μL) was amplified using Fast SYBR® Green Master Mix (Bio-Rad), and the forward/reverse primers for amplifying the cap of the DTMUV CQW1 strain were as follows: Fw: 5′-AGGTTTGTGCTGGCTCTAC-3′ and Rev: 5′-TGTTTGGT CGCCTCATT-3′.

2.10. Statistical analysis

All experiments were reproducible and performed in triplicate. The statistical analyses were carried out using Student's t-test. Data are presented as the mean ± standard deviation (SD). Asterisks (*) in the figures denote statistically significant differences (*, $P < 0.05$; ***, $p < 0.001$). All graphs were generated with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Expression of the Cap-SNase and Cap-Linker-SNase fusion proteins in BHK21 and DF-1 cells

To monitor whether the two fusion proteins were expressed in BHK21 and DF-1 cells, we used Western blot and immunofluorescence assays (IFAs). The Cap-SNase and Cap-Linker-SNase fusion proteins were readily detected as predicted at approximately 30–32 kDa, but differences in their expression levels were not obvious. Neither the negative control nor the BHK21- or DF-1-transfected pcDNA3.1(+) plasmid groups expressed the target protein (β-actin is shown as a reference gene) (Fig. 2A). Accordingly, protein expression was verified by immunostaining, via which we observed cells with green fluorescence in the fusion protein groups but not in the pcDNA3.1(+) group (Fig. 2B), consistent with the Western blot results. Cap-SNase and Cap-Linker-SNase were primarily expressed in the cytoplasm. These data demonstrate that fusion protein expression occurs in BHK21 and DF-1 cells but does not cause distinct differences.

3.2. Nuclease activity of Cap-SNase and Cap-Linker-SNase in vitro

Determining the nuclease activity of the two fusion proteins is pivotal for characterizing their antiviral effects, and we collected plasmid-transfected cells to perform this experiment. Unlike other nucleases, SNase is thermoduric and remains active even when boiled at 100°C, which typically eliminates other cellular nucleases. In addition, the potential nuclease activity of SNase depends on the Ca^{2+} concentration. When Ca^{2+} is available at millimolar levels, SNase is active. The nuclease activity assay of the fusion proteins was examined by

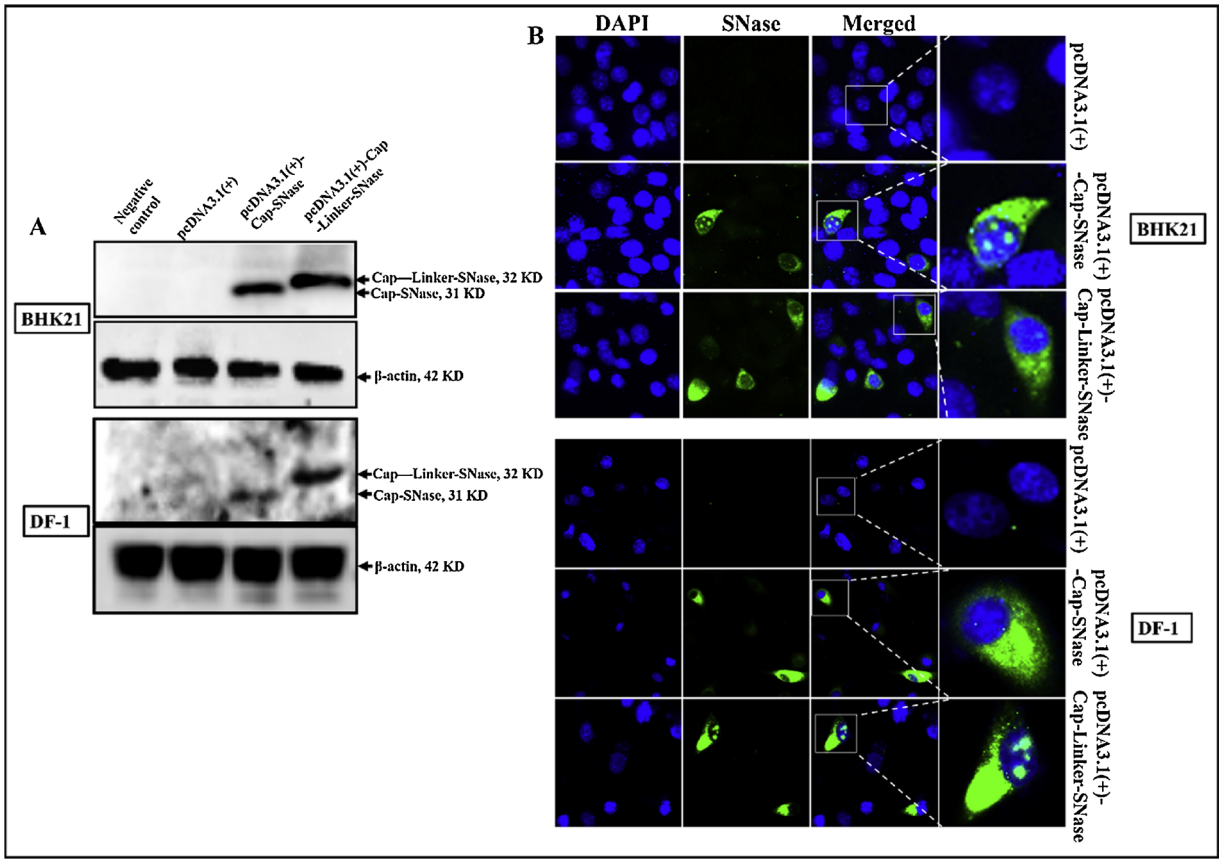

Fig. 2. Expression analysis of the Cap-SNase and Cap-Linker-SNase proteins by Western blot and indirect immunofluorescence. A. Protein lysates from BHK21 and DF-1 cells transfected with the pcDNA3.1(+), pcDNA3.1(+)-Cap-SNase, or pcDNA3.1(+)-Cap-Linker-SNase plasmids; BHK21 and DF-1 cells lacking plasmid transfection served as mocks. In the Western blot assay, mouse anti-SNase serum was used as the primary antibody, and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) was used as the secondary antibody. β-actin was used as a reference gene. **B.** At 48 h post transfection, cells were processed for the direct immunofluorescence assay using the anti-SNase mouse polyclonal antibody and the FITC-tagged (green fluorescence) goat anti-mouse IgG secondary antibody. Nuclear DAPI staining is shown in blue.

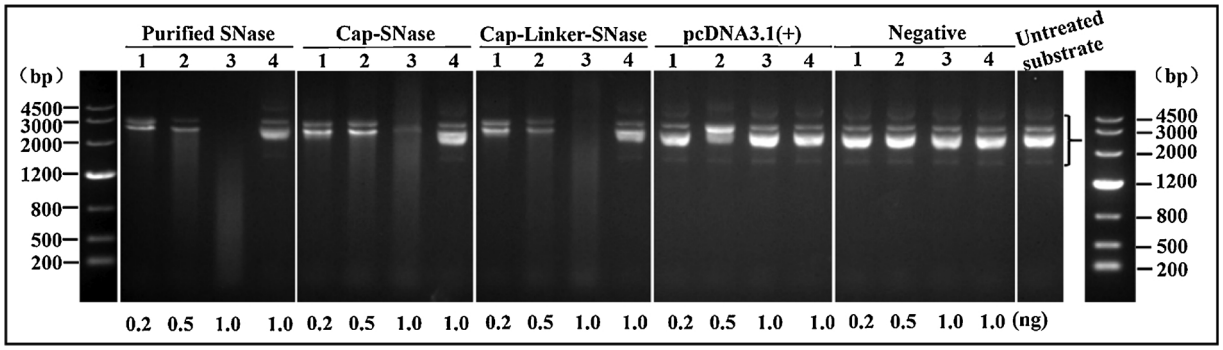

Fig. 3. Nuclease activity analysis of the Cap-SNase and Cap-Linker- SNase proteins by the DNA digestion assay. Different concentrations (0.2, 0.5, 1.0 ng) of samples in lanes 1, 2, 3, and 4 were mixed with the substrate plasmid pcDNA3.1(+). Additionally, 0.5 M EDTA was used to quench the calcium ions (lane 4). Purified SNase protein served as the positive control. The reaction mixture was incubated in SNase assay buffer at 37℃ for 30 min. Plasmid DNA contained covalently closed circles, open circles, and linear DNA (indicated by a curly bracket). The reaction mixtures were electrophoresed on 1% agarose gels.

plasmid DNA degradation analysis. The purified and expressed prokaryotic SNase protein exhibited the best plasmid DNA digestion ability, but remarkably, the Cap-SNase and Cap-Linker-SNase groups also exhibited plasmid DNA digestion abilities. Furthermore, degradation was enhanced as the protein concentrations increased (lanes 1, 2, 3). However, after using EDTA to chelate Ca^{2+}, DNA digestion was prohibited (lane 4), and plasmid DNA degradation was undetected in the pcDNA3.1(+) group at all of the concentrations tested (Fig. 3). These results demonstrate that the expressed Cap-SNase and Cap-Linker-SNase constructs possess Ca^{2+}-dependent nuclease activity.

3.3. Cytotoxicity assessment of Cap-SNase and Cap-Linker-SNase in BHK21 cells

These fusion proteins were shown to be expressed and to have nuclease activity, and whether this activity affects BHK21 cells in the form of cytotoxicity was examined next. Before performing subsequent experiments, we tested whether the fusion proteins exerted cytotoxicity in BHK21 cells using the MTT assay. BHK21 cells were observed every day after transfection, but no differences were detected between the control and experimental groups (including pcDNA3.1(+), pcDNA3.1(+)-Cap-

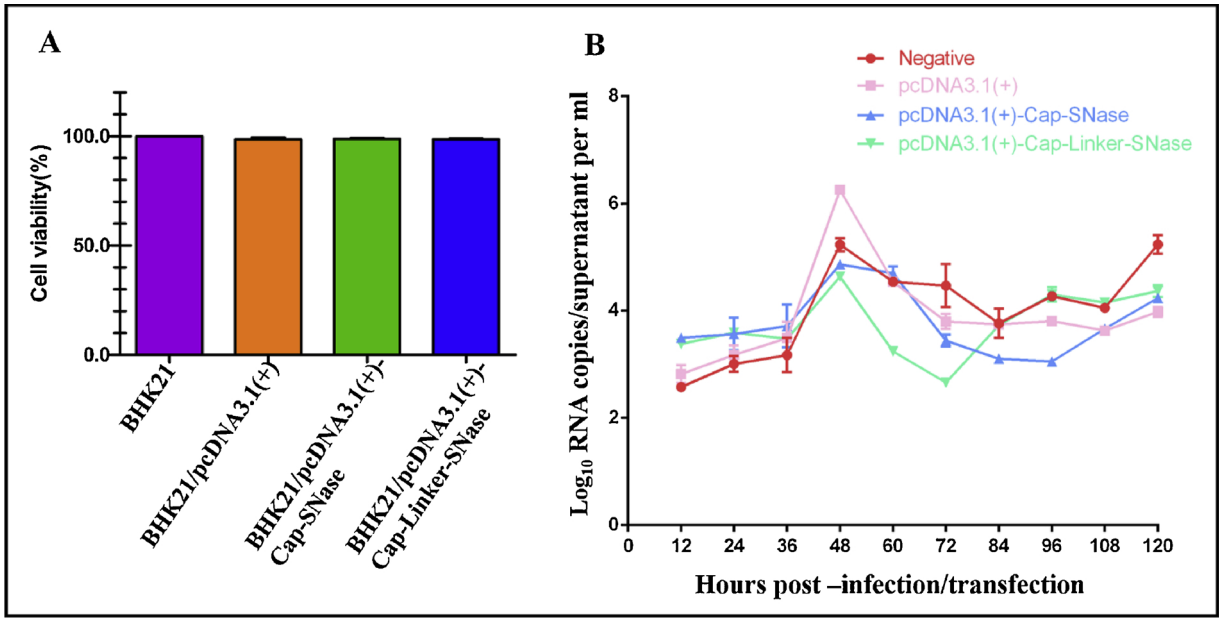

Fig. 4. Cytotoxicity analysis by the MTT assay and detection of viral copies by RT-qPCR. A. Cytotoxicity of the fusion proteins Cap-SNase and Cap-Linker-SNase in susceptible BHK21 cells (mean ± SD, n = 3). No significant differences were observed between the control and experimental groups. **B.** Culture supernatants were collected at 12, 24, 36, 48, and 72 h after infection and transfection (mean ± SD, n = 3) and assayed by real-time fluorescence quantitative PCR (qRT-PCR). The dotted line indicates the detection limit. A *P*-value < 0.05 was considered statistically significant (*, $P < 0.05$; **; $P < 0.01$; ***; $P < 0.001$). Error bars indicate standard deviations.

SNase, pcDNA3.1(+)-Cap-Linker-SNase). No significant differences in cell viability were observed between the control and plasmid-transfected groups ($P = 0.1910$, $P = 0.0602$, and $P = 0.0753$, respectively; mean ± SD, n = 3) (Fig. 4A). Taken together, these data suggest that the target proteins were not cytotoxic and were tolerated by BHK21 cells.

3.4. Antiviral effect of Cap-SNase and Cap-Linker-SNase

Previous experimental results encouraged us to further explore the antiviral capacity of the fusion proteins. Whether the fusion proteins expressed by the cells possess antiviral activity primarily depends on the SNase activity, but expressed SNase was activated in response to extracellular millimolar levels of Ca^{2+}. Thus, antiviral capacity was tested in the extracellular environment, and culture supernatants were collected at the indicated time points. The viral RNA levels in the tested samples were determined by RT-PCR. While the RNA copy numbers in the experimental groups (Cap-SNase and Cap-Linker-SNase) did not obviously differ from those in the control groups at 12, 24, 36 h, the viral RNA levels were higher in the Cap-SNase and Cap-Linker-SNase groups than in the controls. However, from 48 h to 72 h, the viral RNA levels of Cap-Linker-SNase were markedly reduced compared to those of the other groups (Fig. 4B). A *P*-value < 0.05 was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

4. Discussion

CTVI is a novel protein-based antiviral strategy that has been successfully applied to several types of viral species. Compared to other RNA-based antiviral methods, CTVI has several advantages, such as the effector of the fusion protein, which can efficiently and specifically destroy viral components from within. Exogenous effector molecules in this strategy are primarily nucleases, lipases, proteases, and scAbs. The expressed SNase is integrated into the virus as a structural segment of the viral fusion protein upon replication in cells. SNase is a Ca^{2+}-dependent nuclease that requires millimolar levels of Ca2+ to destroy DNA/RNA, whereas 0.5–1.0 millimolar Ca^{2+} facilitates the optimal degradation competence of SNase (Qin, 2005; Wang and QIU, 2007). In most cells, Ca^{2+} is available at approximately nanomolar concentrations, and SNase thus does not exert cytotoxicity on these cells. However, when the virion containing SNase is released into the extracellular space via exocytosis, wherein Ca^{2+} exists at millimolar levels, SNase recovers its capacity to destroy the virion DNA/RNA, resulting in loss of viral infectivity (Zhang et al., 2016). Theoretically, a high viral inactivation efficiency is obtainable despite that only one SNase is synchronously assembled into the virion (Qin et al., 2005), and SNase may thus be an appropriate candidate for CTVI in which a viral structural protein-nuclease fusion protein is targeted for virion inactivation using nuclease activity as the major antiviral mechanism. In this strategy, the cap protein acts as the carrier, which also efficiently prohibits the formation of immune escape mutants (Qin, 2005; Zhou et al., 2010) because the C-terminal portion of the cap protein, which is rich in positively charged arginine and lysine residues, can combine with the negatively charged viral RNA to initiate packaging of the nucleocapsid, indicating that the cap is indispensable for both viral replication and assembly.

The most important results from our studies are presented herein. The antiviral effect of the two fusion proteins studied is also exhibited by other flaviviruses, such as DENV and JEV, and the antiviral effects of CTVI are more ideal (Pang et al., 2013; Qin et al., 2005; Qin and Qin, 2006). The expression, nuclease activity and antiviral effects of Cap-Linker-SNase were comparable or superior to those of Cap-SNase, which we believe was primarily attributable to the linker (15 amino acids $(G_4S)_3$) between the Cap and SNase, which advantageously facilitates two components of fusion protein formation, generation of the correct spatial structure and promotion of better biological activity, but does not affect the respective functions of the target protein. In addition, the antiviral mechanism involves viral genome integration of the exogenous gene together with the fusion gene Cap upon viral replication in cells. However, recombinant plasmids are randomly incorporated into the cellular genome when transfected, but the probability is very low, with most of the incorporations being achieved via nonhomologous recombination, leading to unstable expression of the

desired gene. Thus, the fusion proteins transiently expressed in cells are barely incorporated into the viral genome, resulting in an indistinctive antiviral effect. IFAs showed that the fusion proteins were expressed in the cytoplasm, wherein flavivirus replication and protein synthesis also occur, thus contributing to their use upon viral capsid synthesis. The capsid protein together with the SNase is a packaging component of virus assembly and facilitates internal contact between the genome and SNase to promote its degradation. In future studies, we plan to examine whether we can construct stable cell lines to express the fusion proteins, which may increase both the integration of SNase and the probability of fusion protein incorporation into virions.

Some studies have reported that positive TMUV serum antibodies have been detected in humans from Malaysia and in duck industry workers (Tang et al., 2013; Zhong et al., 2017), and DTMUV exhibits neurovirulence in mammals (Li et al., 2013). Most flaviviruses are zoonotic, meaning that they can be transmitted between animals and humans (Bai et al., 2013). As such, DTMUV may pose a substantial threat to humans in the future. CTVI has been successfully applied to dengue virus and JEV, and DTMUV is a flavivirus with a similar genomic structure, providing theoretical support for CTVI applications regarding DTMUV. Currently, only a few studies have been published concerning vaccines and drugs against DTMUV (Chen et al., 2014; Huang et al., 2018; Li et al., 2014; Ma et al., 2016; Wang et al., 2016), and these results may thus represent a promising method for the development of DTMUV therapy. However, if developed as a therapeutic, the drug delivery route and medication form should be considered. Experiments must be performed to assure that the drug is not passively removed. Initial studies remain in early stages, and many questions remain concerning the use of CTVI for flavivirus infections in the poultry industry. This strategy provides interesting new insight into flavivirus infection, but further experiments are needed for translation in the clinic.

Authors' contributions

Xingcui Zhang designed the experiment and wrote the paper; Renyong Jia conceived the study and designed the experiments; Yuhong Pan helped complete the experiments; Mingshu Wang, Shun Chen, Dekang Zhu, Mafeng Liu, Xinxin Zhao, Qiao Yang, Ying Wu, Shaqiu Zhang, Yunya Liu, Ling Zhang, Zhongqiong Yin, Bo Jing, Juan Huang, Bin Tian, Leichang Pan, Yanling Yu, and Mujeeb Ur Rehman guided the experiments, helped analyse the data and edited the English language in the manuscript; and Anchun Cheng was responsible for revising the manuscript critically for expert content. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors have no competing interests to declare.

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