

Inhibition of viral replication by small interfering RNA targeting of the foot-and-mouth disease virus receptor integrin $\beta 6$

NA-NA HU¹, WENZHI ZHANG¹, LINA WANG¹, YUAN-ZHI WANG² and CHUANG-FU CHEN¹

¹College of Animal Science and Technology, Shihezi University;

²College of Medicine, Shihezi University, Shihezi, Xinjiang 832000, P.R. China

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Abstract. In animals, foot-and-mouth disease (FMD) causes symptoms such as fever, limping and the development of blister spots on the skin and mucous membranes. RNA interference (RNAi) may be a novel way of controlling the FMD virus (FMDV), specifically by targeting its cognate receptor protein integrin $\beta 6$. The present study used RNAi technology to construct and screen plasmids that expressed small interfering RNA molecules (siRNAs) specific for the integrin $\beta 6$ subunit. Expression of green fluorescence protein from the RNAi plasmids was observed following transfection into porcine embryonic fibroblast (PEF) cells, and RNAi plasmids were screened using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. A fragment (5'AAAGGCCAAGTGGCAAACGGG 3') with marked interference activity was ligated into a PXL-EGFP-NEO integration plasmid and transfected into PEF cells. Transfected cells were selected using G418, and interference of the integrated plasmid was subsequently evaluated by FMDV challenge experiments, in which the levels of viral replication were determined using optical microscopy and RT-qPCR. A total of seven interference plasmids were successfully constructed, including the pGsi-Z4 plasmid, which had a significant interference efficiency of 91.7% in PEF cells ($^{**}P<0.01$). Upon transfection into PEF cells for 36 h, a Z4 integration plasmid exhibited significant inhibitory effects ($^{**}P<0.01$) on the integrin $\beta 6$ subunit. Subsequent challenge experiments in transfected PEF cells also demonstrated that viral replication was reduced by 24.2 and 12.8% after 24 and 36 h, respectively. These data indicate that RNAi technology

may inhibit intracellular viral replication in PEF cells by reducing expression of the FMDV receptor integrin $\beta 6$.

Introduction

Foot-and-mouth disease (FMD) is an acute, febrile and contagious disease caused by the FMD virus (FMDV) (1). FMDV infection primarily occurs through the binding of FMDV to receptors on the host cell surface (2). Following cell penetration and uncoating, FMDV undergoes replication, transcription, translation and genome packaging (3). Receptor binding is mediated by an arginine-glycine-aspartate (RGD) sequence in the FMDV major capsid protein 1 (VP1), which acts as the cognate ligand for cell surface receptors (4). Adsorption to host cells involves specific interactions between viral proteins, including VP1, and cell surface membrane receptors, which is a prerequisite for viral infection of host cells (5). Previous results have confirmed the presence of two types of cellular receptors for FMDV: Integrins and heparin sulfate (6,7). In particular, integrins have key roles in mediating viral infection of host cells.

Integrins are protein heterodimers composed of α and β subunits, and occur in at least 20 distinct types. Infection of host cells by FMDV is principally mediated by integrin heterodimers. Currently, four types of receptors are considered to mediate FMDV infection, namely $\alpha\beta 1$, $\alpha\beta 3$, $\alpha\beta 6$ and $\alpha\beta 8$, with $\alpha\beta 6$ having a key role in the infection process (8). It has been demonstrated that the major adsorption receptor for FMDV is cell surface-expressed $\alpha\beta 6$ integrin (9). Loss of the $\beta 6$ subunit has an inhibitory effect on viral infection, as the conserved motif region within the cytoplasmic domain of integrin $\beta 6$ has an important role in the infection process.

In the present study, based on the gene sequence of the porcine integrin receptor $\beta 6$ subunit, small interfering RNA molecules (siRNAs) were designed to target the cytoplasmic domain of the integrin $\beta 6$ subunit, and corresponding expression plasmids were constructed. The inhibitory function of different siRNAs and their effects on viral replication were evaluated in porcine embryonic fibroblasts (PEFs). Ultimately, PEFs were obtained in which viral replication was inhibited, thus providing a basis for therapeutic targeting of the receptor-virus interactions in FMD.

Correspondence to: Dr Chuang-Fu Chen, College of Animal Science and Technology, Shihezi University, 221 Shihez Shibe Four Road, Shihezi, Xinjiang 832000, P.R. China
E-mail: ccf-xb@163.com

Dr Yuan-Zhi Wang, College of Medicine, Shihezi University, 221 Shihez Shibe Four Road, Shihezi, Xinjiang 832000, P.R. China
E-mail: wangyuanzhi621@126.com

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Materials and methods

Materials. The *E. coli* DH5 α strain, PEF cells, green fluorescent protein (GFP)-expressing RNA interference (RNAi) expression vectors pGenesil-1 and pXL-eGFP-Neo and the pGsi-Z4 recombinant expression plasmid were generated in our laboratory as previously described (10). The FMDV virulent strain OS-22 was provided by Ningbo Tecon Biotechnology Co., Ltd. (Ningbo, China). G418 (250 μ g/ml) antibiotic, reverse transcriptase PrimeScript Reverse Transcriptase kit, restriction endonucleases and a fluorescence quantitative polymerase chain reaction (qPCR) reagent kit were purchased from Takara Biotechnology Co., Ltd., (Dalian, China). An X-tremeGENE HP DNA Transfection Reagent kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). TRIzol reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell isolation and culture. The porcine embryonic fibroblast (PEF) cells were proved by Dr Shiwei Ma (College of Animal Science and Technology, Shihezi University, Xinjiang, China) (11). Cells were cultivated for 24 h in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in an atmosphere containing 5% CO₂ at 39°C.

Construction of interference plasmids. Gene sequences of the porcine integrin $\beta 6$ subunit were retrieved from the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). Six pairs of positive short hairpin RNAs shRNAs and one pair of negative (control) shRNAs were designed to target different regions of the cytoplasmic domain of the $\beta 6$ subunit gene; these primer pairs were termed pGsi-Z1, pGsi-Z2, pGsi-Z3, pGsi-Z4, pGsi-Z5, pGsi-Z6 and pGsi-Z7 (negative control). DNA sequences corresponding to the hairpin structure of the shRNA were synthesized (Table I) by Beijing Liuhe Genomics Technology Co., Ltd. (Beijing, China).

Single-stranded shRNA fragments were diluted to 100 μ mol with distilled water. Pairs of single-stranded shRNA that targeted the same DNA sequence were then mixed together (10 μ l each) and the mixture was incubated at 95°C for 3 sec, 72°C for 2 min, 37°C for 2 min and 25°C for 2 min for annealing. The resulting dsRNA sequence was cloned into a pGenesil-1 vector and transformed into DH5 α competent cells for the isolation of recombinant plasmids. Positive plasmids were identified by double-enzyme digestion using the *Bam*HI, *Hind*III, and *Eco*RI enzymes. Plasmids with the correct digestion patterns were sent to Beijing Liuhe Genomics Technology Co. Ltd. for sequencing and identification.

Cell transfection and observation of recombinant plasmids by fluorescence microscopy. Prior to transfection, PEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS in a 5% CO₂ incubator at 39°C. When cell confluence reached 80-90%, recombinant interference plasmids (pXL-U6-Z4) were transfected into PEF cells using the X-tremeGENE HP DNA Transfection Reagent kit, according to the manufacturer's instructions. Cell morphology and the

expression of recombinant plasmids containing the GFP gene were observed under a fluorescence microscope every 12 h starting 24 h after transfection. The transfection efficiency of recombinant interference plasmids was also evaluated using flow cytometry as previously described (12).

Analysis of recombinant plasmid interference. Gene sequences of the porcine integrin receptor $\beta 6$ subunit and the internal control, β -actin, were retrieved from GenBank. Primers for reverse transcription-quantitative polymerase chain reaction PCR (RT-qPCR) were designed using Primer Premier 5.0 primer design software (PREMIER Biosoft, Palo Alto, CA, USA; Table II) and synthesized by Beijing Liuhe Genomics Technology Co., Ltd.

Total cellular RNA was extracted from PEF cells transfected with RNAi vectors using a total RNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNA was synthesized from 2 μ g of total RNA using a reverse transcription kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. $\beta 6$ expression was detected by RT-qPCR, with β -actin as an internal control. The primer sequences for $\beta 6$ are listed in Table II. PCR cycling conditions were as follows: 94°C for 5 min; followed by 40 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and 72°C for 10 min. The results were calculated and analyzed using the 2^{- $\Delta\Delta C_q$} method. Three replicates were performed (13).

Cell construction with an integrated U6-Z4 gene. The recombinant plasmid constructed in the present study, pGsi-Z4, was verified by double-enzyme digestion with *Eco*RI and *Bgl*II. The digested fragment included the U6 promoter and Z4 RNAi fragment sequence; this fragment was termed U6-Z4. The U6-Z4 gene and pXL-EGFP-NEO vector were ligated and transformed into *E. coli* DH5 α competent cells for plasmid isolation. Plasmids were digested with *Mlu*I (Takara Biotechnology Co., Ltd.) and recombinant plasmids were identified by running digestion products on a 1% agarose gel. DNA ladder 10,000 was used (Tiangen Biotech Co., Ltd.). The stored bacterial solutions were sent to Beijing Liuhe Genomics Technology Co. Ltd., for subsequent sequencing and identification.

Positive recombinant plasmid clones expressing U6-Z4 were subjected to single-enzyme digestion using *Apa*LI. DNA fragments were obtained using an agarose gel DNA Recovery kit (CWBIO, Beijing, China). DNA fragments (pXL-U6-Z4 plasmid) were transfected into PEF cells using the X-tremeGENE HP DNA Transfection Reagent kit, according to the manufacturer's protocol. Cells transfected with linearized pX1 without a U6-Z4 sequence were used as a control group. Cells were cultured in DMEM supplemented with 10% FBS in an incubator containing 5% CO₂ at 39°C. When cell confluence reached 60-70%, cell medium was replenished with G418 selection culture medium with DMEM supplemented with 10% FBS (250 μ g/ml). After ~15 days of selection, antibiotic selection was terminated and surviving (G418-resistant PEF) cells were cultured, passaged and amplified.

Validation of integration plasmid interference effects. The level of integrin receptor $\beta 6$ subunit gene expression in PEF cells transfected with pXL-U6-Z4 plasmid was assessed using RT-qPCR. Titers of the OS-22 virulent FMDV strain (Ningbo

Table I. shRNA sequences.

shRNA name	shRNA sequence
pGsi-Z1	5'-GATCCAAATTTGAAGCAGAACGGTCATTCAAGAGATGACCGTTCTGCTTCAAAT TTTTTTTTTACGCGTA-3'
pGsi-Z2	5'-GATCCAAGCAGAACGGTCAAAGGCCATTCAAGAGATGGCCTTTGACCGTTC TGCTTTTTTTTACGCGTA-3'
pGsi-Z3	5'-GATCCAACGGTCAAAGGCCAAGTGGCTTCAAGAGAGCCACTTGGCCTTTGA CCGTTTTTTTTTACGCGTA-3'
pGsi-Z4	5'-GATCCAAAGGCCAAGTGGCAAACGGGTTCAGAGACCCGTTTGCCACTTGG CCTTTTTTTTTTACGCGTA-3'
pGsi-Z5	5'-GATCCAAGTGGCAAACGGGAACCAATTTCAAGAGAATTGGTTCCCGTTTGC CACTTTTTTTTTTACGCGTA-3'
pGsi-Z6	5'-GATCCAACCAATCCACTGTACAGAGGTTCAAGAGACCTCTGTACAGTGGATTGG TTTTTTTTTACGCGTA-3'
pGsi-Z7	5'-GATCCAGTCTGTACAGAGCGACTCTTTCAAGAGAAGAGTCGCTCTGTACA GGACTTTTTTTTTTACGCGTA-3'

shRNA, small hairpin RNA.

Table II. Primer design for reverse transcription-quantitative polymerase chain reaction.

Primer name	No. of amplified bases	Primer DNA sequences
Murine β -actin	98	F, 5'-GCTGTCCCTGTATGCCTCTG-3' R, 5'-GGAGAGCATAGCCCTCGTAG-3'
Murine β 6	186	F, 5'-GTGTCAGTGGCGATCCTG-3' R, 5'-GTGCTTGTAGGTCACGTTTC-3'
Porcine β -actin	196	F, 5'-CATTTGTCATGGACTCTGGGGA-3' R, 5'-CTTCTCCTTGATGTCCCGCA-3'
Porcine β 6	180	F, 5'-GGGGTTTCACTGGCTATTCT-3' R, 5'-GGTTACATTTTAAAGGCGC-3'

F, forward; R, reverse.

Tecon Biotechnology Co., Ltd.) were determined, and the 50% tissue culture infective dose (TCID₅₀) of the virus stock was calculated using the Reed-Muench formula (14). Based on calculations, PEF cells were infected with FMDV at 48 h after transfection with the interference plasmid. PEF cells were monitored for cytopathic effects (CPEs) at 12, 18, 24 and 36 h after virus inoculation, and virus supernatants were collected. Differences in the levels of FMDV replication in the supernatants of integrated plasmid transfection and control groups (including normal PEF cells and PEF cells with FMDV infection without pXL-U6-Z4 plasmid) were preliminarily determined. CPEs within cells were observed using an inverted microscope TH4-200 (Olympus Corporation, Tokyo, Japan) and measured using a semi-quantitative method (20% of cells exhibiting CPEs, +; 40% CPE, ++; 60% CPE, +++; 80% CPE, ++++; and 100% CPE, +++++). Virus supernatants were collected at different time points (12, 18, 24 and 36 h) following the viral challenge, and differences in the levels of viral replication between the integrated plasmid transfection and control groups following FMDV infection

were determined using RT-PCR with three replicates. Total RNA was extracted using a total RNA extraction kit (Tiangen Biotech Co., Ltd.). First-strand cDNA was synthesized from 2 μ g of total RNA using a reverse transcription kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. Expression of FMDV VP1 gene was detected by RT-PCR with GAPDH as an internal control. The RT-PCR final reaction volume was 20 μ l comprised of ddH₂O (7.2 μ l), SYBR Fluorescent dye (10.0 μ l), cDNA Template (2.0 μ l), upstream primer (0.4 μ l), and downstream primer (0.4 μ l). The primers used were as follows: FMDV VP1, forward 5'-GAAGATCTC CCAGTGGAAAGACGCG-3' and reverse 5'-CGGAATTCT TGAAAAAAGCTACAGATCACC-3'; GAPDH, forward, 5'-GTCACCAAGGCTGCTTT-3' and reverse 5'-TGTGCC GTTGAACCTTGC-3'. Primers were designed using Primer Premier 5.0 software. PCR cycling was as follows: 95°C for 5 sec followed by 40 cycles of 95°C for 15 sec, 52°C for 30 sec, 72°C for 20 sec and 72°C for 10 min. The results were calculated and analyzed using the comparative 2^{- $\Delta\Delta$ Cq} method (13).

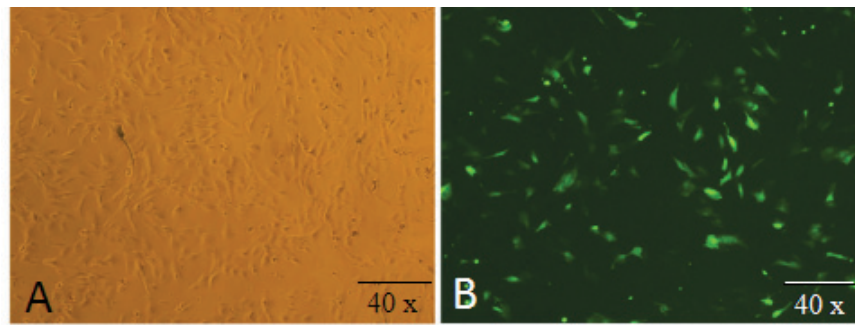


Figure 1. Observation of PEF cells by fluorescence microscopy following transfection. (A) Normal PEF cells. (B) Expression of GFP (green) in PEF cells transfected for 48 h with recombinant RNA interference plasmids expressing a GFP marker. Magnification, x40. PEF, porcine embryonic fibroblast; GFP, green fluorescent protein.

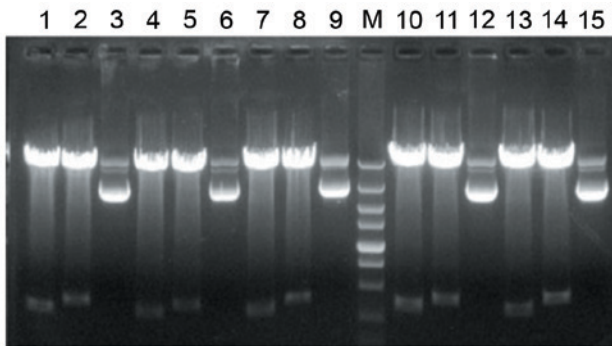


Figure 2. Identification of RNAi recombinant plasmids using enzyme digestion. Lanes 1, 4, 7, 10 and 13 show the full RNAi recombinant plasmids (controls), lanes 2, 5, 8, 11 and 14 show the U6 promoter digestion product (~320 bp) obtained from *Bam*HI and *Eco*RI digestion and lanes 3, 6, 9, 12 and 15 show the the U6 promoter and short hairpin RNA (~390 bp) digestion products obtained from a *Hind*III and *Eco*RI digestion. Lane M indicates the Marker 5,000 DNA ladder. RNAi, RNA interference.

Statistical analysis. Data were analyzed with one-way analysis of variance using SPSS Statistics version 17.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Transfection of PEF cells with recombinant interference plasmids. PEF cells were isolated using an enzyme digestion and tissue block culture method (Fig. 1A). PEF cells were transfected with recombinant interference plasmids expressing a green fluorescent protein marker, and expression of green fluorescent protein was used to identify successful transfectants (Fig. 1B).

Selection of recombinant interference plasmids. Recombinant interference plasmids were digested with enzymes and subjected to agarose gel electrophoresis. It was observed that a 320 bp band obtained from *Bam*HI and *Eco*RI digestion included the U6 promoter, while a 390 bp band obtained from *Hind*III and *Eco*RI digestion contained the U6 promoter and shRNA identified as previously described (15) (Fig. 2). Recombinant plasmids containing the U6 promoter and shRNA were selected as positive clones. Positive plasmids identified by double-enzyme digestion were also sent to the

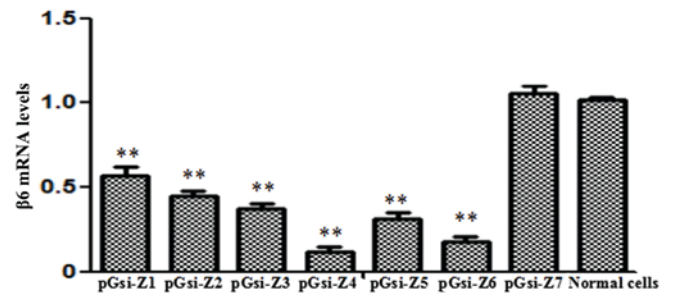


Figure 3. Analysis of integrin $\beta 6$ subunit gene expression. ** $P < 0.01$ vs. normal cells.

Beijing Genomics Institute for sequencing. The sequencing results were consistent with the experimental findings.

Fluorescence RT-qPCR detection of the FMDV integrin receptor $\beta 6$ subunit gene. In PEF cells, the pGsi-Z4 recombinant plasmid exhibited marked inhibitory effects on the expression of the $\beta 6$ subunit gene, whereby inhibition efficiency reached 91.7% ($P < 0.01$ vs. control). The interference effects of the other recombinant plasmids were not significant (Fig. 3).

Linearizing pXL-U6-Z4 and cell transfection. Bacterial supernatant with the U6-Z4 sequencing results was amplified as a pXL-U6-Z4 plasmid in the *E. coli* DH5 α strain for plasmid isolation. PXL-U6-Z4 was linearized using *Apa*LI enzyme digestion and plasmid fragments were detected by electrophoresis. The DNA sequence that was ~1,240 bp in size and contained the Amp resistance gene with a prokaryotic replication initiation site was removed. A linearized pXL-U6-Z4 sequence of ~6,580 bp was recovered (Fig. 4A) and transfected into PEF cells. Subsequent GFP expression was observed under an inverted fluorescence microscope and transfection efficiency was estimated to be ~90%. (Fig. 4B).

Validation of pXL-U6-Z4 interference effects. Fluorescence qPCR indicated that the interference effect of the integrated pXL-U6-Z4 plasmid increased after transfection in a time-dependent manner. The plasmid exhibited inhibitory effects ($P < 0.01$) on the $\beta 6$ subunit gene, with inhibition rates of

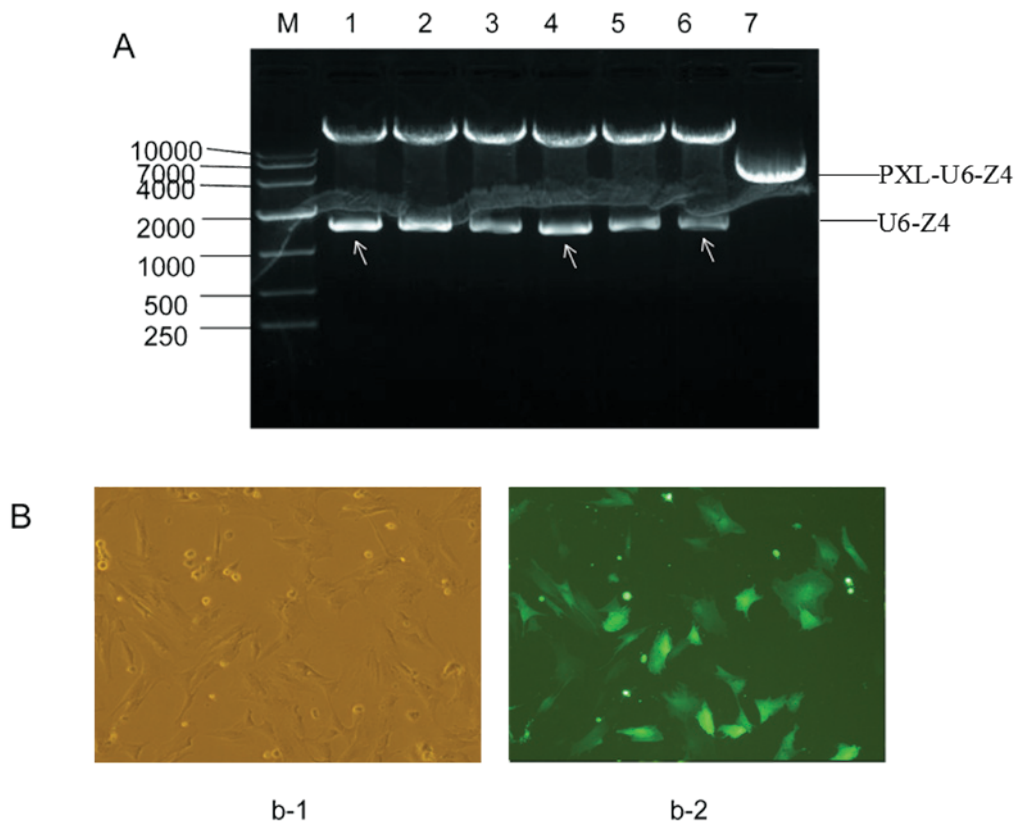


Figure 4. Enzyme digestion and transfection of pXL-U6-Z4 expression plasmid. (A) *Apa*LI enzyme digestion; lanes 1-6, target genes (U6-Z4, indicated by white arrows); lane 7, negative control (pXL-U6-Z4); lane M, Marker 10,000 DNA ladder. (B) Cells 48 h after pXL-U6-Z4 plasmid transfection (magnification, $\times 100$). (B1) Normal PEF cells. (B2) Expression of GFP (green) in PEF cells transfected for 48 h with pXL-U6-Z4 plasmid expressing a GFP marker. PEF, porcine embryonic fibroblast; GFP, green fluorescent protein.

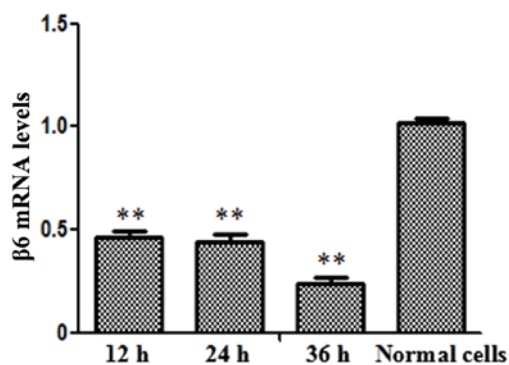


Figure 5. Analysis of $\beta 6$ mRNA expression by reverse transcription polymerase chain reaction in PEF cells. ** $P < 0.01$ vs. normal cells. PEF, porcine embryonic fibroblast.

56.5, 59.5 and 78.5% observed at 12, 24 and 36 h, respectively (Fig. 5).

Detection of integrated plasmid transfection and viral challenge. After dilution of an FMDV stock, a virus challenge assay was performed using 2.1×10^3 TCID₅₀ of virus solution/well. The following three groups were included in the assay: A virus challenge after pXL-U6-Z4-transfection group, a virus challenge without transfection group and a normal (untreated) cell group. At 12, 28, 24 and 36 h after the virus challenge, PEF cells were observed for CPE. Results were determined using a semi-quantitative method according to cell lesion conditions

Table III. CPE of foot-and-mouth disease virus infection in PEF cells.

PEF cell group	Time after transfection, h			
	12	18	24	36
Non-transfected cells	+	++	++++	+++++
Transfected cells	+	+	+++	+
Normal cells	-	-	-	-

CPEs within cells were observed under a microscope and measured using a semi-quantitative method. Proportions of cells exhibiting CPEs were scored as follows: -, no CPE; +, 20% CPE; ++, 40% CPE; +++, 60% CPE; ++++, 80% CPE; and +++++, 100% CPE. PEF, porcine embryonic fibroblasts; CPE, cytopathic effects.

(Table III). Normal cells exhibited regular long spindle morphologies, while virally challenged cells exhibited cellular lesions, a round and/or shrunken morphology and clumped structures (Fig. 6).

Detection of virus replication using RT-qPCR. In a fluorescence RT-qPCR assay (Fig. 7), the level of FMDV VP1 antigen, as an indicator of FMDV levels, measured in normal cells at 36 h post-viral challenge was defined as 1. The levels of FMDV measured in the transfection group was higher

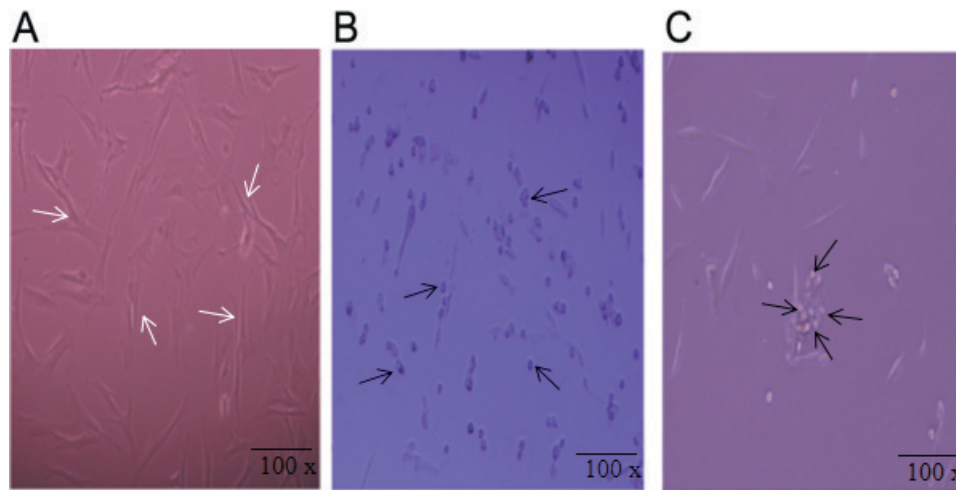


Figure 6. Cytopathic effects of FMDV inoculation in PEF cells transfected with pXL-U6-Z4 plasmid. (A) Normal PEF cells. (B) Untransfected PEF cells at 36 h after virus infection. (C) Transfected PEF cells at 36 h after virus infection. Normal cells exhibited regular long spindle morphologies, while virally challenged cells exhibited cellular lesions, a round and/or shrunken morphology and clumped structures. PEF, porcine embryonic fibroblast; FMDV, foot-and-mouth disease virus. White arrows indicate regular long spindle morphologies. Black arrows indicate cellular lesions.

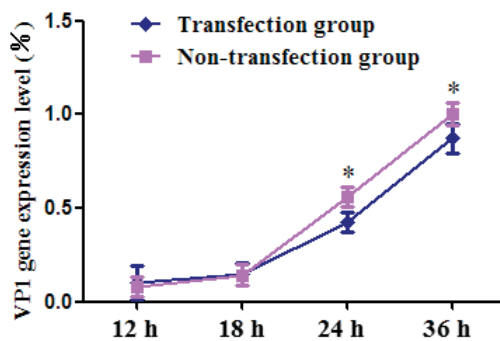


Figure 7. Levels of FMDV VP1 antigen expression. Reverse transcription-quantitative polymerase chain reaction was used to measure the levels of FMDV VP1 expression, as an indicator of FMDV levels, at different time points in cells transfected with or without pXL-U6-Z4 plasmid. * $P < 0.05$ vs. control, FMDV, foot-and-mouth disease virus; VP1, major capsid protein 1.

than that in the non-transfection group at 12 h after the viral challenge, and the non-transfection group had higher levels of virus at 18 h after viral challenge, though these differences were not significant. At 24 and 36 h post-viral challenge, the level of viral replication in the transfection group was lower than that in the non-transfection group ($P < 0.05$; Fig. 7). Viral replication was reduced by 24.2 and 12.8%, after 24 and 36 h, respectively. Collectively, these data indicate that transfection with pXL-U6-Z4 may have inhibited expression of the FMDV integrin receptor, thus reducing the ability of FMDV to invade and replicate within PEF cells. These results suggest that the pXL-U6-Z4 interference plasmid may inhibit intracellular FMDV replication.

Discussion

FMDV expresses five antigenic sites on the surface of its viral capsid. The main viral antigen, VP1, contains a G-H loop on its surface with a highly conserved arginine-glycine-aspartate (Arg-Gly-Asp, RGD) sequence, which promotes viral infection of target cells (4,16). A number of interactions between host

cell integrins, namely $\alpha v \beta 1$, $\alpha v \beta 3$, $\alpha v \beta$, $\alpha v \beta 6$, $\alpha v \beta 8$, $\alpha II \beta 3 b$, $\alpha 5 \beta 1$, $\alpha 1 \beta 8$, and the RGD sequences within the FMDV VP1 antigen have been proposed, though currently only integrins $\alpha v \beta 1$, $\alpha v \beta 3$, $\alpha v \beta 6$ and $\alpha v \beta$ have been confirmed as a receptor for FMDV (17). In particular, the integrins $\alpha v \beta$ and $\alpha v \beta 6$ may have a key role in viral infection (18).

Integrins are a family of cell surface receptor proteins that have important roles in cell development, immune responses, blood coagulation and inflammatory reactions (19). More than 20 combinations of integrin $\alpha \beta$ heterodimers are expressed on the cell-extracellular matrix boundary. These heterodimers participate in signal transduction between cells and also serve intermediary roles in the process of viral invasion of cells (20). The β subunit and a number of α subunits exhibit an α -I subunit structure domain. This is composed of five amino acids and adopts a structure that relies upon metal ion adsorption sites (also known as metal ion-dependent adhesion site, MIDAS) to generate a ligand binding site (21). A previous study of the porcine integrin αv subunit, as a receptor for FMDV, demonstrated that lentiviral RNAi technology inhibited target gene and corresponding protein expression of the α subunit. RT-qPCR analysis also indicated that this approach decreased viral replication by >3 -fold (22). Results in cattle have also suggested that $\alpha v \beta 6$, rather than $\alpha v \beta 3$, has a major role in determining the tropism of FMDV for the epithelia, as the primary organ targeted by the virus (23). Indeed, $\alpha v \beta 6$ is currently considered to function as a primary FMDV receptor that contributes to FMDV tropism and viral invasion (24). Furthermore, $\alpha v \beta 6$ exerts regulatory functions in the process of coagulation (25). Jacobsen *et al* (26) documented that $\beta 6$ -knockout mice exhibit significantly delayed wound healing at the early stage of disease when compared with wild-type diabetic mice. In addition, expression of $\alpha v \beta 6$ in keratinocytes promoted adhesion and migration in cultured cells (26). Sullivan *et al* (27) and Miller *et al* (28) knocked out regions of the $\beta 6$ subunit cytoplasmic domains containing the RGD site, and observed that modified $\alpha v \beta 6$ was unable to mediate FMDV infection. Therefore, the $\beta 6$ subunit was the

focus of the current study due to its potential involvement in FMDV infection. In the current study, the function of the $\beta 6$ subunit was confirmed by evaluating the effect of $\beta 6$ subunit knockdown on FMDV infection. It was observed that RNAi of the $\beta 6$ subunit influenced viral replication, thus indicating that the $\beta 6$ subunit may be required for viral infection.

RNAi is an effective method for inhibiting viral replication, and previous studies have investigated the effects of anti-viral RNAi methods on FMDV (29,30). The initial stage of host cell infection by FMDV involves interaction with a cognate cell-surface receptor, which subsequently enables virus particles to enter cells in a receptor-mediated manner. Thus, cell-surface receptors are key in determining viral host range and tissue tropism (31). The present study targeted the $\alpha\beta 6$ receptor, specifically by designing siRNA molecules and RNAi expression plasmids that targeted the cytoplasmic domain region of the $\beta 6$ subunit. The plasmids were introduced into PEF cells to inhibit the expression of the $\alpha\beta 6$ receptor, as a mediator of FMDV infection. Results of RT-qPCR demonstrated that the pGsi-Z4 recombinant plasmid had an inhibitory effect on the expression of the $\beta 6$ subunit within PEF cells, indicating that RNAi successfully inhibited receptor gene expression. Measurements in PEF cells transfected with the pXL-U6-Z4 integration plasmid indicated that an interference effect was present; however, the interference efficiency was lower than that of cells transfected with the initial pGsi-Z4 interference expression plasmid. This may have been due to random integration effects of the integration plasmid. For instance, expression of the interference fragment or the efficiency of integration into the plasmid target site may have been sub-optimal, thus reducing the interference effect. Following transfection of the pXL-U6-Z4 integration into cells, the interference fragment likely integrated into a random site in the genome. Further study is required to identify these random plasmid integration site(s) within the genome.

Viral challenge experiments were performed on PEF cells transfected with the pXL-U6-Z4 plasmid. Observations of cell morphology identified more marked cellular lesions in the non-transfection group than in the transfection group, indicating that transfection with the integration plasmid may have reduced viral replication. However, results of RT-qPCR at 12 h post-infection demonstrated that the levels of FMDV in the transfection group were higher than that in the non-transfection group. As transfection potentially reduces cell growth, the resistance of transfected cells may have been lower than that of non-transfected cells. Therefore, a number of transfected cells may have died earlier in the assay compared with non-transfected cells, resulting in relatively higher levels of viral replication in remaining transfectants. At 18 h after viral inoculation, levels of viral replication in both groups were similar. Despite a potential loss of damaged transfected cells, surviving transfectants retained the ability to reduce viral replication. This was indicated by lower levels of virus in the transfection group, relative to the non-transfection group, at 24 h after viral inoculation. At 36 h after viral inoculation, the level of FMDV in the transfection group remained lower than that in the non-transfection group.

Using the TCID₅₀ method to inoculate cells with FMDV, it was demonstrated that siRNA had inhibitory effects on the replication capacity of FMDV. This validated that RNAi of

FMDV integrin receptors, including the previously studied porcine integrin αv subunit receptor (8), may exert effects at the cellular level.

Using RNAi technology, the present study successfully inhibited expression of the integrin $\beta 6$ sub-domain I at the mRNA level. This potentially blocked cell invasion by FMDV and prevented viral replication and dissemination. Viral replication was reduced by 24.2 and 12.8%, after 24 and 36 h, respectively. Future studies are now warranted to determine whether RNAi of integrin α subunits, similar to RNAi of the $\beta 6$ subunit, has inhibitory effects on viral replication.

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