

RESEARCH

Open Access

Lentiviral-mediated RNAi to inhibit target gene expression of the porcine integrin αv subunit, the FMDV receptor, and against FMDV infection in PK-15 cells

Jihuai Luo, Junzheng Du, Shandian Gao, Guofeng Zhang, Jingjing Sun, Guozheng Cong, Junjun Shao, Tong Lin and Huiyun Chang*

Abstract

Background: shRNA targeting the integrin αv subunit, which is the foot-and-mouth disease virus (FMDV) receptor, plays a key role in virus attachment to susceptible cells. We constructed a RNAi lentiviral vector, αv pLenti6/BLOCK-iTTM, which expressed siRNA targeting the FMDV receptor, the porcine integrin αv subunit, on PK-15 cells. We also produced a lentiviral stock, established an αv -PK-15 cell line, evaluated the gene silencing efficiency of mRNA using real-time qRT-PCR, integrin αv expression by indirect immunofluorescence assay (IIF) and cell enzyme linked immunosorbent assays (cell ELISA), and investigated the *in vivo* inhibitory effect of shRNA on FMDV replication in PK-15 cells.

Results: Our results indicated successful establishment of the αv U6 RNAi entry vector and the αv pLenti6/BLOCK-iT expression vector. The functional titer of obtained virus was 1.0×10^6 TU/mL. To compare with the control and mock group, the αv -PK-15 group αv mRNA expression rate in group was reduced by 89.5%, whilst IIF and cell ELISA clearly indicated suppression in the experimental group. Thus, αv -PK-15 cells could reduce virus growth by more than three-fold and there was a > 99% reduction in virus titer when cells were challenged with 10^2 TCID₅₀ of FMDV.

Conclusions: αv -PK-15 cells were demonstrated as a cell model for anti-FMDV potency testing, and this study suggests that shRNA could be a viable therapeutic approach for controlling the severity of FMD infection and spread.

Background

Foot-and-mouth disease virus (FMDV) is a picornavirus, which causes a highly contagious disease in cloven-hoofed animals. Foot-and-mouth disease (FMD) is a threat to the worldwide livestock industry, because FMD can have a devastating effect on a country's economy. FMDV shows a high genetic and antigenic variability, which is reflected in the seven serotypes and numerous variants described to date [1]. The FMDV genome is composed of a positive-sense single-stranded RNA molecule of about 8 500 nucleotides, which contains a unique open reading frame. There are seven distinguishable serological types, *i.e.*, O, A, C, Asia1, SAT1, SAT2, and SAT3, and more than 65 subtypes.

Recently, RNAi has shown promise as a therapeutic in many infections, including, viral diseases of animals and humans. RNAi is a process of sequence specific, post-transcriptional gene silencing (PTGS) in animals and plants, which is conducted using 21 to 33 nucleotides (nt) of small interfering RNA (siRNA), which are homologous in sequence to the silenced gene [2]. In eukaryotic organisms, dsRNA is produced *in vivo* or introduced by pathogens and processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) using an enzyme called Dicer, which is a member of the RNase III family of double-stranded RNA-specific endonucleases [3,4]. Each siRNA is then incorporated into an RNA-induced silencing complex (RISC), which is an enzyme complex used to target cellular transcripts complementary to the siRNA for specific cleavage and degradation [5,6]. In addition to dsRNA, other endogenous RNA molecules have been shown to be capable of

* Correspondence: changhuiyun@126.com
State Key Laboratory of Veterinary Etiological Biology, National Foot and Mouth Disease Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China

triggering gene silencing, including, short temporal RNA (stRNA) and microRNA (miRNA) [7].

RNAi has been widely studied as an antiviral technology for combating FMDV [8,9]. FMDV is antigenically variable, undergoing rapid mutation, which allows it to easily escape the host immune system via the high variability of its surface antigens. Existing vaccines and antiviral drugs have limited effectiveness, so the development of new strategies is essential. High genetic variability is a major issue that must be addressed in order to establish RNAi as a viable approach against FMDV [10]. Current measures for the control of FMDV replication include, RNAi plasmid 1D or 3D [11], VP1 [9], IRES [12], all of which are focused on several regions of the FMDV genome [13]. RNAi directed at specific gene sequences of certain FMDV strains might be a risky strategy, especially in the event of an emergent FMD outbreak in the absence of information concerning the serotype or genotype of the isolated pathogen, although early protection is needed. The integrin αv subunit is the FMDV receptor for each serotype or genotype and the integrin αv subunit of the FMDV receptor plays a key role in the attachment of the virus to susceptible cells. Studies have shown a clear correlation between sensitivity of FMDV and expression of integrins $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, and $\alpha v\beta 8$. Most integrin subunits have independent functions and are essential for normal development [14,15]. $\alpha 1$ -null mice are viable, fertile and apparently normal, whereas $\alpha 7$ -null mice develop muscular dystrophy, and $\alpha 9$ -null mice die within 10 day of birth. Mutations in many of the ligands for αv integrins are also viable. Thus, although many of these ligands and αv integrins are widely expressed during development, they do not appear to be essential [16,17]. In this study, we used “knockdown” rather than “knockout” target gene expression of the porcine integrin αv subunit, the FMDV receptor, to establish a cell line that could inhibit FMDV replication. Therefore, our work on RNAi with FMDV is a step forward on all previous work, particularly with respect to the cell model system.

In this study, we constructed an RNAi lentiviral vector that inhibited target gene expression of the porcine integrin αv subunit and the FMDV receptor in PK-15, and FMDV replication in αv -PK-15. The vector was capable of inhibiting viral replication in cultured porcine PK-15 cells.

Methods

Cells and viruses

293T cells and PK-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO™, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (pH 7.4). Cultures were incubated at 37°C with 5% CO₂.

FMDV O/CHA/99 [GenBank accession number AF506822] was used for viral challenge.

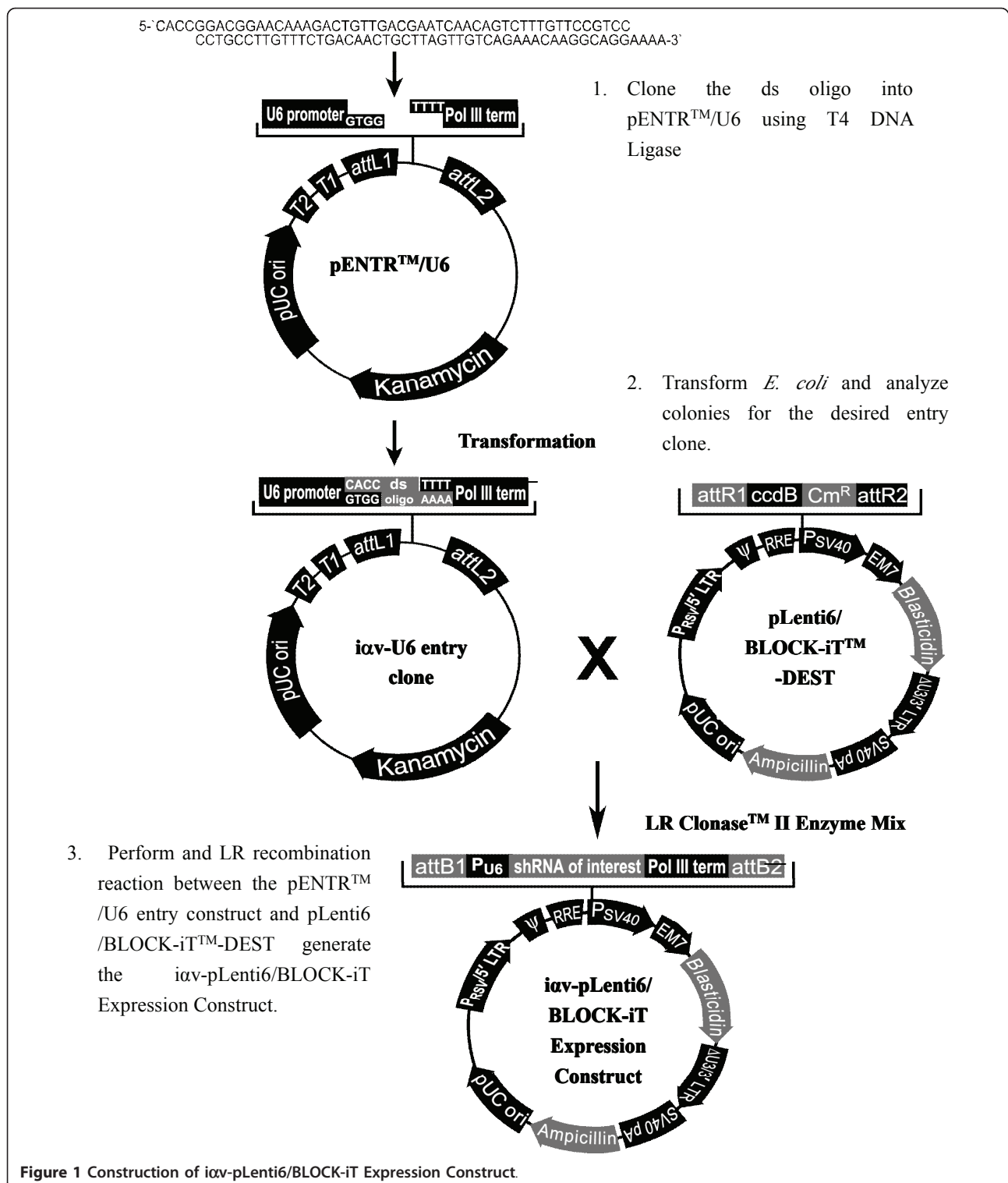
Construction of Lentivirus vector and establishment of the αv -PK-15 cell line

Double-stranded oligo [18] (ds oligo)

5'–CACCGGACGGAACAAAGACTGTTGACGAAT
CAACAGTCTTTGTTCCGTC

CCTGCCTTGTCTGACAACCTGCTTAGTTGTCA-
GAAACAAGGCAGGAAAA–3'

was chemically designed and synthesized by the Invitrogen Corporation, and the ds oligo was cloned into the linearized pENTR™/U6 vector. The pENTR™/U6 entry vector provides a rapid and efficient way to clone oligo duplexes encoding a desired shRNA target sequence into a vector containing an RNA Pol III-dependent expression cassette for use in RNAi analysis. We generate an αv -pLenti6/BLOCK-iT™ expression clone construct using an LR recombination reaction between the αv -pENTR™/U6 entry clone and the pLenti6/BLOCK-iT™-DEST vector (Figure 1). We used the expression clone construct and the LR clonase enzyme mix to produce a lentiviral construct. We co-transfected the 293FT producer cell line with three optimized packaging plasmids (pLP1, pLP2, and pLP/VSVG) and the αv -pLenti6/BLOCK-iT™ expression clone construct, which resulted in production of lentiviral stocks with a suitable titer. Stably transduced PK-15 cells were selected using blasticidin, with the minimum concentration of blasticidin required to kill untransduced PK-15 cells. The following procedure was used to determine the titer of the lentiviral stock using the PK-15 cells. To set up the stable cell line selection, we first needed to produce a lentiviral stock (αv -PK-15 containing the packaged αv pLenti6/BLOCK-iT™-DEST expression construct and a control containing the packaged pLenti6/BLOCK-iT™-DEST expression construct) by co-transfecting the optimized ViraPower™ packaging mix or pLenti6/BLOCK-iT™-DEST expression construct into the 293FT producer cell line. PK-15 cell cultures were incubated at 37°C with 5% CO₂. The day before transduction, cells were trypsinized, diluted with DMEM fresh medium, and seeded into 6-well culture plates. On the day of transduction (day one), when PK-15 cells were about 80% confluent, we thawed the lentiviral stock and diluted the appropriate amount of virus (MOI = 5) into fresh DMEM complete medium. The culture medium was removed from the cells. The medium containing virus was gently mixed by pipetting and added to the cells. Polybrene was added to a final concentration of 6 µg/mL. Cells were incubated for approximately six hours prior to changing the medium. The following day (day two), the medium containing virus was removed and replaced with fresh, complete culture medium. The following day (day three),



the medium was again removed and replaced with fresh, complete medium containing the appropriate amount of 2 µg/mL blasticidin to select for stably transduced cells. Selection took up to two weeks. At some point there was a massive cell death and most of the cells were washed from

the bottom of the dish, leaving colonies of stable cells behind. Blasticidin-resistant colonies were picked using a Gilson pipette with a sterile tip, by lowering the tip to the surface of the colony of interest then scraping and sucking gently until they were pulled into the tip. Colonies were

transferred to a well in a 24-well plate and the process was repeated with other colonies. When wells were confluent, they were split into one well of a 6-well plate (passage number 20) with a lower amount of blasticidin for maintenance (1 µg/mL). Cells were then assayed for knockdown of the target gene in one of the clones.

Assay for knockdown of the αv -PK-15 cells

RNA isolation and real-time qRT-PCR [19]

Targeted gene expression was detected by establishing αv -PK-15 cell lines at passage number 20, which were cultured in DMEM supplemented with 10% heat inactivated FBS, and total RNA was extracted from αv -PK-15 cell cultures using an RNeasy Mini Kit (Invitrogen), before real-time qRT-PCR analysis using the SYBR RT-PCR Kit (TaKaRa, Japan). Specific amplification was confirmed by melting curve analysis of the real-time qRT-PCR products, performed according to the manufacturer's protocol. Fluorescence was measured after each cycle and displayed graphically with a Stratagene Real time PCR Mx3000p (USA). Beta actin was used as a positive control and the αv cDNA was amplified from cDNA preparations of αv -PK-15 cells by PCR using the primers shown in Table 1. The PCR procedures followed the manufacturer's instruction, with the following thermal profile: 10 s at 95°C for pre-denaturation; 40 cycles of 5 s at 95°C; and 20 s at different annealing temperatures, depending on the integrins that were amplified. The specific amplification was confirmed by melting curve analysis of the real-time qRT-PCR products according to the manufacturer's protocol, with the following thermal profile: 1 cycle of 95°C for 60 s; 55°C for 30 s; and 95°C for 30 s. PCR products were run on 3% agarose gel containing ethidium bromide and DNA bands were visualized using a UV transilluminator.

IIF

Indirect immunofluorescence (IIF) assay was carried out to detect expression of the targeted αv protein on the cell surface of an established αv -PK-15 cell line at passage number 20, which was cultured in DMEM supplemented with 10% heat inactivated FBS. Cells were grown on acid-washed cover slips to ensure adherence of cells. The culture media was carefully aspirated from the culture dish using a pipette, because vacuum aspiration

might also suck up cells from the cover slip surface. Cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature and were permeabilized for 5 min with 0.1% triton X-100 in 1× PBS at room temperature. Cells were then incubated in freshly prepared 50 mM NH₄Cl for 15 min to quench free aldehyde groups from PFA, before fixation after washing three times with 1× PBS. Cultures were blocked with 1% BSA in 1× PBS for 30 minutes at room temperature, before incubation with primary antibody diluted in blocking buffer (1% BSA in PBS) for 1 h at room temperature. After primary incubation, cover slips were returned to the dishes and washed in PBS five to six times, for 5 min each wash. Secondary incubation was for 1 h at room temperature. The diluted antibody was centrifuged before use to pellet aggregates and covered in foil to protect from light. A control was included, which was comprised of the secondary antibody alone. The cover slips were returned to the dish and washed five times, for 5 min each wash using PBS, ensuring that samples were protected from the light. Cover slips were mounted by dipping the cover slips in distilled water to remove salt from PBS, before blotting the edge using filter paper to dry off excess water, before placing the cover slip cell-side down on a drop of mounting medium on a glass slide. The mounting medium used was DAPI (USA). The cover slip was stabilized on the slide using nail lacquer and left to dry overnight before viewing.

Indirect cell ELISA for adherent Cells

We added 100 µL of the cell suspension (1.0×10^5 cells in each well of a 96-well culture plate) and incubated in 5% CO₂ at 37°C, for at least 24 h allowing cells to attach. We removed the residual culture medium by inversion and by gently tapping the inverted plate on a paper towel. We dispensed the primary antibody (50 µL), which was diluted in ice-cold ELISA buffer at the optimal concentration, whilst holding the pipette tip against the walls of the wells, then incubated for 1 h at 4°C. The unbound primary antibody was removed by vacuum aspiration, whilst taking care to avoid drying. The plate was washed by gently adding 200 µL of ice-cold washing buffer, whilst holding the pipette tip against the wall of the well to prevent detachment and loss of cells. The washing step was repeated four more times. After the last washing, the washing buffer was removed by inversion, whilst gently tapping the inverted plate on a paper towel. We then added the HRP-conjugated to the specific antibody for the primary antibody (50 µL) diluted in ice-cold ELISA buffer at the optimal concentration before incubating the plate for 1 h at 4°C. After five washings with PBS containing 1% BSA, 100 µL of tetramethylbenzidine (TMB) substrate was added to each well and incubation was continued for an additional 10 min at room temperature. We stopped the enzyme reaction by adding 50 µL of 1.25 M H₂SO₄ and the optical

Table 1 Primer sequences used in this study

Primer sequence	Subunit	Size	Purpose
AF 5'-GATTGTTGTTACTGGCTGTTTTGG-3'	αv	94 bp	Real-time PCR
AR 5'-TGTTCCCTTCCTGTTCTTCTTG-3'			
ACTF 5'-GGACTTCGAGCAGGAGATGG-3'	B-actin	138 bp	Real-time PCR
ACTR 5'-AGGAAGGAGGGCTGGAAGAG-3'			

density (OD) was measured with an ELISA reader (iEMS-Reader; LabSystems, Helsinki, Finland) at $A_{450 \text{ nm}}$.

Viral challenge assay for the αv -PK-15 cells

Virus infectivity was determined by serial dilution of cell samples grown in 96-well plates. The virus titer was calculated as the 50% tissue culture infective dose (TCID₅₀) using the Reed-Muench formula [20]. A viral suspension titrated at $10^{4.41}$ TCID₅₀ was used in the experiment. Monolayers (about 50% confluent) were grown in 96-well plates to assess the capacity of viruses to grow in PK-15 cells expressing siRNAs. Cells in one well of the 96-well plates were infected with 100 TCID₅₀ of virus per 0.1 ml. After 1 h of adsorption, the inoculum was removed and cells were washed twice with DMEM. The infection then proceeded in DMEM supplemented with no-fetal bovine serum and the virus titers were determined after two days. Statistical analysis was performed with the Microsoft Excel program.

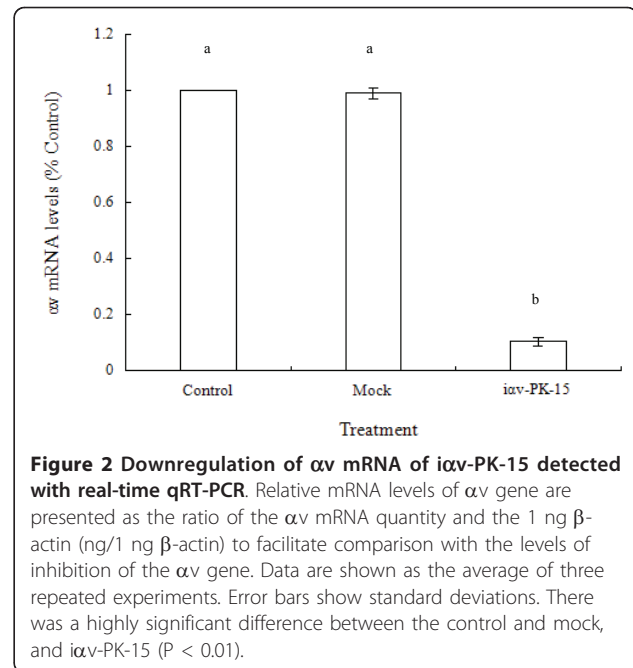
Results

Target gene expression of the porcine integrin αv subunit (the FMDV receptor) constructed with an RNAi lentivirus vector and established as an αv -PK-15 cell line

We constructed the αv -pENTRTM/U6 entry clone and αv -pLenti6/BLOCK-iTTM expression clone by producing a lentiviral stock (containing the packaged αv -pLenti6/BLOCK-iTTM-DEST expression construct, or the packaged pLenti6/BLOCK-iTTM-DEST expression construct) and co-transfecting the optimized ViraPowerTM Packaging Mix (3.0 $\mu\text{g}/\mu\text{l}$) and αv -pLenti6/BLOCK-iTTM-DEST expression construct or pLenti6/BLOCK-iTTM-DEST expression construct (3 μg) into the 293FT producer cell line, then harvested the viral supernatant, and determined the lentiviral stock titer with blasticidin. We picked blasticidin-resistant clones and cultured then in DMEM supplemented with 10% heat-inactivated FBS at passage number 20. The sequencing results demonstrated successful cloning of the αv -pENTRTM/U6 entry clone and the αv -pLenti6/BLOCK-iTTM expression clone construct. The functional titer of the virus obtained was 1.0×10^6 TU/mL. We established stable αv -PK-15 cells line at passage number 20, which inhibited target gene expression in the porcine integrin αv subunit, the FMDV receptor.

Real-time qRT-PCR assay of inhibition of RNA replication

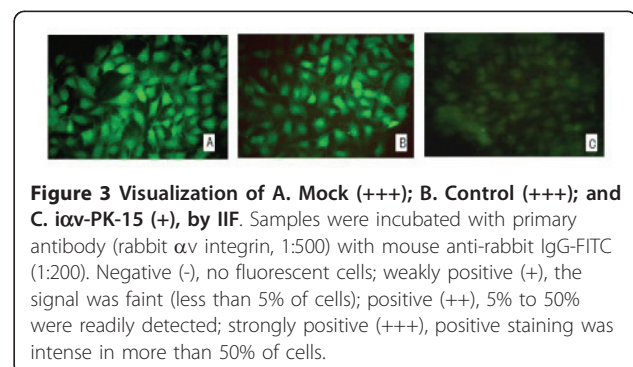
To demonstrate the level of inhibition, αv -PK-15 cells were collected at passage number 20 and real-time qRT-PCR analysis was performed (Figure 2). The level of the αv gene was determined by real-time qRT-PCR and significantly decreased by about 89.5% in αv -PK-15 cells. Our results indicate that αv -PK-15 cells can effectively and specifically target gene expression of the porcine integrin αv subunit, the FMDV receptor.

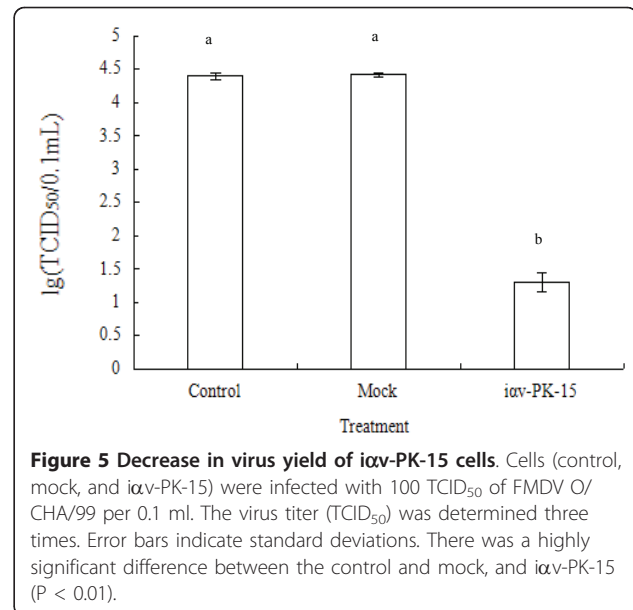
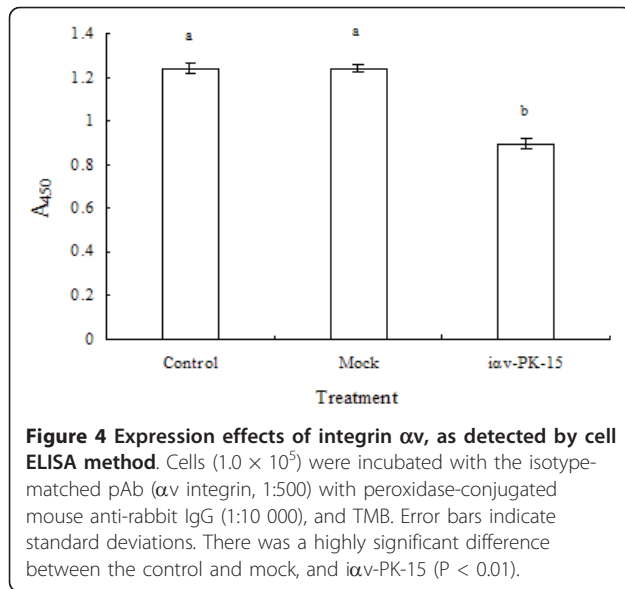


Level of inhibition of integrin αv subunit expression by IIF and indirect cell ELISA for adherent cells

Indirect immunofluorescence was used to visualize the cells under a fluorescence microscope. Control and mock exhibited a lot of green fluorescence, whereas significant reduction was observed in the green fluorescent signal of αv -PK-15, relative to the control and mock, indicating that αv -PK-15 cells had inhibited expression of the surface protein (Figure 3).

Cell ELISA was a useful technique for the quantitative analysis of cell surface antigen expression and was a sensitive, simple and reproducible method for detecting the cell membrane antigen processing machinery components of cells. Inhibition to target the gene expression of porcine integrin αv subunit, the FMDV receptor, was performed to demonstrate that the reactivity of pAb located in the membrane of cells was significantly inhibited (Figure 4).





Inhibition of FMDV O/CHA/99 replication in iav-PK-15 cells

The antiviral activity was tested using cell monolayers in 96-well plates, where 100 μ L of the cell suspension (1.0×10^5 cells in each well of a 96-well culture plate) was incubated in 5% CO₂, at 37°C, allowing at least 24 h for cells to attach. Cells were infected with 100 μ L of 100 TCID₅₀ FMDV O/CHA/99 and observed microscopically after 48 h. Viral infection caused a marked cytopathic effect (CPE) culminating in total cellular detachment, rounding up, and destruction, which was observed by microscopy [21]. The virus in the supernatant was titrated for infectivity using iav-PK-15 cells. After 48 h, viral titers decreased from $10^{4.39}$, $10^{4.41}$ in control and mock to $10^{1.29}$ TCID₅₀ in iav-PK-15 cells. SiRNAs inhibited virus yield by greater than 1000-fold and there was a > 99% reduction in virus titer (Figure 5).

Discussion

FMDV is the prototypic member of the *Aphthovirus* genus of the *Picornaviridae* family. The viral capsid is made up from 60 copies each of four viral encoded proteins, VP1 to VP4: VP1, VP2 and VP3 form the outer clapsed shell; and VP4 makes up the interior surface [22]. FMDV enters the cell by receptor-mediated endocytosis, in a process that begins with the initial attachment of the virus to cell-surface receptors. The G-H loop forms a major antigenic site on the virion, which includes at its apex an Arg-Gly-Asp (RGD) motif [23,24], a sequence that has been implicated in receptor binding on the basis of synthetic peptide inhibition studies [25]. FMDV can enter cells using different pathways; the main pathway is by binding integrins via a conserved RGD located within the GH loop of VP1 [26,27]. One subgroup within the integrin family is the αv integrin, which recognizes RGD

as a binding motif on natural ligands. The αv subunit is subdivided into two groups based on certain structural differences [28]. The first group is formed by α -1, α -2, α -L, α -M, and α -X. The second group is formed by α -3, α -5, α -6, α -7, α -8, α -IIB, α -V and α ^{IEL}. 24 functional integrins have been identified in mammalian species, which result from different pairings among 18 α subunits and 8 β subunits [29]. Different integrins have roles as FMDV receptors ($\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, $\alpha v\beta 8$) [30]. Members of this group all undergo post-translational cleavage of their precursor into a heavy and a light chain. The light chain is composed of the cytoplasmic domain, the transmembrane region, and a part of the extracellular domain (about 25 kDa), whilst the heavy chain contained the rest of the extracellular domain (about 120 kDa) [31].

RNAi is a powerful gene silencing mechanism operating in most eukaryotic cells [32]. The effector molecules comprise short duplex RNA sequences of 21-23 bp that direct inhibition of homologous genes. This natural pathway is important for processing of regulatory micro RNAs (miRs) [33]. FMDV is antigenically highly variable and consists of seven serotypes and multiple subtypes [34] that underlie the rapid spreading nature of the clinical disease as early as 2-3 days post-exposure. This means that there is a need to develop new tools that provide early protection, thereby containing the disease spread. RNAi is used as an antiviral technology and has been widely studied for its affect on FMDV [9,35,36]. A multiple-siRNA expression system was developed that focused on several regions of the viral genome [37]. Focusing on the conserved regions of the viral genome [38,39], or simultaneously targeting several viral sequences are different approaches to resolving this issue. The multiple-siRNA-expressing plasmid, the pCWN11

system, is a feasible strategy to overcome the issue of high genetic variability in FMDV and viral escape [40,41]. This approach may be used to deal with several viruses, which would be especially useful in the treatment of coinfections by multiple pathogens [8]. Current measures for the control of FMD outbreak include routine vaccination, control of animal movement, and slaughter. FMD vaccines based on inactivated virus and adjuvants are effective in eliminating the disease, but risk the escape of live viruses from animal facilities or as a consequence of improper vaccine preparations [33,42]. The development of a recombinant peptide vaccine [43] and a synthetic peptide vaccine [44] both of which are safe and effective, has been reported. The limited effectiveness of existing vaccines and antiviral drugs means the development of new strategies is essential. The RNAi approach has been reported as an ideal tool to inhibit infectious virus replication in host cells, because siRNA can target and silence important viral genes. However, RNA viruses are likely to evolve mechanisms to suppress or escape an RNAi response. Long-term silencing of viral protein expression by siRNAs has been reported to result in the emergence of viruses resistant to RNA interference [40,45]. In particular, integrin αv -heterodimers have been previously shown to mediate cellular entry of field strains of the virus. In contrast, cell surface expression of $\alpha\beta 3$ or mRNA for the $\alpha\beta 1$, $\alpha\beta 3$ or $\alpha\beta 5$ subunits did not appear to contribute to sensitivity of cells to FMDV [46]. We constructed an RNAi lentiviral vector to inhibit target gene expression of the porcine integrin αv subunit, the FMDV receptor.

It was previously shown that siRNAs inhibited virus yield by 10-fold to 1000-fold. The level of viral replication (IND 63/72) inhibition varied depending on the siRNAs produced by the Cocktail Kit. Here, the maximum inhibition (120-fold reduction in virus titer) was observed with the 2A-2C product compared with both VP3-VP1 (100-fold reduction in virus titer) and 3D-3' UTR (40-fold reduction in virus titer) at 24 h. In this case, there was a > 99% reduction in virus titer with all the serotypes up to 24 h [47]. pCWN11 was a plasmid constructed to express siRNAs with multiple-targeting of the VP1 genes in FMDV. To investigate the effect of pCWN11 on FMDV replication, approximately 10^3 TCID₅₀ of HKN/2002 virus progeny was detected in the supernatant and collected from cells transfected with pCWN11 at 19 h p.i. Over 10^5 TCID₅₀ of virus was determined in the supernatant collected from control cells [8]. However, a 1000-fold reduction was found in virus titer 24 h post-infection of BHK cells expressing small hairpin RNA (shRNA) under the control of a mouse U6 promoter from a plasmid construct [35]. We observed a significant cross-inhibition of FMDV replication in BHK-21 cells by siRNAs targeted at various conserved regions of the viral genome (5VNCR, VP4, VPg, POL, and 3VNCR). The results

showed that siRNAs generated *in vitro* by human recombinant dicer enzyme resulted in a viral yield inhibition of 10-fold to 1000-fold in both homologous (HKN/2002) and heterologous (CHA/99) isolates of FMDV serotype O at 48 h post-infection [13]. A sequence corresponding to the critical 30 bp region that forms a stem-loop structure in IRES was also selected for investigation. The regions selected for shRNA were designated as D1, D2, D3, and D4. The virus in the supernatant was titrated in BHK-21 cells to determine infectivity. At 12, 18, and 24 h, the D4-transfected cells yielded a viral titer (10^{-5} TCID₅₀) that was almost equal to the control. The virus titer in the D2- and D3-transfected cells was less than 10^{-2} TCID₅₀ at 18 and 24 h. D1-transfected cells yielded $10^{1.4}$, $10^{1.7}$, and $10^{2.9}$ TCID₅₀ at 12, 18, and 24 h, respectively [48]. Kahana et al. demonstrated 100% inhibition of virus growth in BHK-21 cells transfected with a mixture of several anti-FMDV siRNAs [49]. In this study, there was reduction in virus titer of greater than 1000-fold in αv -PK-15 cell for O/CHA/99. The results indicate that the reduction in virus titer was either better than the previous study or showed the same effect.

In this study, we constructed an RNAi lentiviral vector containing the packaged αv -pLenti6/BLOCK-iTTM-DEST expression construct to inhibit target gene expression of the porcine integrin αv subunit, the FMDV receptor. We co-transfected the ViraPowerTM Packaging Mix and the αv pLenti6/BLOCK-iTTM-DEST expression construct containing the U6 RNAi cassette into 293FT cells to produce a replication-incompetent lentivirus, which was transduced into the mammalian cell line of interest. Once the lentivirus enters the target cell the viral RNA is reverse-transcribed, actively imported into the nucleus, and stably integrated into the host genome. The lentiviral construct contains a deletion in the 3' LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. Once the lentiviral construct has integrated into the genome, the lentivirus can no longer produce packageable virus and the shRNA of interest is constitutively expressed allowing the performance of transient RNAi analysis, or blasticidin selection to generate a stable cell line for long-term knockdown studies. To determine whether this system can be used to inhibit gene expression of the integrin αv subunit gene, the expression construct and packing plasmid were transduced into PK-15 cells, and we then established an αv -PK-15 cell line. We found that co-transduction resulted in a significant reduction in the corresponding integrin αv subunit transcription by real-time qRT-PCR, and integrin αv subunit expression by cell ELISA and IIF. Cell ELISA is a useful technique for the quantitative analysis of cell surface antigen expression, which was developed on the basis of enzyme immunochemistry (EIH) and ELISA. Since its development, which was facilitated by the establishment of

monoclonal antibody technology, a wide range of cell types and surface molecules have been analyzed by cell ELISA, and other methods for the quantitative detection of cell surface molecules. Cell ELISA is not appropriate for analyzing mixed-cell populations. In cell ELISA, labeling of immunoreactant molecules is achieved using an enzyme, such as horseradish peroxidase (HRP). IIF is a rapid sensitive method, which uses cell culture for the detection of integrin αv subunit expression in cell surface. At present, there is no information available; further work is needed to test the relationship between anti-FMDV efficiency and integrin $\beta 1$, $\beta 3$, $\beta 6$ and $\beta 8$.

Conclusion

Our results suggest that the interference integrin αv subunit expressing system is a feasible strategy for overcoming the issue of high genetic variability of FMDV, and viral escape. This approach may be developed to combat several viruses, which would be especially useful in the treatment of co-infections by FMDV. In addition, the new strategy would be beneficial in combating viruses with a high viral mutation rate. Our study provides a foundation for the cultivation of new varieties of transgenic organisms. However, this work represents a significant advancement, describing a new approach to trigger anti-FMDV pathways through inhibiting the FMDV receptor gene expression of the porcine integrin αv subunit.

Acknowledgements

This work was supported by the National Major Special Project on New Varieties Cultivation for Transgenic Organisms (2009ZX08006-002B, 2009ZX08007-008B and 2008ZX08011-004).

Authors' contributions

JD, SG and JL participated in planning of the study. JL carried out the majority of the experiments and drafted the manuscript. HC conceived the study and helped to draft the manuscript. GZ and JS produced the construction vector. GC, JS and TL participated in cell and virus cultures. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 7 June 2011 Accepted: 7 September 2011

Published: 7 September 2011

References

1. Bachrach HL: Foot-and-mouth disease virus. *Annu Rev Microbiol* 1968, **22**:201-224.
2. Bass BL: Double stranded RNA as a template for gene silencing. *Cell* 2000, **101**:235-238.
3. Bernstein E, Caudy AA, Hammond SM, Hannon GJ: Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference. *Nature* 2001, **409**:363-366.
4. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH: Dicer Functions in RNA Interference and in Synthesis of Small RNA Involved in Developmental Timing in *C. elegans*. *Genes Dev* 2001, **15**:2654-2659.
5. Hammond SM, Bernstein E, Beach D, Hannon GJ: An RNA-Directed Nuclease Mediates Genetic Interference in *Caenorhabditis elegans*. *Nature* 2001, **404**:293-296.
6. Nykanen A, Haley B, Zamore PD: ATP Requirements and Small Interfering RNA Structure in the RNA Interference Pathway. *Cell* 2001, **107**:309-321.
7. Ambros V, MicroRNAs: Tiny Regulators with Great Potential. *Cell* 2001, **107**:823-826.
8. Wei Cong, Shaoqiang Cui, Jiulian Chen, Xiaoping Zuo, Yonggan Lu, Weiyao Yan, Zhaoxin Zheng: Construction of a multiple targeting RNAi plasmid that inhibits target gene expression and FMDV replication in BHK-21 cells and suckling mice. *Vet Res Commun* 2010, **34**:335-346.
9. Ke Lv, Yingjun Guo, Yiliang Zhang, Kaiyu Wang, Ka Li, Yan Zhu, Shuhan Sun: Transient inhibition of foot-and-mouth disease virus replication by siRNAs silencing VP1 protein coding region. *Research in Veterinary Science* 2009, **86**:443-452.
10. Grubman M, Santos T: Rapid control of foot-and-mouth disease outbreaks: is RNAi a possible solution? *TRENDS in Immunology* 2005, **26**:65-68.
11. Weizao Chen, Mingqiu Liu, Ye Jiao, Weiyao Yan, Xuefeng Wei, Jiulian Chen, Liang Fei, Yang Liu, Xiaoping Zuo, Fugui Yang, Yonggan Lu, Zhaoxin Zheng: Adenovirus-Mediated RNA Interference against Foot-and-Mouth Disease Virus Infection both In Vitro and In Vivo. *JOURNAL OF VIROLOGY* 2006, **3559**-3566.
12. Francisco Martínez-Azorín, Miguel Remacha, Encarnación Martínez-Salas, Juan PGBallesta: Internal translation initiation on the foot-and-mouth disease virus IRE is affected by ribosomal stalk conformation. *FEBS Letters* 2008, **582**:3029-3032.
13. Mingqiu Liua, Weizao Chena, Zheng Ni, Weiyao Yana, Liang Fei, Ye Jiaoa Jun Zhang, Qingyun Du, Xuefeng Wei, Jiulian Chen, Yumei Liuc, Zhaoxin Zhenga: Cross-inhibition to heterologous foot-and-mouth disease virus infection induced by RNA interference targeting the conserved regions of viral genome. *Virology* 2005, **336**:51-59.
14. Richard O, Hynes : Targeted Mutations in Cell Adhesion Genes: What Have We Learned from Them? *DEVELOPMENTAL BIOLOGY* 1996, **180**:402-412.
15. Gardner H, Kreidberg J, Koteliansky V, Jaenisch R: Deletion of integrin $\alpha 1$ by homologous recombination permits normal murine development but gives rise to a specific deficit in cell adhesion. *Dev Biol* 1996, **175**:301-313.
16. Hirsch E, Gullberg D, Balzac F, Altruda F, Silengo L, Tarone G: αv Integrin subunit is predominantly located in nervous tissue and skeletal muscle during mouse development. *Dev Dyn* 1994, **201**:108-120.
17. Yamada S, Brown KE, Yamada KM: Differential mRNA regulation of integrin subunits αv , $\beta 1$, $\beta 3$, and $\beta 5$ during mouse embryonic organogenesis. *Cell Adhesion Commun* 1995, **3**:311-325.
18. Ji-huai LUO, Jun-zheng DU, SHan-dian GAO, Guo-feng ZHANG, Hui-yun CHANG: The optimum siRNA screening of targeting porcine integrin αv subunit gene as FMDV receptor against FMDV replication. *Acta Veterinaria et Zootechnica Sinica* 2011.
19. Junzheng Du, Huiyun Chang, Shandian Gao, Shuang Xue, Guozheng Cong, Junjun Shao, Tong Lin, Zaixin Liu, Xiangtao Liu, Xuepeng Cai: Molecular characterization and expression analysis of porcine integrins $\alpha v \beta 3$, $\alpha v \beta 6$ and $\alpha v \beta 8$ that are potentially involved in FMDV infection. *Molecular and Cellular Probes* 2010, **24**:256-265.
20. Reed LJ, Muench HA: A simple method of estimating fifty percent end points. *Am J Hyg* 1938, **27**:493-497.
21. Dumbell KR, Jarrett JO, Mautner V, Minson AC, Pereira HG, Pereira MS, Porterfield JS, Wilkinson PJ: Picornaviridae in Porterfield JS (ed) *Andrewes' viruses of vertebrates*. Cambridge University Press, Cambridge, United Kingdom, 5 1989, 120-145.
22. Manson PW, Grubman MJ, Baxt B: Molecular basis of pathogenesis of FMDV. *Virus Res* 2003, **91**:9-32.
23. Burman A, Clark S, Abrescia NG, Fry EE, Stuart DI, Jackson T: Specificity of the VP1 GH loop of foot-and-mouth disease virus for alpha-v integrins. *J Virol* 2006, **80**:9798-9810.
24. Fry EE, Stuart DI, Rowlands DJ: The structure of foot-and-mouth disease virus. *Curr Top Microbiol Immunol* 2005, **288**:71-101.
25. Alcalá P, Feliu JX, Arís A, Villaverde A: Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins. *Biochem Biophys Res Commun* 2001, **285**:201-206.
26. Dicara D, Burman A, Clark S, Berryman S, Howard MJ, Hart IR, Marshall JF, Jackson T: Foot-and-mouth disease virus forms a highly stable, EDTA-resistant complex with its principal receptor, integrin $\alpha v \beta 6$: implications for infectiousness. *J Virol* 2008, **82**(3):1537-1546.

27. Jackson T, King AMQ, Stuart DI, Fry E: **Structure and receptor binding.** *Virus Res* 2003, **91**:33-46.
28. Ruiz-Sáenz J, Goez Y, Tabares W, López-Herrera A: **Cellular Receptors for Foot and Mouth Disease Virus.** *Intervirology* 2009, **52**:201-212.
29. van der Flier Sonnenberg: **A Function and interactions of integrins.** *Cell and Tissue Research* 2001, **305**:285-298.
30. Takada Y, Ye X, Simon S: **The integrins.** *Genome Biol* 2007, **8**:215.
31. Humpries MJ: **Integrin structure.** *Biochem Soc Trans* 2000, **28**:311-339.
32. Aagaard L, Rossi JJ: **RNAi therapeutics: principles, prospects and challenges.** *Adv Drug Deliv Rev* 2007, **59**:75-86.
33. Vreeswijk J: **Developments in foot-and-mouth disease vaccines.** *Vaccine* 1991, **9**:75-88.
34. Grubman MJ, Baxt B: **Foot and mouth disease.** *Clinical Microbiology Reviews* 2004, **17**:465-493.
35. Chen W, Yan W, Du Q, Fei L, Liu M, Ni Z, Sheng Z, Zheng Z: **RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice.** *J Virol* 2004, **78**:6900-6907.
36. Mohapatra JK, Sanyal A, Hemadri D, Tosh C, Kumar RM, Bandyopadhyay SK: **Evaluation of in vitro inhibitory potential of small interfering RNAs directed against various regions of foot-and-mouth disease virus genome.** *Biochem Biophys Res Commun* 2005, **329**:1133-1138.
37. Nishitsujii H, Kohara M, Kannagi M, Masuda T: **Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration.** *J Virol* 2006, **80**:7658-7666.
38. Chang LJ, Liu X, He J: **Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1.** *Gene Ther* 2005, **12**:1133-1144.
39. Dave RS, Pomerantz RJ: **Antiviral effects of Human Immunodeficiency Virus type 1-specific small interfering RNAs against targets conserved in select neurotropic viral strains.** *J Virol* 2004, **78**:13687-13696.
40. Gitlin L, Stone JK, Andino R: **Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches.** *J Virol* 2005, **79**:1027-1035.
41. Geisbert TW, Hensley LE, Kagan E, Yu EZ, Geisbert JB, Daddario-DiCaprio K, Fritz EA, Jahrling PB, McClintock K, Phelps JR, Lee ACH, Judge A, Jeffs LB, MacLachlan I: **Postexposure protection of guinea pigs against a lethal Ebola virus challenge is conferred by RNA interference.** *J Infect Dis* 2006, **193**:1650-1657.
42. King AM, Underwood BO, McCahon D, Newman JW, Brown F: **Biochemical identification of viruses causing the outbreaks of foot-and-mouth disease in the UK.** *Nature* 1981, **293**:479-480.
43. Li G, Chen W, Yan W, Zhao K, Liu M, Zhang J, Fei L, Xu Q, Sheng Z, Lu Y, Zheng Z: **Comparison of immune responses against foot-and-mouth disease virus induced by fusion proteins using the swine IgG heavy chain constant region or β -galactosidase as a carrier of immunogenic epitopes.** *Virology* 2004, **328**:274-281.
44. Wang CY, Chang TY, Walfield AM, Ye J, Shen M, Chen SP, Li MC, Lin YL, Jong MH, Yang PC, Chyr N, Kramer E, Brown F: **Effective synthetic peptide vaccine for foot-and-mouth disease in swine.** *Vaccine* 2002, **20**:2603-2610.
45. Das AT, Brummelkamp TR, Westerhout EM, Vink M, Madiredjo M, Bernards R, Berkhout B: **Human Immunodeficiency Virus type 1 escapes from RNA interference-mediated inhibition.** *J Virol* 2004, **78**:2601-2605.
46. Donald PK, Alison B, Sarah G, Andrew ES, Terry J, Nigel P, Ferris : **Integrin sub-unit expression in cell cultures used for the diagnosis of foot-and-mouth disease.** *Veterinary Immunology and Immunopathology* 2011, **140**:259-265.
47. Jajati Keshari Mohapatra, Aniket Sanyal, Divakar Hemadri, Chakradhar Tosh, Manoj Kumar R, Santanu Kumar Bandyopadhyay: **Evaluation of in vitro inhibitory potential of small interfering RNAs directed against various regions of foot-and-mouth disease virus genome.** *Biochemical and Biophysical Research Communications* 2005, **329**:1133-1138.
48. Joyappa DH, Sarika Sasi, Ashok KC, Reddy GR, Suryanarayana WS: **The plasmid constructs producing shRNA corresponding to the conserved 3D polymerase of Foot and Mouth Disease virus protects guinea pigs against challenge virus.** *Vet Res Commun* 2009, **33**:263-271.
49. Kahana RL, Kuznetsova A, Rogel KC, Shemesh D, Hai H, Yadin YStram: **Inhibition of foot-and-mouth disease virus replication by small interfering RNA.** *J Gen Virol* 2004, **85**:3213-3217.

doi:10.1186/1743-422X-8-428

Cite this article as: Luo *et al.*: Lentiviral-mediated RNAi to inhibit target gene expression of the porcine integrin αv subunit, the FMDV receptor, and against FMDV infection in PK-15 cells. *Virology Journal* 2011 **8**:428.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

