Robust Protection against Highly Virulent Foot-and-Mouth Disease Virus in Swine by Combination Treatment with Recombinant Adenoviruses Expressing Porcine Alpha and Gamma Interferons and Multiple Small Interfering RNAs

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ABSTRACT
Because the currently available vaccines against foot-and-mouth disease (FMD) provide no protection until 4 to 7 days postvaccination, the only alternative method to halt the spread of the FMD virus (FMDV) during outbreaks is the application of antiviral agents. Combination treatment strategies have been used to enhance the efficacy of antiviral agents, and such strategies may be advantageous in overcoming viral mechanisms of resistance to antiviral treatments. We have developed recombinant adenoviruses (Ads) for the simultaneous expression of porcine alpha and gamma interferons (Ad-porcine IFN-α/γ) as well as 3 small interfering RNAs (Ad-3siRNA) targeting FMDV mRNAs encoding nonstructural proteins. The antiviral effects of Ad-porcine IFN-α/γ and Ad-3siRNA expression were tested in combination in porcine cells, suckling mice, and swine. We observed enhanced antiviral effects in porcine cells and mice as well as robust protection against the highly pathogenic strain O/Andong/2010 and increased expression of cytokines in swine following combination treatment. In addition, we showed that combination treatment was effective against all serotypes of FMDV. Therefore, we suggest that the combined treatment with Ad-porcine IFN-α/γ and Ad-3siRNA may offer fast-acting antiviral protection and be used with a vaccine during the period that the vaccine does not provide protection against FMD.

IMPORTANCE
The use of current foot-and-mouth disease (FMD) vaccines to induce rapid protection provides limited effectiveness because the protection does not become effective until a minimum of 4 days after vaccination. Therefore, during outbreaks antiviral agents remain the only available treatment to confer rapid protection and reduce the spread of foot-and-mouth disease virus (FMDV) in livestock until vaccine-induced protective immunity can become effective. Interferons (IFNs) and small interfering RNAs (siRNAs) have been reported to be effective antiviral agents against FMDV, although the virus has associated mechanisms of resistance to type I interferons and siRNAs. We have developed recombinant adenoviruses for the simultaneous expression of porcine alpha and gamma interferons (Ad-porcine IFN-α/γ) as well as 3 small interfering RNAs (Ad-3siRNA) to enhance the inhibitory effects of these antiviral agents observed in previous studies. Here, we show enhanced antiviral effects against FMDV by combination treatment with Ad-porcine IFN-α/γ and Ad-3siRNA to overcome the mechanisms of resistance of FMDV in swine.

Foot-and-mouth disease (FMD) is one of the most prevalent and costly diseases affecting livestock globally. It is a highly contagious disease that affects cloven-hoofed animals, such as cattle, swine, and sheep (1). The FMD virus (FMDV) belongs to the Aphthovirus genus in thePicornaviridae family (2). The virus consists of 7 serotypes (A, Asia1, C, O, SAT1, SAT2, and SAT3), and numerous subtypes have evolved within each serotype (3, 4).

The use of current commercial FMD vaccines to induce rapid protection provides limited effectiveness because the protection does not develop until a minimum of 4 days (partial protection) or 7 days (complete protection) after vaccination (5). The recombinant adenovirus (Ad) FMDV subunit vaccine also requires 7 days to induce seroprotection (6). Therefore, during outbreaks antiviral agents remain the only available treatment to confer rapid protection and reduce the spread of FMDV in livestock until vaccine-induced protective immunity can become effective (7). In the absence of adequate means for early control, the virus spreads rapidly; pigs release high numbers of copies of airborne FMDV, and airborne FMDV can spread from pigs to cattle, sheep, and goats (8–10). Therefore, it is important to control the transmission of FMDV from pigs to other animals.

Combination treatment strategies have been used to enhance the effects of several antiviral agents against various viruses (11–14). Our group has also reported enhanced antiviral effects against FMDV in cells by combination treatment with interferon (IFN)
and antiviral agents (15). FMDV elicits a type I interferon suppression mechanism (16, 17), although FMDV has been reported to be quite sensitive to interferons (18). Viruses can eventually develop resistance mechanisms through point mutations to evade targeted small interfering RNAs (siRNAs), although siRNAs can offer rapid antiviral treatment (19, 20). We have developed recombinant adenoviruses that simultaneously express porcine alpha interferon (IFN-α) and IFN-γ (referred to here as Ad-porcine IFN-αγ) and multiple (n = 3) siRNAs targeting nonstructural proteins of FMDV (referred to here as Ad-3siRNA) to enhance the inhibitory effects of these antiviral agents observed in previous studies (21, 22). Importantly, both classes of antiviral agents operate through distinct mechanisms and are effective against a broad range of FMDV serotypes.

In this study, we show that the combined use of Ad-porcine IFN-αγ and Ad-3siRNA provides enhanced antiviral effects and that the combination is effective against the seven FMDV serotypes in porcine cells and suckling mice. Furthermore, we confirm that robust antiviral effects of the combination treatment might be achieved against the highly pathogenic FMDV strain O/Andong/SKR/2010 in swine. Therefore, we suggest that the combination therapy described herein is a novel, rapid, and widely effective antiviral approach for controlling FMDV infection in swine.

MATERIALS AND METHODS

Cells, viruses, and virus titrations. Human embryonic kidney cells containing human adenovirus type 5 E1 envelope (293A cells) and porcine kidney (IBRS-2) cells were propagated in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. FMDV strains O/SKR/2002 (Genbank accession numbers AY312589 and AY312588) was used for viral challenges in cells and suckling mice. Strains O/Andong/SKR/2010 (Genbank accession number KC509397), A22/IRQ 24/64 (Genbank accession number AY593763), A/Pocheon/SKR/2010 (Genbank accession number KC88943), Asia1/MOG/05 (Genbank accession number EF614458), C3/Resende (Genbank accession number AY593807), SAT1/BOT1/68, SAT2/ZIM5/81, and SAT3/ZIM4/81 were used to test the antiviral effects of the combination treatment against each of the 7 FMDV serotypes in cell culture. FMDV strains O/SKR/2002, O/Andong/SKR/2010, and A/SKR/Pochen/2010 were isolated in our laboratory and were derived from swine and cattle specimens obtained in the Republic of Korea. The Asia1/MOG/05 strain was isolated in our laboratory using a sample provided by the State Central Veterinary Laboratory in Mongolia. Strains C3/Resende, SAT1/BOT1/68, SAT2/ZIM5/81, and SAT3/ZIM4/81 were isolated by the Pirbright Institute, OIE/FAO, FMD Reference Laboratory, in the United Kingdom. FMDV strain O/Andong/SKR/2010 was passaged 2 times in pigs and used for viral challenges in swine experiments. All infections using FMDV were conducted in a biosafety level 3 containment facility at the Animal and Plant Quarantine Agency (QIA). The preparation of recombinant adenoviruses expressing 3 different siRNA sequences targeting mRNA encoding the nonstructural proteins 2B and 3C (Ad-3siRNA) or porcine IFN-α and IFN-γ proteins (Ad-porcine IFN-αγ) has been described previously (21, 22). Amplified adenoviruses were purified using a ViraBind adenovirus purification kit (Cell Biolabs, San Diego, CA, USA) or a Vivapure AdenoPack 500 kit (Sartorius, Goettingen, Germany). FMDV titers were determined in IBRS-2 cells, and recombinant adenovirus titers were determined in 293A cells. The 50% tissue culture infective doses (TCID50) were calculated using the formula of Reed and Muench (23).

Determination of antiviral effects in IBRS-2 cells. IBRS-2 cells were seeded overnight in separate wells of 96-well plates at a density of 3.5 × 104 cells per well so that they were 90% confluent at the time of infection on the following day. Cells were inoculated with recombinant adenoviruses at 10-fold serial dilutions. After a 24-h absorption period, the recombinant adenoviruses were removed, and the cells were washed twice with DMEM. IBRS-2 cells were immediately infected with 200 TCID50 of FMDV. After a 1-h absorption period, the inocula were removed, 100 μl culture medium with 2% FBS was added, and the cells were incubated at 37°C for 48 h. Supernatants were collected at 48 h postinfection (p.i.) and analyzed in replication assays. To assay the effects of the recombinant adenoviruses on FMDV RNA replication, RNA was extracted and quantitative real-time reverse transcription-PCR (RT-PCR) was performed. Viral RNA was extracted using a MagNA Pure 96 system (Roche, Basel, Switzerland). Real-time RT-PCR was conducted as previously described (21).

Determination of antiviral effects in suckling mice. All mouse experiments conducted in this study were approved by the Animal Care and Use Committee of QIA. To investigate antiviral effects in vivo, 6-day-old CD-1 (ICR) suckling mice weighing 4 to 5 g (Orient Co. Ltd., Republic of Korea) were used. The 50% lethal dose (LD50) of FMDV was determined in 10-fold serial dilutions of the virus and estimated by the method of Reed and Muench (23). Suckling mice were inoculated by intraperitoneal (i.p.) injection of 4 × 102 TCID50 of recombinant adenovirus. At 24 h p.i. with recombinant adenoviruses, the suckling mice were challenged with 125 LD50 or 250 LD50 of FMDV (O/SKR/2002 strain, 0.05-ml volume) by i.p. injection. The survival of the animals was monitored for 7 days.

Determination of antiviral effects against 7 serotypes of FMDV in IBRS-2 cells. IBRS-2 cells were plated in separate wells of 96-well plates at a density of 3.5 × 104 cells per well. On the following day, the cells were 90% confluent at the time of infection. In experiments involving combination treatment, cells were inoculated with recombinant adenoviruses at a multiplicity of infection (MOI) of 0.01 or 0.05. After an 18-h absorption period, the recombinant adenoviruses were removed and the cells were washed twice with DMEM. IBRS-2 cells were immediately infected with 100 TCID50 of FMDV strain O/Andong/SKR/2010, A22/IRQ 24/64, A/Pocheon/SKR/2010, Asia1/MOG/05, C3/Resende, SAT1/BOT1/68, SAT2/ZIM5/81, or SAT3/ZIM4/81. After a 1-h absorption period, the inocula were removed and 100 μl of culture medium with 2% FBS was added. The cells were incubated at 37°C for 48 h. Supernatants were collected at 48 h p.i. and analyzed in replication assays. To assay the effects on FMDV RNA replication, RNA extraction and quantitative real-time RT-PCR were performed. Viral RNA was extracted using the MegNA Pure 96 system, and real-time RT-PCR was performed as previously described (21).

Determination of antiviral effects in swine. Four independent experiments with pigs were performed in this study. All swine experiments in this study were approved by the Animal Care and Use Committee of QIA. In these experiments, Yucatan specific-pathogen-free (SPF) minipigs weighing 10 to 15 kg were purchased from OptiFarm Solution Medi-Pig, Inc. (Republic of Korea). The pigs used in this study were FMDV structural protein antibody negative because an FMDV vaccination program for cattle and pigs has been implemented on a nationwide scale since December 2010 in the Republic of Korea (24). After injection of recombinant adenoviruses, FMDV challenge was performed by infection by direct contact. The donor pigs were inoculated by intradermal injection at 4 sites in the hind heel bulb with 105 TCID50 of the O/Andong/KOR/2010 strain that had been passed 2 times in pigs (25). When vesicles were visualized at 48 h postinoculation with FMDV, the donor pigs were placed into direct contact with recipient pigs. At 18 h postcontact, the donor pigs were euthanized (26). Each group of pigs was housed in separate sections of the room after contact challenge, and the pigs showing signs of clinical disease were immediately removed from the room to limit the severity of challenge for the remaining animals.

Swine experiment 1 (assaying for combination effects). Twelve pigs were divided into 5 groups, including a control group of 2 animals, 2 groups of 2 animals each receiving a single adenovirus preparation (single adenovirus group), and 2 groups of 3 animals each receiving the combination of adenovirus preparations (combination adenovirus group). The
pigs were inoculated by the intramuscular (i.m.) route with $3 \times 10^8$ (single adenovirus group) or $6 \times 10^8$ (combination adenovirus group) TCID$_{50}$ of recombinant adenovirus. The combination groups were divided into two groups according to the ratios of the Ad-porcine IFN-α/γ to Ad-3siRNA titers of 1:1 and 1:5 that they received. These are referred to as the 1:1 and 1:5 combination groups. At 24 h p.i. with recombinant adenoviruses, the pigs were challenged by infection by direct contact with 2 donor pigs.

**Swine experiment 2 (evaluating injection route efficacy).** Eight pigs were divided into 4 groups, including a control group of 2 animals. Pigs were inoculated by the aerosol route, the i.m. route, or a combination of routes (i.m. and aerosol) with $3 \times 10^8$ TCID$_{50}$ of Ad-porcine IFN-α/γ and $3 \times 10^8$ TCID$_{50}$ of Ad-3siRNA. Aerosol inoculations were performed using an AeroMask aerosol delivery system (Trudell Medical International, Ontario, Canada) and an Aeroneb Pro nebulizer (Aerogen, Galway, Ireland), as described previously (27). A combination route of inoculation was performed, with the i.m. route being used for Ad-porcine IFN-α/γ injection and the aerosol route being used for Ad-3siRNA injection. At 48 h p.i. with recombinant adenoviruses, the pigs were challenged by infection by direct contact with 2 donor pigs.

**Swine experiment 3 (determining effective Ad titers).** Eight pigs were divided into 3 groups, including a control group of 2 animals. Pigs were inoculated by the i.m. route with $4 \times 10^9$ or $1.2 \times 10^{10}$ TCID$_{50}$ of a combination of the Ad-porcine IFN-α/γ and Ad-3siRNA vectors at the same titer. At 24 h p.i. with the adenoviruses, the pigs were challenged by infection by direct contact with 2 donor pigs.

**Swine experiment 4 (measuring the duration of antiviral effects).** Twenty-one pigs were divided into 6 groups, including a control group of 6 animals. Pigs were inoculated by the i.m. route with $7.2 \times 10^8$ or $1.75 \times 10^{10}$ TCID$_{50}$ of a combination of the adenovirus vectors (ratio of Ad-porcine IFN-α/γ titer to Ad-3siRNA titer, 1:5). At 2, 4, or 7 days p.i. with the recombinant adenoviruses, the pigs were challenged by infection by direct contact with 4 donor pigs.

**Clinical observation, sample collection, and analysis of swine experiments.** Clinical observations, mouth swab specimen collections, and blood collections were performed daily. Animals were monitored for clinical signs of lameness and vesicles on the snout, mouth, tongue, and digits, and the lesion score was determined, with a maximum score of 12 (28).

**Cytokine assays in swine.** In experiments where cytokine production was measured, 12 Yucatan SPF minipigs that weighed 10 to 15 kg each and that were verified to be negative for FMDV structural protein antibodies were purchased from OptiFarm Solution Medi-Pig, Inc. (Republic of Korea). Pigs were inoculated by i.m. injection of 1.2 $\times 10^9$ TCID$_{50}$ of Ad-porcine IFN-α/γ and $6 \times 10^9$ TCID$_{50}$ of Ad-3siRNA at 2, 4, or 7 days before blood collection. The control group was inoculated by i.m. injection of $6 \times 10^9$ TCID$_{50}$ of the nonrecombinant adenovirus control (Ad-null control) at 2 days before blood collection. At 2, 4, and 7 days after adenovirus infection, plasma was separated from blood collected by venipuncture (anterior vena cava) and placed into Vacutainer serum tubes (BD Biosciences) until 10 days postchallenge. Plasma samples were stored at $-80^\circ$C until analysis. Viral RNA extractions and quantitative real-time RT-PCR experiments were performed to detect FMDV in serum and mouth swab samples.

**Enhancement of antiviral effects in suckling mice following combination treatment with Ad-porcine IFN-α/γ and Ad-3siRNA.**

Enhancement of antiviral effects in suckling mice following combination treatment with Ad-porcine IFN-α/γ and Ad-3siRNA vectors shows enhanced antiviral effects compared to the effects of treatment with the individual vectors (Fig. 1). The antiviral effects observed following combination treatment were significantly enhanced compared to those observed with individual adenoviruses ($P < 0.05$, t test). The FMDV titer produced aftercombination treatment with Ad-porcine IFN-α/γ and Ad-3siRNA (MOI = 0.001) was decreased by at least 3 log units compared to that for an Ad-null control. FMDV titers were also significantly decreased compared to those in cells infected with Ad-porcine IFN-α/γ or Ad-3siRNA alone ($P < 0.05$, t test).

Enhanced antiviral effects in suckling mice following combination treatment with Ad-porcine IFN-α/γ and Ad-3siRNA. To test the anti-FMDV effects of Ad-porcine IFN-α/γ and Ad-3siRNA in vivo, we injected the recombinant adenoviruses into suckling mice, followed by a challenge with 125 LD$_{50}$ or 250 LD$_{50}$ of FMDV O/SKR/2002 (Fig. 2). The recombinant adenoviruses inhibited FMDV replication in suckling mice ($P < 0.005$, log-rank test). The survival rates of mice pretreated with Ad-porcine IFN-α/γ, Ad-
Enhancement of survival rates after FMDV challenge in suckling mice injected with Ad-porcine IFN-α and Ad-3siRNA in combination. Suckling mice were inoculated with 4 × 10⁵ TCID₅₀ of Ad-porcine IFN-α and Ad-3siRNA, or both adenoviral vectors in combination by intraperitoneal injection 24 h before FMDV infection. Suckling mice were challenged with 125 LD₅₀ (A) or 250 LD₅₀ (B) of FMDV (O/SKR/2002). Animals were monitored for a 7-day period postinfection with FMDV.

3siRNA, and their combination were 100% following exposure to 125 LD₅₀ of FMDV (Fig. 2A). However, enhanced survival rates were observed in mice injected with Ad-porcine IFN-α and Ad-3siRNA in combination compared with those observed in mice injected with Ad-porcine IFN-α or Ad-3siRNA alone at 3 to 7 DPC with 250 LD₅₀ of FMDV (Fig. 2B). The survival rate of the combination group was significantly higher than that of the Ad-porcine IFN-α group (P = 0.0001, log-rank test) or Ad-3siRNA group (P = 0.048, log-rank test). In addition, the survival rates of the Ad-3siRNA groups were higher than those of the Ad-porcine IFN-α groups (P = 0.0011, log-rank test).

Combination treatment with Ad-porcine IFN-α and Ad-3siRNA exerts antiviral effects against 7 serotypes of FMDV. To test the antiviral effect of combination treatment against 7 FMDV serotypes, we used various FMDV strains, such as O/Andong/SKR/2010, A22/IRQ 24/64, A/Pochen/SKR/2010, Asia1/MOG/05, C3/Resende, SAT1/BOT1/68, SAT2/ZIM5/81, and SAT3/ZIM4/81 (Fig. 3). The replication of all FMDV strains was decreased in cells treated with the combination of Ad-porcine IFN-α and Ad-3siRNA (Fig. 3A; MOI = 0.01; P < 0.05, t test). Each FMDV strain was also efficiently inhibited in cells pretreated with both adenoviruses in combination, using an MOI of 0.05 (Fig. 3B).

Enhancement of antiviral activity in swine following combination treatment with Ad-porcine IFN-α and Ad-3siRNA. Pigs were pretreated with 3 × 10⁷ TCID₅₀ of Ad-porcine IFN-α, Ad-3siRNA, or a combination and challenged with the FMDV O/Andong/SKR/2010 strain at 1 day postinoculation with the recombinant adenoviruses. The results of these experiments showed that the pigs inoculated with the combination showed fewer clinical signs than the groups inoculated with Ad-porcine IFN-α or Ad-3siRNA alone and that the amount of virus produced in the combination treatment group was less than that observed in the groups inoculated with Ad-porcine IFN-α or Ad-3siRNA alone (Fig. 4). The FMDV RNA levels observed in sera from the group receiving the combination with a higher titer of Ad-3siRNA (ratio of Ad-porcine IFN-α titer/Ad-3siRNA titer = 1:5) were significantly lower than those observed in sera from the control group (P < 0.05; Fig. 4A). When measuring the maximum amount of FMDV in saliva swabs as an indicator of FMDV excretion, lower viral loads were observed in both the 1:1 and 1.5 combination groups than in the control group (P < 0.05; Fig. 4B). Comparisons of the times required for the first detection of FMDV in saliva swabs or sera in individual subjects clearly indicated that FMDV infection was delayed much longer in the 1:5 combination group containing a high concentration of Ad-3siRNA than in the control group (P < 0.05; Fig. 4C and D). At 7 DPC, lesion scores in the 1:5 combination group were significantly lower than those in the control group and the groups administered either Ad-porcine IFN-α or Ad-3siRNA alone (P < 0.05; Fig. 4E). One of three pigs in the 1:1 combination group showed a high lesion score.

Intramuscular injection as an effective route for delivering recombinant adenovirus in swine. To determine the most effective injection route for delivering Ad-porcine IFN-α and Ad-3siRNA (ratio of the Ad-porcine IFN-α titer to the Ad-3siRNA titer in combination, 1:1), pigs were inoculated by the i.m. route, the i.m. route, and the aerosol and i.m. routes, followed by administration of a challenge dose of FMDV (O/Andong/SKR/2010 strain) after 2 days. When the FMDV RNA level in the blood reached the maximum level, the aerosol, aerosol plus i.m., and i.m. groups all showed lower viral loads than the control group (P < 0.005). However, the i.m. group showed a lower viral load in sera than the aerosol (P < 0.005) or aerosol plus i.m. (P < 0.05) group (Fig. 5A). Additionally, the i.m. group showed lower levels of production of FMDV in saliva than the control group, aerosol group, and aerosol plus i.m. group (P < 0.05; Fig. 5B). In terms of the time taken for the virus to first appear in saliva, FMDV infection was delayed for longer durations in the i.m. and the aerosol...
FIG 3  Antiviral effects of combined adenoviral vector treatment against 7 FMDV serotypes in IBRS-2 cells. IBRS-2 cells were inoculated with recombinant adenoviruses at an MOI of 0.01 (A) or 0.05 (B) 18 h before FMDV infection. Following the adenoviral infections, the medium was removed and cells were infected with 100 TCID₅₀ of various FMDV strains (O/Andong/SKR/2010, A22/IRQ 24/64, A/Pocheon/SKR/2010, Asia1/MOG/05, C3/Resende, SAT1/BOT1/68, SAT2/ZIM5/81, or SAT3/ZIM4/81). Supernatants were collected at 48 h postinfection with FMDV and analyzed by extracting RNA from infected cells and performing real-time RT-PCR. Cells not infected with adenoviruses were used as negative controls. Error bars indicate SDs from the mean. A t test was performed to identify statistically significant differences. *, P < 0.05.
Enhancement of antiviral effects against FMDV challenge in swine following combined treatment with the Ad-porcine IFN-α/γ and Ad-3siRNA. Pigs were treated by i.m. injection of $3 \times 10^9$ (single adenovirus group) or $6 \times 10^9$ (combination adenovirus group) TCID$_{50}$ of recombinant adenovirus. The combination groups were infected with Ad-porcine IFN-α/γ and Ad-3siRNA at combination ratios of 1:1 and 1:5. At 24 h postinjection of the recombinant adenoviruses, the pigs were challenged with O/Andong/SKR/2010 by direct contact with donors. The maximum amounts of FMDV detected in serum (A) and saliva swab (B) specimens were measured by real-time RT-PCR from the day that FMDV was first detected in serum (C) or saliva swab (D) specimens to 7 DPC. The symbols (▲, ▼, ◇, ○, and ■) represent duplicate values of viral copy number measurements. (E) Clinical signs at 7 DPC are indicated as clinical scores. Mean values are indicated with bars. A t test was performed to identify statistically significant differences. *, $P < 0.05$; **, $P < 0.005$. 

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FIG 5 Evaluating the efficacy of the different injection routes for delivering Ad-porcine IFN-α and Ad-3siRNA in combination in swine. Pigs were inoculated by aerosol, i.m. injection, or aerosol and i.m. injection combined with 3 × 10^9 TCID₅₀ each of Ad-porcine IFN-α and Ad-3siRNA. At 48 h postinjection with the recombinant adenoviruses, pigs were challenged with O/Andong/SKR/2010 by direct contact with donors. The maximum amount of FMDV detected in serum (A) and saliva swab (B) specimens was measured by real-time RT-PCR from the day that FMDV was first detected in serum (C) or saliva swab (D) specimens to 7 DPC. The symbols (▼, ▲, ◆, ♦, and □) represent duplicate values of viral copy numbers. (E) Clinical signs at 7 DPC are indicated as clinical scores. Mean values are indicated with bars. A t test was performed to identify statistically significant differences. *, P < 0.05; **, P < 0.005.
plus i.m. groups than in the control group (P < 0.05; Fig. 5D). However, the results for the group receiving recombinant adenovirus by the aerosol route were similar to those for the control group. The difference in the times required to detect FMDV in serum to the aerosol and control groups was not statistically significant (Fig. 5C). In terms of clinical signs, the i.m. group showed a lower lesion score than the control or aerosol group (P < 0.05), whereas the aerosol plus i.m. group did not show a significant decrease in lesion scores compared to the control or aerosol group (Fig. 5E).

Protection against the highly virulent FMDV strain O/Andong/SKR/2010 by combination treatment with Ad-porcine IFN-α-γ and Ad-3siRNA in pigs. We tested the antiviral effects of 4 × 10^9 or 1.2 × 10^10 TCID_{50} of adenovirus combination treatment (ratio of Ad-porcine IFN-α-γ titer to Ad-3siRNA titer, 1:1) against FMDV O/Andong/SKR/2010 in pigs for up to 10 days. The group treated with 1.2 × 10^{10} TCID_{50} of adenovirus tested negative for clinical signs, viral RNA in the blood, and virus in saliva, confirming that protection against FMDV was achieved (Table 1). In the group treated with the lower titer (4 × 10^{10} TCID_{50}) of adenovirus, 1 out of 3 pigs showed evidence of protection against FMDV. The 2 remaining pigs in that group had lower clinical scores than the control group, and the time before the first detection of FMDV in serum and saliva samples was delayed by 2 to 7 days.

Induction of cytokines in pigs by combination treatment with Ad-porcine IFN-α-γ and Ad-3siRNA. To study the time-dependent changes in cytokine production levels in pigs after combination adenovirus treatment, plasma samples were collected and assayed at 2, 4, and 7 days postinoculation (dpi) with adenovirus. When porcine IFN-α, IFN-γ, and IL-12 levels were measured, a time-dependent cytokine induction pattern was observed, where, at 4 dpi in particular, a dramatic increase was observed for all 3 cytokines (Fig. 6). In addition, samples obtained from the groups at 2, 4, and 7 dpi showed an increase in porcine IFN-α production in 1 out of 3, 3 out of 3, and 2 out of 3 pigs, respectively (Fig. 6A), and porcine IFN-α expression levels in the groups sampled at 4 and 7 dpi were significantly higher than those in the control group (P < 0.05). In the groups sampled at 2 and 7 dpi, no increase in porcine IFN-γ was observed in any of the pigs, except for 1 pig in the group sampled at 2 dpi (Fig. 6B). In contrast, all 3 pigs in the group sampled at 4 dpi showed higher IFN-γ expression levels, and the increase in the porcine IFN-γ level in that group was significantly different from that in all other groups, including the control group (P < 0.05). Further, only 1 of the 3 pigs from the group sampled at 2 dpi showed an increase in IL-12 production, while 2 of the 3 pigs within each of the groups sampled at 4 and 7 dpi showed increases (Fig. 6C). In particular, the amount of IL-12 in the group sampled at 4 dpi was significantly larger than that in the control group (P < 0.05).

Duration of protection against FMDV in pigs by combination treatment with Ad-porcine IFN-α-γ and Ad-3siRNA. Following inoculation with 7.2 × 10^9 TCID_{50} of the adenovirus combination (ratio of Ad-porcine IFN-α-γ titer to Ad-3siRNA titer, 1:5), 2 out of 3 pigs in the group sampled at 2 dpi did not show clinical signs, whereas 1 out of 3 pigs in the group sampled at 4 dpi and none in the group sampled at 7 dpi showed clinical signs (Table 2). However, the level of virus excretion, the delay in the time to the onset of the FMDV RNA level in the blood, and the reductions in virus levels in saliva were lower in the adenovirus-treated groups sampled at 2, 4, and 7 dpi than the control group. When the adenovirus titer was increased ~2-fold (1.75 × 10^{10} TCID_{50}), 3 out of 3 pigs in the group sampled at 4 dpi and 1 of 3 pigs in the group sampled at 7 dpi did not show clinical signs, and a delayed onset of FMDV infection compared to the time of onset of FMDV infection in pigs treated with 7.2 × 10^9 TCID_{50} of adenovirus was observed.

**DISCUSSION**

Although several antiviral agents have been shown to suppress the rapid spread of FMDV, the development of a novel antiviral FMDV agent that is safe, long lasting, and effective against different FMDV serotypes and subtypes is still required. We previously developed two recombinant adenoviruses, namely, Ad-porcine IFN-α-γ, which simultaneously expresses type I and type II interferons, and Ad-3siRNA, which simultaneously expresses 3 siRNAs, to maximize the efficacy of interferon and siRNA (21, 22). In this study, we tested a more effective antiviral strategy involving combination adenovirus treatment and administration to a natural host that can satisfy the fundamental requirements needed for

<table>
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<th>Group</th>
<th>Treatment</th>
<th>Ad titer (TCID_{50})</th>
<th>Animal no.</th>
<th>Serum (DPC of first detection)</th>
<th>Mouth swab (DPC of first detection)</th>
<th>Maximum clinical score (DPC of first detection)</th>
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<td>1</td>
<td>Ad-null control</td>
<td>6 × 10^9</td>
<td>10</td>
<td>6.2 × 10^9 ± 0.2 × 10^9 (3)</td>
<td>6.6 × 10^9 ± 0.3 × 10^9 (3)</td>
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<td>1.4 × 10^9 ± 0.1 × 10^9 (2)</td>
<td>8.3 × 10^9 ± 0.4 × 10^9 (2)</td>
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<td>Ad-porcine IFN-α-γ and Ad-3siRNA</td>
<td>2 × 10^9 and 2 × 10^9</td>
<td>12</td>
<td>4.5 × 10^9 ± 0.7 × 10^9 (5)</td>
<td>2.0 × 10^9 ± 0.1 × 10^9 (4)</td>
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<td></td>
<td>13</td>
<td>1.1 × 10^9 ± 0.2 × 10^9 (9)</td>
<td>2.7 × 10^9 ± 0.2 × 10^9 (8)</td>
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<td>14</td>
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<td>3</td>
<td>Ad-porcine IFN-α-γ and Ad-3siRNA</td>
<td>6 × 10^9 and 6 × 10^9</td>
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<td></td>
<td>17</td>
<td>ND</td>
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</table>

* Recombinant adenoviruses were inoculated 24 h before FMDV challenge.
* The data (FMDV RNA copy number per milliliter) represent means ± standard deviations from triplicate real-time RT-PCR experiments.
* Neg, no viral RNA and no clinical symptoms were detected.
* ND, not detected.
effective FMDV antiviral therapy to confirm the efficacy of treatment. Although it has previously been demonstrated that Ad-porcine IFN-α and Ad-3siRNA are promising antiviral agents, suppressing FMDV replication via a single mechanism, they have limitations, and their efficacy has not been proven in a natural host, swine. In addition, previous studies involving combination therapy against FMDV have achieved greater protection at lower doses. Compared to the results observed when testing individual antiviral agents, we observed an increase in antiviral effects in IBRS-2 cells, suckling mice, and swine models using a combination of siRNAs and interferons without antagonism between the two antiviral agents (Fig. 1, 2, and 4). These results agree with our previous results, where the combined use of siRNA and IFN-α enhanced the suppression of viral replication in cells (15).

The Ad-3siRNA component of the combination treatment is a recombinant adenovirus that simultaneously expresses 3 siRNAs targeting the nonstructural protein region of the FMDV genome. The antiviral effect Ad-3siRNA offers an improvement over that of single siRNAs, which can be rendered ineffective by viral escape mutations or may target only a narrow range of virus serotypes or strains (21). The improvement in FMDV suppression was confirmed using combined adenovirus treatment, and FMDV suppression is proposed to be a fast-acting defense against a broad range of FMDV serotypes. We observed that the suppression of FMDV replication by Ad-3siRNA occurred within 2 h of treatment in IBRS-2 cells and was faster than that achieved with Ad-porcine IFN-α in IBRS-2 cells and mice (data not shown). Accordingly, we also suggest that Ad-3siRNA might play a role in conferring rapid resistance to FMDV in natural hosts.

Because porcine IFN-α was reported to be an effective antiviral agent against FMDV in pigs (29), several studies investigated the antiviral effect of IFNs (15, 30, 31). A mixture of adenoviruses expressing porcine IFN-α and porcine IFN-γ had synergistically enhanced anti-FMDV effects compared with the effect of an adenovirus expressing a single IFN (31). The Ad-porcine IFN-α component in the treatment combination is a recombinant adenovirus that simultaneously expresses type I and type II IFNs (22). Ad-porcine IFN-α can suppress FMDV more effectively in animals through a synergistic effect involving IFN-α, which directly suppresses FMDV, and IFN-γ, which plays an important role in T-cell-mediated immunity. The efficacy of Ad-porcine IFN-α in controlling FMDV replication has been proven indirectly as well, by inducing various IFN-stimulated genes (ISGs) (22). Ad-porcine IFN-α not only has a direct suppressive effect on FMDV but also exerts an indirect antiviral effect through the induction of an immune response.

In this study, the suppressive effect of the combination of Ad-3siRNA and Ad-porcine IFN-α was confirmed using 7 FMDV serotypes (Fig. 3). Treatment of cells with both adenoviruses at an MOI of 0.01 inhibited the replication of all 7 FMDV strains tested (P < 0.05, t test), but the extent of inhibition of each virus varied. The extent of inhibition of replication of serotype O viral RNA was 1 to 2 log units higher than that of RNA of strains belonging to the samples were measured by ELISA. The concentration of each protein studied was determined by interpolation from standard curves. The squares represent duplicate values for each sample tested. Mean values are indicated with bars. A t test was performed to identify statistically significant differences. *, P < 0.05; ***, P < 0.005.

FIG 6 Measurement of cytokine induction in swine by treatment with a combination of Ad-porcine IFN-α and Ad-3siRNA. Pigs were inoculated by i.m. injection of 1.2 × 10⁹ TCID₅₀ of Ad-porcine IFN-α and 6 × 10⁹ TCID₅₀ of Ad-3siRNA. Plasma samples were collected from the pigs at 2, 4, or 7 days postinoculation with the recombinant adenoviruses. Porcine IFN-α (A), porcine IFN-γ (B), and porcine IL-12 (C) protein expression levels in the plasma
other 6 serotypes (Fig. 3A). In the case of Ad-3siRNA, suppression was confirmed with all 7 FMDV serotypes, but differences in antiviral effects were observed (21). This result demonstrates that the number of mismatches in the viral target sequence is inversely proportional to the silencing effect. We observed sequence mismatches in the O/Andong/SKR/2010 (1 mismatch), A/Pocheon/SKR/2010 (2 mismatches), A22/IRQ 26/64 (0 mismatches), Asia1/MOG/05 (1 mismatch), C3/Resende (2 mismatches), SAT1/BOT1/68 (7 mismatches), SAT2/ZIM5/81 (7 mismatches), and SAT3/ZIM4/81 (7 mismatches) strains in this study, although the regions targeted by the 3 siRNAs, 2B and 3C, are the most conserved regions in several types of FMDVs. However, variations in the extent of inhibition could not be explained by mismatches in siRNA sequences alone. Dias et al. reported that type I interferon can rapidly protect pigs against multiple serotypes of FMDV (32); they demonstrated the antiviral effect of recombinant adenovirus expressing porcine IFN-α against the O, A, and Asia1 serotypes of FMDV. However, differences in the sensitivity to type I or type II IFNs have not been reported for the 7 serotypes and various strains of FMDV. Therefore, the possibility that the use of Ad-porcine IFN-α also influences the results cannot be ignored. However, when the adenovirus inoculum was increased (MOI = 0.05), all FMDV strains tested were suppressed, thereby demonstrating the possibility that with a sufficiently high dose, the adenovirus can be used against all the FMDV strains tested (Fig. 3B).

In pigs, we observed a higher degree of suppression accompanied by an increase in the duration of protection and a decrease in the level of FMDV in saliva in the group receiving the 1:5 combination, in which the ratio of the Ad-3siRNA titer was higher than that in the other combination group (Fig. 4). The combination groups were divided into two groups according to the ratio of the Ad-porcine IFN-α titer to the Ad-3siRNA titer (1:1 and 1:5) in order to elucidate the most efficient titer ratio. Ad-3siRNA and not Ad-porcine IFN-α was assigned to be present at the higher titer because the production efficiency of Ad-porcine IFN-α is much lower than that of Ad-3siRNA. This may be explained by the fact that the IFN protein from Ad-porcine IFN-α interrupts the replication of recombinant adenoviruses to some extent. Due to the fast-acting effects of siRNAs, the 1:5 combination group had the fast-acting effects of siRNAs, the 1:5 combination group had

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<tr>
<th>Group</th>
<th>Treatment</th>
<th>Ad titer (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Time of challenge&lt;sup&gt;a&lt;/sup&gt; (dpi)</th>
<th>Animal no.</th>
<th>Maximum amt of virus detected in (DPC of first detection)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Maximum clinical score (DPC of first detection)</th>
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<td>Serum</td>
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<td>Ad-null control</td>
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<td>7</td>
<td>23</td>
<td>2.8 × 10&lt;sup&gt;5&lt;/sup&gt; ± 0.8 × 10&lt;sup&gt;5&lt;/sup&gt; (1)</td>
<td>6.8 × 10&lt;sup&gt;7&lt;/sup&gt; ± 0.6 × 10&lt;sup&gt;7&lt;/sup&gt; (1)</td>
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<td>Ad-porcine IFN-αγ and Ad-3siRNA</td>
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<td>29</td>
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<td>32</td>
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<td>6</td>
<td>Ad-porcine IFN-αγ and Ad-3siRNA</td>
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<td>1.2 × 10&lt;sup&gt;7&lt;/sup&gt; ± 0.7 × 10&lt;sup&gt;7&lt;/sup&gt; (3)</td>
<td>2.6 × 10&lt;sup&gt;5&lt;/sup&gt; ± 0.1 × 10&lt;sup&gt;5&lt;/sup&gt; (3)</td>
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<sup>a</sup> Pigs were challenged with FMDV at 2, 4, or 7 dpi with adenovirus (or PBS).

<sup>b</sup> The data (FMDV RNA copy number per milliliter) represent means ± standard deviations from triplicate real-time RT-PCR experiments.

<sup>c</sup> Neg, no viral RNA and no clinical signs were detected.
occurs via the respiratory tract, applying adenovirus to the respiratory tract is expected to be advantageous in quickly preventing FMDV infection. In this study, infection by direct contact was used as the challenge method, as this method of delivery is similar to the means of FMDV infection seen clinically, which occurs via the respiratory tract. However, contrary to the findings presented in previous reports (34, 35), we observed similar results with the negative-control (Ad-null control) group for most clinical factors, and only the levels of FMDV in serum decreased compared with those for the negative-control group when both Ad-porcine IFN-α and Ad-3siRNA were administered by the aerosol route (Fig. 5). When Ad-3siRNA infection was administered by the aerosol route and Ad-porcine IFN-α infection was delivered by i.m. injection, the suppression effect observed was greater than that observed in the Ad-null control group, with the protective effect observed being reduced compared with that observed when both Ad-3siRNA and Ad-porcine IFN-α infections were administered by i.m. injection. In addition, the extent of individual differences in clinical signs was greater in the aerosol plus i.m. injection group. From these results, it is plausible that aerosol infection of pigs may show different delivery efficiencies, depending on the condition of the respiratory tract of the animal at the time of inoculation, and particularly with Ad-porcine IFN-α, the impact on systemic immunity was expected to be lower with aerosol infection than with i.m. infection. Because the FMDV challenge in this study occurred 2 days after adenovirus inoculation, a large amount of adenovirus may have been removed from the respiratory tract at the time of aerosol infection. Previous studies of FMDV have demonstrated that recombinant human adenovirus type 5 expressing siRNA or IFNs can be delivered effectively via i.m. injection in pigs (29, 31, 36). Therefore, we propose that regardless of the various factors involved, i.m. injection is the most appropriate for stable delivery. However, we also suggest that the number of injections be limited because repetitive i.m. injection could decrease the efficiency by changing the conformation of the antibody on recombinant human adenovirus type 5.

When a dose level of $4 \times 10^9$ TCID$_{50}$ was used with the combination of Ad-3siRNA and Ad-porcine IFN-α, only some of the pigs in the group were protected from infection, but when the dose was increased three times to $1.2 \times 10^{10}$ TCID$_{50}$, protection was observed in all pigs (Table 1). The reason for the lack of protection in the test animals, as seen in Fig. 4 and 5, is believed to be due to the inoculation dose being lower than $10^{10}$ TCID$_{50}$.

The measurement of cytokines in swine plasma indicated that IFN-α, IFN-γ, and IL-12 were upregulated by inoculation with Ad-3siRNA and Ad-porcine IFN-α (Fig. 6). IL-12 is a major cytokine in cell-mediated immunity (CMI) and is produced by macrophages to protect the body from pathogens (37). The production of IL-12 is stimulated by IFN-γ, and the induction of Th1 immunity is mediated by IL-12. Type I IFN also stimulates the expression of T-cell functional receptors for IL-12, whereby the increase in IL-12 is believed to have mediated the CMI portion of the antiviral role of IFN-α in this study. These findings suggest that the method used in the current study was effective in inducing CMI. Thus, there is great potential to induce cytokines with Ad-porcine IFN-α. However, because Ad-3siRNA has also been shown to induce ISGs, it is possible that Ad-3siRNA can promote the induction of IFN as well (21). The levels of IFN-α, IFN-γ, and IL-12 were at their peak on the 4th day after inoculation. IFN-α production was maintained up to the 7th day; however, the levels of IFN-γ and IL-12 subsequently decreased, and the concentration of IFN-γ dropped to the level in the control group. In a previous study, when pigs were injected with $10^9$ PFU of recombinant adenovirus expressing porcine IFN-α, the antiviral activity of IFN-α in plasma increased from 12 h to 4 days after injection and then decreased (38), similar to our results in the present study. However, the previous results showed that the antiviral effects of interferon nearly disappeared 5 days following adenovirus injection, implying that the sustained time of IFN-α activity measured was shorter than that in our study. Moreover, in terms of the duration of the antiviral effects in swine, FMDV suppression by induced cytokines can be expected by 4 to 7 days after injection of $9 \times 10^9$ TCID$_{50}$. From the challenge results, antiviral effects, such as the reduction in the level of FMDV RNA in the blood and lowering of the clinical scores, were observed for up to 7 days after adenovirus injection (Table 2). However, in comparison to the group challenged at 2 dpi, the number of pigs with protection in the groups challenged at 4 and 7 dpi showed a gradual decrease, which confirmed that the direct effects of siRNA and interferon are time dependent. The mRNA of recombinant human adenovirus type 5 has been reported to be at its highest level at 24 h or 48 h postinfection, with protein expression peaking at 48 h p.i. (39). Therefore, the highest levels are maintained at 24 to 48 h and 48 h p.i. with Ad-3siRNA and Ad-porcine IFN-α, respectively, and there is a possibility that the most effective direct suppression of the virus could appear right before or after such periods. Other effects may result from cytokine induction. Since the injected dose can influence how long the effects of the exogenous siRNAs and proteins can be maintained in animals, in the group that was administered the adenovirus injection, the titer was 2-fold higher (Table 2). During the period in which siRNA and protein levels were slightly increased in animals in the adenovirus challenge group at 4 dpi and 7 dpi, protection against FMDV was observed. To extend this time, an increase in titer or adjustment of the ratio of IFN to siRNA may be needed.

In addition, the O/Andong/SKR/2010 strain used in this swine study shows an extremely high rate of propagation, as well as high pathogenicity, and caused widespread disease in the Republic of Korea in 2010 and 2011 (24). The delivery of protection against this highly pathogenic FMDV strain suggests that it is plausible that similar protection against other highly pathogenic strains can be expected. Moreover, as confirmed by the antiviral effects observed in IBRS-2 cells, this strategy could be applied to all other FMDV serotypes (Fig. 3). The combined use of Ad-porcine IFN-α and Ad-3siRNA not only showed rapid antiviral effects but also may provide a measure of protection when used with a vaccine during the 7 days of the immunity gap before the vaccine provides protection. Additionally, because IFN-α and IFN-γ are recognized to be vaccine adjuvants, it can be expected that use of the combination treatment evaluated in this study with an FMD vaccine would facilitate improved immune responses and antibody formation (40–42). Use of a combination of IFN inducers and the FMDV vaccine has been reported previously, and enhanced inhibition of FMDV was observed in those studies (43, 44). In future studies, we propose to use a combination of Ad-porcine IFN-α and Ad-3siRNA with a vaccine to evaluate their combined effects.
ACKNOWLEDGMENT
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REFERENCES


