Antiviral Research 104 (2014) 128–135

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

In vitro inhibition of the replication of classical swine fever virus by porcine Mx1 protein

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ARTICLE INFO

Article history:
Received 22 September 2013
Revised 1 December 2013
Accepted 24 January 2014
Available online 2 February 2014

Keywords:
Classical swine fever virus (CSFV)
Porcine Mx1 (poMx1)
Antiviral activity

ABSTRACT

Classical swine fever virus (CSFV) is the causative pathogen of classical swine fever (CSF), a highly contagious disease of swine. Mx proteins are interferon-induced dynamin-like GTPases present in all vertebrates with a wide range of antiviral activities. Although Zhao et al. (2011) have reported that human MxA can inhibit CSFV replication, whether porcine Mx1 (poMx1) has anti-CSFV activity remains unknown. In this study, we generated a cell line designated PK-15/EGFP–poMx1 which expressed porcine Mx1 protein constitutively, and we observed that the proliferation of progeny virus in this cell line was significantly inhibited as measured by virus titration, indirect immune fluorescence assay, Q-PCR and Western blot. Furthermore, when PTD–poMx1 fusion protein expressed in *Escherichia coli* (Zhang et al., 2013) was used to treat CSFV-infected PK-15 cells, the results showed that PTD–poMx1 inhibited CSFV replication in a dose-dependent manner. Additionally, the proliferation of progeny virus was inhibited as measured by virus titration and Q-PCR. Overall, the results demonstrated that poMx1 effectively inhibited CSFV replication, suggesting that poMx1 may be a valuable therapeutic agent against CSFV infection.

1. Introduction

Classical swine fever virus (CSFV), a member of the genus *Pestivirus* within the family *Flaviviridae*, is a small enveloped positive-strand RNA virus (Meyers et al., 1989; Wengler, 1991). Classical swine fever (CSF) caused by CSFV is an acute and highly contagious disease of swine. Although conventional vaccines, including the attenuated lapinized Chinese strain (C strain), provide complete protection to control CSF (Graham et al., 2012), they do not adhere to the principle of differentiating infected from vaccinated animals (DIVA) and consequently they are not often used in areas where CSF has been eradicated. However, DIVA-adherent vaccines, including subunit vaccines, provide incomplete protection compared with conventional vaccines (Ahrens et al., 2000). Vaccination for CSF control is a complicated issue (Newcomer and Givens, 2013). It is, therefore, necessary to develop novel antiviral treatments against CSFV infection.

Type I interferon (IFN) is produced abundantly in the virus-infected cells soon after infection. Mx proteins are interferon-induced dynamin-like GTPases that are present in all vertebrates, such as mammals, birds and fish (Chaleston and Stewart, 1993; Hailer and Kochs, 2002; Haller et al., 2007), and have a broad range of antiviral activities (Mitchell et al., 2013). Murine Mx1 is a nuclear protein with specific activity against *Orthomyxoviruses*, whereas human MxA is a cytoplasmic protein and has a broad antiviral spectrum against many RNA viruses, including *Orthomyxoviridae, Rhabdoviridae and Bunyaviridae* (Haller et al., 2007). Porcine Mx 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 (poMx1) gene was mapped on chromosome 13 (Rettenberger et al., 1996) and the full-length poMx1 gene is 1992 bp. Previous studies have shown that poMx1 can confer resistance to vesicular stomatitis virus (VSV) (Asano et al., 2002; Zhang et al., 2013) and influenza virus (Nakajima et al., 2007; Palm et al., 2010), suggesting that poMx1 is an important mediator of the IFN-induced antiviral state. Endogenous Mx genes are not expressed without IFN (Haller et al., 1998). Previous studies have demonstrated that the lack of IFN-α/β in CSFV-infected cells leads to the absence of expression of dozens of genes responsible for executing antiviral effects, including the porcine endogenous Mx1 (Seago et al., 2007; Durand et al., 2009; Xia et al., 2005). Therefore, we strengthened the expression of Mx1 in CSFV-infected cells to investigate whether exogenous Mx1 harbors an antiviral activity against CSFV. In this study, under G418 selection, a mammalian cell line PK-15 stably expressing EGFP-poMx1 fusion protein was developed and the anti-CSFV activity of EGFP–poMx1 was determined by means of a series of assays. Moreover, PTD–poMx1 fusion protein expressed previously was used to treat the CSFV-infected PK-15 cells in order to confirm the anti-CSFV ability of poMx1.

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http://dx.doi.org/10.1016/j.antiviral.2014.01.020
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The results provided a support that poMx1 could inhibit CSFV replication and be a novel antiviral therapeutic agent against CSFV infection.

2. Materials and methods

2.1. Cells, virus and plasmid

PK-15 cells were purchased from ATCC (CCL-33), and propagated in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin (GIBCO, Invitrogen). Virulent CSFV Shimen strain was obtained from the National Institute of Veterinary Drug Control (Beijing, China; GenBank accession number: AF092448). Plasmid pT-poMx1 which contains porcine Mx1 gene was reported previously (Zhang et al., 2013).

To construct poMx1-expressing plasmid, the 1992 bp of poMx1 gene was amplified using a pair of primers (P1: 5′-AGGGCTCGAGCTGTTTATTCCAGCTGTG-3′;

P2: 5′-AGGGATCCGCCTGGGAACTTG-3′) according to a protocol described previously (Zhang et al., 2013), PCR products were cloned in-frame with EGFP into the vector pEGFP-C1 at *Xho* I and *BamH* I sites. The resulting plasmid was confirmed by DNA sequencing.

2.2. Development of stable cell line

PK-15 cells with 80% confluence in a 24-well plate were transfected with pEGFP-poMx1 using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instructions. Twenty-four hours after transfection, fresh DMEM containing 700 μg/ml G418 was added to cells, the medium was changed every 3–5 days until G418-resistant cell foci appeared. The antibiotic-resistant cell foci were pooled and expanded in DMEM containing 350 μg/ml G418. The cell line stably expressing poMx1 was designated as PK-15/EGFP–poMx1. Expression of EGFP–poMx1 fusion protein was confirmed by fluorescence microscopy and Western blot.

2.3. Reverse transcription (RT)-PCR

RT-PCR was carried out to further confirm the poMx1 gene contained in the developed cells. Briefly, total RNA was isolated from cracked PK-15/EGFP–poMx1 cells using TRIzol (Invitrogen, CA, USA). The RNA was treated with DNase, and transcription of the EGFP-poMx1 gene from the cell line was detected by RT-PCR. The poMx1 gene was detected by a pair of primers P1 and P2 as described above, the EGFP gene was detected using a pair of primers P3 and P4 as follows: P3:5′-CATGGTGAGCAAGGGCGAGG-3′; P4: 5′-GCTTTACTTGTACAGCTCGT-3′. Amplification of EGFP gene consisted of 94 °C for 5 min for pre-denaturing, then 35 cycles of 94 °C, 1 min for denaturing, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresing 5 μL aliquots through 1% agarose gel in 1 × TAE buffer.

2.4. Western blot analysis

Protein concentration of the cell lysates was determined by the Coomassie blue dye-binding assay (Bio-Rad, CA, USA). Equal amounts of lysates were boiled in SDS sample buffer for 5 min and the proteins were separated on 12% SDS–polyacrylamide gels followed with being transferred onto nitrocellulose membrane (Millipore, MA, USA) by a semi-dry transfer method (Maniatis et al., 1989). In this study, three Western blot analyses were carried out as follows: (i) The expression of poMx1 in PK-15/EGFP–poMx1 was confirmed. The membranes were blocked with TBST buffer containing 5% skim milk overnight at 4 °C, and incubated with anti-poMx1 mAb (Abcam, USA) (diluted 1:1000 in PBS plus 2% BSA) at room temperature for 2 h. The bound antibody was detected by being incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Santa Cruz, USA) and visualized with enhanced chemiluminescence (ECL, GE Healthcare Life Science) (Pang et al., 2013). (ii) The lack of poMx1 in CSFV-infected PK-15 cells was confirmed. PK-15 cells were seeded into a 6 well plate. When 90% confluence, the cells were infected with 200 50% tissue culture infectious dose ($TCID_{50}$) CSFV Shimen strain and further cultured in 2% DMEM for 24 h. Alternatively, the cells were stimulated with IFN-α (1000 U/ml) and further cultured in 2% DMEM for 24 and 48 h. Then, the lysates of cells were transferred onto a nitrocellulose membrane. The IFN-α-induced poMx1 protein was probed by anti-poMx1 mAb according to the above procedure. (iii) As for CSFV-infected PK-15/EGFP–poMx1 and PK-15 cells, the total lysates were separated on 12% SDS–polyacrylamide gels and transferred onto nitrocellulose membrane as described above. The membrane was then probed with anti-CSFV-E2 mAb (WH303), and the anti-β-actin antibody (Santa Cruz) was used as a loading control. Bound antibodies were visualized using ECL as described above.

2.5. Immunofluorescence assay

Transfected cells that had been passaged more than 15 times and mock-transfected cells were collected on coverslips, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100. Cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Green fluorescent spots of poMx1 were directly observed with fluorescence microscope. Images were recorded with a confocal microscope (Leica Sp5 AOBS confocal system) with a 63 × HCX PL Apo 1.4 oil immersion objective (Zhang et al., 2013).

To examine whether poMx1 interfered with viral protein in CSFV-infected cells, we performed immunofluorescence staining as described previously (Zhou et al., 2010). Briefly, PK-15/EGFP–poMx1 cells grown on glass coverslips were infected with 200 $TCID_{50}$ CSFV Shimen strain. At 24 h post-infection (hpi), the cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100. Anti-CSFV-E2 mAb (WH303) was diluted 1:100 and reacted with the permeabilized cells. After thorough washing, the coverslips were reacted with goat anti-mouse immunoglobulin G (IgG) conjugated to TRITC (Cell Signaling Tech, USA). After washing, the cells were visualized using a confocal microscope (Leica Sp5 AOBS confocal system) with a 63 × HCX PL Apo 1.4 oil immersion objective.

2.6. GTPase activity of EGFP–poMx1 fusion protein

The Mx1-associated GTPase activity of EGFP–poMx1 fusion protein expressing in PK-15/EGFP–poMx1 cell line was performed using QuantiChrom™ ATPase/GTPase Assay Kit (BioAssay Systems, USA) according to a protocol described previously (Zhang et al., 2013). Briefly, PK-15/EGFP–poMx1 and PK-15 cells were washed three times with ddH_2O, the cell pellet was re-suspended in ddH_2O and lysed by sonication on ice for 1 min with 2-s intervals. The lysates were centrifuged at 10,000×*g* for 15 min, and the GTPase activity of the supernatant was examined. Cell lysates from PK-15 cells were used as a negative control.

2.7. MTT assay

Cell viability was determined by MTT assay (in triplicate) (Yoo et al., 2009). First, the different generations of PK-15/EGFP–poMx1 cells were seeded into a 96-well plate and then maintained in DMEM containing 10% FBS for 5 days, and PK-15 cells were used

as the control. 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). The results were expressed relative to the optical density of wells containing untreated control cells, defined as 100% viability. Second, PK-15 cells seeded into a 96-well plate were incubated with different concentrations of PTD–poMx1 fusion protein (20, 40, 80, 160 and 320 μg/ml) for 72 h. Another MTT assay was performed according to the above procedure.

2.8. Real-time fluorescence quantitative PCR (Q-PCR)

To quantify CSFV RNA in PK-15 cells and PK-15/EGFP–poMx1 cells, Q-PCR was carried out using a pair of primers for amplifying the CSFV gene. At 24, 48, and 72 hpi, viral RNA was extracted from cell culture supernatants and cells by using TRIzol reagent. RNA pellets were suspended in 20 μl DEPC-treated H_2O 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 a 157 bp fragment of CSFV E2 gene (GenBank Accession AF092448.2) and a 114 bp fragment of PK-15 cell (GenBank Accession AY550069.1) in the conserved region by utilizing the DNAMan software, respectively (CSFV-E2-F: 5′-GAGGGATTTGACTAGGGTCTGGAAT-3′; CSFV-E2-R:5′-GTACCTGTAATCTTCCTTGCAGGCT-3′; β-actin 1F: 5′-CTCCATCATGAAGTGCGACGT-3′, β-actin 2R:5′-GTGATCTCCTTCTGCATCCTGTC-3′), Q-PCR was performed by using SYBRGreen PCR master mix (Takara, Dalian, China), 0.25 mM each primer and 20 μl RT product, following the manufacturer's protocols (Applied Biosystems). Samples were heated for 30 s at 95 °C and a two-step cycle (5 s at 95 °C, 31 s at 60 °C) was repeated for 40 cycles. Relative quantification of CSFV RNA related the PCR signal of the target transcript in a treated group to that of another sample such as an untreated control. The results of $2^{-\triangle\triangle Ct}$ showed the difference between experimental group and control group (Livak and Schmittgen, 2001).

$$\Delta\Delta Ct = \text{PK-15/EGFP—poMx1}(Ct_{csfv} - Ct_{\beta\text{-actin}}) - \text{control PK-15}(Ct_{csfv} - Ct_{\beta\text{-actin}}).$$

2.9. Virus yield reduction assay

PK-15/EGFP–poMx1 or PK-15 cells were infected with 100 $TCID_{50}$ CSFV Shimen strain for 1 h. At 24, 48 and 72 hpi, the supernatants were collected and the cells were lysed by three freezing–thawing cycles. Extracellular virus and intracellular virus yields were titrated according to a protocol described previously (Shi et al., 2013). The data were presented as $TCID_{50}$ from quadruplicate.

2.10. Preparation of PTD–poMx1 fusion protein

The expression and purification of the PTD–poMx1 fusion proteins were carried out as described previously (Zhang et al., 2013). The freshly purified proteins were treated with Detoxi-Gel-TM Endotoxin Removing Gel (Thermo, USA) to remove endotoxin derived from bacterial culture in the fusion proteins. The transduction and GTPase activity of fusion proteins were confirmed as described previously (Zhang et al., 2013).

2.11. PTD–poMx1 fusion protein inhibited CSFV replication

The anti-CSFV activity of PTD–poMx1 was determined as follows. First, after inoculation of 200 $TCID_{50}$ CSFV Shimen strain in PK-15 cells for 1 h at 37 °C, the cells were washed with PBS and incubated in DMEM with PTD–poMx1 (5, 10, 20, 40, 80 and 160 μg/ml) for 24 h. Total RNA was extracted from PK-15 cells using TRIzol reagent, then viral RNA was amplified by Q-PCR according to the above protocol. Second, PK-15 cells were seeded to a confluent monolayer in a 96-well plate and then infected with 100 $TCID_{50}$ CSFV Shimen strain for 1 h at 37 °C. At the end of adsorption, the supernatant of each well was removed, and PK-15 cells were incubated in DMEM with 80 μg/ml PTD–poMx1. Virus yield reduction assay was carried out as described above. A $TCID_{50}$ assay was performed to detect the virus in the supernatant every day after infection. Finally, another Q-PCR reaction was carried out to quantify intracellular CSFV RNA in PTD–poMx1-treated and untreated PK-15 cells. At 24, 48 and 72 hpi, viral RNA was extracted using TRIzol reagent and amplified by Q-PCR according to the above protocol.

2.12. Statistics analysis

All data were presented as means ± standard deviation (S.D.) from multiple experiments. Statistics analysis was performed with ANOVA (*F* test) software. A p value <0.05 was considered statistically significant.

3. Results

3.1. The loss of endogenous poMx1 in CSFV-infected cells

As shown in Fig. 1, under the stimulation of IFN-α, PK-15 cells could produce the endogenous poMx1 protein (lane 2 and 3), but no inner poMx1 protein was produced in CSFV-infected PK-15 cells (lane 4). The results demonstrated that high virulent CSFV Shimen strain could inhibit the pathway of IFN-α, causing the loss of endogenous interferon-induced proteins, such as poMx1.

3.2. Stable expression of EGFP-poMx1 fusion protein

The constructed pEGFP–poMx1 plasmid was transfected into the PK-15 cells, cultured in the presence of G418, and antibiotic-resistant cells were selected. The resulting cell line designated as PK-15/EGFP–poxM1 was passaged for 15 generations or more in the presence of G418, and the cell viability of each passage was examined by MTT assay. As shown in Fig. 2A, there was no difference in the cell viability among different passages of PK-15/EGFP–poMx1 cells. Constitutive expression of EGFP–poMx1 fusion protein from this cell line was confirmed by the following experiments. (i) Fluorescence microscopy. PK-15/EGFP–poMx1 cells or non-transfected PK-15 cells were fixed as described in material and methods and examined with confocal fluorescence microscopy. As shown in Fig. 2B, EGFP–poMx1 fusion protein localized

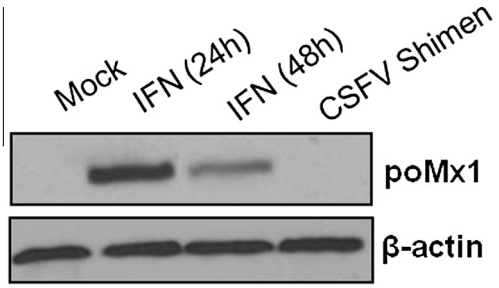

Fig. 1. CSFV inhibited the production of poMx1 protein. Lane 1: the lysates of PK-15 cells; Lanes 2 and 3: the lysates of PK-15 cells stimulated by IFN-α (1000 U/ml) for 24 and 48 h, respectively; Lane 4: the lysates of PK-15 cells infected with CSFV Shimen strain for 24 h.

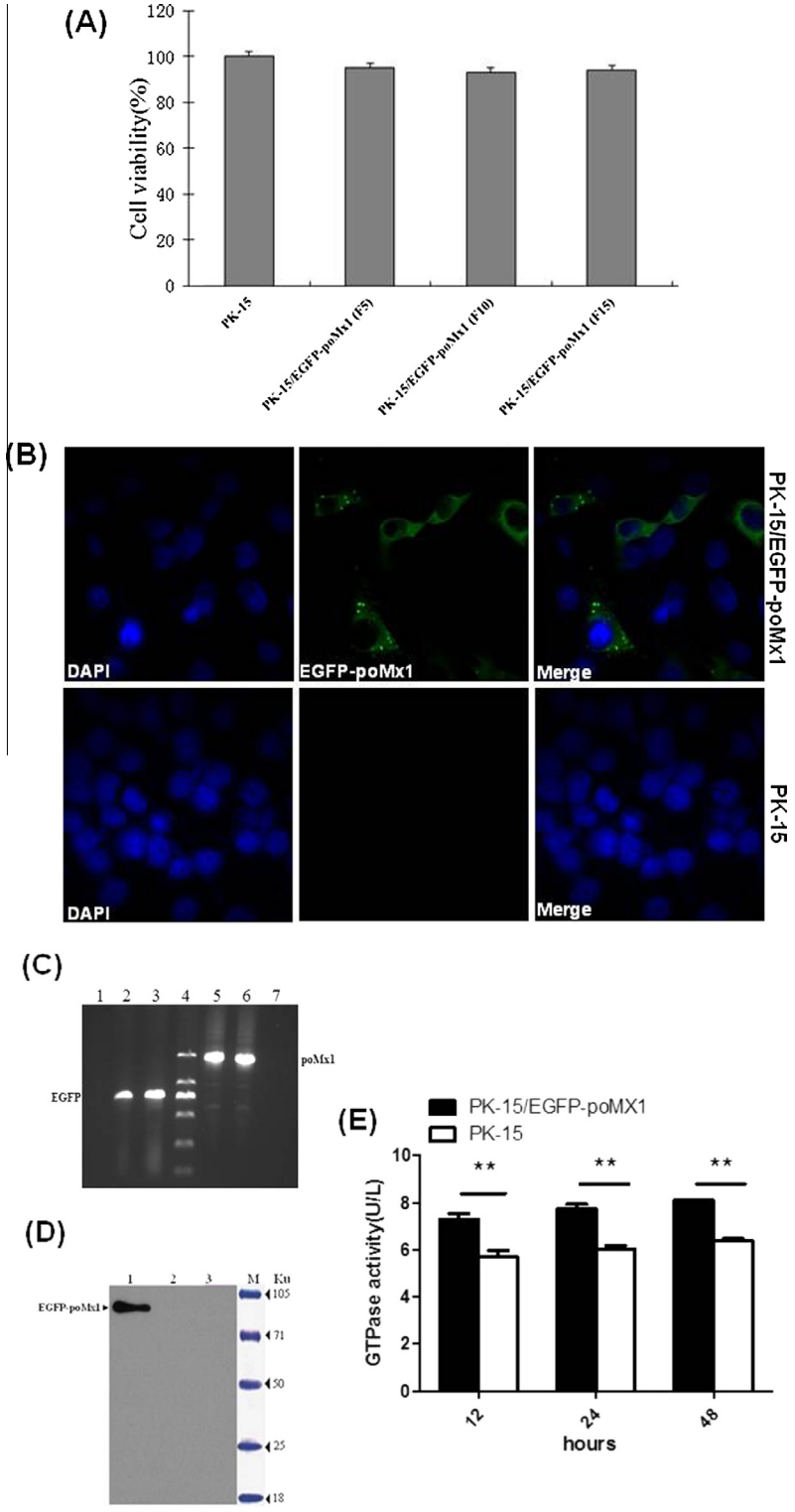

Fig. 2. Generation of PK-15/EGFP–poMx1 cell line stably expressing poMx1. (A)Viability of PK-15/EGFP–pMx1 cells of various passages. The 5th, 10th, 15th passages of PK-15/EGFP–poMx1 cells were grown in a 96-well plate for 72 h, and the cell viability was determined by MTT assay. The cell viability of PK-15 was used as control. Data are expressed as mean (% of control mean) ± S.D. from three independent experiments. (B) Fluorescence microscopy examination of PK-15/EGFP–poMx1 cells or PK-15 cells. Cells grown on glass coverslips were fixed with 4% paraformaldehyde and examined with confocal microscopy. (C) RT-PCR analysis of mRNA in PK-15/EGFP–poMx1 cell line. Lanes 1 and 7: Water as a negative control; lanes 2 and 5: plasmid pEGFP–poMx1 as a positive control; lanes 3 and 6: total RNA extracted from PK-15/EGFP–poMx1 cells; and lane 4: DL 2000 Marker. (D) Western blot analysis of PK-15/EGFP–poMx1 cell lysates with anti-poMx1 mouse monoclonal antibody. Lane1: lysates of PK-15/EGFP–poMx1 cells; lane 2: lysates of PK-15 cells transfected with pEGFP-C1; lane3: lysates of PK-15 cells; and M: Prestained Protein Ladder. (E) PK-15/EGFP–poMx1 showed GTPase activity. PK-15/EGFP-poMx1 or PK-15 cells, grown for 12, 24, and 48 h, respectively, were washed with ddH_2O, and lyzed in RIPA buffer with sonication. The GTPase activity of the cell lysates was examined by QuantiChrom™ ATPase/GTPase Assay Kit (BioAssay Systems, USA) according to a protocol.

exclusively in the cytoplasm of PK-15 cells. (ii) RT-PCR. Fig. 2C showed that both poMx1 gene (1992 bp) and EGFP (724 bp) gene were amplified from the total RNA of PK-15/EGFP–poMx1 cells, but not from that of PK-15 cells. (iii) Western blot. Fig. 2D showed

that a protein with a molecular weight of approximately 99 kD was detected from the lysates of PK-15/EGFP–poMx1 cells using anti-poMx1 monoclonal antibody (Fig. 1D, lane 1), whereas there was no band from the lysates of PK-15/EGFP-C1 and PK-15 cells (lane 2 and 3). Finally, the Mx1-associated GTPase activity in PK-15/EGFP–poMx1 cell was determined by QuantiChrom™ ATPase/

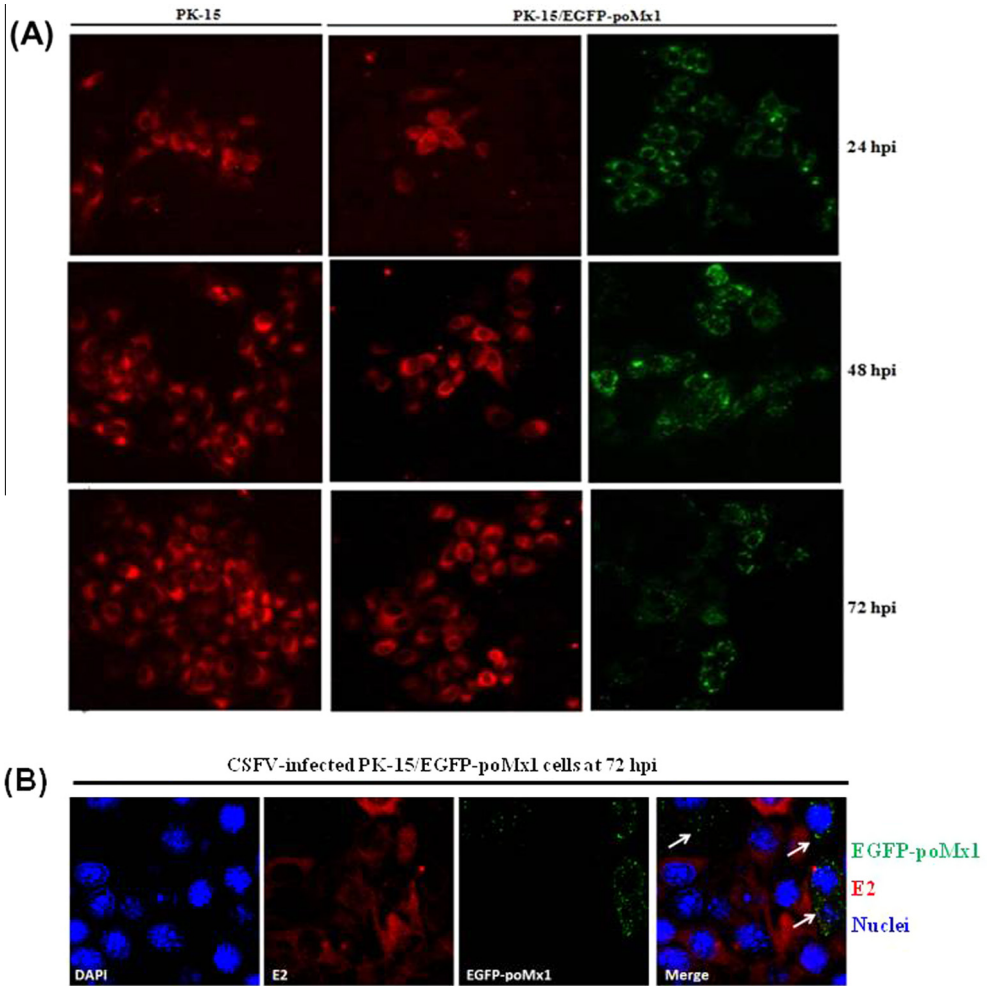

Fig. 3. Inhibition of CSFV replication in PK-15/EGFP–poMx1 cell line was detected by immunofluorescence assay. (A) PK-15/EGFP–poMx1 cells grown on glass coverslips in a 6-well plate were infected with 200 $TCID_{50}$ CSFV Shimen strain. At 24, 48, 72 h post infection (hpi), the cells were fixed with 4% PFA (W/V), reacted with anti-CSFV E2 specific antibody and visualized with fluorescence microscopy. (B) Immunofluorescence analysis of EGFP–poMx1 (in green) and viral E2 (in red) protein expression in CSFV-infected cells at 72 hpi. The cell nucleus was counterstained with DAPI (blue). White arrows in the fourth panel pointed to cells with high EGFP–poMx1 expression level and with no or very low CSFV E2 staining. These results showed that inhibition of CSFV replication by EGFP–poMx1 resulted in the reduction in the viral protein level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

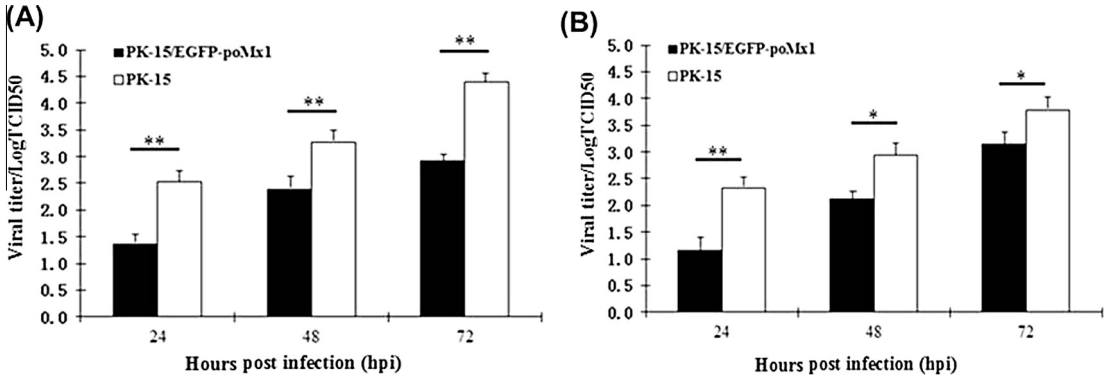

Fig. 4. Inhibition of CSFV replication in PK-15/EGFP–poMx1 cell line resulted in a decrease of progeny virus. At 24, 48 and 72 hpi, PK-15/EGFP–poMx1 cell culture supernatants were collected and cells were lysed by three freezing-thawing cycles. The viral titers in cell culture supernatant (A) and intracellular virus (B) were determined. Data are expressed as the mean ± S.D. of three independent experiments. The results indicated that titers of progeny virus in PK-15/EGFP–poMx1 cells decreased by 3.4∼30.2-fold over the 72 hpi compared with PK-15 cells used as a control (p value <0.05 and p value <0.01 represent significant differences).

GTPase Assay Kit. As shown in Fig. 2E, the GTPase activity of PK-15/EGFP–poMx1 cells (solid bar) was significantly greater than that of PK-15 cells (empty bar), suggesting that intracellular EGFP-poMx1 displayed detectable GTPase activity.

3.3. EGFP–poMx1 fusion protein inhibited CSFV replication

To determine whether EGFP–poMx1 expressed in the PK-15/EGFP–poMx1 cell line interferes with CSFV replication, we performed various experiments and the results are as follows.PK-15/EGFP–poMx1 cells and normal PK-15 cells were infected with CSFV Shimen strain, and then titers of the progeny virus in cell culture supernatants and cells were determined by IFA, respectively. As shown in Fig. 3A, the results showed that the numbers of red fluorescent signals on behalf of virus decreased at 24, 48 and 72 hpi, compared with normal PK-15 cells, suggesting EGFP–poMx1 inhibited CSFV replication in PK-15 cells within 72 h. In addition, as shown in Fig. 3B, fewer red fluorescent signals on behalf of virus were observed in poMx1-positive cells (green fluorescent signals on behalf of EGFP–poMx1). We found that the titers of progeny virus in PK-15/EGFP–poMx1 cells decreased by 3.4∼14.8-fold over the 72 hpi compared with control PK-15 cells (Fig. 4A). Yields of infectious virus in culture supernatants of PK-15/EGFP–poMx1 cells decreased by 7.8∼30.2-fold compared with controls (Fig. 4B).

The ability of inhibiting CSFV replication in PK-15/EGFP–poMx1 cell line was determined by Q-PCR. Relative quantification was performed using β-actin as an endogenous control gene. These results clearly demonstrated significant differences between poMx1-expressing and control PK-15 cell populations at three different periods (24, 48 and 72 hpi). At 24, 48, and 72 hpi, the copy numbers of viral genome in poMx1-expressing cell culture supernatants decreased by 9.2-fold (p value <0.01), 9.9-fold (p value <0.01), and 3.5-fold (p value <0.05), respectively, compared with normal PK-15 cells (Fig. 5A). Then, the copy numbers of viral genome in poMx1-expressing cells decreased by 3.6-fold (p value <0.05), 3.2-fold (p value <0.05), and 3.9-fold (p value <0.05) compared with normal PK-15 cells, respectively (Fig. 5B). This illustrated that the PK-15/EGFP–poMx1 cell line harbored the antiviral activity *in vitro*. To further validate the results obtained from Q-PCR, Western blot analysis was performed as described above. Viral envelope glycoprotein (E2) level also decreased significantly at 24, 48 and 72 hpi (Fig. 6) due to the reduction of viral load. Altogether, these results demonstrated that EGFP–poMx1 fusion protein effectively suppressed CSFV proliferation by decreasing the levels of both viral RNA and protein.

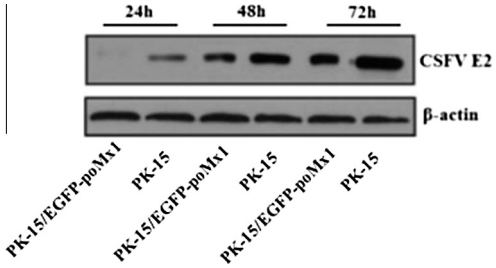

Fig. 6. Levels of CSFV glycoprotein E2 were determined by Western blot analysis. Cell lysates with anti-CSFV E2 mAb (WH303) were specific to the indicated protein. An anti-β-actin antibody was used as an internal loading control. The difference of CSFV E2 protein bands at 24, 48 and 72 hpi could be relatively distinguished, respectively, suggesting EGFP–poMx1 fusion protein effectively inhibited the proliferation of CSFV.

3.4. Antiviral activity of PTD–poMx1 fusion protein

To assess whether PTD–poMx1 fusion protein expressed by *Escherichia coli* had any effect on CSFV replication, four experiments were performed as described above. First, we assessed the cytotoxicity of the PTD–poMx1 fusion protein using MTT assay. PK-15 cells were treated with increasing concentrations of PTD–poMx1 for 72 h. As shown in Fig. 7A, PTD–poMx1 showed no significant cytotoxicity up to a concentration of 80 μg/ml. Cell viability, however, decreased by approximately 5% and 13% at the concentration of 160 and 320 μg/ml, respectively. Second, to determine if PTD–poMx1 was correlated with inhibition of CSFV replication, PK-15 cells were incubated with PTD–poMx1 from 5 to 160 μg/ml for 24 h. As shown in Fig. 7B, we found that PTD–poMx1 and inhibition of CSFV replication in a dose-dependent manner. In fact, CSFV mRNA level of PK-15 cells treated with the highest concentration of PTD–poMx1 (160 μg/ml) was reduced about 86.4% compared with that of untreated PK-15 cells. Third, to determine the reduction of viral load *in vitro*, CSFV-infected PK-15 cells were incubated with 80 μg/ml PTD–poMx1 and were subjected to the virus yield reduction assay as described above. It shown that virus titers were reduced in PTD–poMx1-treated cells. As shown in Fig. 7C, the results showed that the mean of $TCID_{50}$ values produced by PTD–poMx1-treated infected cells was $10^{0.8}$-fold lower

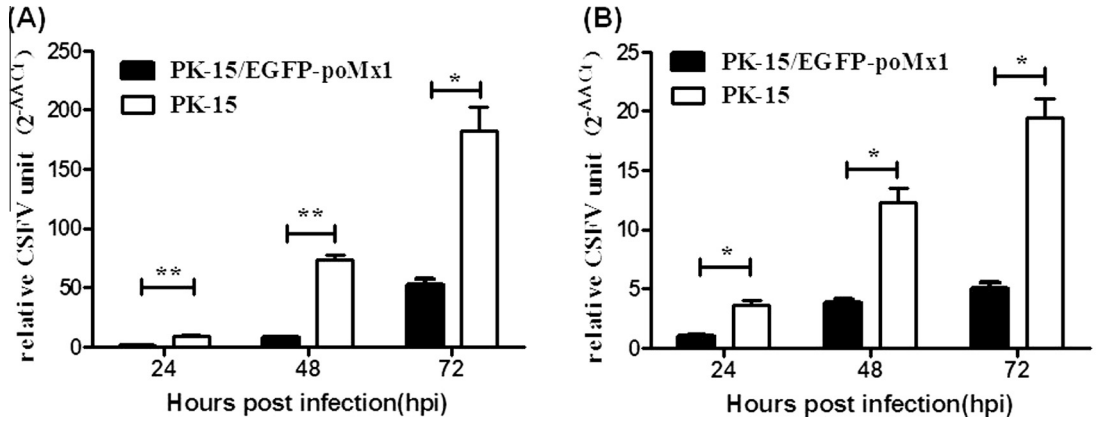

Fig. 5. Inhibition of CSFV mRNA expression in PK-15/EGFP–poMx1 cell line was determined by Q-PCR. At 24, 48 and 72 hpi, PK-15/EGFP–poMx1 cell culture supernatants were collected and cells were lysed by three freezing-thawing cycles. The viral mRNA in cell culture supernatant (A) and intracellular virus (B) were determined by Q-PCR. Relative quantification was performed using β-actin as endogenous control gene. Q-PCR was performed in triplicate and data are shown as mean ± S.D. of three independent experiments (p value <0.05 and p value <0.01 represent significant differences). These results clearly demonstrated significant changes between poMx1-expressing PK-15 cell line and control PK-15 cells at three different periods (24, 48 and 72 hpi).

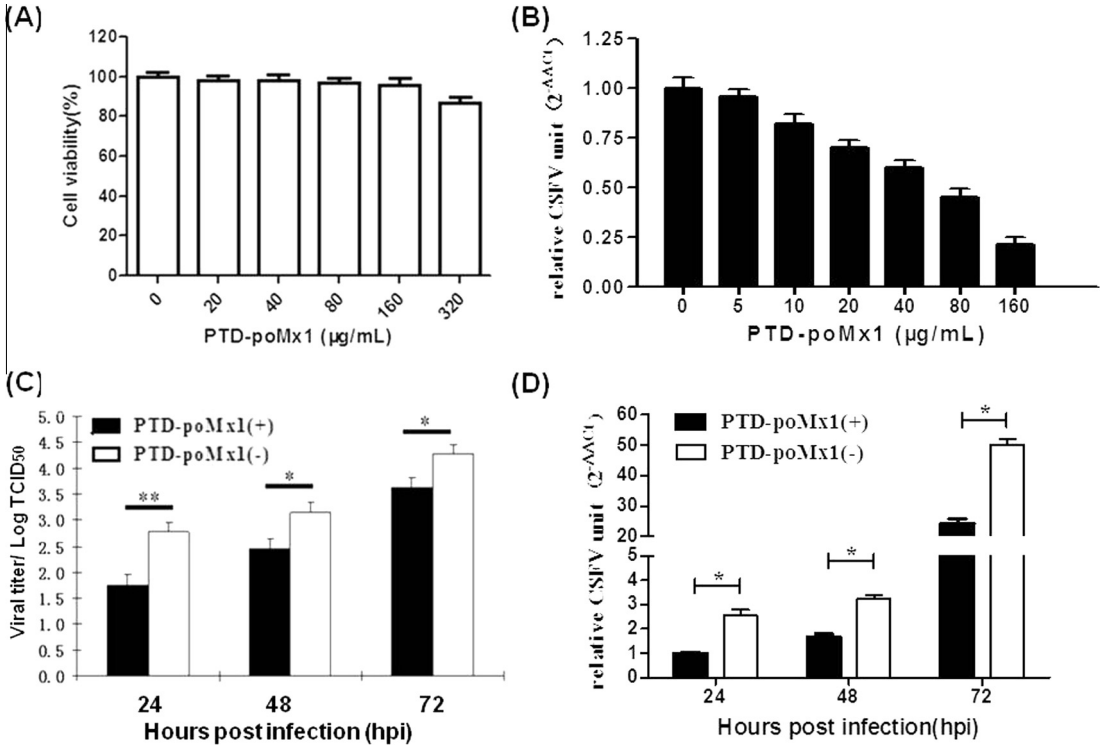

Fig. 7. The anti-CSFV effect of PTD–poMx1 fusion protein in PK-15 cells. (A) Cytotoxicity of PTD–poMx fusion protein. PK-15 cells in a 96-well plate were treated with various concentrations of PTD–poMx fusion protein for 72 h. Three independent MTT assays were performed in triplicate. Data are presented as mean (% of control mean) ±S.D. of three independent experiments. (B) Q-PCR assay was performed to assess the antiviral activity of various concentrations of PTD–poMx1 fusion protein, and DMEM-treated cells were taken as the positive control. The results showed that PTD–poMx1 inhibited CSFV replication in a dose-dependent manner. (C) Effect of PTD–poMx1 on the proliferation of progeny virus at three different periods. At 24, 48 and 72 hpi, CSFV-infected PK-15 cells treated with 80 μg/ml PTD–poMx1 fusion protein were collected and cells were lysed by three freezing–thawing cycles. The viral titers of intracellular virus were determined by virus yield assay. Data are expressed as the mean ± S.D. of three independent experiments (p value <0.05 and p value <0.01 represent significant differences). The result showed that inhibition of CSFV replication resulted in a decrease of progeny virus. (D) Effect of PTD-poMx1 on CSFV mRNA expression at three different periods. At 24, 48 and 72 hpi, CSFV-infected PK-15 cells treated with 80 μg/ml PTD–poMx1 fusion protein were collected and total RNA was extracted using TRIzol reagent, then viral RNA was amplified by Q-PCR. Relative quantification was performed using β-actin as an endogenous control gene. Q-PCR was performed in triplicate and data are shown as the mean ± S.D. of three independent experiments (p value <0.05 and p value <0.01 represent significant differences). These results clearly demonstrated significant differences between PTD–poMx1-treated and untreated infected cells at three different periods (24, 48 and 72 hpi).

than that of untreated infected cells from 24 to 72 hpi (p value <0.05). Finally, the ability of inhibiting CSFV replication in PTD–poMx1-treated cells was determined by Q-PCR. PK-15 cells were treated as described above. Relative quantification was performed using β-actin as an endogenous control. These results clearly demonstrated significant differences between 80 μg/ml-treated and untreated cells at 24, 48 and 72 hpi. The copy numbers of viral genome in PTD–poMx1-treated cells decreased by 2.5-fold (p value <0.05), 1.9-fold (p value <0.05), and 2.1-fold (p value <0.05), respectively, compared with that of untreated PK-15 cells (Fig. 7D). Taken together, these data showed that the expressed PTD–poMx1 clearly harbored the anti-CSFV activity *in vitro*.

4. Discussion

This is the first report that poMx1 protein constitutively expressed in PK-15 cell has antiviral activities against CSFV infection. Moreover, purified PTD-poMx1 fusion protein can inhibit CSFV replication in a dose dependent manner. At present, CSF is still an epidemic disease that could severely threaten the swine industry worldwide. To our knowledge, although the C strain is still considered one of the best CSFV vaccines, the inability to serologically differentiate vaccinated from infected pigs has resulted in the ban of prophylactic vaccination in the European Union (EU). On the contrary, vaccination with subunit vaccines and chimeric vaccines does not present these problems, but their incomplete protection and safety concerns necessitate further study. Therefore, depending on vaccination alone may not be sufficient to control CSF and the development of an antiviral strategy may supplement current control strategies. Capsid-targeted virus inactivation (Zhou et al., 2010), RNA-hydrolyzing recombinant antibody (Jun et al., 2010), RNA interference (Li et al., 2011), Imidazo[4,5-c]pyridines (Vrancken et al., 2008) and uridine derivatives of 2-deoxy sugars (Krol et al., 2010) have been reported in previous studies as anti-CSFV therapies, but their clinical effect and practical application for CSF control need further study and development. In addition, despite reports that human MxA can inhibit CSFV replication (Zhao et al., 2011), little is known about the anti-CSFV activity of poMx1. In that respect, our findings demonstrating the anti-CSFV activity of poMx1 provided significant additional information for the potential development of an antiviral therapy.

Given the nature of the PK-15/EGFP-poMx1 cell line, in vivo administration to CSF-infected pigs may be difficult. Thus, the fusion protein, PTD–poMx1 (poMx1 binding to HIV-1 Tat protein transduction domain (PTD) expressed in *E. coli*), was constructed as an alternative approach and was evaluated in vitro. Results of the virus yield and viral mRNA reduction assay showed that PTD-poMx1 was able to significantly inhibit CSFV replication in vitro. Hence, this approach, by transduction of poMx1 protein in cells after injection in CSFV-infected pigs, may be a more suited approach for clinical therapy.

Previous studies have revealed the antiviral mechanisms of human MxA and murine Mx1. Interaction with viral nucleoprotein (NP) is the most likely common pathway for MxA to perform its antiviral function against RNA viruses, in particular for influenza A virus (Zimmermann et al., 2011). Whereas, the anti-influenza mechanism of murine Mx1 protein is blocking primary transcription of *Orthomyxoviruses* via molecular interaction with the PB2 subunit of the viral polymerase (Stranden et al., 1993). Although the results showed that poMx1 could effectively inhibit CSFV replication, the mechanistic details of the poMx1-mediated anti-CSFV effects are unknown to date. In this study, immunofluorescence assays demonstrated that poMx1 was localised in the cytoplasm of PK-15 cells assuming that poMx1 exerts its anti-CSFV activity entirely in the cytoplasm of the host cell. In addition, Palm et al. (2010) have demonstrated that poMx1 significantly alters binding of early endosome autoantigen 1 (EEA1) to early endosomes and/or early endosome size and spatial distribution. EEA1 is primary endocytic vesicles and early endosomes at the cell periphery. A similar mechanism may be hypothesized for CSF in EGFP-poMx1 in PK-15/EGFP-poMx1 cells.

In conclusion, we confirmed that these two fusion proteins, EGFP-poMx1 expressed by the cell line and PTD-poMx1 expressed by *E. coli*, both dispaly an anti-CSFV ability *in vitro*. Although identifying the anti-CSFV mechanism of poMx1 requires further studies, the present results have suggested poMx1 as a potential inhibitor of CSFV replication.

Acknowledgements

This work was supported by a Grant from the National Natural Science Foundation of China (31001062) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). Also, the authors show tremendous gratitude towards Dr. Kui Yang and Ms. Elizabeth G. Wills, both from department of microbiology and immunology at Cornell University, for critically reading the manuscript and revising it.

References

Ahrens, U., Kaden, V., Drexler, C., Visser, N., 2000. Efficacy of the classical swine fever (CSF) marker vaccine Porcilis Pesti in pregnant sows. Vet Microbiol. 77 (1–2), 83–97.

Asano, A., Ko, J.H., Morozumi, T., Hamashima, N., Watanabe, T., 2002. Polymorphisms and the anti viral property of porcine Mx1protein. J. Vet. Med. Sci. 64, 1085–1089.

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

Durand, S.V., Hulst, M.M., de Wit, A.A., Mastebroek, L., Loeffen, W.L., 2009. Activation and modulation of antiviral and apoptotic genes in pigs infected with classical swine fever viruses of high, moderate or low virulence. Arch. Virol. 154, 1417–1431.

Graham, S.P., Everett, H.E., Haines, F.J., Johns, H.L., Sosan, O.A., Salguero, F.J., Clifford, D.J., Steinbach, F., Drew, T.W., Crooke, H.R., 2012. Challenge of pigs with classical swine fever viruses after C-strain vaccination reveals remarkably rapid protection and insights into early immunity. PLoS One 7 (1), e29310.

Hailer, O., Kochs, G., 2002. Interferon-induced M x proteins:dynamin-like GTPases with anti viral activity. Traffic 3, 710–714.

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

Haller, O., Frese, M., Kochs, G., 1998. Mx proteins: mediators of innate resistance to RNA viruses. Rev. Sci. Technol. 17, 220–230.

Li, J.N., Dai, Y.J., Liu, S., Guo, H.C., Wang, T.D., Ouyang, H.S., Tu, C.C., 2011. In vitro inhibition of CSFV replication by multiple siRNA expression. Antiviral Res. 91 (2), 209–216.

Krol, E., Wandzik, I., Szeja, W., Grynkiewicz, G., Szewczyk, B., 2010. In vitro antiviral activity of some uridine derivatives of 2-deoxy sugars against classical swine fever virus. Antiviral Res. 86 (2), 154–162.

Jun, H.R., Pham, C.D., Lim, S.I., Lee, S.C., Kim, Y.S., Park, S., Kwon, M.H., 2010. An RNA-hydrolyzing recombinant antibody exhibits an antiviral activity against classical swine fever virus. Biochem. Biophys. Res. Commun. 395 (4), 484–489.

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.

Meyers, G., Rumenapf, T., Thiel, H.J., 1989. Molecular cloning and nucleotide of the genome of hog cholera virus. Virology 171, 555–567.

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.

Mitchell, P.S., Emerman, M., Malik, H.S., 2013. An evolutionary perspective on the broad antiviral specificity of MxA. Curr. Opin. Microbiol. 16 (4), 493–499.

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.

Newcomer, B.W., Givens, M.D., 2013. Approved and experimental countermeasures against pestiviral diseases: bovine viral diarrhea, classical swine fever and border disease. Antiviral Res. 100, 133–150.

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.

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.

Seago, J., Hilton, L., Reid, E., Doceul, V., Jeyatheesan, J., Moganeradj, K., McCauley, J., Charleston, B., Goodbourn, S., 2007. The Npro product of classical swine fever virus and bovine viral diarrhea virus uses a conserved mechanism to target interferon regulatory factor-3. J. Gen. Virol. 88, 3002–3006.

Shi, Z., Sun, J., Guo, H., Yang, Z., Ma, Z., Tu, C., 2013. Down-regulation of cellular protein heme oxygenase 1 inhibits proliferation of classical swine fever virus in PK-15 cells. Virus Res. 173, 315–320.

Stranden, A.M., Staeheli, P., Pavlovic, J., 1993. Function of the mouse Mx1 protein is inhibited by overexpression of the PB2 protein of influenza virus. Virology 197, 642–651.

Vrancken, R., Paeshuyse, J., Haegeman, A., Puerstinger, G., Froeyen, M., Herdewijn, P., Kerkhofs, P., Neyts, J., Koenen, F., 2008. Imidazo[4,5-c]pyridines inhibit the in vitro replication of the classical swine fever virus and target the viral polymerase. Antiviral Res. 77 (2), 114–119.

Wengler, G., 1991. Family Flaviviridae. In: Francki, R.I.B., Fauquet, C.M., Knudson, D.L., Brown, F. (Eds.), Classication and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses. Springer, Berlin, pp. 223–233.

Xia, C., Dan, W., Wu, W.X., Wan, J.Q., Li, W., Yang, T.Y., Wang, Q., Ning, Y.B., 2005. Cloning and expression of interferon-alpha/gamma from a domestic porcine breed and its effect on classical swine fever virus. Vet. Immunol. Immunopathol. 104, 81–89.

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.M., He, D.N., Zhou, B., Pang, R., Liu, K., Zhao, J., Chen, P.Y., 2013. In vitro inhibition of vesicular stomatitis virus replication by purified porcine Mx1 protein fused to HIV-1 Tat protein transduction domain (PTD). Antiviral Res. 99, 149–157.

Zhao, Y., Pang, D., Wang, T., Yang, X., Wu, R., Ren, L., Yuan, T., Huang, Y., Ouyang, H., 2011. Human MxA protein inhibits the replication of classical swine fever virus. Virus Res. 156, 151–155.

Zhou, B., Liu, K., Wei, J.C., Mao, X., Chen, P.Y., 2010. Inhibition of replication of classical swine fever virus in a stable cell line by the viral capsid and *Staphylococcus aureus* nuclease fusion protein. J. Virol. Methods 167, 79–83.

Zimmermann, P., Mänz, B., Haller, O., Schwemmle, M., Kochs, G., 2011. The viral nucleoprotein determines Mx sensitivity of influenza A viruses. J. Virol. 85, 8133–8140.