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In vitro inhibition of vesicular stomatitis virus replication by purified porcine Mx1 protein fused to HIV-1 Tat protein transduction domain (PTD)

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ABSTRACT

Vesicular stomatitis virus (VSV) is the causative agent of Vesicular stomatitis (VS), a highly contagious fatal disease of human and pigs. Few effective antiviral drugs are currently available against VSV infection. Mx proteins are interferon (IFN)-induced dynamin-like GTPases present in all vertebrates with a range of antiviral activities. Previous studies have shown that the transfected cell lines expressing either porcine Mx1 or human MxA acquired a high degree of resistance to VSV. To explore the feasibility of taking porcine Mx1 protein expressed in *Escherichia coli* as an antiviral agent, we applied the pCold system to express this fusion protein (PTD-poMx1), which consisted of an N-terminal HIV-1 Tat protein transduction domain (PTD) and the full-length porcine Mx1, and investigated its effects on the replication of VSV in Vero cells. The results demonstrated that the purified PTD-poMx1 fusion proteins could transduct into cells after incubated for 5 h and had no cytotoxic. Furthermore, plaque reduction assay, determination of $TCID_{50}$, real-time PCR and Western blot analyses were carried out to confirm the antiviral activity of purified fusion proteins in VSV-infected Vero cells. Altogether, these data suggested that PTD-poMx1 fusion proteins might be applicable to inhibit VSV replication as a novel antiviral therapeutic agent.

1. Introduction

In the early phase of virus infection in vivo, type I interferon (IFN) is produced abundantly in the infected cells. Mx proteins are interferon (IFN)-induced dynamin-like GTPases present in all vertebrates, such as mammals, birds and fish (Chaleston and Stewart, 1993; Hailer and Kochs, 2002), with a range of antiviral activities. Murine Mx1 is a nuclear protein with specific activity against orthomyxoviruses, while human MxA is a cytoplasmic protein with a broad antiviral spectrum against many families of RNA viruses, including Orthomyxoviridae, Rhabdoviridae and Bunyaviridae (Haller et al., 2007). Porcine Mx cDNA was firstly isolated from German Landrace breed, and the existence of two Mx genes, Mx1and Mx2, was reported (Müller et al., 1992). The porcine Mx1 gene was mapped on chromosome 13 (Rettenberger et al., 1996) and the nucleotide length of the open reading frame (ORF) of the porcine Mx1 gene is 1992 bp. Moreover, polymorphism in porcine Mx1 exon 14 sequence was found (Morozumi et al., 2001). Thomas et al. (2006) recently identified the promoter region of the porcine Mx1 gene from PK15 cultured cells. The porcine Mx1 mRNA was expressed in cells infected with porcine reproductive and respiratory syndrome virus (PRRSV) (Zhang et al., 1999). Morozumi et al. (2009) firstly reported the structure of the intact porcine Mx2 gene having an open reading frame of 2136 bp. Moreover, a weak constitutive expression of porcine Mx2 mRNA and endogenous Mx2 protein were observed in interferon-untreated cells. However, assays using NIH3T3 cells transfected with Mx genes showed that porcine Mx2 possessed antiviral activity against influenza, although this activity was lower than that of human MxA.

Vesicular stomatitis virus (VSV) is an envelope, negativesense, RNA virus in the Rhabdoviridae family. VSV causes acute disease in a wide range of mammals including cattle, horses and pigs (Letchworth et al., 1999; Johnson et al., 1969). Infection in human is typically asymptomatic or results in a mild flu-like syndrome (Plakhov et al., 1995). The disease is notable because VS in cattle and pig is clinically indistinguishable from foot-and-mouth disease (FMD), which requires different control strategies and entails enormous economic loss (Rainwater-Lovett et al., 2007). Human MxA inhibits primary transcription of the VSV genome by the parental viral transcriptase in transfected mouse 3T3 and U937 cells (Schnorr et al., 1993; Staeheli and Pavlovic, 1991). The porcine Mx1 protein has been observed to accumulate in the cytoplasm (Horisberger and Gunst, 1991) and shows antiviral activity against VSV

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(Asano et al., 2002). But, in the previous studies, when porcine Mx1 or human MxA protein was expressed in the cytoplasm of stably transfected cell lines, they all interfered with the accumulation of VSV viral RNA and proteins. Therefore, the purpose of this study was to applied the prokaryotic expression system to produce porcine Mx1 as clinical antiviral agent against VSV infection. In the present study, we applied the pCold I vector and successfully expressed fusion protein in *Escherichia coli* cells, then purified them in a good yield. The purified porcine Mx1 fusion proteins had good antiviral activity against VSV according to the results of plaque reduction assay, determination of $TCID_{50,}$ real-time PCR and Western blot analysis in VSV-infected Vero cells.

2. Materials and methods

2.1. Construction of pCold-PTD-poMx1 vector

For construction of the vector expressing the fusion proteins, a pair of specific primers (P1: 5′-AAG CTC GAG TAT GGT CGT AAA AAG CGT CGT CAG CGT CGT CGT GTT TAT TCC AGC TGT G-3′; P2: 5′-GAG AAG CTT TCA GCC TGG GAA CTT GGC GA-3′) was designed and used to amplify the PTD-poMx1 gene from the plasmid pT-poMx1, which included an N-terminal HIV-1 Tat protein transduction domain (PTD) (Vives et al., 1977) and porcine Mx1, attached with Xho I and Hind III sites, respectively (Fig. 1A). The 2025 bp PTD-poMx1 fragment was cloned into the pMD18-T vector (Takara Bio., Dalian, China) by the T/A cloning strategy and subcloned into pCold I between the Xho I and Hind III sites.

2.2. Expression and purification of PTD-poMx1 fusion proteins

Rosetta2 (DE3) cells (Novagen, Shanghai, China) transformed with pCold-PTD-poMx1 were cultured in LB medium supplemented with 100 μg/ml ampicillin, grew at 37 °C until the logarithmic phase (at OD_{600} of 0.6) and induced by Isopropyl-thio-galactoside (IPTG) at a final concentration of 0.1 mM for 24 h at 15 °C. The re-suspended cells were lysed by sonication on ice for 8 × 1 min with 30-s intervals. The lysate was centrifuged at 10,000*g* for 15 min, and the supernatant and the pellet resuspended in PBS were both analyzed by SDS–PAGE to observe the solubility of target protein. The fusion proteins in the inclusion bodies were denatured (8 M urea overnight) and remained in the supernatant after centrifugation. His-tagged PTD-poMx1 fusion proteins were bound to Ni–NTA resin (Qiagen, Hilden, Germany) and purified following the manufacturer's instructions (Fig. 2A). Finally, freshly purified proteins were treated with Detoxi-GelTM Endotoxin Removing Gel (Thermo, USA) to remove endotoxin derived from bacterial culture in the fusion proteins. Aliquots of purified protein were stored at −80 °C.

2.3. Western blot analysis

An equal volume of samples were boiled for 5 min and proteins were separated by 12% SDS–PAGE, and transferred to PolyScreen PVDF transfer membrane (PALL, USA) by a semi-dry transfer method (Maniatis et al., 1989). The membranes were blocked with TBST buffer (10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20) containing 5% skim milk over night, then incubated with anti-poMx1 mAb (Abcam, USA) or anti-his mAb (Cell Signaling Technology) for 2 h, and followed by the addition of goat anti mouse IgG HRP conjugate antibody (Santa Cruze, Canada) for 1 h and detection was performed using enhanced chemiluminescence (ECL; GE Healthcare Life Sciences).

2.4. Refolding of PTD-poMx1 fusion proteins

Refolding of PTD-Mx1 fusion proteins was achieved by dialysis in PBS against urea concentration gradient which was 8, 6, 4, 2, 0 M Urea. The dialysis buffer was changed every 3 h and the whole process should be carried out at low temperatures to prevent protein degradation. Protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard. PTD-Mx1 fusion proteins were stored at −80 °C.

2.5. Transduction of fusion proteins

Protein transduction domain (PTD) derived from HIV Tat protein was shown to efficiently deliver macromolecular cargo in various cell types (Schwarze et al., 1999; Wheeler et al., 2003). For

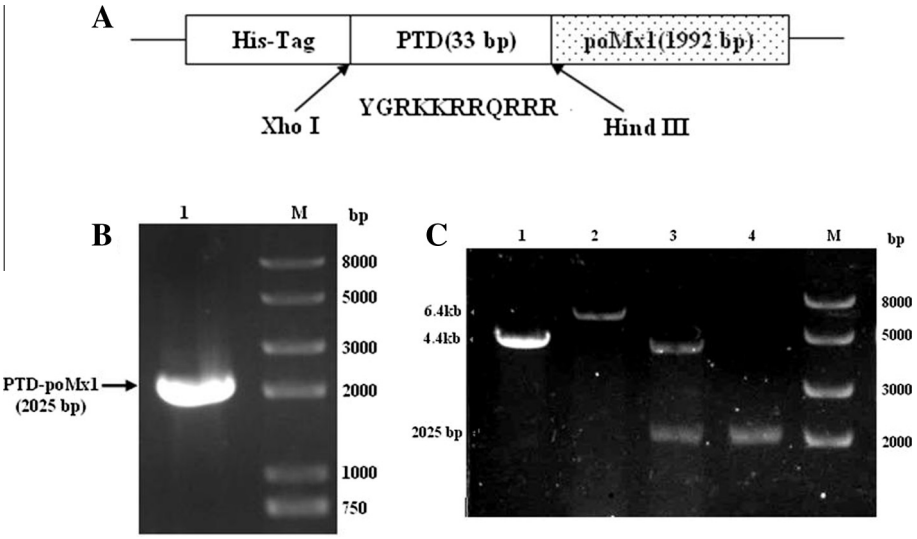

Fig. 1. Construction of the recombinant protein. (A) Schematic diagram of the constructs encoding PTD-poMx1. PTD, protein transduction domain of HIV-1 Tat; poMx1, porcine interferon-induced antiviral protein; His-tag, 6× His-tag. (B) Agarose gel electrophoresis (1%) confirming amplification of the full-length PTD-poMx1 gene (lane 1). (C) Agarose gel electrophoresis (1%) confirming construction of the recombinant plasmid. Lane 1: control pCold I plasmid digested by Xho I and Hind III; Lane 2: recombinant plasmid digested by Hind III; Lane 3: recombinant plasmid digested by Xho I and Hind III; Lane 4: the PTD-poMx1 gene of recombinant plasmid amplified by PCR. M: Marker DL8000 (TaKaRa).

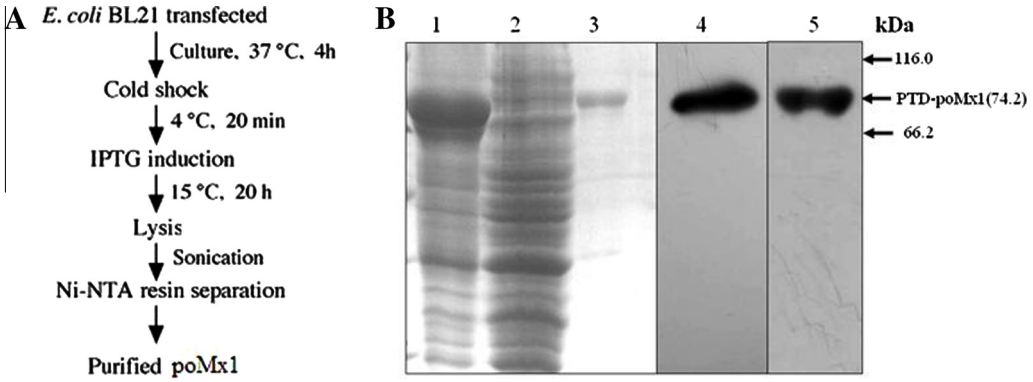

Fig. 2. Procedure for PTD-poMx1 preparation using the pCold vector. (A) Scheme for the PTD-poMx1 preparation. The expressed PTD-poMx1 in *E. coli* cells were purified by Ni^{2+}–NTA resin chromatography. (B) Expression of PTD-poMx1 fusion proteins by SDS–PAGE and Western blot analyses. lane 1, whole cells induced with IPTG; lane 2, control cells induced with IPTG; lane 3, PTD-poMx1 fusion proteins after Ni^{2+}–NTA resin separation; lane 4, Western blot analysis of PTD-poMx1 fusion proteins probed by anti-poMx1 mAb; lane 5, Western blot analysis of PTD-poMx1 fusion proteins probed by anti-his mAb. The proteins were transferred to PVDF membrane and detected with anti-poMx1 mAb and anti-his mAb followed by HRP-conjugated goat anti-mouse IgG. Molecular size of the protein is indicated on the left.

determination of protein transduction efficiency, PTD-poMx1 internalization was assessed by Western blot analysis. Vero cells (ATCC/CCL-81) were cultured as exponentially growing subconfluent monolayers on 6-well plates in DMEM (Gibco) with 10% fetal bovine serum (FBS, Invitrogen). Vero cells were chosen because their genetic defect in type I IFN production prevents activation of endogenous type I IFN-dependent effectors upon infection, thus allowing an artefact-free functional evaluation of exogenous Mx proteins (Emeny and Morgan, 1979). PTD-poMx1 fusion proteins were added to the cells at a concentration of 80 μg/ml (Vives et al., 1994). Every hour from 1 to 6 and 12, 24 h, cells in a well of plate were washed three times with PBS and lysed in pre-cold lysis buffer (1% Triton X-100, 1 mM PMSF in PBS) for 10 min. Lysates were clarified by centrifugation at 12,000*g* for 10 min. Whole cell extracts were separated by SDS–PAGE and transferred onto PVDF membrane. PTD fused to poMx1 protein translocated through the plasma membrane and accumulated in cells, which was detected by anti-poMx1 mAb with Western blot analysis performed as described above. To investigate the stability of PTD-poMx1 in Vero cells, another Western blot analysis was carried out as described previously. Briefly, PTD-poMx1 fusion proteins were added to the cells at a concentration of 80 μg/ml for 6 h. After washing, cells in DMEM free from PTD-poMx1 were incubated for 4, 8, 12, 16, 20 h, respectively. Intracellular PTD-poMx1 in the cell lysates were detected by anti-poMx1 mAb.

2.6. Fluorescence microscopy

Vero cells were treated with PTD-poMx1 as described above. The cells were fixed in acetone for 15 min at −20 °C and washed three times with PBS (pH 7.4). Then anti-poMx1 mAb was applied for 1.5 h at 37 °C. After washing three times with PBS for 5 min, respectively, the cells were incubated with FITC-goat anti-mouse IgG (Pierce) for half an hour at 37 °C. The cells of fluorescent spots were observed under fluorescence microscopy equipped with a digital camera. To better understand the poMx1-mediated antiviral action, VSV receptor integrity at the cell surface was examined after treatment of PTD-poMx1. Briefly, Vero cells treated with or without PTD-poMx1 were incubated with VSV for 1 h on ice. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained for VSV with anti-VSV-G antibody (GenScript, Nanjing, China) and FITC-labeled goat anti-rabbit IgG (Santa Cruze). Cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Images were recorded with a confocal microscope (Leica Sp5 AOBS confocal system) using a 63× HCX PL Apo 1.4 oil immersion objective.

2.7. MTT assay

Cell viability following PTD-poMx1 treatment was determined by MTT assay (in triplicates) (Yoo et al., 2009). Vero cells seeded into a 96-well plate were incubated with different concentrations of PTD-poMx1 fusion proteins for 72 h. Cells were then maintained in DMEM containing 10% FBS for 48 h. Thereafter, 50 μl of MTT (Sigma–Aldrich) solution (2 mg/ml) was added to the culture medium and plates were incubated for 4 h at 37 °C. Formazan crystals were dissolved in 500 μl dimethyl sulfoxide (Sigma–Aldrich), and the values of OD_{560} were detected by ELx800 (Bio-TEK). Results were expressed relative to the optical density of wells containing untreated control cells, defined as 100% viability.

2.8. GTPase activity of PTD-poMx1 fusion proteins

The Mx1-associated GTPase activity after internalization of PTD-poMx1 fusion proteins in Vero cells was performed by QuantiChrom™ ATPase/GTPase Assay Kit (BioAssay Systems, USA) according to the manufacturer's instructions. Briefly, PTD-poMx1 fusion proteins were added to the Vero cells at a concentration of 80 μg/ml. After being incubated for 6, 12, 24 h, cells were washed three times with ddH_2O, and then the re-suspended cells were lysed by sonication on ice for 1 min with 2-s intervals. The lysate was centrifuged at 10,000*g* for 15 min, and the GTPase activity of the supernatant was performed (untreated Vero cells as negative control).

2.9. Antiviral activity

2.9.1. Virus plaque assay

Interference of PTD-poMx1 fusion proteins with the replication of VSV was proved by virus plaque assay in Vero cells. Confluent Vero cells in 6-well plates were incubated for 6 h in DMEM with 160, 80 and 20 μg/ml proteins respectively before infection. After washes twice, the cells were incubated with 100 $TCID_{50}$ VSV (Indiana serotype) for 1 h. At the end of adsorption, the supernatant of each well was removed, and then each well was overlaid with 1.5 ml of 1% molten agarose (Sigma–Aldrich, USA) in DMEM containing 2% FBS, and the plates were incubated for 72 h at 37 °C. At the end of incubation period monolayers were fixed with formalin, then the agarose was gently removed and the cells were

stained using 1% crystal violet. Three independent observers counted the plaques using a hand lens. All the experiments were run in triplicates and plaques were counted.

2.9.2. Virus yield reduction assay

Virus yield reduction assay was carried out as described previously (Li et al., 2012).Briefly, Vero cells were seeded to a confluent monolayer in a 96-well plate and then infected with 100 $TCID_{50}$ VSV for 1 h at 37 °C. At the end of adsorption, the supernatant of each well was removed, Vero cells were incubated in DMEM with 80 μg/ml PTD-poMx1. A 50% tissue culture infective dose test ($TCID_{50}$) was performed to detect the virus in the supernatant every day after infection. Infectious titers were calculated according to the method of Reed and Muench (1938). In addition, to quantify the reduction of VSV G protein, Western blot analysis was carried out to investigate the antiviral activity of poMx1. Briefly, 15 μg of protein from each sample was separated by 12% SDS–PAGE and transferred by electro blotting onto PVDF membrane. The membrane was then treated sequentially with blocking solution (PBST containing 5% non-fat skim milk), then probed with rabbit anti-VSV-G polyclonal antibody (GenScript, Nanjing, China), or anti-β-actin antibody (Santa Cruz) as described above. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Cell Signaling), and visualized using enhanced chemiluminescence.

2.9.3. Real-time PCR

To quantify intracellular VSV RNA in PTD-poMx1-treated and untreated Vero cells, real-time PCR was carried out using a pair of primers for amplifying the VSV gene. At 24, 48, 72 h post-infection (hpi), viral RNA was extracted from each well by using TRIzol reagent. RNA pellets were suspended in 25 μl DEPC-treated water and a reverse transcription (RT) reaction was performed by utilizing an RT reaction kit (Takara, Dalian, China). Two pairs of specific primers were designed to amplify VSV G gene and β-actin gene of Vero cell by utilizing the DNAMan software, respectively (VSV-G-1F:5′-CGGAGGATTGACGACTAATGC-3′, VSV-G-2R:5′-ACCATCCGAGCCATTCGA-3′; β-actin-1F:5′-TCTTCCAACCTTCCTTCCT-3′, β-actin-2R (5′-GTCCACGTCACACTTCAT-3′), real-time PCR was performed by using SYBRGreen PCR master mix (Takara, Dalian, China), 0.25 mM each primer and 20 μl of the RT reaction, following the manufacturer's protocol (Applied Biosystems). These two segments were cloned into pMD-18T vector to construct the standard plasmid respectively, in order to obtain a relative standard curve. Relative quantification of VSV RNA relates the PCR signal of the target transcript in a treated group to that of another sample such as an untreated control. The $2^{-\Delta\Delta Ct}$ method was a convenient way to analyze the relative changes in gene expression in real-time quantitative PCR experiments (Livak and Schmittgen, 2001; Pang et al., 2013). The results of $2^{-\Delta\Delta Ct}$ showed the difference between treated and untreated cells.

$$\Delta\Delta Ct = \text{Vero/PTD} - \text{poMx1}(Ct_{VSV} - Ct_{\beta-\text{actin}}) - \text{control Vero}(Ct_{VSV} - Ct_{\beta-\text{actin}})$$

2.10. Statistics

All data were presented as means ± standard deviation (S.D.) for the number of experiments indicated. The ANOVA (*F* test) was used to determine the significance of differences between treated and control groups. A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Construction of the prokaryotic expression plasmid

PTD-poMx1 gene was successfully amplified from the plasmid pT-poMx1 that contained the full-length poMx1 segment (Fig. 1B). PCR products for PTD-poMx1 were digested with Xho I and Hind III, respectively and inserted into pCold-I expression vector digested with the same enzymes to yield the recombinant plasmid pCold-PTD-poMx1. Plasmid constructs were verified using restriction enzyme digestion, PCR and DNA sequencing (Fig. 1C).

3.2. Expression of fusion proteins

After incubation for 24 h at 15 °C, the cells were lysed in the presence of ADP and several protease inhibitors. The result of SDS–PAGE assay showed that PTD-poMx1 fusion proteins (74.2 kDa) were contained within the insoluble pellet fraction as inclusion bodies (Fig. 2B, lane 1). In contrast, noninduced recombinant vector showed the same electrophoresis results as control groups (Fig. 2B, lane 2).

3.3. Purification and refolding of fusion proteins

Complete solubilization of the inclusion bodies was achieved in urea buffer. Then, these fusion proteins were purified by Ni^{2+}–NTA resin under denaturing conditions and eluted in elution buffer with a good yield (Fig. 2B, lane 3). As shown in Fig. 2B (lanes 4 and 5), the 74.2 kDa protein band was confirmed as poMx1 by immunoblotting with anti-poMx1 mAb and anti-his mAb, respectively. These results strongly indicated that a large number of purified poMx1 proteins were achieved by prokaryotic expression system. To determine whether the PTD mediated fusion proteins were transducted into cells, Vero cells were incubated at a concentration of 80 μg/ml. Whole cell extracts from 1 h to 24 h were separated by SDS–PAGE and Western blot analyses. After adding PTD-poMx1 to the cells, live fluorescent cell images were observed under a fluorescence microscope. Remarkably, more and more fluorescent spots showed in the cells with the increasing incubated hours (Fig. 3A), indicating that the PTD-poMx1 fusion proteins could efficiently penetrate cell membranes within a few hours and localize in the cytoplasm. At the same time, as shown in Fig. 3B, anti-poMx1 mAb could probe the fusion proteins in the cells incubated from 5 h to 24 h. The results indicated that the fusion proteins began to enter into cells after at least 5 h. Furthermore, after washing, the stability of PTD-poMx1 fusion proteins in Vero cells was examined using anti-poMx1 mAb. As shown in Fig. 3C, the results indicated that intracellular PTD-poMx1 in the cell lysates were detected within 12 h, but not since 16 h. It was suggested that the fusion proteins could be maintained within 12 h in cells. We next assessed the cytotoxicity of the PTD-poMx1 fusion proteins using MTT assay. Vero cells were treated with increasing concentrations of PTD-poMx1 for 72 h. As shown in Fig. 3D, PTD-poMx1 showed no significant cytotoxicity up to a concentration of 80 μg/ml. Cell viability, however, decreased by approximately 7% and 12% at a concentration of 160 and 320 μg/ml, respectively. Finally, the Mx1-associated GTPase activity after internalization of PTD-poMx1 fusion proteins in Vero cells was determined by QuantiChrom™ ATPase/GTPase Assay Kit. After being incubated from 6 h to 24 h, with specific GTPase activities varying from 3.15 to 9.51 U/L, more significant activity in the lysate of Vero cells (PTD-poMx1) was observed following 80 μg/ml, as compared to control Vero cells (Fig. 3E, *p* value <0.05), suggesting that preparations of PTD-poMx1 showed detectable GTPase activity.

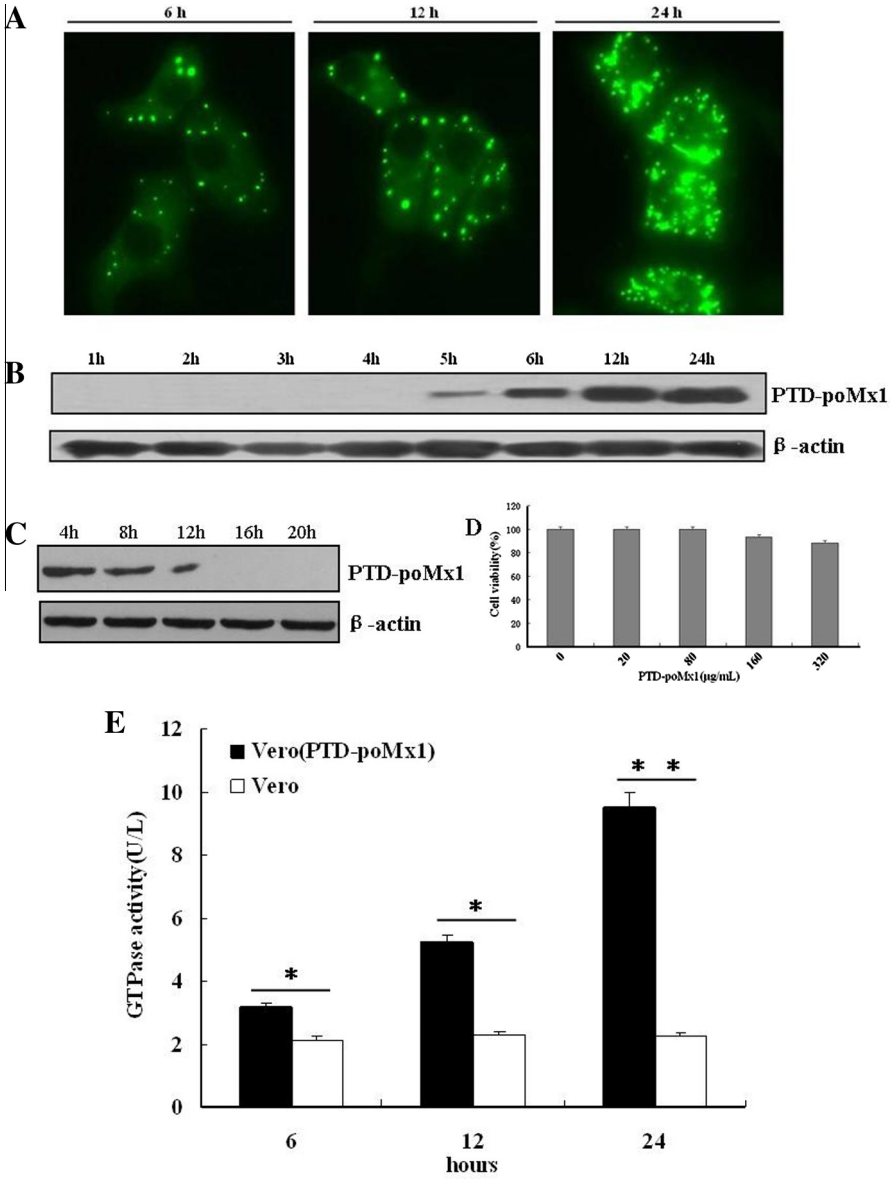

Fig. 3. Internalization of PTD-poMx1 fusion proteins in Vero cells. (A) Indirect immunofluorescence assay (IFA) analysis of intracellular localization of PTD-poMx1 fusion proteins in Vero cells. 6 h after transduction, the fluorescence images were captured under the fluorescence microscope (400×). Moreover, more and more fluorescent spots showed in the cells with the increasing incubated hours, indicating that the PTD-poMx1 fusion proteins could efficiently penetrate cellmembranes within a few hours and localize in the cytoplasm. (B) The transduction kinetics of fusion proteins was determined using anti-poMx1 mAb by Western blot analysis. The results showed that PTD-poMx1 fusion proteins began to transduct into cells after incubated for 5 h. More and more fusion proteins entryed into Vero cells with the increasing incubated hours. (C) The stability of PTD-poMx1 fusion proteins in Vero cells was determined using anti-poMx1 mAb by Western blot analysis. The results showed that intracellular PTD-poMx1 in the cell lysates were detected within 12 h, but not since 16 h. It was suggested that the fusion proteins could be maintained within 12 h in cells. (D) Cytotoxicity of PTD-poMx fusion proteins. Vero cells in a 96-well plates were treated with various concentrations of PTD-poMx fusion proteins for 72 h. Three independent MTT assays were performed in triplicate. Data are mean (% of control mean) ± S.D. of three independent experiments. (E) GTPase activity of PTD-poMx fusion proteins. The data showed that more significant activity in the lysate of Vero cells (PTD-poMx1) was observed following 80 μg/ml, as compared to control Vero cells after incubated from 6 to 24 h, suggesting that preparations of PTD-poMx1 showed detectable GTPase activity.

3.4. Antiviral activity of PTD-poMx1 fusion proteins

To assess whether PTD-poMx1 fusion proteins had any effect on reduction of viral load *in vitro*, Vero cells, which were incubated with 160, 80 and 20 μg/ml fusion proteins, respectively, were subjected to plaque assay as described above. It was shown obviously the number of plaque was dramatically reduced in PTD-poMx1-treated cells. Antiviral activity of high concentration (160 μg/ml) presented more protection than other two concentrations (80 and 20 μg/ml) and DMEM control (Fig. 4A). At 2 dpi, more significant reduction in viral titer was observed following 160 and 80 μg/ml, as compared to DMEM (p value <0.01), however, significant reduction in viral titer was observed following 20 μg/ml, as compared to DMEM (p value <0.05) (Fig. 4B). In addition, to quantitatively compare the antiviral activity of fusion proteins against VSV, the supernatants of the above 80 μg/ml-treated and untreated virus-infected cell cultures were collected at different time points for VSV titer in Vero cells. The results showed that from 2 to 5 dpi, the mean of $TCID_{50}$ values produced by PTD-poMx1-treated infected cells was mean $10^{1.5}$ times lower than that of untreated infected cell (p value <0.05) (Fig. 4C).

The ability of inhibiting VSV replication in PTD-poMx1-treated cells was determined by real-time PCR. Vero cells were treated as described above. Relative quantification was performed using

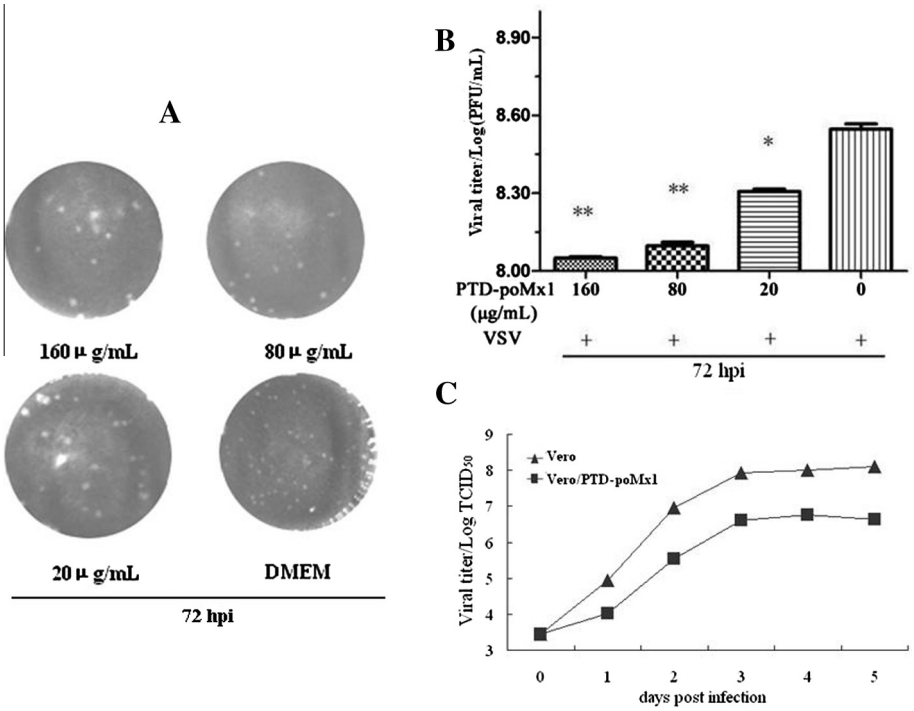

Fig. 4. Inhibition of virus plaque formatuon by PTD-poMx fusion proteins. (A) Plaque assay to subject the effect on reduction of viral load in vivo due to various concentrations of PTD-poMx fusion proteins (160, 80 and 20 μg/ml), and DMEM-treated cells were as positive control.The results showed that antiviral activity rised with the increasing of protein concentration. (B) Plaque forming units were counted in VSV-infected cells treated with different concentration of PTD-poMx1 fusion proteins, p value <0.05 (*) and p value <0.001 (**) compared to controls(DMEM). (C) The antiviral effect of PTD-poMx1 fusion proteins in Vero cells was evaluated by the growth curves of VSV from control Vero cells (triangles) and PTD-poMx1-treated cells (squares). The infectious titers from culture supernatants were titrated on Vero cells using a 50% tissue culture infective dose ($TCID_{50}$) test. The results showed the differences between two groups were significant after 2 dpi.

β-actin as endogenous control gene. These results clearly demonstrated significant changes between 80 μg/ml-treated and untreated cells at three different periods (24, 48 and 72 h). At 24 hpi, the copy numbers of viral genome in treated cells were dramatically decreased compared with that of untreated cells (Fig. 5A, p value <0.01). Then, at 48 and 72 hpi, it were significantly less, respectively (Fig. 5A, p value <0.05). These results illustrated that PTD-poMx1 fusion proteins had the exactly antiviral activity *in vitro*. To further validate the results obtained from real-time PCR, Western blot analysis was performed as described above. Viral envelope (G) protein level also decreased significantly at both 48 and 72 hpi (Fig. 5B) due to the reduction of viral load. Altogether, these results demonstrated that PTD-poMx1 fusion proteins effectively suppressed VSV proliferation by decreasing the levels of both viral RNA and protein.

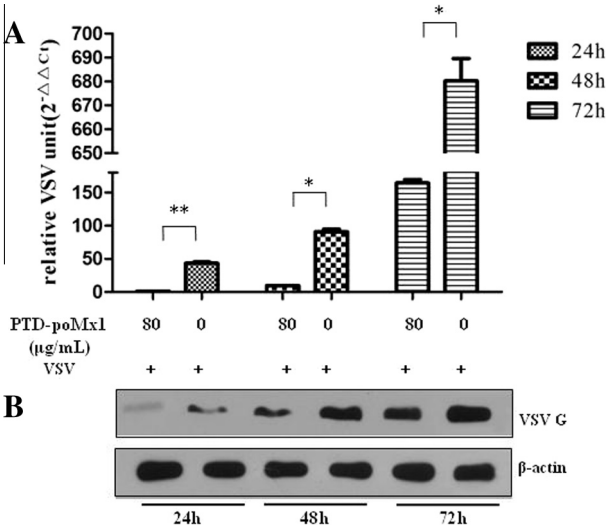

Fig. 5. Reduction of intracellular viral genome and protein levels by the inhibition of PTD-poMx1 fusion proteins. (A) Inhibition of VSV mRNA expression in Vero cells was determinded by real-time PCR. At 24 hpi, inhibition of virus proliferation was oberversed more significant (p value <0.01). At 48 and 72 hpi, inhibition of virus proliferation were considered significant (p value <0.05). The real-time PCR was performed with triplicates and data shown are the mean ± S.D. of three independent experiments. (B) Levels of d envelope (G) proteins were determined by immunoblotting cell lysates with antibodies specific for the indicated protein. An anti-β-actin antibody was used as an internal loading control. The difference of G protein bands in 24, 48 and 72 hpi could be relatively distinguish, respectively.

3.5. PTD-poMx1 did not affect VSV cell entry

We evaluated the effect of commonly used PTD-Mx1 on VSV cell entry by a confocal microscope. As shown in Fig. 6A, no matter whether Vero cells were treated with or without PTD-poMx1, the fluorescence spots of VSV in cell surface were both detected, suggesting PTD-Mx1 fusion proteins did not affect virus binding, uptake, and virus- membrane interaction. Two similar images clearly demonstrated VSV receptor integrity at the cell surface after treatment of PTD-poMx1 (Fig. 6B). It was suggested that PTD-poMx1 treatment would not affect VSV cell entry, resulting in reduction of receptors concentration for VSV infection.

4. Discussion

From previous studies of transfected cells expressing poMx1 it is clear that this protein can confer resistance to VSV (Asano et al., 2002) and Influenza virus (Nakajima et al., 2007; Palm et al., 2010), which suggests that poMx1 is an important mediator of the IFN-induced antiviral state. The application of this antiviral

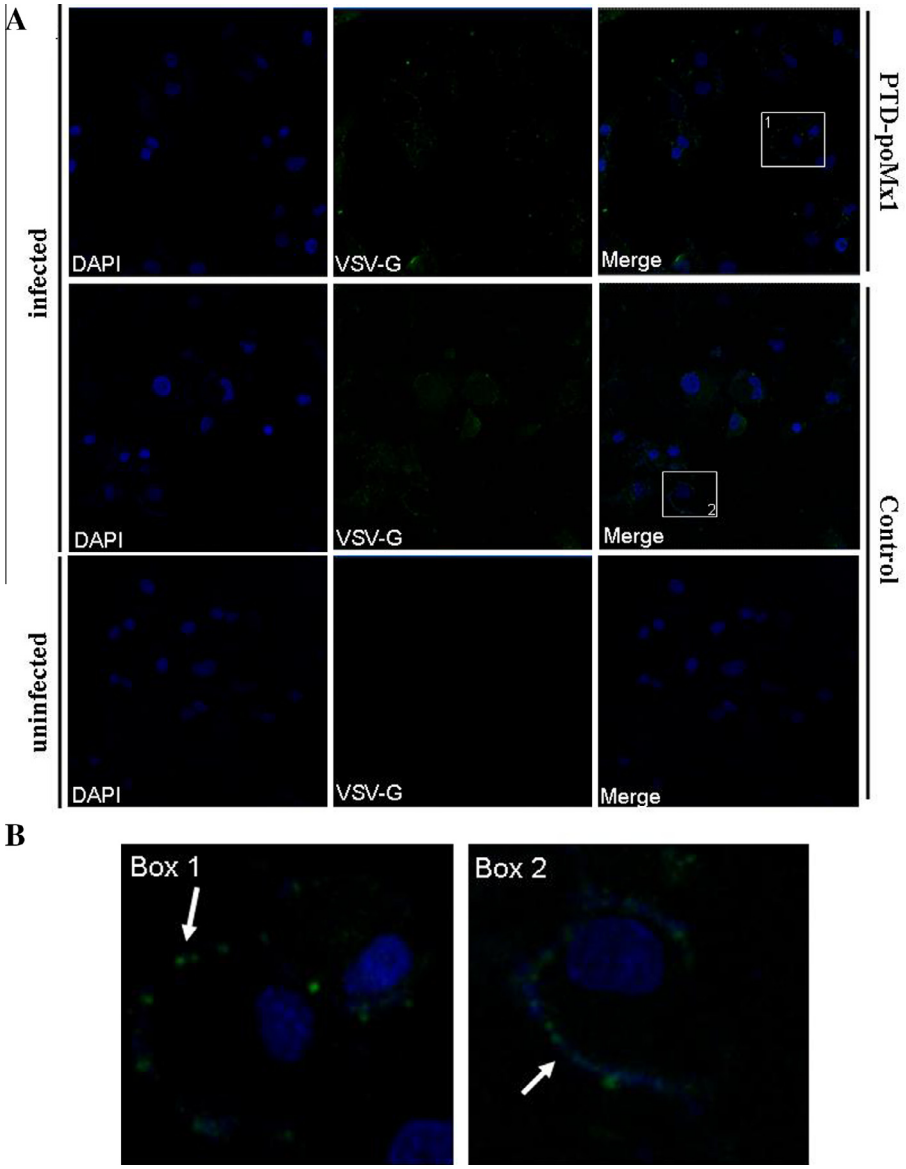

Fig. 6. VSV binding to cell surface receptor. Cells were fixed and processed for immunofluorescence with DAPI, anti-VSV-G antibody, and FITC-labeled goat anti-rabbit IgG and analyzed by confocal microscopy. (A) Whether Vero cells were treated with or without PTD-poMx1, the fluorescence spots of VSV in cell surface were both detected, respectively. (B) Two similar boxes clearly showed that VSV binded to cell surface treated with or without PTD-poMx1.

protein to VSV-infected cells is an important initial goal of this project, but it is still considered to be difficult for practical application. Therefore, a more complex project involving application of poMx1 to treat VSV-infected pigs or other animals is needed. In this report we firstly demonstrated that purified recombinant poMx1 protein could inhibit the VSV proliferation in VSV-infected cells transducted with these fusion proteins due to PTD.

The human immunodeficiency virus type 1 (HIV-1) Tat protein transduction domain (PTD), which contains rich arginine and lysine residues, is responsible for the highly efficient transduction of protein through the plasma membrane. Moreover, it can be secreted from infected cells and has the ability to enter neighboring cells (Becker-Hapak et al., 2001; Yang et al., 2002). When the PTD of Tat is fused to proteins and exogenously added to cells, the fusion proteins can cross plasma membranes (Hakansson et al., 2001). In addition, pCold is a cold shock vector developed in 2004 by Qing et al. (2004), and has been used to express many proteins that are difficult to produce using regular plasmids (Tamura et al., 2011). pCold vector has the promoter for csp A gene, a cold shock expression gene of *E. coli*, followed by lac operator, cspA 5́UTR, multi-cloning site, and cspA 3′ UTR. Here we designed a pair of specific primers to clone PTD gene into the upstream of porcine Mx1 gene (Wang et al., 2010) and constructed the recombinant expression vector. PTD-poMx1 fusion proteins purified with a Ni-chelating resin could transduct into cells after incubated for 5 h and not be cytotoxic to cells. The data showed transducted fusion proteins could effectively inhibit the replication of VSV in infected cells. What is more, it was easy to produce porcine Mx1 in bacterial expression systems in good yields, which had tremendous application potential in the future as an antiviral agent, compared with proteins expressed in eukaryotic expression systems.

The mechanistic details of the Mx-mediated antiviral effects are presently unknown. It is unclear, for example, how the GTPase activity of Mx proteins may relate to their antiviral properties (Horisberger, 1992; Pavlovic et al., 1993). Furthermore, GTPase activity was a reliable marker for MxA quality control, the capabilities of preparations of purified wild-type MxA to inhibit the VSV *in vitro* (Schwemmle et al., 1995). In this study, the Mx1-associated

GTPase activity of PTD-poMx1 fusion proteins was proved, which was a reliable and convenient marker for monitoring the purification of active PTD-poMx1, after they were purified and refolded. It is suggested that the characteristic of porcine Mx1 on VSV inhibition is close to that of human MxA.

In previous studies, Mx proteins could clearly inhibit VSV replication, such as human MxA (Schwemmle et al., 1995; Staeheli and Pavlovic, 1991), chicken Mx (Li et al., 2012), bovine Mx1 (Baise et al., 2004) and mouse Mx2 (Zurcher et al., 1992). It is well-known that human cytoplasmic MxA inhibits VSV, whose replication takes place entirely in the cytoplasm, but the human cytoplasmic MxB lacks antiviral activity. MxA could inhibit both leader RNA and mRNA synthesis of VSV, suggesting that it interfered with transcription initiation. However, little is known about the antiviral activity of porcine Mx1 protein. To our knowledge, poMx1 and huMxA (human MxA) all localize to the cytoplasm, which prevents VSV by interfering with transcription, like murine Mx1. Consequently, in this study, the level of VSV mRNA synthesis was determined by real-time PCR, which caused the reduction of VSV virions. Then, the reduction of VSV protein was determined by Western blot analysis. These results could clearly demonstrate that PTD-pomx1 could inhibit the VSV replication. Moreover, previous study had shown that virus entry and the initiation of the early stages of the viral life cycle were not affected by MxA (Netherton et al., 2009). Strikingly, our results had clearly revealed that PTD-poMx1 treatment would not affect VSV cell entry, resulting in reduction of receptors concentration for VSV infection. Asano et al. (2002) firstly reported the antiviral effect against VSV in the stable Mx1 mRNA-expressing cell clones (PK15 and LLC-PK1 cell lines). However, it was difficult to apply the cell clones to clinically treat the infected pigs in vivo. Therefore, it would be a fairly good clinical treatment measure where mass porcine Mx1 proteins were injected into body and transducted into virus-infected cells. Then, these antiviral proteins would effectively suppress VSV proliferation and cure infected pigs in vivo.

In summary, we applied the pCold system to express porcine antiviral protein Mx1 binding to HIV-1 Tat protein transduction domain in *E. coli* and purified them with Ni-chelating resin for preparations of an antiviral agent. After these antiviral proteins transducted into the virus-infected cells, viral titers reduced due to inhibition of the replication of VSV. Therefore, purified PTD-poMx1 fusion proteins might be applicable to VSV inhibition as a novel antiviral therapeutic strategy in clinically cases.

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References

Asano, A., Ko, J.H., Morozumi, T., Hamashima, N., Watanabe, T., 2002. Polymorphisms and the antiviral property of porcine Mx1 protein. J. Vet. Med. Sci. 64, 1085–1089.

Baise, E., Pire, G., Leroy, M., Gérardin, J., Goris, N., De Clercq, K., Kerkhofs, P., Desmecht, D., 2004. Conditional expression of type I interferon-induced bovine Mx1 GTPase in a stable transgenic vero cell line interferes with replication of vesicular stomatitis virus. J. Interferon Cytokine Res. 24 (9), 513–521.

Becker-Hapak, M., McAllister, S.S., Dowdy, S.F., 2001. TAT-mediated protein transduction into mammalian cells. Methods 24 (3), 247–256.

Chaleston, B., Stewart, H.J., 1993. An interferon induced Mx protein: cDNA sequence and high-level expression in the endometrium of pregnantsheep. Gene 137, 321–327.

Emeny, J.M., Morgan, M.J., 1979. Regulation of the interferon system: evidence that Vero cells have genetic defect in interferon production. J. Gen. Virol. 43, 247–252.

Hailer, O., Kochs, G., 2002. Interferon-induced Mx proteins: dynamin-like GTPases with antiviral activity. Traffle 3, 710–714.

Hakansson, S., Jacobs, A., Caffrey, M., 2001. Heparin binding by the HIV-1 tat protein transduction domain. Protein. Sci 10, 2138–2139.

Haller, O., Stertz, S., Kochs, G., 2007. The Mx GTPase family of interferon-induced antiviral proteins. Microbes Infect. 9, 1636–1643.

Horisberger, M.A., 1992. Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins. J. Virol. 66, 4705–4709.

Horisberger, M.A., Gunst, M.C., 1991. Interferon-induced proteins: identification of Mx proteins in various mammalian species. Virology 180, 185–190.

Johnson, K.M., Tesh, R.B., Peralta, P.H., 1969. Epidemiology of vesicular stomatitis virus: some new data and a hypothesis for transmission of the Indian serotype. J. Am. Vet. Med. Assoc. 155, 2133–2140.

Letchworth, G.J., Rodriguez, L.L., Barrera, J.D.C., 1999. Vesicular stomatitis. Vet. J. 157, 239–260.

Li, B., Fu, D., Zhang, Y., Xu, Q., Ni, L., Chang, G., Zheng, M., Gao, B., Sun, H., Chen, G., 2012. Partial antiviral activities of the Asn631 chicken Mx against newcastle disease virus and vesicular stomatitis virus. Mol. Biol. Rep. 39, 8415–8424.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25, 402–408.

Maniatis, T., Fritsch, E.F., Sambrook, J., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, 888–897.

Morozumi, T., Sumantri, C., Nakajima, E., Kobayashi, E., Asano, A., Oishi, T., Mitsuhashi, T., Watanabe, T., Hamasima, N., 2001. Three types of polymorphisms in exon 14 in porcine Mx1 gene. Biochem. Genet. 39, 251–260.

Morozumi, T., Naito, T., Lan, P.D., Nakajima, E., Mitsuhashi, T., Mikawa, S., Hayashi, T., Awata, T., Uenishi, H., Nagata, K., Watanabe, T., Hamasima, N., 2009. Molecular cloning and characterization of porcine Mx2 gene. Mol. Immunol. 46, 858–865.

Müller, M., Winnacker, E.L., Brem, G., 1992. Molecular cloning of porcine Mx cDNAs: new members of a family of interferon-inducible proteins with homology to GTP-binding proteins. J. Interferon Res. 12, 119–129.

Nakajima, E., Morozumi, T., Tsukamoto, K., Watanabe, T., Plastow, G., Mitsuhashi, T., 2007. A naturally occurring variant of porcine Mx1 associated with increased susceptibility to influenza virus in vitro. Biochem. Genet. 45, 11–24.

Netherton, C.L., Simpson, J., Haller, O., Wileman, T.E., Takamatsu, H.H., Monaghan, P., Taylor, G., 2009. Inhibition of a large double-stranded DNA virus by MxA protein. J. Virol. 83, 2310–2320.

Palm, M., Garigliany, M.M., Cornet, F., Desmecht, D., 2010. Interferon-induced Sus scrofa Mx1 blocks endocytic traffic of incoming influenza A virus particles. Vet. Res. 41, 29.

Pang, R., He, D.N., Zhou, B., Liu, K., Zhao, J., Zhang, X.M., Chen, P.Y., 2013. In vitro inhibition of Japanese encephalitis virus replication by capsid-targeted virus inactivation. Antiviral Res. 97, 369–375.

Pavlovic, J., Schröder, A., Blank, A., Pitossi, F., Staeheli, P., 1993. Mx proteins: GTPases involved in the interferon-induced antiviral state. Ciba Found. Symp. 176, 233–243.

Plakhov, I.V., Arlund, E.E., Aoki, C., Reiss, C.S., 1995. The earliest events in vesicular stomatitis virus infection of the murine olfactory neuroepithelium and entry of the central nervous system. Virology 209 (1), 257–262.

Qing, G., Ma, L.C., Khorchid, A., Swapna, G.V., Mal, T.K., Takayama, M.M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G.T., Ikura, M., Inouye, M., 2004. Cold-shock induced high-yield protein production in *Escherichia coli*. Nat. Biotechnol. 22, 877–882.

Rainwater-Lovett, K., Pauszek, S.J., Kelley, W.N., Rodriguez, L.L., 2007. Molecular epidemiology of vesicular stomatitis New Jersey virus from the 2004–2005 US outbreak indicates a common origin with Mexican strains. J. Gen. Virol. 88, 2042–2051.

Reed, L.J., Muench, H., 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Rettenberger, G., Bruch, J., Fries, R., Archibald, A.L., Hameister, H., 1996. Assignment of 19 porcine type I loci by somatic cell hybrid analysis detects new regions of conserved synteny between human and pig. Mamm. Genome 7, 275–279.

Schnorr, J.J., Schneider-Schaulies, S., Simon-Jödicke, A., Pavlovic, J., Horisberger, M.A., ter Meulen, V., 1993. MxA-dependent inhibition of measles virus glyceprotein synthesis in a stably transfected human monocytic cell line. J. Virol. 67, 4760–4768.

Schwarze, S.R., Ho, A., Vocero-Akbani, A., Dowdy, S.F., 1999. In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285, 1569–1572.

Schwemmle, M., Weining, K.C., Richter, M.F., Schumacher, B., Staeheli, P., 1995. Vesicular stomatitis virus transcription inhibited by purified MxA protein. Virology 206, 545–554.

Staeheli, P., Pavlovic, J., 1991. Inhibition of vesicular stomatitis virus mRNA synthesis by human MxA protein. J. Virol. 65, 4498–4601.

Tamura, M., Ito, K., Kunihiro, S., Yamasaki, C., Haragauchi, M., 2011. Production of human b-actin and a mutant using a bacterial expression system with a cold shock vector. Protein Expr. Purif. 78, 1–5.

Thomas, A.V., Palm, M., Broers, A.D., Zezafoun, H., Desmecht, D.J., 2006. Genomic structure, promoter analysis, and expression of the porcine (*Sus scrofa*) Mx1 gene. Immunogenetics 58, 383–389.

Vivès, E., Brodin, P., Lebleu, B., 1977. A truncated HIV 1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J. Biol. Chem. 272, 16010–16017.

Vives, E., Charneau, P., van Rietschoten, J., Rochat, H., Bahraoui, E., 1994. Effects of the tat basic domain on human immunodeficiency virus type 1 transactivation,

using chemically synthesized tat protein and tat peptides. J. Virol. 68, 3343–3353.
Wang, Y.F., Wang, Z.H., Li, Y., Zhang, X.J., Sun, Y., Li, M., Qiu, H.J., 2010. In vitro inhibition of the replication of classical swine fever virus by capsid-targeted virus inactivation. Antiviral. Res. 85, 422–424.
Wheeler, D.S., Dunsmore, K.D., Wong, H.R., 2003. Intracellular delivery of HSP70 using HIV-1 tat protein transduction domain. Biochem. Biophys. Res. Commun. 301, 54–59.
Yang, Y., Ma, J., Song, Z., Wu, M., 2002. HIV-1 TAT-mediated protein transduction and subcellular localization using novel expression vectors. FEBS Lett. 532, 36–44.
Yoo, J.S., Kim, C.M., Kim, J.H., Kim, J.Y., Oh, J.W., 2009. Inhibition of Japanese encephalitis virus replication by peptide nucleic acids targeting cis-acting elements on the plus- and minus-strands of viral RNA. Antiviral Res. 82, 122–133.
Zhang, X., Shin, J., Molitor, T.W., Schook, L.B., Rutherford, M.S., 1999. Molecular responses of macrophages to porcine reproductive and respiratory syndrome virus infection. Virology 262, 152–162.
Zurcher, T., Pavlovic, J., Staeheli, P., 1992. Mouse Mx2 protein inhibits vesicular stomatitis virus but not influenza virus. Virology 187, 796–800.