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Expression of exogenous IFN-α by bypassing the translation block protects cells against FMDV infection

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is the most contagious pathogen of cloven-hoofed animals. Previous studies have demonstrated that type I interferons [alpha/beta interferons (IFN-α/βs)] can suppress FMDV replication and spread. Conversely, FMDV can also inhibit IFN-α expression in infected cells by blocking cap-dependent translation. To overcome the blockade on IFN-α mRNA translation during FMDV infection, we generated an IRES-IFN construct that carries FMDV's internal ribosome entry site (IRES) cDNA sequence between the promoter and porcine IFN-α gene. ELISA assays indicated that expression of IFN-α regulated by wild-type IRES increased to 125% of pre-infection level after infection for 24 h, but the expression of IFN-α regulated by nonfunctional IRES mutants were only ~50% of pre-infection level. Correspondingly, the former could suppress the replication of FMDV to 20% of the latter and protect cells against FMDV for a longer time. Therefore, these findings provide a new strategy to anti-FMDV therapy.

1. Introduction

Foot-and-mouth disease virus (FMDV) is the prototype species of the *Aphthovirus* genus of the *Picornaviridae* family and is the causative agent of FMD, one of the most devastating viral diseases of wild and domestic cloven-hoofed animals. Upon infection, viral RNA is translated into a polyprotein that is further co-translationally processed by a virus-encoded protease into four structural proteins and eight non-structural proteins, which function in various aspects of the viral replication cycle (Mason et al., 2003). The first protein translated, Lpro, is a papain-like protease (Mason et al., 2003; Kleina and Grubman, 1992; Piccone et al., 1995) that cleaves itself from the polyprotein precursor. This protease also cleaves the translation initiation factor eIF4G, preventing host cap-dependent mRNA translation without affecting viral protein synthesis (Devaney et al., 1988; Kirchweger et al., 1994). But viral RNA can be translated by a cap-independent mechanism that utilizes an internal ribosome entry site (IRES). Therefore, FMDV RNA does not require intact eIF4G for viral protein production. The virus takes over the host cell's protein synthesis machinery for its own benefit to produce progeny virions (Belsham and Brangwyn, 1990). Previous studies have shown that FMDV IRES is a *cis*-acting element that is 462 nucleotides long and consists of five structural domains. Studies on the IRES elements of picornavirus family demonstrate that the IRES do not show extensive primary sequence conservation (Martínez-Salas et al., 2002). However, viral IRES elements do have intricately folded structures, in part because the 5′UTR plays a critical role in replication and packaging of viral RNAs (Kozak, 2003). The most conserved regions are the GNRA loop, GNRA hairpin, RAAA loop, ACCCC loop, and Domain 3 stem. Mutating different regions leads to a variable reduction of translational efficiency. Notably, mutating the GNRA loop and RAAA loop results in almost complete loss of translation efficiency (Martínez-Salas et al., 2002).

The first host cell responses to viral infections include expression and secretion of interferons (IFNs), which establish an antiviral state and contribute to the induction and regulation of the antiviral immune response (Höhle et al., 2005). Type I interferons (alpha/beta interferons, IFN-α/β) are rapidly induced after viral infection. Via a series of events, including paracrine and autocrine processes, IFN-α/β lead to the expression of hundreds of gene products, some of which exhibit antiviral activity (Der et al., 1998). Recent studies have shown that IFN-α have an important role in the outcome of FMDV infection (Moraes et al., 2007). However, current IFN therapy has limitations. It was reported that IFN-α/β protein was rapidly cleared and higher concentrations of IFN-α or IFN-β had little or no more effect during a single treatment (Moraes et al., 2007). Thus, the clinical use of IFN-α/β requires multiple inoculations of high doses over a prolonged period of time to be effective, which can lead to adverse systematic effects (Dusheiko, 2003).

In attempts to utilize IFN-α to treat FMDV, many researchers have focused on co-expressing IFN-α with other proteins to enhance antiviral activity, such as a combination of adenoviruses expressing IFN-α and a foot-and-mouth disease virus subunit (Moraes et al., 2003) or type I and II porcine interferons (Moraes

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et al., 2007). Recent work has also demonstrated that the activity of IFN-α can be enhanced by fusing IFN-α with multiple epitopes of FMDV (Du et al., 2008).

FMDV can inhibit IFN-α/β responses (Kleina and Grubman, 1992) and reduce the transcriptional level of IFN-β mRNA by Lpro (de los Santos et al., 2006). However, a method to overcome the translational block by FMDV has not been explored previously. In this study, we investigate the utilization of FMDV IRES to overcome the translational block on the host's production of endogenous IFN-α after FMDV infection. The use of FMDV IRES *in cis* was found to stabilize the cellular production of exogenous IFN-α after infection and could confer long-term protection from FMDV infection. Therefore, these findings provide a new strategy for anti-FMDV therapy.

2. Materials and methods

2.1. Construction of recombinant plasmids

Standard DNA cloning procedures (Maniatis et al., 1989) were used except where noted. All primers used in this study are listed in Table 1. FMDV RNA was extracted using Trizol® reagents (Invitrogen) and the first strand cDNA was generated using the reverse transcription primer R-IRES and M-MLV enzyme (TaKaRa) following the manufacturer's instructions. FMDV IRES was amplified by PCR using primers R-IRES, F-IRES and *LA-Taq*® polymerase (TaKaRa). The reaction was run for 32 cycles of 30 s at 94 °C, 30 s at 54 °C, and 1 min at 72 °C. PCR product was purified and digested by *Nhe*I and *Eco*RI, and then ligated into *Nhe*I- and *Eco*RI-digested pcDNA 3.1(+) vector (Invitrogen). The resulting plasmid was named pc-IRES.

The porcine IFN-α gene (GenBank Accession no. X57191) was amplified by PCR from genomic DNA. Porcine liver was purchased from a local market, and genomic DNA was extracted using Tissue DNA Extraction Kit (Omega). PCR was performed using the primers F-IFN and R-IFN for 32 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. PCR product was purified by gel electrophoresis and digested with *Eco*RI and *Xho*I, and then inserted into *Eco*RI- and *Xho*I-digested pc-IRES. The resulting plasmid was named pc-IRES-IFN.

Nonfunctional IRESΔG and IRESΔT mutants were generated by overlapping PCR. Primers F-GNRA and R-GNRA were used to generate the GNRA loop-deletion IRESΔG mutant, whereas primers F-TTT and R-TTT were used to generate the TTT loop-deletion IRESΔT mutant. Two mutated IRES constructs were used to replace the wild-type IRES in pc-IRES and pc-IRES-IFN, and the new plasmids were named pc-IRESΔG, pc-IRESΔT, pc-IRESΔG-IFN, and pc-IRESΔT-IFN, respectively (Fig. 1). All PCR-cloned DNA sequences were confirmed by DNA sequencing.

Table 1
Primers used for PCR and real-time RT-PCR.

Name[a]	Sequence (5′–3′)	Concentration (nm)[b]
F-IRES	GCGCTAGCGGTAACCACATGCTTATTACC	20
R-IRES	GCGAATTCTGGTAAAGTAGTTGTAGAG	20/50
F-infa	GCGAATTCATGGCCCCAACC	20
R-infa	GCCTCGAGTCACTCCTTCTTCC	20
F-IFN516-RT	CATGACTTCTGCCCTGACGATC	50
F-GNAR	CCCTCCTTGCAAGGACCCGCGGGGCC	20
R-GNAR	GGGTCCTTGCAAGGAGGGATTCC	20
F-TTT	CAAGCCACGTCGACCCAACATGTGTGC	20
R-TTT	CATGTTGGGTCGACGTGGCTTGCGG	20
F-PKR	GAATGCTGACCCACCATCCTC	20
R-PKR	GTTCCAATGTCCCTTTATCACAG	20/50
F-OAS	GCGTCTCCAAAGTTGTGAAGG	20
R-OAS	GCTCCCATGCATAGACTGTCAGG	20/50
F-GAPDH	TGGCAAGTTCAAAGGCACA	20
R-GAPDH	AGATCCACGACGGACACG	20/50

[a] F, forward primers; R, reverse primers.
[b] 20 used for PCR, 50 used for first stand synthesis.

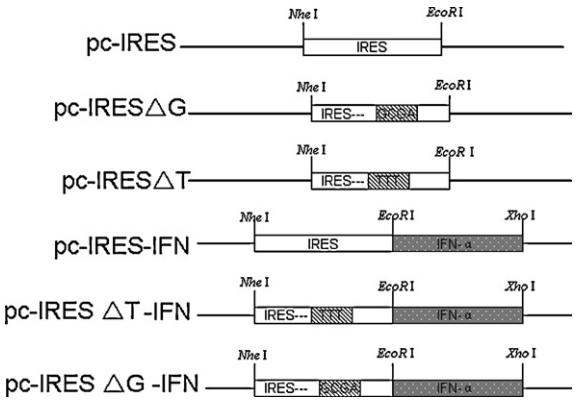

Fig. 1. Schematic diagram of IRES, IRES-IFN and their mutant constructs in the pcDNA3.1(+) vector. IRESΔG and IRESΔT mutants were generated to serve as controls.

2.2. Cells and viruses

Porcine kidney (PK) cells were provided by the China Center for Type Culture Collection (CCTCC). All cells were maintained in MEM containing 10% calf serum and incubated at 37 °C in 5% CO_2. Lipofectamine™ 2000 (Invitrogen) was used to transfect PK cells. The virus strain of serotype O FMDV and vesicular stomatitis virus (VSV) used in the study were also provided by CCTCC.

2.3. Viral infection and RNA extraction

Cells were washed for three times with PBS and infected with FMDV. After virus adsorption for 1 h at 37 °C, cells were washed for 1 min with 0.1 M phosphate buffer (pH 6.0) to remove free virus, and washed again extensively with MEM. The infection was allowed to proceed in MEM supplemented with 2% fetal bovine serum.

Cells were homogenized with the Trizol® reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions and dissolved in RNase-free H_2O.

2.4. RT-PCR analysis of gene transcription

Total RNA was treated with RQ1 DNase I (Promega) for 1 h at 37 °C and then extracted with phenol–chloroform and precipitated with ethanol. Primer R-IFN-516 was used to synthesize the first strand and IFN-α cDNA was amplified by PCR using primers R-IFN-516 and F-IFN. PCR was carried out for 30 cycles of 20 s at 94 °C, 20 s at 54 °C, and 45 s at 72 °C with a *Premix Taq*® DNA polymerase (TaKaRa). GAPDH was amplified similarly to serve as an internal control. The transcription of RNA-dependent protein kinase (PKR) gene and the 2′, 5′-oligoadenylate synthetase (OAS) gene (Goodbourn et al., 2000; Charles, 2001) was also analyzed by RT-PCR using primer pairs R-PKR/F-PKR and R-OAS/F-OAS, respectively. PCR products were resolved by electrophoresis on a 2% agarose gel.

2.5. IFN-α ELISA assay

According to the instruction, supernatants of each sample were collected and centrifuged for 10 min at 3000 × *g* to remove cell debris. Following centrifugation, 100 μl of supernatant from each sample was used to measure the amount of IFN-α protein by ELISA using the Porcine IFN-α ELISA Kit (CUSABIO Biotech).

2.6. One-step real-time RT-PCR

Transfected cells were infected with FMDV. Total RNA was isolated and RT-PCR was performed using the Platinum® Quantitative RT-PCR ThermoScript™ One-step Mastermix Reagents Kit (Invitrogen). Details of the reaction procedure, as well as PCR primers and fluorogenic probes, have been described previously (Gu et al., 2007). The reaction was optimized to a volume of 30 μl.

2.7. Plaque assay

PK cells were cultured in 6-well plates and transfected with the plasmids pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN prior to infection. After incubation for 48 h, cells were stained and viral titer was assessed using a plaque assay (Sabara and Larence, 2003).

3. Results

3.1. IFN-α expression in cells transfected with different IRES-IFN constructs

To achieve long-term protection from FMDV infection, we asked whether the introduction of the FMDV IRES element to the 5′ end of the IFN-α gene would help stabilize the translation of IFN-α mRNA when the host translation initiation factor eIF4G was inactivated by the virus. We used the IRES sequence located from 830 nt to 1428 nt in the FMDV genome (GenBank Accession no. DQ478936.1) to generate a plasmid pc-IRES-IFN that has the 599 nt IRES cDNA sequence inserted between the CMV promoter and the IFN-α gene in pcDNA3.1(+) (Fig. 1). These sequences mainly constitute the stem-loop structures as predicted by DNA Mfold server and it is unknown if this large piece of DNA containing multiple loop structures will impact on transfection or transcription. As a control in the FMDV infection experiments, two additional plasmid constructs were generated containing the IRESΔG and IRESΔT mutants. The pc-IRESΔG-IFN construct contains a GNRA loop deletion whereas pc-IRESΔT-IFN carries a RTTT loop deletion, both of which are similar in length to the IRES but have completely lost their function.

Before FMDV infection, most host mRNAs were translated using a cap-dependent mechanism. To investigate whether the introduction of FMDV IRES in pc-IRES-IFN would affect IFN-α production, we firstly examined the transcription of the IFN-α gene in PK cells transfected with the pc-IRES-IFN, pc-IRESΔG-IFN, pc-IRESΔT-IFN by RT-PCR. As shown in Fig. 2A, transcribed mRNA of IFN-α can be detected in cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN but not in mock-transfected cells. This indicated that the IRES element in pc-IRES-IFN construct did not block IFN-α transcription, and that IRESΔG and IRESΔT mutants were also unable to block IFN-α transcription. Furthermore, the introduction of the IRES, or IRES mutants alone, did not lead to detectable transcription of the endogenous IFN-α gene (Fig. 2A), indicating that endogenous IFN-α mRNA was either not transcribed or transcribed very little. This is consistent with the notion that, under physiological conditions, very little or no endogenous IFN-α was produced (Cheng et al., 2006).

We observed that the transcribed IFN-α mRNA was translated into functional protein, as IFN-α protein derived from exogenous IFN-α gene could be detected by ELISA in PK cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN (Fig. 2B). We also found very low production of IFN-α from endogenous gene and this expression was not steady. Additionally, two IFN-α/β-stimulated genes (ISGs), RNA-dependent protein kinase (PKR) gene and 2′,5′-oligoadenylate synthetase (OAS) gene (Goodbourn et al., 2000),

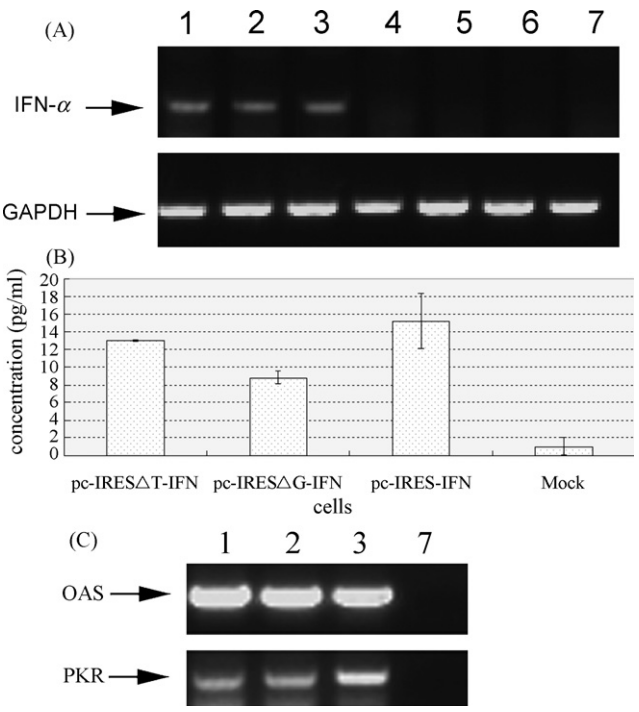

Fig. 2. IFN-α expression in PK cells transfected with different IRES-IFN constructs. (A) Detection of IFN-α mRNA level by RT-PCR. Lane 1–7, cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, pc-IRESΔT-IFN, pc-IRES, pc-IRESΔG, pc-IRESΔT, and pcDNA3.1(+), respectively. GAPDH serves as an internal control. (B) The expression level of IFN-α protein derived from exogenous IFN-α gene was determined by ELISA. PK cells were cultured in a 12-well plate and were transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN. Supernatants were collected 24 h later and the amount of IFN-α was assayed by ELISA. Each experiment was performed independently for more than one time. Data was shown as the average of three repeats. Error bars refer to standard deviations. To measure the expression of exogenous IFN-α gene, the expression level of mock-transfected cells was set as a blank control according to the instruction of ELISA kit. The difference in IFN-α expression levels could be due to variation in transfection efficiency. (C) Detection of PKR and OAS gene transcription by RT-PCR. The sample number in B is the same as in A.

were found to be transcriptionally active in PK cells transfected with either pc-IRES-IFN, pc-IRESΔG-IFN, or pc-IRESΔT-IFN, but not in mock-transfected cells (Fig. 2C). This finding indicated that there should be IFN-α protein present in cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN.

3.2. Exogenous IFN-α production during VSV infection

To elucidate whether the IFN-α expressed by the cells carrying the pc-IRES-IFN construct had antiviral activity, we firstly examined the cellular impact of VSV infection. The result showed that, after infection for 16 h, untransfected PK cells developed a cytopathic effect (CPE), but none of the cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN developed CPE (Fig. 3A). Those results demonstrated that IFN-α expressed by exogenous IFN-α gene was active.

We also performed the infection experiments with VSV. After a 12 h infection, PK cells transfected with pc-IRESΔG-IFN and pc-IRESΔT-IFN displayed a little increase in IFN-α production. But cells transfected with pc-IRES-IFN showed a 20% decrease in IFN-α productions (Fig. 3B). However, after 24 h, IFN-α production in all transfected cells increased to pre-infection level or above (Fig. 3B). Those also demonstrated that the expression levels of exogenous IFN-α displayed the same trends after infection by VSV. It suggested both wild-type and mutant IRES had no distinct effect on the expression of IFN-α.

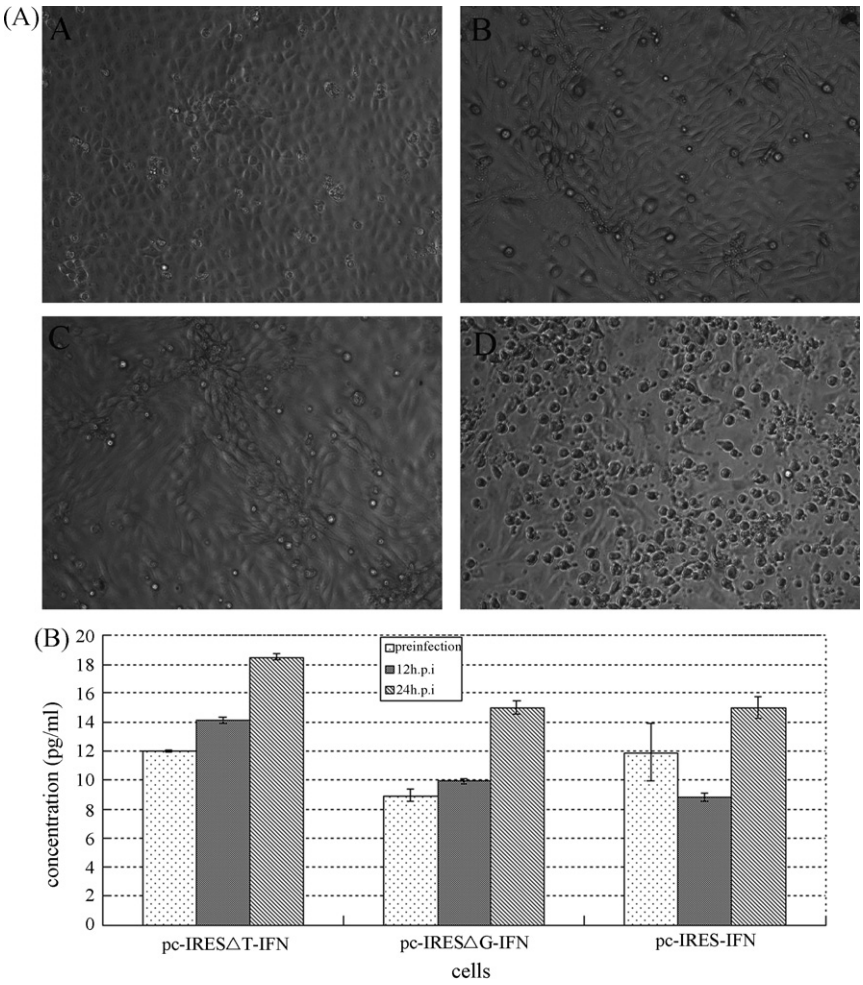

Fig. 3. Exogenous IFN-α production can protect cells against VSV infection. (A) Cell morphology after VSV infection. PK cells with or without IRES-IFN constructs were infected with VSV at 0.1 pfu/cell. Pictures were taken at 16 h.p.i. A: cells transfected with pc-IRES-IFN. B: cells transfected with pc-IRESΔG-IFN. C: cells transfected with pc-IRESΔT-IFN. D: PK cells. (B) Exogenous IFN-α production in transfected cells after VSV infection. PK cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN, along with an untransfected control, were cultured in a 12-well plate for 24 h before infection with VSV at 0.1 pfu/cell. Culture supernatants were collected at 12, and 24 h.p.i. to determine the amount of IFN-α by ELISA. Each experiment was performed independently for more than one time. Data was shown as the average of three repeats. Error bars refer to standard deviations. To measure the expression of exogenous IFN-α gene, the expression level of mock-transfected cells was set as a blank control according to the instruction of ELISA kit.

3.3. Exogenous IFN-α expression in cells transfected with different IRES-IFN constructs after FMDV infection

As mentioned earlier, before FMDV infection, host cells relied on a cap-dependent translation mechanism to synthesize all proteins. We speculated that, in cells transfected with pc-IRESΔG-IFN and pc-IRESΔT-IFN, which carried nonfunctional FMDV IRES mutants, there should be no distinct difference in the production of exogenous IFN-α compared with cells transfected with pc-IRES-IFN prior to infection. However, after FMDV infection, the production of exogenous IFN-α in cells transfected with pc-IRESΔG-IFN and pc-IRESΔT-IFN could be down-regulated along with other host proteins, but IFN-α production in cells transfected with pc-IRES-IFN might continue due to the function of the *cis*-acting IRES in mRNA translation.

To investigate whether the IRES-IFN construct could stabilize IFN-α production after FMDV infection, PK cells were transfected with the plasmids pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN 24 h prior to FMDV infection. Supernatants from each culture were collected at 12 and 24 h post-infection (h.p.i) to measure the amount of IFN-α. We found that all cultures displayed a reduction of IFN-α production after infection for 12 h, and after 24 h, IFN-α production in cells transfected with pc-IRESΔG-IFN and pc-IRESΔT-IFN, continued to decline to almost 50% of the pre-infection level (Fig. 4A). In contrast to this decrease in production, IFN-α from cells transfected with pc-IRES-IFN increased to 150% of the pre-infection level (Fig. 4A). These findings demonstrated that the FMDV IRES element could not only help stabilize IFN-α expression after FMDV infection, but also augment the expression of IFN-α.

3.4. The anti-FMDV activity of IRES-regulated IFN-α expression

To evaluate whether IRES-regulated expression of IFN-α could confer protection against FMDV infection of cells, we examined the amount of viral RNA after FMDV infection. PK cells were transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN, prior to infection with FMDV at 0.1 pfu/cell. Cells were harvested after 4 h, and total RNA was extracted to measure viral RNA by real-time RT-PCR analysis. The result showed that the amount of viral RNA in cells transfected with pc-IRES-IFN was fivefold lower than that in cells transfected with pc-IRESΔG-IFN and pc-IRESΔT-IFN. Further, all of them are outclassed by the mock-transfected cells (Fig. 4B). To address the impact of those differences directly, plaque assays were performed to confirm that cells transfected with pc-IRES-IFN were more resistant to FMDV infection than those transfected with pc-IRESΔG-IFN and pc-IRESΔT-IFN (Fig. 4C). The data showed that

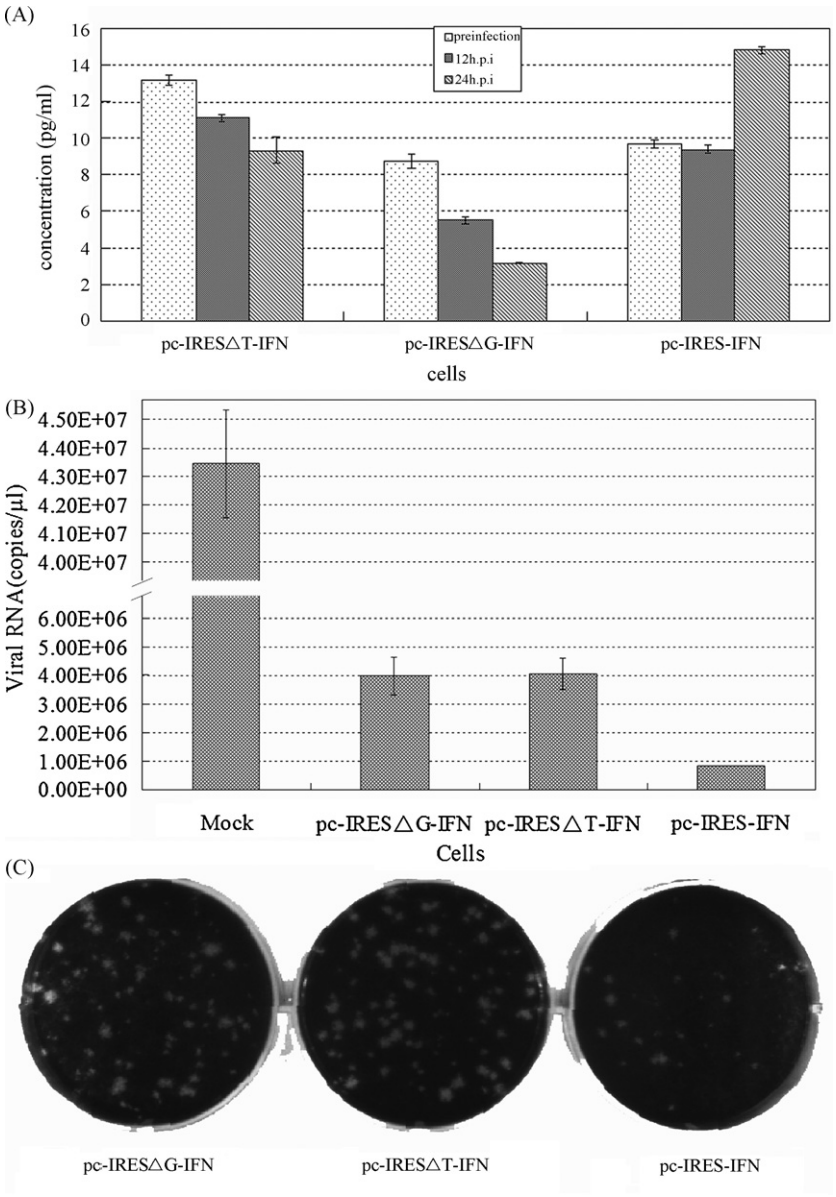

Fig. 4. IRES-regulated expression of exogenous IFN-α in cells could offer protection from FMDV infection. (A) PK cells transfected with pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN, along with the untransfected control, were cultured in a 12-well plate for 24 h prior to infection with FMDV. Supernatants from each cell culture were collected at 12 and 24 h.p.i to evaluate the amount of IFN-α by ELISA. Each experiment was performed independently for more than one time. Data was shown as the average of three repeats. Error bars refer to standard deviations. To measure the expression of exogenous IFN-α gene, the expression level of mock-transfected cells was set as a blank control according to the instruction of ELISA kit. (B) The level of viral RNA after infection is shown. Transfected PK cells were cultured in a 12-well plate and infected with FMDV at 0.1 pfu/cell. RNA was extracted 4 h later to determine the amount of FMDV RNA by real-time RT-PCR. Each experiment was performed independently for more than one time. Data was shown as the average of three repeats. Error bars refer to standard deviations. (C) A plaque assay is shown. PK cells were cultured in a 6-well plate and transfected with the plasmids pc-IRES-IFN, pc-IRESΔG-IFN, and pc-IRESΔT-IFN. Once cells were a confluent monolayer, cells were infected with FMDV at 1000 pfu/well. After a 48 h incubation, cells were stained to be observed. We performed at least one more independent experiment and obtained results consistent with those shown.

the inhibition efficiency of IFN-α was almost 80% in cells transfected with mutant IRES-IFN construct and 95% in cells transfected with pc-IRES-IFN.

Additionally, we examined the morphology of cells after FMDV infection. Cells were cultured in a 12-well plate and infected with FMDV at 1 pfu/cell. Cytopathic effects were observed in PK cells (Fig. 5 panel I) transfected with pc-IRESΔG-IFN (Fig. 5 panel C), pc-IRESΔT-IFN (Fig. 5 panel D), and pcDNA3.1(+) (Fig. 5 panel H), as well as untransfected cells (Fig. 5 panel A), 8 h post-infection (Fig. 5). In contrast, CPE was not observed in cells transfected with pc-IRES-IFN (Fig. 5 panel B) in the same timeframe. In fact, these cells did not exhibit CPE until 16 h post-infection (data not shown). Notably, cells transfected with pc-IRES-IFN received long-term protection from FMDV infection.

4. Discussion

It's obviously that IFN-α expression under the regulation of FMDV IRES can provide enhanced protection to transfected cells. Interferon is a powerful tool to prevent and cure viral infection. However, it can also inhibit protein synthesis in host cells (Roberts et al., 1976; Robertson and Mathews, 1973). A wide array of adverse effects associated with interferon over-expression has been reported (Valentine et al., 1998; Dusheiko, 2003). In order to balance the protective and deleterious effects of IFN-α, the expression of exogenous IFN-α prior to FMDV infection must be low, whereas expression during a productive FMDV infection must be robust and prolonged. So far, all the methods available are not able to solve this problem. In this study, we report a new method to protect cells from

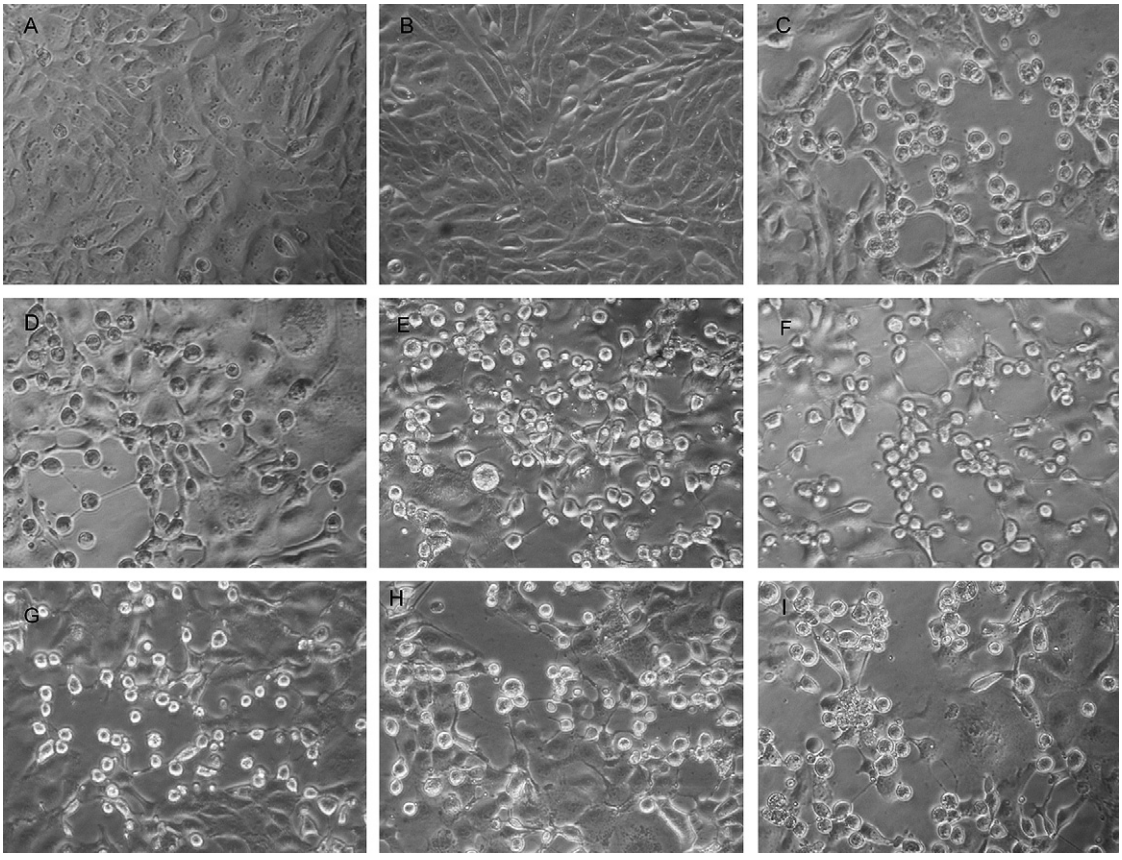

Fig. 5. Cell morphology after FMDV infection is shown. Transfected PK cells were infected with FMDV at 1 pfu/cell. Pictures were taken at 8 h.p.i. Panel A: PK cells without infection. Panel B: PK cells transfected with pc-IRES-IFN. Panel C: PK cells transfected with pc-IRESΔG-IFN. Panel D: PK cells transfected with pc-IRESΔT-IFN. Panel E: PK cells transfected with pc-IRES. Panel F: PK cells transfected with pc-IRESCΔT. Panel G: PK cells transfected with pc-IRESCΔG. Panel H: PK cells transfected with pcDNA3.1(+). Panel I: untransfected PK cells.

FMDV infection by FMDV IRES-modulated ectopic expression of IFN-α. This new method takes advantage of a key element of FMDV, its IRES, to prolong the expression of exogenous IFN-α after FMDV infection, which successfully leads to protection against FMDV.

It has been demonstrated that IFN-α gene delivered by replication-defective adenovirus vector could successfully protected against FMDV (Moraes et al., 2003; Moraes et al., 2007; Chinsangaram et al., 2003). First, infection of adenovirus will stimulate the protective state of host; second, expression of exdogenous IFN-α gene can enhance the antiviral state of host. In addition, IFN-α protects against all serotypes and sub-type of FMDV. But it's still a problem to prolong the expression of IFN-α to provide a long-term protection. On the base of our finding, introduction of FMDV IRES is an ideal choice. Furthermore, previous study showed that monocistronic mRNA containing FMDV IRES was translated significantly better than a standard cellular mRNA (Paulous et al., 2003).

We introduced the FMDV IRES element into a CMV promoter-controlled IFN-α expression cassette. PK cells transfected with this construct grew normally (date not shown), suggesting that the amount of ectopically expressed IFN-α did not cause unendurable adverse effect to host cells. To determine whether the IRES element in the construct may interfere or enhance the expression of IFN-α in the absence of FMDV, we compared the effect of wild-type and mutated nonfunctional IRES on IFN-α expression. We found that the expression levels of IFN-α regulated by wild-type and mutated IRES were similar, indicating that IRES did not function in the absence of FMDV infection. On the other hand, when cells were infected with FMDV, we found that wild-type IRES-regulated IFN-α expression was increased compared with mutated IRES-regulated IFN-α expression. This suggests that the FMDV IRES-regulated expression of IFN-α could prolong the expression of exogenous IFN-α by escaping the translational blockade by FMDV. Therefore, FMDV IRES appears to be an ideal modulator that not only allows proper expression of IFN-α to avoid adverse effect when FMDV is absent, but also increases the expression of IFN-α after FMDV infection.

Interferon is a cytokine, which acts as an alarm to arouse a host's antiviral immunity. So the duration of interferon is very important for its activity. Our result indicated that IRES-modulated IFN-α could express continually before and after FMDV infection. As a non-coding sequence, IRES causes no or minimal immunoreactions. In our study, when cells were transfected with pc-IRES, pc-IRESΔG, pc-IRESΔT as well as mock-transfected cells, no transcription of IFN-α mRNA was detected. Therefore, FMDV IRES appears to be a good choice to regulate IFN-α expression in response to FMDV infection.

In this study, we also evaluated the antiviral activity of IFN-α against VSV. VSV is routinely used in WISH-VSV (WISH cells infected with VSV) assay to evaluate the antiviral activity of IFN-α (Peng et al., 2006). We found that IFN-α expression regulated by wild-type and mutated IRES both could protect PK cells from VSV infection, and the expression level of IFN-α increased as the incubation prolongs. This effect could be due to two reasons. First, VSV did not shut off the host's translation as FMDV did, and the expression of exogenous IFN-α continued. Second, VSV infection greatly stimulated the expression of endogenous IFN-α (Belardelli, 1995). Compared the effect of wild-type IRES and mutated IRES on IFN-α expression after VSV infection, a similar trend of increase in IFN-α expression was observed. By contrast, the trend was no long similar when infected by FMDV, as our result showed that only wild-type IRES-regulated IFN-α could be expressed constantly. It's clear that only wild-type

IRES could help to stabilize and augment the expression of IFN-α after FMDV infection.

Many researches focused on using IRES to generate bicistronic constructs in order to co-express various proteins or to explore IRES function (Martinez-Salas, 1999). As mentioned early, combination of two antiviral proteins can increase the antiviral activity against FMDV (Moraes et al., 2003; Moraes et al., 2007; Du et al., 2008). Future studies will focus on how to utilize FMDV IRES to construct a polycistronic vector, with which two or more anti-FMDV proteins can be expressed simultaneously. Together, they may offer better protection against viral infection. *In vivo* tests are also planed to be carried on when adenovirus vector mediated gene delivering system is completed. Based on ours findings, adenovirus vector delivered IRES-IFN construct or polycistronic construct will play an important role in anti-FMDV therapy and immunity.

However, some problems still remain. The antiviral activity of IFN-α is dependent on the function of interferon-stimulated genes (ISGs) (Billiau, 2006). Although IFN-α can be produced consistently under the regulation of IRES after FMDV infection, the expression of ISGs could not escape the translational block mediated by FMDV. Without the help of ISGs, the antiviral power of IFN-α cannot be fully unleashed.

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