RESEARCH REPORT
SWINE HEALTH

Title: Improvement of Interferon Biotherapeutics for Foot-and-mouth Disease in Swine – NPB #14-014

Investigator: James J. Zhu (Email: james.zhu@ars.usda.gov.com; Tel: 631-323-3340)

Institution: USDA-ARS, FADRU, Plum Island Animal Disease Center, Orient, NY 11957.

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Industry Summary:

Vaccination is one of the best strategies to prevent viral diseases; however, it usually takes 1 - 2 weeks for vaccines to induce protective immunity. Animals may become sick if they come into contact with the pathogens before the establishment of protective immunity. Foot-and-mouth disease virus (FMDV) is one of the most contagious animal viruses that could have devastating effects on livestock industries. There are commercial FMD vaccines available. The elapsed time of full protection for FMD vaccines is about one week. Development of a countermeasure with rapid onset of immunity will greatly facilitate the control of this disease. Biotherapeutics using a replication-defective recombinant adenovirus carrying an interferon gene can fully protect pigs from FMDV infection 24 hours after treatment. However, the disadvantages of this biotherapeutics are the requirement of high dose and short-lasting effect. The objective of this research is to enhance the potency of this adenovirus-based interferon biotherapeutics to overcome these drawbacks. We applied several strategies to produce three new recombinant adenoviruses. All these recombinant viruses induced higher anti-FMDV activity in cell culture than the adenovirus previously tested. We then selected the top two new and the previously tested adenoviruses to test in pigs. Based on the antiviral activities induced in the sera of treated pigs, the best adenovirus is greater than 20 fold more potent than the previous recombinant virus. It induced not only significantly higher but also longer lasting anti-FMDV activity even at a dose ten times lower than the previous virus. Our future plans are to determine the protective dose of this new biotherapeutics against viral challenges in pigs and to develop a system to produce this recombinant virus efficiently. There are still approaches that can be used to improve this biotherapeutics further and eventually make it feasible for commercial production.

Keywords: Interferons, biotherapeutics, Foot-and-mouth disease virus, antiviral activity, recombinant adenovirus, porcine

Scientific Abstract:

Foot-and-mouth disease virus (FMDV) is one of the most contagious animal viruses with potential devastating economic effect. There are commercial FMD vaccines available; however, it usually takes a week for the vaccines to induce protective immunity. Biotherapeutics using a replication-defective adenovirus inserted with an interferon gene can completely protect pigs from FMDV infection. However, the disadvantages of this biotherapeutics are the requirement of high dose and short-lasting effect. To improve it, we used two strategies: (1) to identify the most potent interferon gene for use in the biotherapeutics, (2) to enhance interferon production and (3) to develop novel biotherapeutics using interferon regulatory factor (IRF) genes. Thirty-seven porcine interferon genes were transiently expressed in cell culture and their anti-FMDV activities were compared using a CPE reduction assay. The highest and the lowest antiviral activities of genes differed more than one thousand times. Adenovirus inserted with the top interferon gene induced an anti-FMDV activity four-fold higher in cell culture than the previous one. To increase interferon production, an adenovirus tripartite sequence and a porcine SOCS1 gene with an EF1a promoter were inserted into the adenovirus. This new recombinant virus induced up to 170-fold higher anti-FMDV activity in cell culture than the previous adenovirus. Among three constitutively active IRF genes tested, IRF2 induced the highest anti-FMDV activity. The recombinant adenovirus inserted with IRF2 also induced a higher anti-FMDV activity than the one previously tested. These two new and the previous tested recombinant adenoviruses were compared in pigs. The results of anti-FMDV activity in the sera of treated pigs indicated that the adenovirus inserted with the best IFN and SOCS1 gene is the best biotherapeutics with the improvement of greater than 20 fold in potency. This new recombinant adenovirus not only enhanced the magnitude of antiviral activity but also prolonged the duration of the activity.

Introduction:

Vaccination is one of the best strategies to prevent viral diseases; however, it usually takes 1 - 2 weeks for vaccines to induce protective immunity. Animals may become sick if they come into contact with the pathogens before the establishment of protective immunity. Foot-and-mouth disease virus (FMDV) is a positive-sense, single-stranded RNA virus belonging to the Aphthovirus genus of the Picornaviridae family and is capable of causing an acute vesicular disease in cloven-hoofed animals including cattle, swine, goats and sheep. It is one of the most contagious animal viruses and could have a devastating economic effect on livestock industries if outbreaks occurred, especially in FMD-free countries. Although there are commercial FMD vaccines available, it takes approximately one week for the vaccines to induce protective immunity. Development of a countermeasure with a rapid onset of immunity will greatly facilitate the control of this disease.

FMDV has been known to be very sensitive to the inhibition of type I interferons (IFN) (Chinsangaram et al., 1999; Sellers, 1963). Because of their rapid and potent antiviral effects, type I IFN genes have been used to induce rapid onset of immune protection against FMDV in swine. Pigs can be completely protected against FMDV challenge 24 h after injection with a replication-defective human adenovirus 5 vector (Ad5) inserted with an IFNα gene (Chinsangaram et al., 2003; Dias et al., 2011; Moraes et al., 2003). However, this biotherapeutics requires much higher protecting doses than Ad5-based FMDV vaccines (Dias et al., 2011; Pena et al., 2008) and the protective activity lasted less than a week, which limits its field application. A feasible biotherapeutics that can induce rapid and long lasting protection against FMDV can significantly facilitate the control of the disease during the outbreaks.

The type I IFN gene family consists of several subtypes in all mammalian species, and some subtypes contain multiple genes (Roberts et al., 1998). The antiviral activities of the genes differ a great deal (Moll et al., 2011). In pigs, seven subtypes (α, αω, β, δ, ε, κ and ω) have been reported (Sang et al., 2010), and the antiviral activities against PRRSV and VSV infection differ significantly among genes and in different cell lines (Sang et al., 2010; Zanotti et al., 2015). There are substantial polymorphisms in the genes among individuals, which account for significant differences in antiviral activity among the genes (Sang et al., 2011). These results indicate that it is important in terms of biotherapeutic potency to screen a large number of genes and to test in multiple cell lines to identify genes with the highest virus-specific antiviral activity.

To improve IFN biotherapeutics, we hypothesized that the potency could be greatly enhanced if the IFN gene with the highest anti-FMDV activity was inserted into the Ad5 vector. Additionally, this biotherapeutics could be improved further by increasing IFN expression from the gene inserted in recombinant adenovirus. Because IFN signaling can shut down protein translation in IFN-stimulated cells (Ivashkiv and Donlin, 2014) and SOCS1 inhibits type 1 IFN signaling (Fenner et al., 2006), we hypothesized that inserting SOCS1 gene into recombinant adenoviruses containing IFN gene can enhance IFN production from the gene in the infected cells.

Because type I IFN and other antiviral immune effectors can be induced by some activated transcription factors such as activated interferon regulatory factors (IRFs)(Honda and Taniguchi, 2006), these genes could also be used as an alternative approach to developing antiviral biotherapeutics. IRF1, IRF2, IRF3 and IRF7 have been reported to be the major transcription regulators of type I IFN genes (Gu et al., 2015; Harada et al., 1989; Lin et al., 2000; Lin et al., 1994; Miyamoto et al., 1988; Morin et al., 2002). Constitutively active IRFs using phosphorylation mimicking could induce IFN expression as indicated in IFNβ promoter reporter assays (Lin et al., 2000; Lin et al., 1994). Given that there are multiple type I IFN genes in animal genomes, using these constitutively active IRFs in adenovirus-based IFN biotherapeutics may enhance the potency.

We have developed a colorimetric cytopathic effect reduction assay (MTT-CPER assay) to measure anti-FMDV activity (Ramanathan et al., 2015). The MTT-CPER assay is more cost-effective, higher throughput, less labor intensive and more sensitive than the plaque reduction assay. FMDV-susceptible porcine cell lines are used in the assay to measure anti-FMDV specific activity. In this study, we used this assay to screen for the IFN gene with the highest anti-FMDV activity, to test the effect of SOCS1 on IFN expression and to develop alternative anti-FMDV biotherapeutics to improve the existing IFN biotherapeutics.

Objectives: The goal of this proposed research is to develop more effective Ad5-based biotherapeutics in terms of cost and potency for the pork industries to control foot-and-mouth disease (FMD). The overall objective of this research is to improve the potency of the existing IFN biotherapeutics developed by Dr. Grubman. In this proposed research, we used *in-vitro* studies (1) to identify the best porcine type I IFN gene for the biotherapeutics, (2) to increase the magnitude and duration of IFN production of the IFN gene, (3) to develop a new biotherapeutics using an immune activator gene, and (4) to test the potency of new biotherapeutics developed in this study in pigs to validate *in-vitro* results.

Materials & Methods:

1. Cells and viruses

Immortalized LFBK-αvβ6 kidney cells (LaRocco et al., 2013; Swaney, 1988), IBRS-2 kidney cells (House et al., 1988) and HEK 293 cells (Graham et al., 1977) were obtained from the Foreign Animal Disease Diagnostic Laboratory at the Plum Island Animal Disease Center. The LFBK-αvβ6 cells were cultured in Dulbecco's Modified Eagle medium (DMEM, GIBCO, Grand Island, NY) containing high glucose, 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and supplemented with 1% Antibiotics-Antimycotic 100X (GIBCO) and 1% Sodium Pyruvate 100X (GIBCO). The IBRS-2 and HEK 293 cells were grown in Minimum Essential Medium (MEM, GIBCO) with 10% FBS, 1% L-Glutamine, 1% Antibiotics, and Non-Essential Amino Acids 100X (GIBCO). All cell culture media and reagents were purchased from Life Technologies (Carlsbad, CA) unless specified otherwise. Titrated FMDV type A24 Cruzeiro strain was used in this study.

2. Interferon Expression

Full-length coding sequences of 19 porcine IFNα and 5 β genes were identified from the pig genome sequences released in Aug. 2011 (SGSC Sscrofa10.2/susScr3 Assembly) using the UCSC Genome Browser. Additionally, 13 IFNα coding sequences of miniature pigs were retrieved from the genomic sequences deposited in NCBI GenBank from Accession #: PRJNA176189, AJKK01153980, AJKK01221111, AJKK01220487, AJKK01240321, AJKK01266018, AJKK01153977, and AJKK01148380. The coding DNA sequences were cloned into pcDNA3.1-vector (Life Technologies) between the NheI and NotI restriction sites. The plasmids containing the inserts of interests as well as a vector only control were transiently transfected into LFBK-αvβ6 for expression of their respective porcine proteins using Lipofectamine 2000 (ThermoFisher Scientific). The cell culture supernatants were harvested 2 and 4 days post-transfection and stored at -70°C until assayed. The expressed IFN proteins were detected by Western blotting using a rabbit anti-porcine IFN-α as a primary antibody and the WesternDot 625 Goat Anti-rabbit Western Blot Kit (ThermoFisher Scientific). The imaging was performed using a GelDoc imager (BioRad).

3. MTT-CPER assay

The anti-FMDV activity of the harvested cell culture supernatants was measured with an MTT-CPE reduction (MTT-CPER) assay we developed (Ramanathan et al., 2015). Briefly, IBRS-2 and/or LFBK-αvβ6 cells were plated in 96-well flat-bottomed tissue culture plates. After overnight incubation, the cells were treated with two-fold serially diluted cell culture supernatants harvested from the cells transfected with plasmid DNA or infected with Ad5 recombinant viruses. Then the IFN-containing media were removed and the cells were inoculated with FMDV A24 Cruzeiro at a multiplicity of infection (MOI) of 0.4. After overnight incubation, an MTT substrate (ATCC, Manassas, VA) was added to each well and the plates were stored in the dark for at least 3 hours. Then a detergent reagent (ATCC, Manassas, VA) was added to each well for another 3 hours incubation at room temperature before spectrometry. The absorbance or optical density (OD) readings in each well was measured using an ELx808 Absorbance Microplate Reader at 570 nm with the reference filter set at 650 nm (BioTek, Winooski, VT). The blank values were subtracted from the absorbance values and the average of the three replicates are reported. Each assay was repeated at least twice. In this study, OD readings were used as the indicator of anti-FMDV activity (cells protected by IFN against FMDV infection).

4. Effect of non-coding sequences (NCS) on IFNα expression

To test the effect of non-coding sequences on IFN expression, an adenovirus tripartite NCS (Logan & Shenk, 1984; Kaufman, 1985) and the 3'-end NCS of ACTA1 (NM_174225) was placed in the 5' or 3'-ends of an IFNα coding sequence, respectively, and inserted into pcDNA3.1 plasmid between NheI and NotI restriction sites. The plasmid inserted with IFNα coding sequence only was used as the control. These plasmids were

transfected with an equal amount of DNA into LFBK-αvβ6 cells. After DNA transfection, the supernatants were harvested for MTT-CPER assay as described above at days 2, 4 and 6 post transfection.

5. Co-transfection of SOCS1 and IFNα

To test the effect of SOCS1 on IFN expression, the porcine SOCS1 coding sequence (NM_001204768) was inserted into the pcDNA3.1 plasmid between NheI and NotI restriction sites. The plasmid containing a Socs1 gene was used to co-transfect LFBK-αvβ6 cells with a pcDNA3.1 plasmid inserted with an interferon α gene at 1:1 and 1:3 (pcDNA3.1-IFNα vs pcDNA3.1-SOCS1) of DNA using the transfection procedure described earlier. A co-transfection with the same amount of plasmid pcDNA3.1 vector and the plasmid inserted with the interferon gene was used as the control to assess the effect of the SOCS1 gene on the interferon expression. After DNA transfection, the supernatants were harvested for MTT-CPER assay as described above.

6. EF1α Promoter

To insert two genes into the Ad5-blue vector, we constructed a promoter using upstream the sequence of bovine and porcine EF1α coding sequence. These sequences were synthesized with flanking restriction sites of MfeI and NheI by GenScript. The pcDNA3.1 plasmid inserted with a coding sequence of an interferon α gene between NheI and NotI sites was digested with MfeI and NheI restriction enzymes and ligated with MfeI and NheI restriction enzyme digested bovine and/or porcine promoter DNA fragments, which replaced the hCMV promoter of the pcDNA3.1 plasmid vector (pcDNA3.1_hCMV-IFNα). These two plasmids containing bovine and porcine promoters were named as pcDNA3.1_bEF1α-IFNα and pcDNA3.1_sEF1α-IFNα, respectively. These three plasmid DNA samples were used to transfect HEK293 and LFBK-αvβ6 cells to measure anti-FMDV activity induced in the culture supernatants as described earlier.

7. IRF Expressing Plasmids

The coding DNA sequences of bovine and/or porcine IRF2, 3, and 7 and RELA (NM_001205793, NM_001029845, NM_001105040, NM_001080242, AK238995, NM_001114281) were downloaded from GenBank. Constitutively active bovine IRF2, 3 and 7 and porcine IRF2 were constructed according to Lin et al. (Lin et al., 2000; Lin et al., 1994). These coding sequences containing a NheI and NotI restriction site sequence in the 5'- and 3'-ends, respectively, were synthesized by GenScript (Piscataway, NJ). After restriction digestion, these DNA fragments were sub-cloned into pcDNA3.1- vector (Life Technologies) between NheI and NotI restriction sites. The plasmid DNA was isolated using Qiagen miniprep kit (Valencia, CA). The antiviral activities of these three genes were compared with the same methods described for the IFN genes.

8. Adenovirus production

Ad5 Blue plasmid vector (Moraes et al., 2001) was used to produce recombinant adenoviruses expressing the genes of interest. A porcine IFNα gene previously tested in pigs (Chinsangaram et al., 2003) and the IFN and IRF genes with the highest anti-FMDV activity identified in this study were cloned into the vector using ClaI and XbaI cloning sites. The plasmids with the correct inserts were used to transfect HEK293 cells with Lipofectamine in Opti-MEM after linearization with PacI restriction enzyme (New England Biolabs, Ipswich, MA). The recombinant viruses were isolated from the plaques, propagated in HEK293 cells and purified by CsCl gradient centrifugation. The recombinant adenoviruses were produced by Welgen Inc (Worcester, MA). To construct recombinant adenovirus containing two inserted genes, a poly-A termination sequence, the promoter of porcine EF1α gene and the coding sequence of porcine SOCS1 were inserted at the

3'-end of the coding sequence of the identified IFN gene. This fragment was inserted into the Ad5-blue vector using ClaI and XbaI sites to produce recombinant adenovirus as described earlier.

9. Adenovirus titration

Titers of recombinant adenoviruses were determined based on tissue culture infectious dose (TCID50) using HEK293 cell monolayer in 96 well plates according to Moraes et al. (Moraes et al., 2002). Briefly, the cells were plated at a density of $1x10^4$ cells per well, incubated at 37°C with 5% CO_2 for 3 days or 95-100% confluency. Tenfold serial dilutions starting at 10^{-5} to 10^{-12} in Minimum Essential Medium (MEM, GIBCO) were prepared in 1.7 ml sterile micro-centrifuge tubes. Prior to inoculation, the cell culture media was removed and 100 µl per well of the diluted samples was added. Sixteen replicates per dilution and eight dilutions per titration with two independent replications were performed. The plates were incubated at 37°C, 5% CO_2 and checked for the presence of CPE daily for 10 days. Spearman-Karber 50% endpoint viral titers were calculated for TCID50.

10. Ad5-virus-produced antiviral activity

To measure the anti-FMDV activity of the recombinant adenoviruses, LFBK-αvβ6 cells cultured in 24-well plates were infected at different MOIs of recombinant adenoviruses (2-fold serial dilution with the serum-free medium) for 1 hour. After the infection, the viruses were removed and the wells were washed three times with medium. Fresh growth medium was added to each well and cell culture supernatants were collected at different time points after the infection and filtered using Centricon® 30 filters (Millipore, Billerica, MA). The anti-FMDV activity of the supernatants was measured with the MTT-CPER assay as described earlier.

11. Statistical analysis

For the determination of anti-FMDV activity of the genes and the recombinant adenovirus, the OD readings were fit to a sigmoid dose-response curve using GraphPad Prism software package (PBL Assay Science, Piscataway, NJ). The half maximal effective concentration (EC50) used as the indicator of anti-FMDV activity were calculated using the software based on the fold dilutions of the supernatants and the OD readings of the culture wells. IFN gene GQ415066/IFN19 was used as a reference in all DNA transfections and antiviral assays for comparison among the genes. The gene with the highest anti-FMDV activity was given an arbitrary index number of 1 as an anti-FMDV activity. The EC50 of other genes was divided by the gene with the highest activity to normalize the antiviral activity. Differences in anti-FMDV activity between the Ad5 viruses created in this study and the Ad5 viruses tested previously were calculated by taking both MOI and EC50 into account.

12. Animal testing

Commercial pigs (body weight at 50-60 LB) were injected subcutaneously with one of three recombinant adenoviruses (the existing adenovirus: Ad5-IFNα and two new adenoviruses: Ad5-IRF2 and Ad5-IFN19+SOCS1 also named as Ad5IFN19+) at 10^{10} (FMD protective dose) and/or 10^9 plaque forming unit (PFU) per pig. There were four pigs per treatment group including a control group injected with PBS only. The available amount of Ad5-IFN19+ was enough only for three pigs at 10^9 PFU per animal. Blood samples (5 ml) were collected at one day before injection and days 1, 2, 3, 4, 5, 6, and 7 post injection. Sera were prepared from the blood samples for measuring anti-FMDV activity using MTT-CPER assay as described earlier. Two-fold serial dilutions of serum samples (2^1 to 2^{12}) were used in the assay. Three replications per sample were

conducted in the MTT-CPER assay. The animal use protocol was reviewed and approved by Animal Use Review Committees at the University of Nebraska and the *in-vivo* experiment was performed in the animal facility located at the College of Veterinary Medicine, University of Nebraska. The Serum samples were shipped to Plum Island Animal Disease Center on dry ice for the MTT-CPER assays.

Results:

1. Identification of the best porcine IFN gene

1a. Protein expression of porcine IFNα and β genes

All porcine IFNα proteins were highly expressed in the cell line using Lipofectamine mediated transient DNA transfection with significant variations in expression levels based on Western blotting results (Figure 1). Each IFN displayed two bands (one with and another without glycosylation) as expected. DNA transfection with pcDNA3.1-vector alone also induced low IFN expression visible on the Western blot (Lane 8 in Figure 1); however, there was detectable low anti-FMDV activity in the supernatants from transfection of plasmid vector only based on the MTT-CPER assays (data not shown). Supernatants harvested from two transfections using two independent plasmids with identical coding sequences displayed nearly identical OD reading in the MTT-CPER assay (data not shown), indicating the approach is accurate and specific regarding assessing the antiviral activity of specific porcine IFN genes.

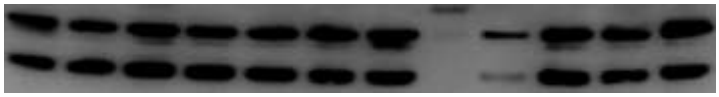


Figure 1. Western blotting of expressed porcine interferon α (Lane 1-7 and 10-12: IFNα; Lane 8: molecular weight markers, Lane 9: plasmid vector)

1b. Antiviral activity of porcine IFNα and β genes

Among the IFN genes tested, Gene GQ415066 (named IFN19 in this study) consistently displayed the highest anti-FMDV activity in MTT-CPER assays using two cell lines (IBRS-2 and LFBK-αvβ6) and cell culture supernatants harvested at two different time points (2 and 4 days post-DNA transfection) (data not shown). Table 1 listed the normalized EC50 of tested IFN genes with indexes of anti-FMDV activity relative to the best gene. The activity at days 2 and 4 were highly correlated (r^2 = 0.95 among IFNα and r^2 = 0.99 among IFNβ genes). Interestingly, the decreases in anti-FMDV activity from day 2 to 4 were much greater for IFNβ (averaging ~200 fold) than those for IFNα (averaging ~30 folds). Based on the EC50, the differences among the genes tested ranged from approximately 2 to more than 1,000 fold.

2. Enhancement of IFN expression

2a. Effect of non-coding sequences on IFN expression

Figures 2 shows that the transfection of plasmid inserted with IFNα containing the adenovirus tripartite NCS induced higher (approximately two fold) antiviral activity than that of plasmid inserted with IFNα without the NCS in the supernatants harvested at days 2 (Figure 2a),
4 (Figure 2b) and 6 (Figure 2c) post transfection. In contrast, there were no differences between the supernatants from transfections with plasmids inserted with IFNα containing 3' end NCS of

Table 1. The EC50 of porcine interferons α and β genes expressed from IBRS-2 cells transfected with pcDNA3.1 inserted with interferon genes

		Day 2		Day 4	
Gene	Type	EC50	Index	EC50	Index
GQ415066	α	537025	1.000	21901	1.000
AOCR01001737	α	329558	0.614	12083	0.552
AJKK01220487	α	307724	0.573	7711	0.352
GQ415060	α	296130	0.551	9772	0.446
AJKK01153977	α	200159	0.373	5059	0.231
XM_003480504	α	143237	0.267	9696	0.443
AOCR01194172	α	101432	0.189	2670	0.122
GQ415056	α	97004	0.181	3814	0.174
NM_001195375	α	96768	0.180	4049	0.185
AJKK01148380	α	83962	0.156	1568	0.072
AJKK01240321	α	83327	0.155	3194	0.146
AJKK01153980	α	80698	0.150	4180	0.191
XM_003480505	α	76442	0.142	4762	0.217
AOCR01194178	α	74862	0.139	1421	0.065
DQ872659	α	67009	0.125	2988	0.136
AOCR01194172-2	α	61482	0.114	1220	0.056
NM_001166319	α	53063	0.099	2464	0.113
XM_003480507	α	45761	0.085	2787	0.127
XM_005660067	α	45205	0.084	2747	0.125
AOCR01027891	α	43572	0.081	1337	0.061
NM_001195377	α	31527	0.059	1490	0.068
XM_003353504	α	18092	0.034	574	0.026
NM_001164855	α	17022	0.032	836	0.038
NM_001166311	α	16282	0.030	666	0.030
XM_003480495	α	7586	0.014	468	0.021
AJKK01266018	α	7015	0.013	235	0.011
X57191	α	3219	0.006	313	0.014
XM_003353507	α	905	0.002	69	0.003
GQ415061	α	ND*		ND	
NM_001164860	α	ND		ND	
XM_003121882	α	ND		ND	
AJKK01221111	α	ND		ND	
AY687281	β	83637	0.156	514	0.023
GQ415073	β	29304	0.055	143	0.007
KF414741	β	7336	0.014	57	0.003
EF104599	β	357	0.001	ND	
JF906509	β	ND		ND	

ND: EC50 could not be reliably estimated with the MTT-CPER assay due to low anti-FMDV activity

ACTA1 (data not shown). Based on these results, two recombinant adenoviruses were produced from the Ad5 virus inserted with the IFNα genes with and without the NCS (Ad5-NCS-IFNα and Ad5-IFNα, respectively). Ad5-NCS-IFNa induced approximately 2 fold higher antiviral activity than Ad5-IFNa did in the supernatants harvested at days 2, 4 and 6 from the cells infected at MOI of 20 (only day 6 shown in Figure 2d). Therefore, both *in-vitro* tests show that the adenovirus tripartite NCS enhanced the expression of the recombinant IFN from the Ad5 virus.

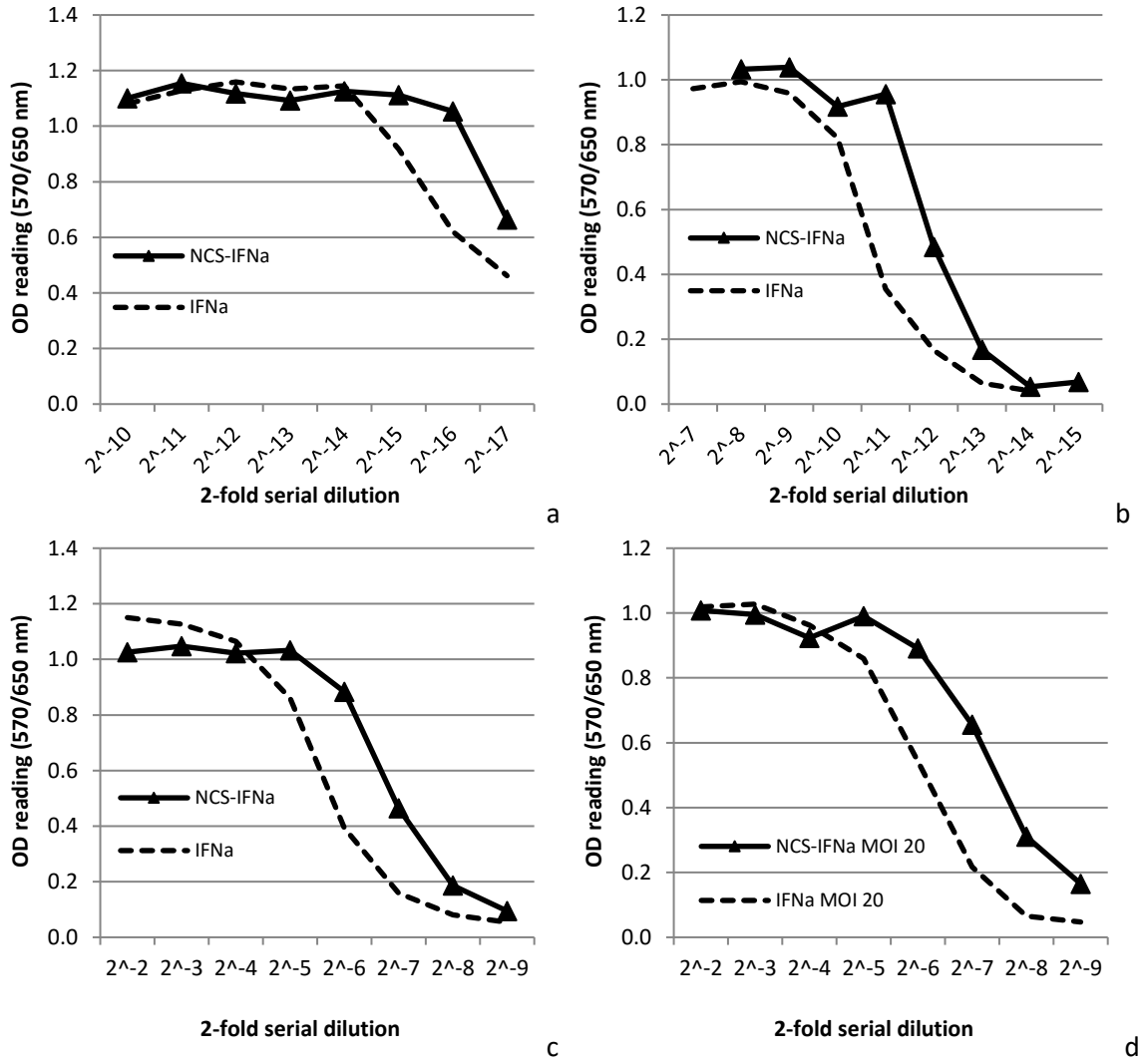


Figure 2. The OD readings of MTT-based CPER assay using supernatants harvested from LFBK-αvβ6 cells co-transfected with plasmid DNA containing an IFNα gene without or with an adenovirus tripartite sequence (NCS) in the 5'-end of the coding sequence at days 2 (a), 4 (b) and 6 (c) post-transfection and using supernatants from the cells infected with adenoviruses containing an IFNα gene with or without the NCS at day 6 post infection (d)

2b. Effect of SOCS1 on IFN expression

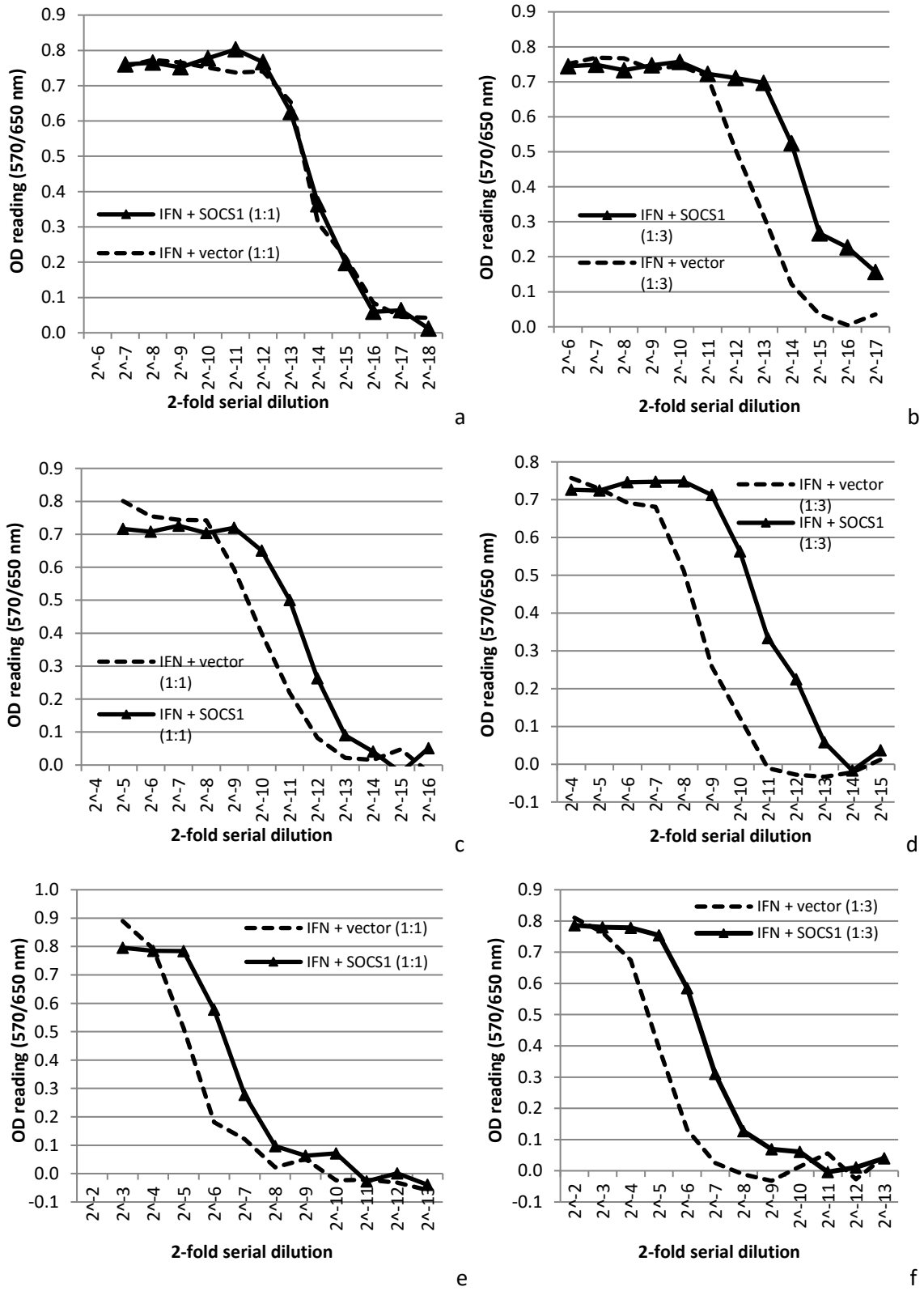


Figure 3. The OD readings of MTT-based CPER assay using LFBK-αvβ6 and supernatants harvested from LFBK-αvβ6 cells co-transfected with different ratios of plasmid DNA inserted with IFNα and SOCS1 or vector only at days 2 (a,b), 4 (c,d) and 6 (e,f) post-transfection

Co-transfection of pcDNA3.1-IFNα and pcDNA3.1-SOCS1 at 1:1 ratio increased the antiviral activity of the supernatants harvested on days 4 and 6 but not on day 2 when it was compared to the co-transfection with pcDNA3.1 vector only at the same ratio (Figure 3a, 3c, 3e). Interestingly, when the ratios (IFNα:SOCS1) were increased to 1:3, the increases in anti-FMDV activity were observed on days 2, 4 and 6 with greater differences (Figure 3b, 3d, 3f). These results indicate that SOCS1 genes delivered together with an IFNα gene could increase the interferon expression and the effect was dose-dependent.

2c. Transcriptional activity of EF1α gene promoters

The transfection of pcDNA3.1-hCMV-IFNα produced the highest anti-FMDV activity among the three plasmids transfected in HEK293 (Figure 4a); however, the anti-FMDV activity of the supernatants from the transfection of the pcDNA3.1-pEF1α-IFNα was greater than those from the pcDNA3.1-bEF1α-IFNα or pcDNA3.1-hCMV-IFNα transfections in LFBK-αvβ6 cells (Figure 4b), indicating that the porcine EF1α promoter is an excellent promoter to express another gene of interest in the Ad5 vector in porcine cells.

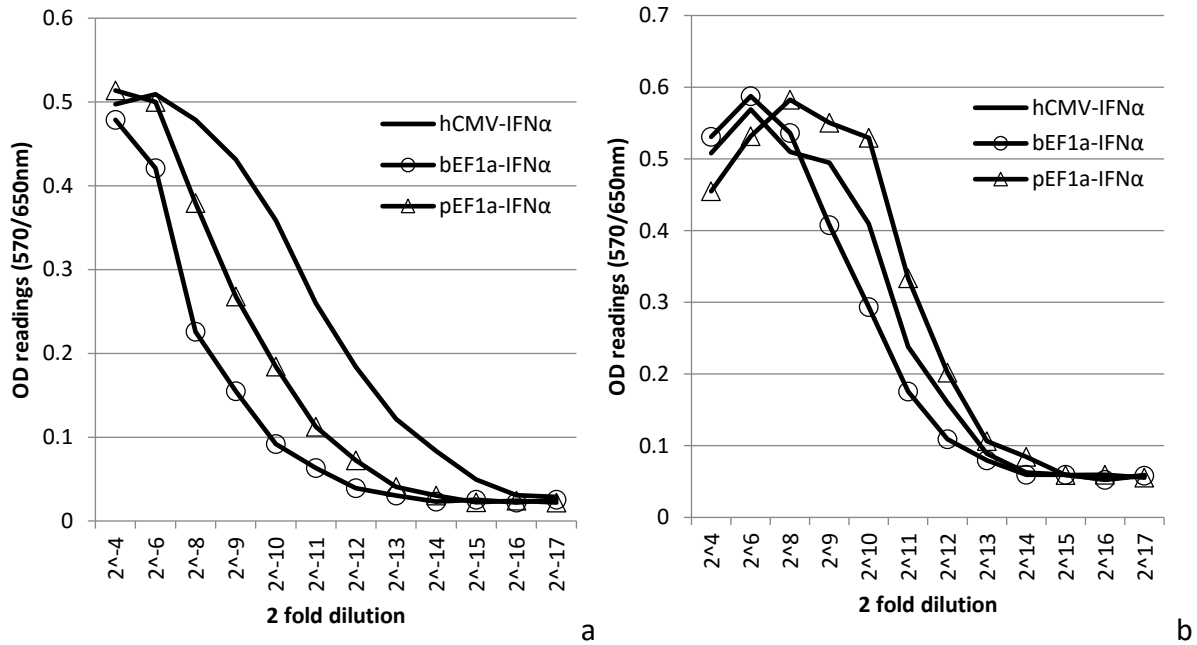


Figure 4. The OD readings of MTT-based CPER assay using LFBK-αvβ6 and supernatants harvested from HEK293 (a) and LFBK-αvβ6 (b) cells transfected with plasmid DNA containing an IFNα gene inserted after CMV or EF1α promoters at day 1 post-transfection

3. Development of new recombinant adenoviruses

3a. Anti-FMDV activity of adenoviruses with the best IFN

A recombinant Ad5 virus containing IFN19 gene (Ad5-IFN19) was produced and validated by DNA sequencing. The Ad5 virus inserted with the IFNα gene previously tested in pigs (Ad5-IFNα) served as a benchmark control. These Ad5 viruses were titrated and used to infect LFBK-αvβ6 cells at different MOI (six 2-fold serial dilutions starting at MOI of 46). The anti-FMDV activities of the supernatants from Ad5-IFNα and Ad5-IFN19 infections decreased linearly with the dilutions (data not shown). No CPE was observed in the cells infected with these two viruses at the MOI tested. At the same MOI, the EC50 values for the Ad5-IFN19 virus were approximately four-fold higher than those of Ad5-IFNα (only two MOI shown in Figure 5), which suggests that the anti-FMDV activity of the best porcine IFN gene is about four-fold higher than that of the one previously tested and using IFN19 improved the potency by four folds.

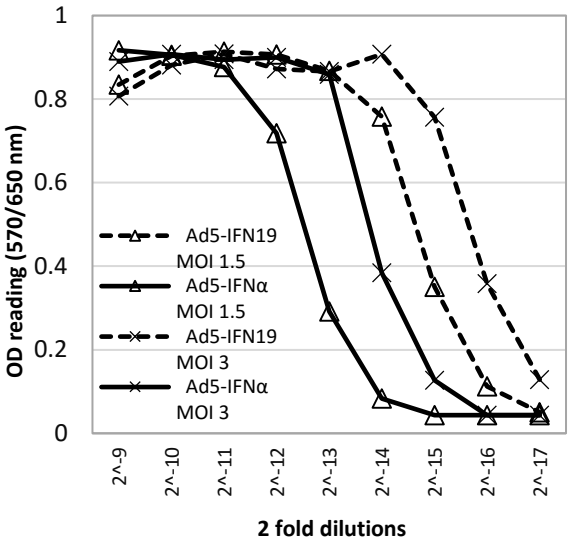


Figure 5. The OD readings of MTT-based CPER assay using LFBK from supernatants harvested from LFBK cells infected with different MOI of recombinant adenoviruses Ad5-IFN19 and Ad5-IFNα at Day 1 post infection

3b. Anti-FMDV activity of adenovirus containing NCS-IFN19 and EF1α-SOCS1

A recombinant adenovirus inserted with IFN19 containing the adenovirus tripartite NCS (NCS-IFN19) and SOCS1 transcribed by EF1a promoter (EF1α-SOCS1) was produced and names as Ad5-IFN19+. To compare Ad5-IFN19+ with Ad5-IFNα, we infected LFBK-αvβ6 cells with the viruses at MOI of 46. Unlike Ad5-IFNα and Ad5-IFN19 infections, Ad5-IFN19+ infection induced CPE in the cells after infection at MOI of 46. Figure 6 shows the antiviral activities induced by Ad5-IFN19+ infection at days 1, and 2 post-infection was approximately 16 times (2^4 fold) higher than those by Ad5-IFNα infection, whereas the differences at day 3 post infection were much smaller (~four times), presumably due to CPE. These results demonstrated that SOCS1 significantly enhanced IFN expression.

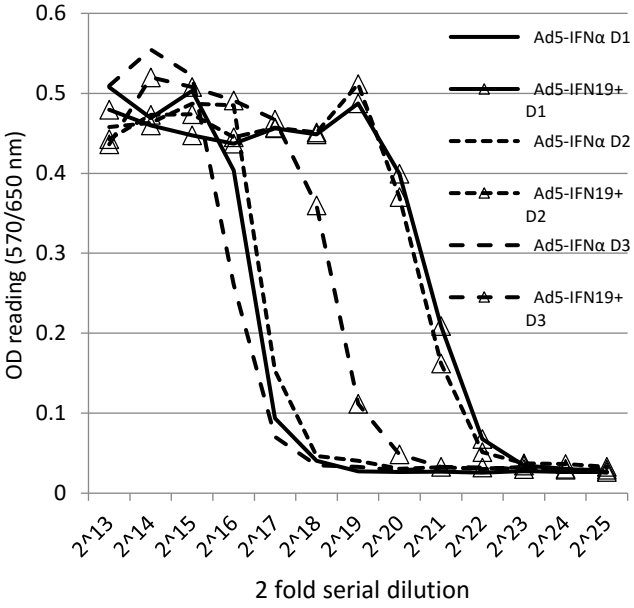


Figure 6. The OD readings of MTT-CPER assays using IBRS-2 and supernatants harvested from LFBK-αvβ6 cells infected with Ad5-IFN19+ or Ad5-IFNα virus at MOI of 46 at Days (D) 1, 2 and 3 post infection

To reduce CPE of Ad5-IFN19+ on LFBK-αvβ6, we infected the cells with 2-fold serially diluted Ad5-IFN19+ staring at MOI of 20. Ad5-IFN19+ virus infection caused CPE at MOI of 10 and 20 with a dose effect. To assess the differences in the antiviral activity between Ad5-IFNα and Ad5-IFN19+, we calculated EC50 for the antiviral activity of all supernatants harvested from different MOIs of infection. The differences in MOI and OD readings were also taken into account for the antiviral activity. Figure 7 shows that Ad5-IFN19+ virus infection produced approximately 16-30 fold higher antiviral activity in day 1 post-infection than Ad5-IFNα. The fold differences increased at days 2/3 and 4 ranging from approximately 50 to 170 fold except for MOIs of 20 and 46. The differences in anti-FMDV activity decreased when MOI was greater than 10. The differences in antiviral activity among infection doses suggest that the effect of SOCS1 and the cytotoxicity were correlated with induced anti-FMDV activity.

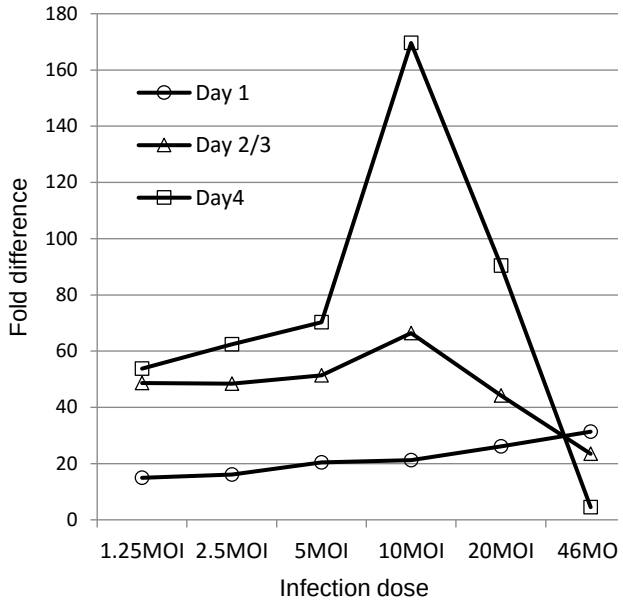


Figure 7. The differences in anti-FMDV activity between Ad5-IFN19+ and Ad5-IFNα at Days 1, 2-3 and 4 post infections of different MOI

3c. Anti-FMDV activity of adenovirus containing IRF2

The induction of antiviral activity differed a great deal among the DNA transfection of a plasmid containing three bovine IRF genes. After transfection of the plasmid DNA samples, the anti-FMDV activity of the supernatants harvested from the plasmid inserted with the constitutively active IRF2 produced the highest anti-FMDV activity at days 1 and 2-3 (Figure 8). The supernatants harvested at day 1 post transfection of the plasmid containing IRF2 gene displayed an antiviral activity approximately 8 (2^3) fold higher than that of the transfection of the plasmid containing IRF7 gene, whereas the differences between IRF2 and IRF3 was much greater, which was approximately 512 (2^9) fold (Figure 8a). The differences in antiviral activity between the IRF2 and IRF7 genes at day 2-3 were greater (~32 fold) than those at day 1 (<8 fold). The antiviral activity at day 2-3 was not detectable for the IRF3 gene (Figure 8b). These results indicate that the IRF2 gene is the best gene regarding the magnitude and duration of inducing anti-FMDV activity.

To test differences in anti-FMDV activity between constitutively active bovine (bIRF2) and porcine (pIRF2) IRF2, DNA transfection of plasmids containing these genes was performed as stated. Figure 9 shows that DNA transfection of the bovine IRF2 gene induced higher anti-FMDV activity (approximately two-fold at days 1 and 2-3) than the transfection with the porcine IRF2 gene, whereas the bovine IRF7 gene induced the lowest anti-FMDV activity among the

three genes as observed earlier (Figure 8). Therefore, the bovine IRF2 gene was selected to be cloned into the Ad5-blue plasmid vector for recombinant adenovirus production.

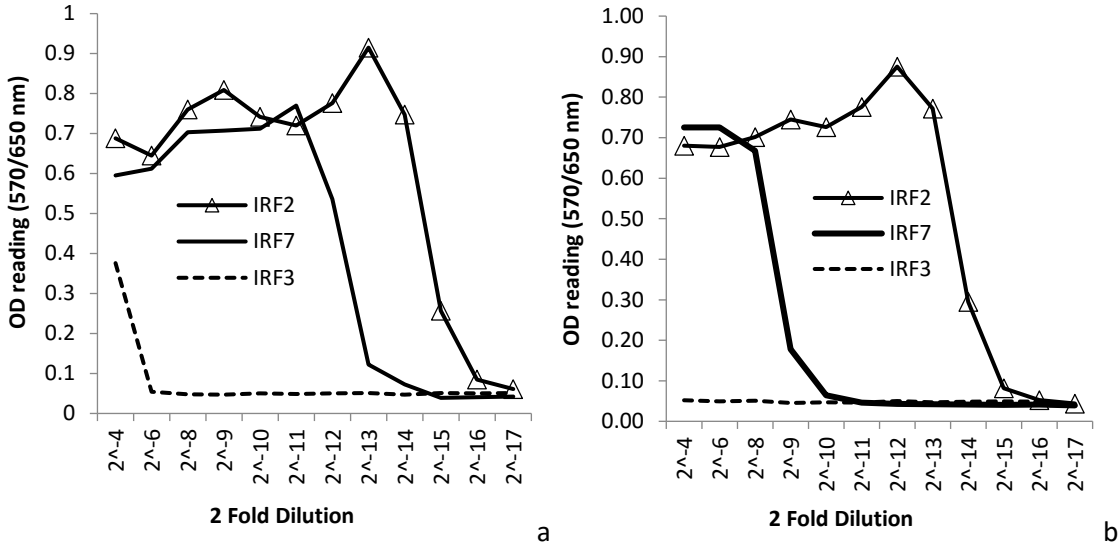


Figure 8. The OD readings of MTT-based CPER assay using supernatants harvested from LFBK-αvβ6 cells transfected with pcDNA3.1 plasmid inserted with constitutively active bovine IRF2, 3 or 7 at day 1 (a) and day 2-3 (b) post DNA transfection

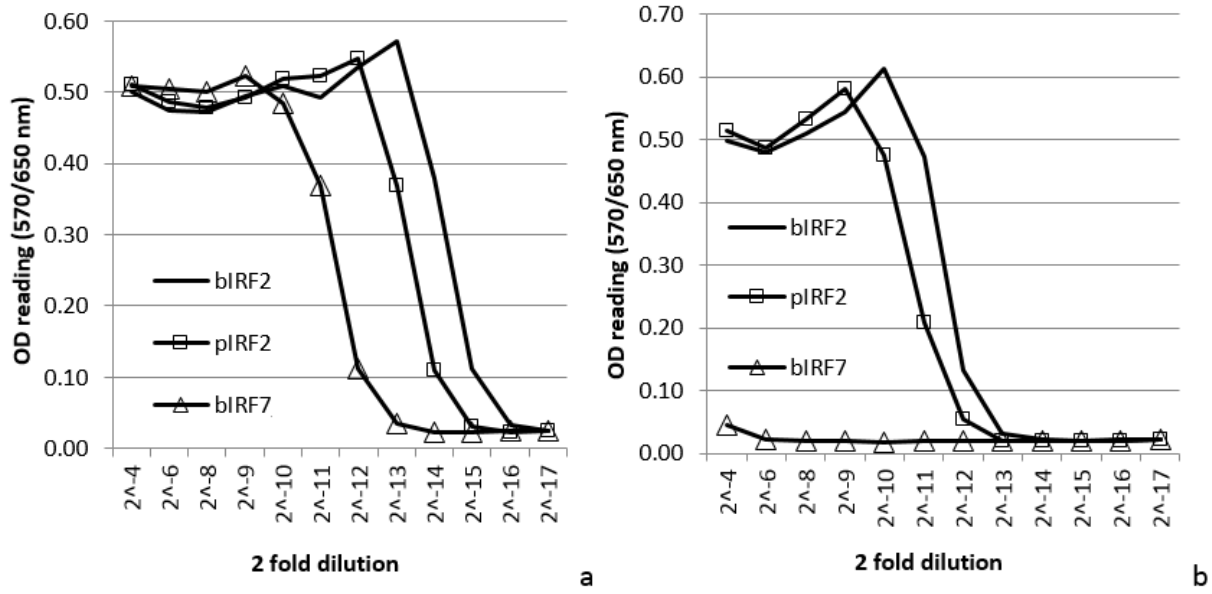


Figure 9. The OD readings of MTT-based CPER assay using IBRS-2 and supernatants harvested from LFBK-αvβ6 cells transfected with pcDNA3.1 plasmid inserted with constitutively active bovine/porcine IRF2 or bovine IRF7 at day 1 (a) and days 2-3 (b) post DNA transfection

An Ad5-IRF2 recombinant virus was produced from an Ad5 plasmid inserted with the bIRF2 gene. After titration, this virus was used to infect the LFBK-αvβ6 cells at MOI of 46. The CPE was observed in the cells infected with the Ad5-bIRF2 adenovirus, whereas no CPE was observed in the Ad5-pIFNα at this dose of infection. The anti-FMDV activity of the supernatants

from the Ad5-IFNα virus was very similar at days 1, 2 and 3 post infections (Figure 10). The anti-FMDV activity of Ad5-bIRF2 infection was nearly the same at days 1 and 2, which was approximately two-fold higher than that of the Ad5-pIFNα infection. However, the activity decreased by approximately 32 fold at day 3 compared to days 1 and 2. The results suggest that the Ad5-bIRF2 virus could produce higher antiviral activity than the Ad5-pIFNα virus within the first two days at this MOI but lower activity at day 3 due to CPE induced by the infection.

To test the anti-FMDV activity at lower MOI, we infected LFBK-αvβ6 cells with 2-fold serially diluted Ad5-bIRF2 virus starting at MOI of 24. The CPE was observed in cells infected at MOIs of 6, 12 and 24 in a dose effect manner. To compare the antiviral activity of Ad5-IRF2 virus with the virus tested in pigs, we calculated EC50 for the antiviral activity of all supernatants. The differences in MOI and OD readings were taken into consideration of antiviral activity. Figure 11 shows that cells infected with Ad5-IRF2 virus produced higher anti-FMDV activity than that of Ad5-IFNα at all doses and both days except day 1 at MOI of 1.5. The differences ranged from 1.5 to 7.5 fold. As observed for Ad5-IFN19+, Ad5-IRF2 also showed a dose effect on differences between Ad5-IRF2 and the Ad5-IFNα due to CPE; however, Ad5-IRF2 displayed a greater cytotoxicity than Ad5-IFN19+.

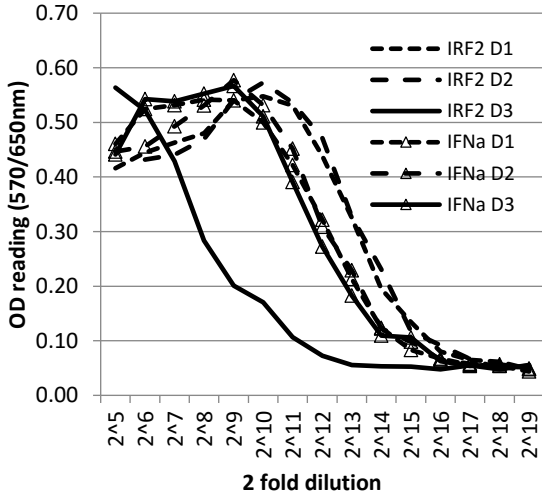


Figure 10. The OD readings of MTT-based CPER assay using IBRS-2 cells and supernatants harvested from LFBK-αvβ6 cells infected at MOI of 46 recombinant adenoviruses inserted with constitutively active bovine IRF2 and porcine IFNα at days 1, 2 and 3 post DNA transfection

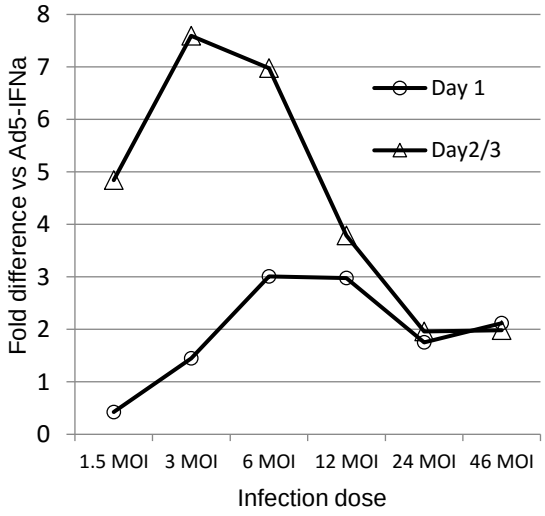


Figure 11. The differences (fold = EC50 of Ad5-bIRF2/EC50 of Ad5-pIFNα) in anti-FMDV between Ad5-bIRF2 and Ad5-pIFNα at Days 1 and 2-3 post infections at different MOIs

4. Animal testing of the new adenoviruses

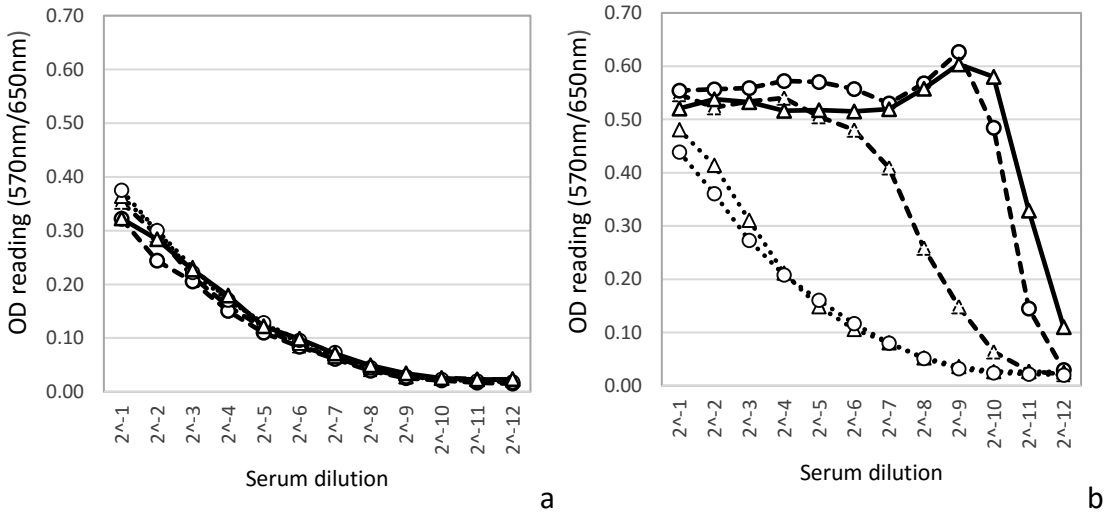
Before the injection of recombinant adenoviruses, the sera in all pigs showed very similar background anti-FMDV activities (Figure 12a). All background activities could not fully protect the tested cells from FMDV infection at the highest tested serum concentration (2 fold

dilution) based on the OD readings of the positive controls (wells without FMDV, OD readings at 0.60). The anti-FMDV activity in the sera of the group injected with PBS remained practically the same throughout the entire tested period (data not showed). One pig injected with Ad5-IFNα and all with Ad5-IFN19+ showed jaundice. All pigs injected with Ad5-IFN19+ also displayed other symptoms of sickness and one had to be euthanized at day 2 post injection. The pigs with jaundice in Ad5-IFNα group had the highest anti-FMDV activity in its group, likewise the antiviral activity of the euthanized pig was the highest in its group. The results show that jaundice and sickness after injection were highly correlated with anti-FMDV activity in the sera.

Anti-FMDV activity induced by Ad5-IFNα displayed a positive dose effect equivalent to the difference between the doses. The antiviral activity at day 1 post injection was the highest and then decreased by at least two-fold each day (Figure 12). The anti-viral activity fell below the full protection level at day 4 post injection with 10^9 PFU (Figure 12e) and at day 6 with 10^{10} PFU (Figure 12g). There was a difference of more than four-fold in antiviral activity between individuals with the highest and the lowest anti-FMDV activity in this group.

Interestingly, the pigs injected with Ad5-IFN19+ displayed the highest anti-FMDV activity among the groups at all days post injection with no decrease at day 2 (Figure 12b and Figure 12c). Then the antiviral activity gradually decreased but remained at very close to the full protection level at day 7 post injection (Figure 12h). It appears that the differences between the groups injected with Ad5-IFN19+ and Ad5-IFNα s started at day 1 post injection and the differences increased up to day 4 and then decreased afterward, indicating antiviral effect induced by Ad5-IFN19+ not only was greater but also lasted longer if taking both dose and the activity into consideration.

Unexpectedly, the pigs treated with Ad5-IRF2 induced only slightly higher anti-FMDV activity by OD readings of approximately 0.05 to 0.1 without a significant dose effect (Figure 11a-h, dotted lines). The antiviral activity induced by Ad5-IRF2 injection could not fully protect the cultured cells based on all OD readings well below 0.60, indicating this biotherapeutics cannot protect pigs at the tested doses.



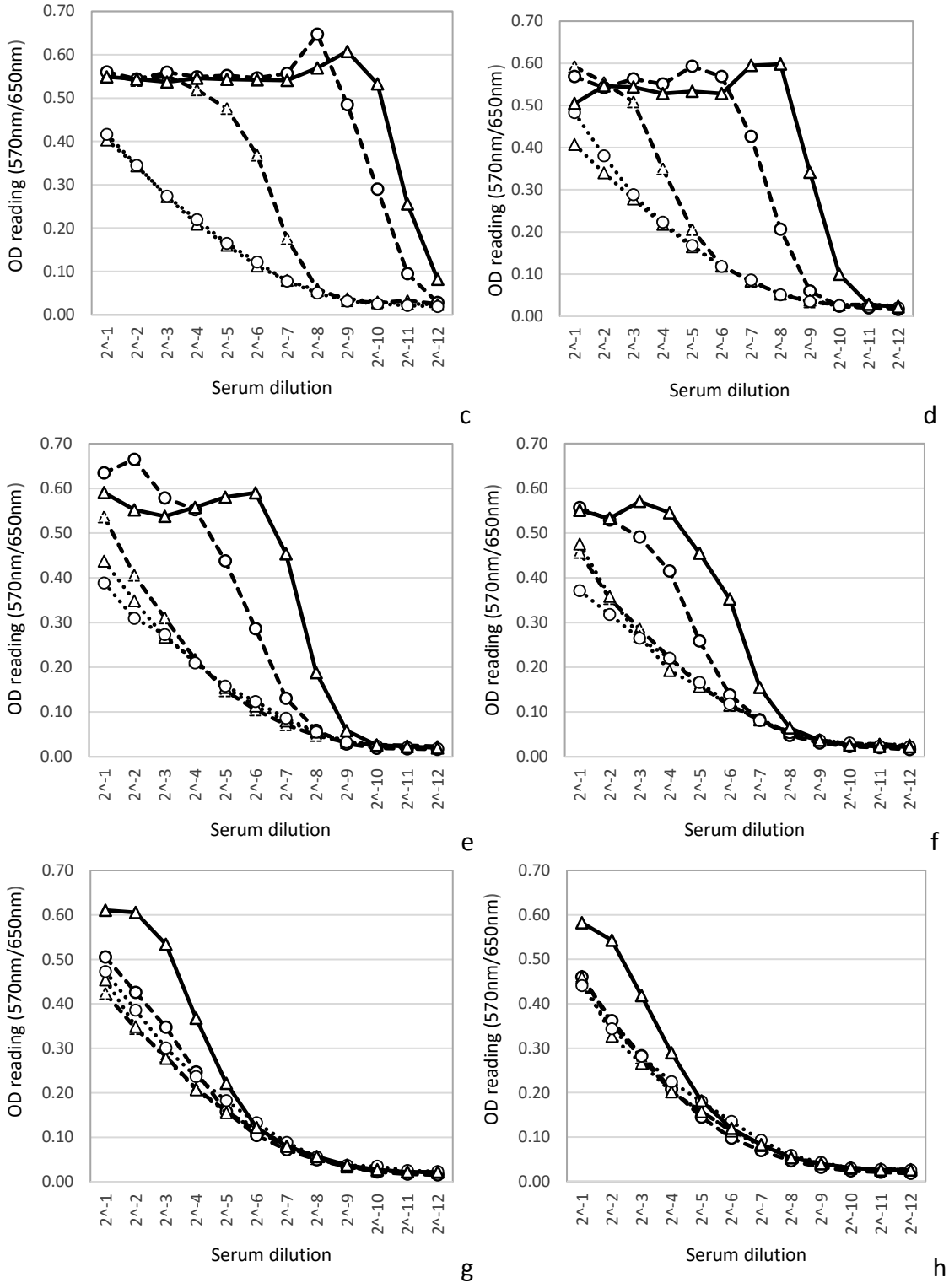


Figure 12. Anti-FMDV activities (OD readings) in sera of pig treated with Ad5-IFNα (dash lines), Ad5-IRF2 (dotted lines) and Ad5-IFN19+ (solid line) at a dose of 10^9 PFU (triangle) or 10^{10} PFU (circle) one day before treatment (a) and days 1, 2, 3, 4, 5, 6, and 7 post-treatment (b, c, d, e, f, g and h, respectively)

In summary, we, based on our *in-vitro* and *in-vivo* results, have improved the potency of an existing IFN biotherapeutics using four elements: (1) the IFN gene with the highest anti-FMDV activity, (2) the adenovirus tripartite sequence, (3) the EF1α promoter and (4) the SOCS1 gene. The improvement includes increases in the magnitude and duration of induced anti-FMDV activity as showed both in the *in-vitro* and *in-vivo* results though the differences between Ad5-IFNα and Ad-IFN19+ *in-vivo* were smaller than those *in-vitro*. Ad5-IRF2 induced not only strong anti-FMDV activity but also cell death even at low MOI in cell culture; however, the treatment of Ad5-IRF2 barely induced anti-FMDV activity in pigs. Therefore, Ad5-IFN19+ recombinant virus is currently the best IFN biotherapeutics. Taking both dose and antiviral activity into consideration, we have improved the IFN biotherapeutics more than 20 fold as compared to the one previously tested.

Discussion:

Adenovirus-based IFN biotherapeutics is very effective in protecting pigs against FMDV infection (Chinsangaram et al., 2003; Dias et al., 2011; Moraes et al., 2003); however, this approach requires a protective dose approximately 100 time higher than adenovirus-based vaccines. Reducing the protective dose is critical for making the biotherapeutics feasible. We have successfully applied several approaches to enhance the potency of the biotherapeutics. The first approach identified the most potent IFN genes for use in the biotherapeutics. This approach accounted for an improvement of approximately four folds. To identify the best IFN gene, we applied an approach very similar to the method used by Zanotti et al. (2015) to produce IFN for antiviral activity assays using a colorimetric MTT assay we developed (Ramanathan et al., 2015). Our tests yielded nearly identical results, indicating these assays are highly reproducible.

The differences in the antiviral activity of porcine type I interferons have been tested against PRRSV and VSV infection in different cell lines (Sang et al., 2010; Zanotti et al., 2015). There were substantial differences among the genes tested. In the study by Sang et al (2010), porcine IFNα6 (GQ415060) was the top interferon against PRRSV and VSV in MARC-15 cells, whereas IFNα12 (GQ415066 or IFN19 in our study) was the best interferon against VSV in PK-15 cells. Similarly, in the study by Zanotti et al. (2015), IFNα6 was the interferon with the highest anti-VSV activity and IFNα12 was the second best; however, it is unknown if the coding sequence of the IFNα12 is identical to GQ415066. IFN19 has also been demonstrated to be the most potent interferon against CSFV in swine macrophages (Fernandez-Sainz et al., 2015). Our results together with other reports indicate that IFN19 or IFNα12 is the best anti-FMDV interferon.

The second approach enhanced the expression of IFN genes. Using the adenovirus tripartite sequence, the SOCS1 gene and the EF1α promoter in addition to IFNα12 or IFN19, we constructed a new Ad5 virus containing two inserted porcine genes. This recombinant adenovirus induced an *in-vitro* anti-FMDV activity up to 170 fold higher than the previous adenovirus. We observed very similar patterns of SOCS1 effect on induced antiviral activity in

in-vitro and *in-vivo* tests though much smaller differences were observed in pigs than those in cell culture. The differences probably were due to hundreds-fold higher IFN concentrations induced in cell culture than those in animals, which provided an environment for SOCS1 to play a bigger role *in-vitro* than *in-vivo*. The effect of SOCS1 could be explained by reducing both apoptotic effect of IFN on IFN-producing cells according to Chinsangaram et al. (2003) and Thyrell et al. (2002) and inhibitory effect of IFN on protein translation based on Ivashkiv and Donlin (2014) via suppressing IFN signaling (Fenner et al., 2006). We also observed the effect of the adenovirus tripartite sequence on the expression of the recombinant protein in our *in-vitro* testing very similar to those reported by Logan & Shenk (1984) and Kaufman (1985). Slower decreases in the anti-FMDV activity induced by Ad5-IFN19+ both *in-vitro* and *in-vivo* than those by Ad5-IFNα also support these explanations.

Taking the differences in injection doses, serum antiviral activities and the euthanized pig (having highest antiviral activity in its group) into consideration; we estimate that this new biotherapeutics has a potency more than 20 time higher than the one previously tested. Other clinical observations also supports the increase of the potency. Jaundice has been observed as a side-effect associated with high IFN concentrations in pigs (Chinsangaram et al., 2003; Dias et al., 2011; Moraes et al., 2003). Even at a dose ten-fold lower than the reported protective dose, all pigs injected with Ad5-IFN19+ showed more severe jaundice and other symptoms of sickness than other pigs in this study. In this study, these symptoms were positively correlated with the anti-FMDV activity in the sera. The Ad5-IFN19+ also showed higher cytotoxicity in our *in-vitro* studies than the Ad5-IFNα. Therefore, the dose of this new biotherapeutics for pigs needs to be optimized.

The third approach did not result in the improvement of the biotherapeutics in pigs at the tested doses though the recombinant virus containing IRF2 also induced higher anti-FMDV activity in cell cultures than the previous recombinant virus. This may be due to the high cytotoxicity of Ad5-IRF2 as we observed in cell culture (Figure 10). There were no differences in the anti-FMDV activity induced in the sera by two tested doses of Ad5-IRF2. Lower doses may be tested in pigs. It has been reported that constitutively active IRF2 induced higher antiviral activity than the constitutively active IRF1 (Lin et al., 2000; Lin et al., 1994) and IRF1 induced a broad spectrum of genes that can promote the adaptive immune response, such as IL-12 and IL15 (Liu et al., 2004; Maruyama et al., 2003; Ogasawara et al., 1998). Ad5-IRF2 may also induce other immune cytokines whose antiviral activity could not be measured with the MTT-CPER assay. These cytokines may enhance vaccine responses. IRF1 has been reported to be a potent adjuvant for a DNA vaccine (Castaldello et al., 2010). Therefore, the antiviral and adjuvant effects of the IRF-based biotherapeutics need further testing in pigs.

In summary, we have used four biological elements to improve the potency of the existing IFN biotherapeutics for pigs by >20 folds. This new biotherapeutics can induce higher and longer anti-FMDV activity than the one previously tested. Because the Ad5-IFN19+ can induce such a high antiviral activity, it is difficult to produce this recombinant virus. Our future plan is to develop a system to produce Ad5-IFN19+ virus efficiently. We also plan to test this biotherapeutics in protecting pigs against viral challenges, e.g. FMDV, CSFV, ASFV, etc. There

are still approaches that can be used to improve this biotherapeutics further and eventually make it feasible for commercial production.

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References

Castaldello, A., Sgarbanti, M., Marsili, G., Brocca-Cofano, E., Remoli, A.L., Caputo, A., Battistini, A., 2010. Interferon regulatory factor-1 acts as a powerful adjuvant in tat DNA based vaccination. J Cell Physiol 224, 702-709.

Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, Borden EC. 2003. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. Apoptosis 8(3):237-49.

Chinsangaram, J., Moraes, M.P., Koster, M., Grubman, M.J., 2003. Novel viral disease control strategy: adenovirus expressing alpha interferon rapidly protects swine from foot-and-mouth disease. J Virol. 77, 1621-1625.

Chinsangaram, J., Piccone, M.E., Grubman, M.J., 1999. Ability of foot-and-mouth disease virus to form plaques in cell culture is associated with suppression of alpha/beta interferon. J Virol. 73, 9891-9898.

Dias, C.C.A., Moraes, M.P., Segundo, F.D.-S., de Los Santos, T., Grubman, M.J., 2011. Porcine Type I Interferon Rapidly Protects Swine Against Challenge with Multiple Serotypes of Foot-and-Mouth Disease Virus. Journal of Interferon & Cytokine Research 31, 227-236.

Fenner, J.E., Starr, R., Cornish, A.L., Zhang, J.G., Metcalf, D., Schreiber, R.D., Sheehan, K., Hilton, D.J., Alexander, W.S., Hertzog, P.J., 2006. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. Nat Immunol 7, 33-39.

Graham, F.L., Smiley, J., Russell, W.C., Nairn, R., 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36, 59-74.

Gu, M., Lin, G., Lai, Q., Zhong, B., Liu, Y., Mi, Y., Chen, H., Wang, B., Fan, L., Hu, C., 2015. Ctenopharyngodon idella IRF2 plays an antagonistic role to IRF1 in transcriptional regulation of IFN and ISG genes. Dev Comp Immunol 49, 103-112.

Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., Taniguchi, T., 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell 58, 729-739.

Harada, H., Willison, K., Sakakibara, J., Miyamoto, M., Fujita, T., Taniguchi, T., 1990. Absence of the type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. Cell 63, 303-312.

Honda K., Taniguchi T., 2006. IRFs: master regulators of signaling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol. 6:644-58.

House, J.A., House, C., Llewellyn, M.E., 1988. Characteristics of the porcine kidney cell line IB-RS-2 clone D10 (IB-RS-2 D10) which is free of hog cholera virus. In Vitro Cell Dev Biol 24, 677-682.

Ivashkiv L.B., Donlin L.T., 2014. Regulation of type I interferon responses. Nat Rev Immunol. 14:36-49.

Kaufman R.J. 1985. Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors. Proc Natl Acad Sci U S A. 82:689-93.

LaRocco, M., Krug, P.W., Kramer, E., Ahmed, Z., Pacheco, J.M., Duque, H., Baxt, B., Rodriguez, L.L., 2013. A continuous bovine kidney cell line constitutively expressing bovine alphavbeta6 integrin has increased susceptibility to foot-and-mouth disease virus. J Clin Microbiol 51, 1714-1720.

Lin, R., Genin, P., Mamane, Y., Hiscott, J., 2000. Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7. Mol Cell Biol. 20, 6342-6353.

Lin, R., Mustafa, A., Nguyen, H., Gewert, D., Hiscott, J., 1994. Mutational analysis of interferon (IFN) regulatory factors 1 and 2. Effects on the induction of IFN-beta gene expression. J Biol Chem 269, 17542-17549.

Liu, J., Guan, X., Tamura, T., Ozato, K., Ma, X., 2004. Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. J Biol Chem 279, 55609-55617.

Logan J, Shenk T. 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc Natl Acad Sci U S A. 81:3655-9.

Maruyama, S., Sumita, K., Shen, H., Kanoh, M., Xu, X., Sato, M., Matsumoto, M., Shinomiya, H., Asano, Y., 2003. Identification of IFN regulatory factor-1 binding site in IL-12 p40 gene promoter. J Immunol 170, 997-1001.

Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., Taniguchi, T., 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell 54, 903-913.

Moll, H.P., Maier, T., Zommer, A., Lavoie, T., Brostjan, C., 2011. The differential activity of interferon-[alpha] subtypes is consistent among distinct target genes and cell types. Cytokine 53, 52-59.

Moraes, M.P., Chinsangaram, J., Brum, M.C., Grubman, M.J., 2003. Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit vaccine. Vaccine. 22, 268-279.

Moraes, M.P., Mayr, G.A., Grubman, M.J., 2001. pAd5-Blue: direct ligation system for engineering recombinant adenovirus constructs. Biotechniques 2001 Nov;31, 1050.

Moraes, M.P., Mayr, G.A., Mason, P.W., Grubman, M.J., 2002. Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. Vaccine 20, 1631-1639.

Morin, P., Braganca, J., Bandu, M.T., Lin, R., Hiscott, J., Doly, J., Civas, A., 2002. Preferential binding sites for interferon regulatory factors 3 and 7 involved in interferon-A gene transcription. J Mol Biol 316, 1009-1022.

Ogasawara, K., Hida, S., Azimi, N., Tagaya, Y., Sato, T., Yokochi-Fukuda, T., Waldmann, T.A., Taniguchi, T., Taki, S., 1998. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. Nature 391, 700-703.

Pena, L., Moraes, M.P., Koster, M., Burrage, T., Pacheco, J.M., Segundo, F.D., Grubman, M.J., 2008. Delivery of a foot-and-mouth disease virus empty capsid subunit antigen with nonstructural protein 2B improves protection of swine. Vaccine 26, 5689-5699.

Ramanathan, P., Zhu, J.J., Bishop, E.A., Puckette, M.C., Hartwig, E., Grubman, M.J., Rodriguez, L.L., 2015. A colorimetric bioassay for high-throughput and cost-effectively assessing anti-foot-and-mouth disease virus activity. Vet Immunol Immunopathol 164, 74-78.

Roberts, R.M., Liu, L., Guo, Q., Leaman, D., Bixby, J., 1998. The evolution of the type I interferons. J Interferon Cytokine Res 18, 805-816.

Robinson, N., McComb, S., Mulligan, R., Dudani, R., Krishnan, L., Sad, S., 2012. Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. Nat Immunol 13, 954-962.

Sang, Y., Rowland, R.R., Blecha, F., 2011. Porcine type I interferons: polymorphic sequences and activity against PRRSV. BMC 5, S8.

Sang, Y., Rowland, R.R., Hesse, R.A., Blecha, F., 2010. Differential expression and activity of the porcine type I interferon family. Physiol 42, 248-258.

Sellers, R.F., 1963. Multiplication, interferon production and sensitivity of virulent and attenuated strains of the virus of foot-and-mouth disease. Nature. 198, 1228-1229.

Swaney, L.M., 1988. A continuous bovine kidney cell line for routine assays of foot-and-mouth disease virus. Vet Microbiol. 18, 1-14.

Tanaka, N., Kawakami, T., Taniguchi, T., 1993. Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. Mol Cell Biol 13, 4531-4538.

Thyrell L, Erickson S, Zhivotovsky B, Pokrovskaja K, Sangfelt O, Castro J, Einhorn S, Grandér D. 2002. Mechanisms of Interferon-alpha induced apoptosis in malignant cells.Oncogene. 21(8):1251-62.

Zanotti, C., Razzuoli, E., Crooke, H., Soule, O., Pezzoni, G., Ferraris, M., Ferrari, A., Amadori, M., 2015. Differential Biological Activities of Swine Interferon-alpha Subtypes. J Interferon Cytokine Res 35, 990-1002.