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ORIGINAL ARTICLE

Inhibition of porcine endogenous retrovirus in PK15 cell line by efficient multitargeting RNA interference

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inhibition, multitargeting, PK15 cells, porcine endogenous retrovirus, RNA interference.

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Conflict of interest
The authors declare that there is no conflict of interest.

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Summary

To effectively suppress porcine endogenous retroviruses (PERV)s, RNAi technique was utilized. RNAi is the up-to-date skill for gene knockdown which simultaneously multitargets both gag and pol genes critical for replication of PERVs. Previously, two of the most effective siRNAs (gag2, pol2) were found to reduce the expression of PERVs. Concurrent treatment of these two siRNAs (gag2+pol2) showed knockdown efficiency of up to 88% compared to negative control. However, despite the high initial knockdown efficiency 48 h after transfection caused by siRNA, it may only be a transient effect of suppressing PERVs. The multitargeting vector was designed, containing both gag and pol genes and making use of POL II miR Expression Vector, which allowed for persistent and multiple targeting. This is the latest shRNA system technique expressing and targeting like miRNA. Through antibiotics resistance characteristics utilizing this vector, miRNA-transfected PK15 cells (gag2-pol2) were selected during 10 days. An 88.1% reduction in the level of mRNA expression was found. In addition, we performed RT-activity analysis and fluorescence in situ hybridization assay, and it demonstrated the highest knockdown efficiency in multitargeting (gag2+pol2) miRNA group. Therefore, according to the results above, gene knockdown system (siRNA and shRNA) through multitargeting strategy could effectively inhibit PERVs.

Introduction

Pig organs and tissues are well suited for transplantation of human [1,2] and are approximately the same size as human organs. However, all pigs always have the porcine endogenous retrovirus (PERV) genome inserted in the pig germ line and transmitted to offsprings. Until now, the fact that PERV-A and PERV-B could infect human cells *in vitro* has been revealed without direct evidence [3,4]. Furthermore, PERVs are known to have wide host range including mouse, chimpanzee, dog, cat, horse, mink, and cow as well as pig [5]. Recombination rate of PERV-A/C is much higher than PERV-A to infect HEK293 cells. It is very difficult to eliminate PERV from pigs [1,6]. Therefore, PERV is one of the major threats in xenotransplantation because of potential special risk. Recently, RNA interference technology was developed to knockdown gene expression, and it can be a good alternative to increase the safety of xenotransplantation [7–9]. In many RNAi technologies, small interfering RNAs (siRNA) could induce high knockdown efficiency. It prevents protein translation by disrupting the mRNA encoding the same sequence of RNAi [10]. Also, short hairpin RNAs (shRNA) could target multiple of specific genes [11]. This vector system could be transfected into primary pig's fibroblast and allow to produce PERV-controlled transgenic pigs [12,13] in which PERV

expression would be suppressed for a long time [14]. Therefore, shRNA vector-based system should be considered for long-term inhibition of PERV [11,14]. Through these technologies, PK15 cell lines derived from pigs that have PERV-A and PERV-B [6] will be available on the several inhibition studies through *in vitro* experiments for safety xenotransplantation [15].

Indeed, three functional genes of PERV encode capsid protein (*gag*), reverse transcriptase (*pol*), and envelope glycoprotein (*env*) which are different in the copy number of *gag, pol, and env* from the organs of pig [16,17]. The copy number of *pol* gene of PERV was higher than *gag, envA, envB, and envC* [18]. The pol-targeting RNAi could prevent infection through reduced reverse transcription and replication because of the most important role on PERV processing. Also, the mRNA level of gag, which encodes capsid protein essential for budding, was reduced by RNAi, blocking viral particles from shedding [3,19]. Therefore, if gag and pol genes of PERV can be suppressed simultaneously, it will be a great strategy for significant inhibition of PERV expression.

This could significantly inhibit PERV expression for the first time using RNAi strategy by multitargeting of PERV gag and pol genes in PK15 cells.

Material and methods

Cell culture and transfection

Transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect the siRNAs and miRNAs into PK15 cells (ATCC CCL-33) according to the manufacturer's reverse transfection method. A preliminary experiment was conducted to determine the best transfection condition with highest efficiency, and it was 30 000 cells/well (6-well plate), and 5 μl lipofectamine for 100 pmol siRNAs. For miRNA (1.6 μg), it was 10 000 cells/well (12-well plates) and 4 μl lipofectamine. We followed the reverse transfection method as it was more efficient than forward transfection. Using these conditions, we got 70% transfection efficiencies into PK15 cell for both siRNA and miRNA (Fig. 1). Transfected PK15 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), and without antibiotic, in 5% CO_2 incubator [11].

The siRNAs design

The siRNAs targeting gag, pol and the negative control siRNAs were purchased from Invitrogen (Table 1). Each of target gene and negative control could be designed automatically through Invitrogen Web (siRNAs design software) which did not affect experiment results.

The miRNAs design and delivered plasmids

Through the screen of siRNAs, two efficient siRNAs were recently selected to inhibit expression of PERV: gag2 miRNA and pol2 miRNA. A multitargeting vector of pol and gag gene of PERV was made through shRNA vector system (Table 2). The sequence of miRNA (gag2-pol2) was designed

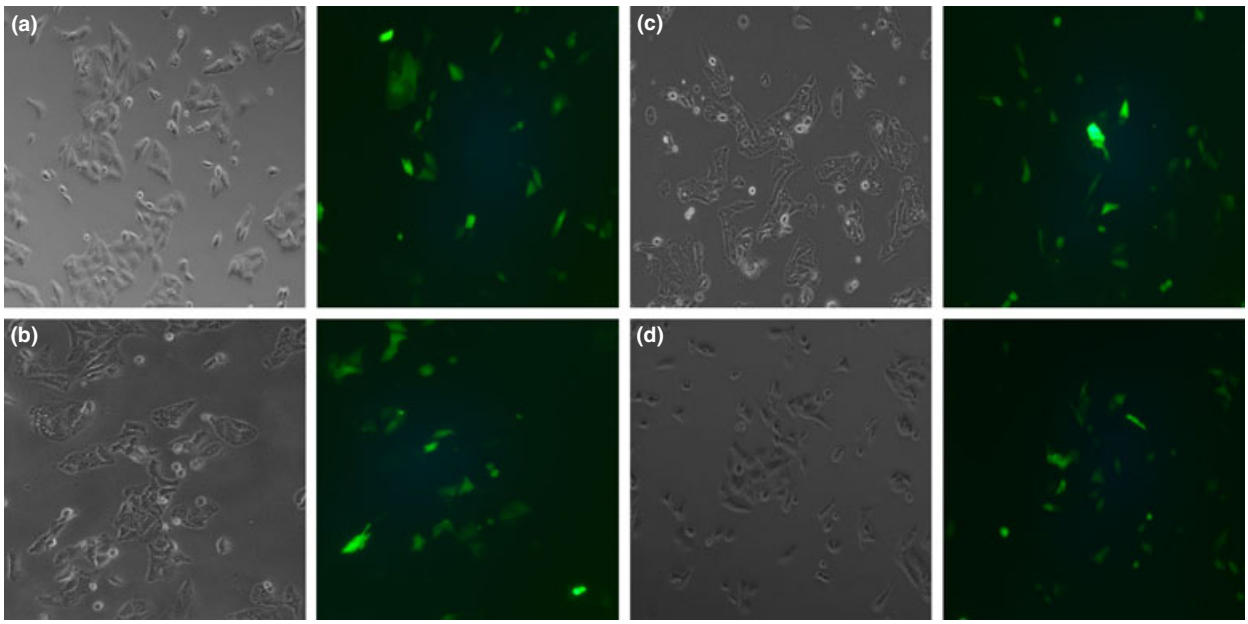

Figure 1 The miRNAs transfection. Lipofectamine2000 (Invitrogen) Transfection reagent was used to transfect the plasmid into PK15 cells (a) gag2 miRNA transfection (b) pol2 miRNA transfection (c) gag2-pol2 miRNA transfection (d) Neg-vector transfection.*Images were taken 24 h post-transfection using fluorescence microscope ×50 (4:1 reagent-to-DNA ratio).

Table 1. The siRNAs targeted against porcine endogenous retroviruses (PERVs) in PK15 cells.

No	Gene*	GenBank†	Target site‡	Forward sequences	Reverse sequences
1	gag 1	AF038600.1	1167~1185	GGAUAUCAGAAAGGAACUUTT	AAGUUCCUUUCUGAUAUCCT
2	gag 2	AF038600.1	1499~1517	GGACUACAACACGGCUGAA	UUCAGCCGUGUUGUAGUCC
3	pol 1	AF038600.1	3587~3605	UUUAGUUACGUCAGGGAGG	CCUCCCUGACGUAACUAAA
4	pol 2	AF038600.1	4093~4111	GCUAUGUGGUGGAAGGUAATT	UUACCUUCCACCACAUAGCTT

*Designed to target siRNAs of PERV gene.
†GenBank: AF038600.1; Sus scrofa porcine endogenous retrovirus PERV-MSL mRNA, complete sequence.
‡Targeting mRNA of PERV by siRNAs in PK15 cells.

Table 2. The miRNAs select oligos designed to contain all of these sequence elements for miR RNAi Vector.

				Pre-miRNA oligo		
No	Gene*	GenBank†	Target site‡	Antisense target sequences	Loop sequences	Sense target sequences
1	gag2	AF038600.1	1499~1517	CCTTCAGCCGTGTTGTAGTCC	GTTTTGGCCACTGACTGAC	GGACTACAACGGCTGAAGG
2	pol2	AF038600.1	4093~4111	TCTTACCTTCCACCACATAGC	GTTTTGGCCACTGACTGAC	GCTATGTGGGAAGGTAAGA

*Designed to target mRNA of PERV gene.
†GenBank: AF038600.1 ; Sus scrofa porcine endogenous retrovirus PERV-MSL mRNA, complete sequence.
‡Targeting mRNA of PERV by miR-Vector in PK15 cells.

to be the same as siRNA targeting gag2 and pol2. Both gag2 and pol2 targeting sequences were cloned through POL II miR RNAi Expression Vector Kits (Invitrogen) (Fig. 2a).

Generating a stable inhibition of PERV in PK15 cells

Concentration of blasticidin used was sufficient to kill untransfected PK15 cells (5 μg/ml). Transfected PK15 cells were plated at 60% confluence in a set of 24-well plates, and cells were allowed to adhere to it overnight. The next day, the culture medium was replaced with DMEM containing 5 μg/ml concentrations of blasticidin. The DMEM containing blasticidin was replenished every 2 days, and the percentage of surviving cells was observed until blasticidin-resistant colonies could be identified (generally at 10–14 days after selection). At least 10 blasticidin-resistant colonies per construct were picked and identified each clone. Then, the assay was performed to find the target-gene knockdown, comparing uninduced cells with cells stably transfected with negative control plasmid [20].

Quantification of RT activity

C-type RT activity kit (Cavidi) was intended for quantifying RT activity of PERV pol gene according to the manufacturer's protocol. Then, RT activity was determined for wells giving an A_{405} within the linear range of the reading. Also, standard curve for C-type RT activity kit was obtained through the serial dilutions of MMuLV rRT against the concentration of MMuLV present (LOT number 11071).

RNA extraction and quantitative real-time RT-PCR

Cellular RNA was extracted from PK15 cells (ATCC CCL-33) using the RNA Plus Kit (Qiagen) according to the manufacturer's protocol. Then, RNA concentrations were measured with a UV spectrophotometer at 260 nm. Total RNA concentration was adjusted to be 500 ng/20 μl. Then, contaminated genomic DNA was removed by adding DNase I (Fermentas). Step one is 37 °C, 30 min after which was inactivated by incubating step two at 67 °C, 10 min. Oligo dTs primer (100 pmol) and 2 μl of RNA was mixed, heated at 95 °C for 5 min, and chilled on ice immediately [16]. The remaining reagents including 5× first strand buffer, 10 mM DTT, and 0.3 mM each dNTP were added in a final volume of 20 μl. To analyze the expression of PERV gag and pol mRNA, a SYBER Green qPCR was performed with the qPCR Kit (Fermentas) master mix. To enhance the sensitivity and accuracy, the specific primer was designed as follows: (GAPDH for: 5′- CACCCTGTTGC TGTAGCCA AA, GAPDH rev: 5′- CGACCACTTCGTCA AGCTCAT) the shRNA expression cassette (pol for: 5′- CATCCTCTTAC CTTCCACCACAT, pol rev: 5′- GAC TGGAGAAGTGCTAA CCTGGTT) and (gag for: 5′-CCT ACCTTCAGCCGTGTTG TAGT, gag rev: 5′-AGATTG ACATGGGATTTCCCTTAA) under the following temperature conditions: 95 °C, 10 min; 40 cycles (95 °C, 30 s; 60 °C 30 s; 72 °C, 30 s). Each of the expression levels of target genes was calculated as relative quantity (RQ) values by comparing the PERV gag, pol, both gag and pol, cellular GAPDH RNA expression through real-time qRT-PCR (Stepone plus-applied Biosystems and Thermo) [14,21].

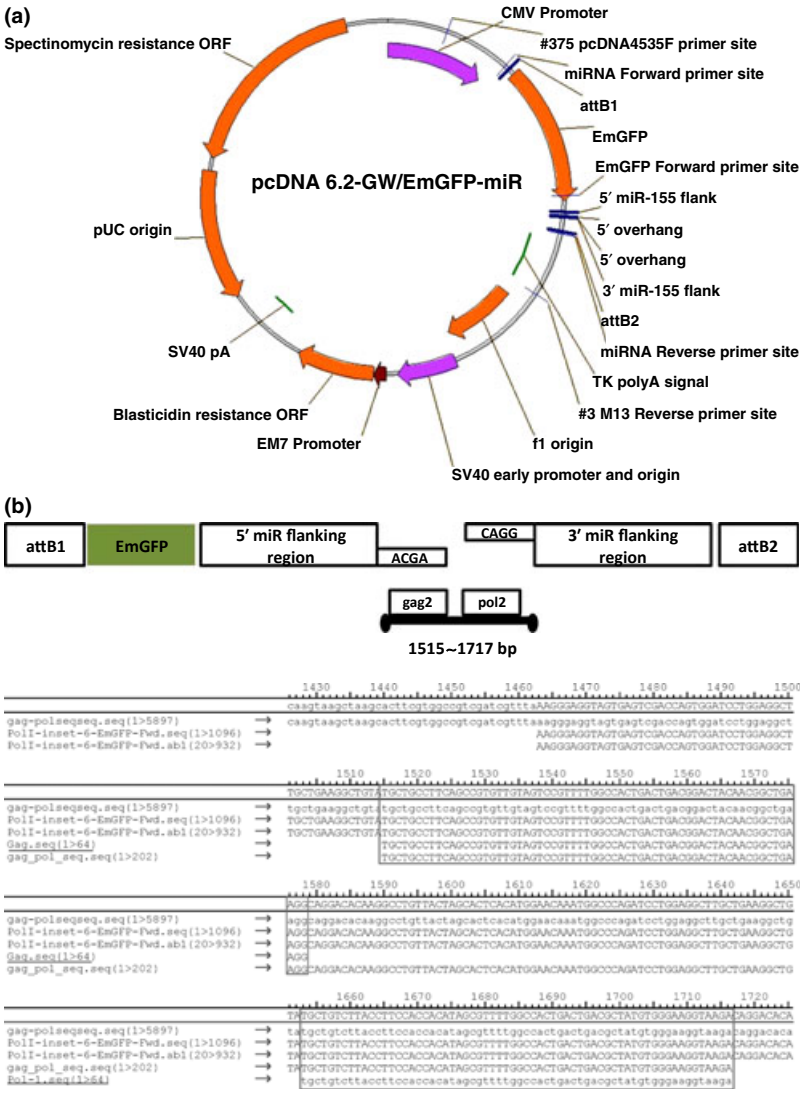

Figure 2 Constructs design. (a) Map and features: pcDNA 6.2-GW/EmGFP-miR Expression Vector for targeting of porcine endogenous retroviruses (PERV). CMV promoter, green fluorescent protein coding sequence (GFP), PERV multitargeting genes inserted into the attB2 sites, EM7 promoter, and blasticidin resistance gene; (b) The strategy of designing the constructs: Both of gag2 (1515~1579 bp) and pol2 (1653~1717 bp) were inserted to pcDNA 6.2-GW/EmGFP-miR Expression Vector (Invitrogen). *Confirmed the sequence of both gag and pol genes ligated in transformed E.coli (DH5a strain).

FISH (fluorescence in situ hybridization) assay

The slides were fixed by ethanol and 4% formaldehyde, using the QuantiGene View RNA FISH kit (Affymetrix), and View RNA Probe Set (Affymetrix) is designed to specifically hybridize to pol gene (accession AF038600_2) probe (Cy5 650-nm filter set) of PERV and Pig B2M gene (accession NM_213978) probe (FITC 488-nm filter set) of PK15 cells according to the manufacturer's protocol.

In vitro cocultivation inhibition of PERV in PK15 cells with HEK293 cells

PK15 and HEK293 cells (ATCC CRL-1573) were cultured in insert plate and 24-well plate (Nunc Cell Culture Inserts and Carrier Plates, Thermo Scientific), respectively. PK15 cells used in this study were already transfected with miRNA (gag2+pol2) and showed stable inhibition of PERV. PK15 cells transfected with neg-vector were used as a negative control. After one night, the insert plate was

transferred to a 24-well plate for cocultivation using 10% FBS in a mixture of DMEM and MEM (1:1) and incubated at 37 °C in a CO_2 incubator for 24, 48, 72, and 96 h. After incubation for different time periods, insert plates that included PK15 cell and supernatant were completely removed from the well and the cells remaining (HEK293 cells) were harvested with trypsin 0.25%. Genomic DNA and mRNA were extracted from HEK293 cells, and quantitative real-time RT-PCR assay was performed to identify the PERV inhibitory effect of miRNA in cocultivated human cell.

Results

Knockdown of PERV mRNA expression in PK15 cells by siRNAs

Four siRNAs targeting gag and pol genes of PERV were designed, which could inhibit the mRNA expression. The knockdown efficiency could be found up to 72% of PERV gag2 mRNA when the target region was between nucleotides 1499 to 1517bp of PERV gag mRNA sequence. Pol2-targeting siRNA between nucleotides 4093 to 4111bp showed 84.7% of knockdown efficiency. Also, it could bear knockdown efficiency up to 79.7% (gag site), 88% (pol site) of PERV mRNA when the target regions were both gag and pol compared to the negative siRNAs control. It was found that the transfection of both pol2 and gag2-targeting siRNAs into PK15 cells at the same time led to further suppression. However, each of single gag1-targeting and pol1-targeting siRNAs of PERV showed only 53%, 33% of knockdown efficiency, respectively. According to the results of the statistical analysis, four siRNAs targeting gag regions of PERV showed significant inhibition relative to negative control (Fig. 3a). Also, targeting pol regions of PERV certainly proved that pol2 siRNA is more effective than pol1 siRNA in terms of inhibition efficiency (Fig. 3b).

Changes of siRNAs knockdown efficiency in PK15 cells

The knockdown efficiency of siRNAs has changed in transfected PK15 cells over time. Although PK15 cells transfected with both gag and pol siRNAs were subsequently subcultured, they could not maintain knockdown efficiency permanently. After 48 h, siRNA knockdown efficiency was up to 83%. As time went by, knockdown efficiency was reduced more and more. The inhibition of effi-

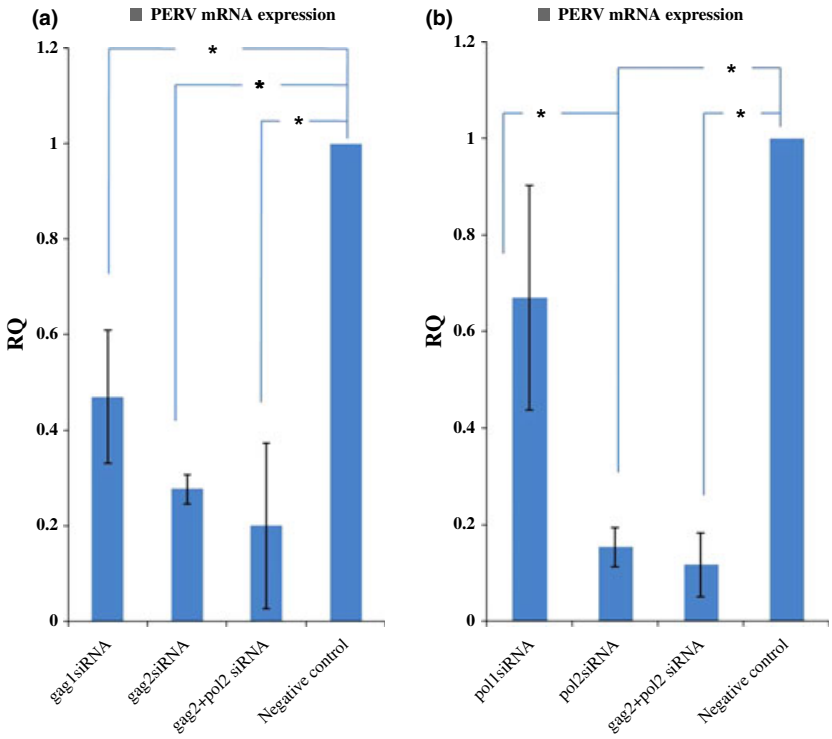

Figure 3 Efficiency of siRNA in reduction of porcine endogenous retroviruses (PERV) mRNA expression in PK15 cells. The control did not affect any experiment results through Invitrogen Web because the sequence of siRNAs did not target any gene product as standard control (RQ = 1), comparisons showed differences in suppression efficiency among siRNA targeting different sites of (a) gag and (b) pol genes. Porcine endogenous retrovirus expression was measured by a two-step quantitative real-time PCR. All experiments were repeated three times. *Marks indicate the statistical differences between targeting site groups (SPSS program Kor 12.0.1 paired *t*-test, $P < 0.05$). relative quantity RQ represents relative quantity.

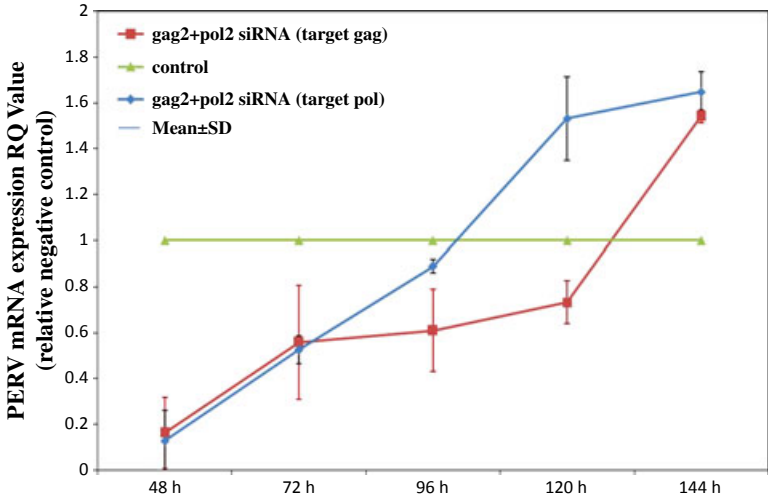

Figure 4 Knockdown efficiency of siRNAs (gag2+pol2) expression in transfected PK15 cells. Real-time PCR was used to detect the expression level of PERV mRNA in PK15cells relative to standard negative control (RQ = 1). *RQ represents relative quantity.

ciency for up to 96 h was maintained on some extent. However, 144 h after transfection, siRNA knockdown efficiency disappeared when compared to negative control (Fig. 4). They originally exceeded the amount of PERV mRNA expression of the control. It was confirmed that using siRNA just showed a transient inhibition effect.

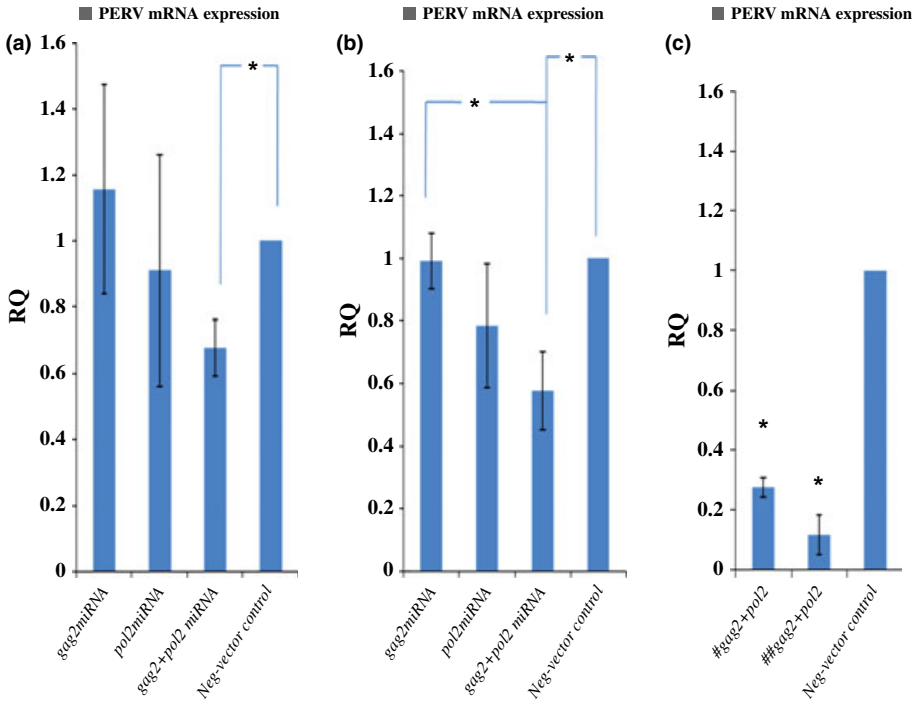

Figure 5 Suppression efficiency among miRNAs targeting different sites (a) gag, (b) pol, and (c) after transfection, selected blasticidin-resistant colonies; gag2-pol2 miRNAs (#) targeting gag region, (##) targeting pol region. The neg-vector-transfected control is regarded as standard control (RQ = 1), the multitargeting shRNA vector was then designed for expressing miRNAs targeting both gag and pol gene sites. It showed the most knockdown efficiency among RNAi groups. Porcine endogenous retroviruses mRNA expression was measured by a two-step quantitative real-time PCR. All experiments were repeated three times. *Marks indicate the statistical differences among targeting site groups (SPSS program Kor 12.0.1 paired *t*-test, $P < 0.05$). RQ represents relative quantity.

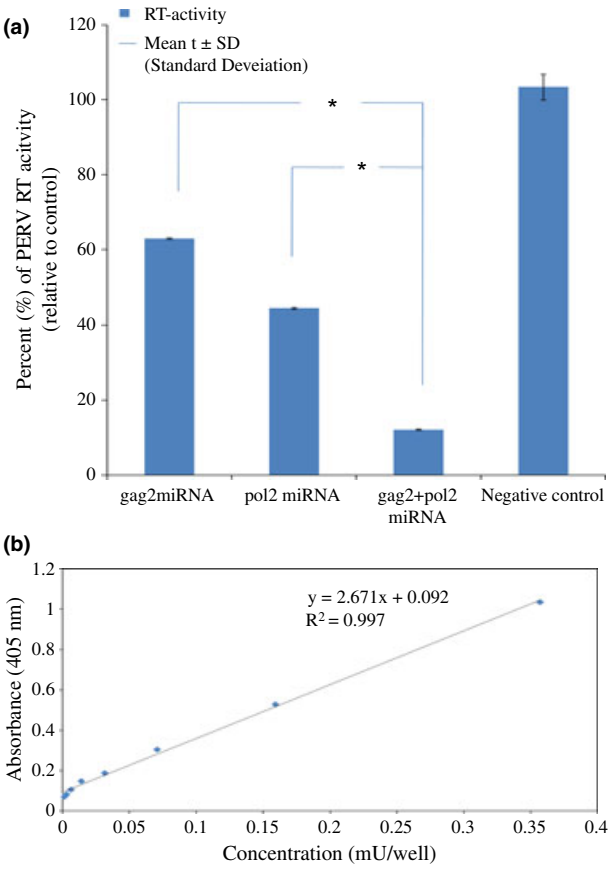

Figure 6 Porcine endogenous retroviruses reverse transcriptase activity among miRNA targeting different sites. (a) Indicating a practical value of negative control as a standard, each of sample values was presented as above by % level. (b) Standard curve for C-type RT activity kit was obtained with the serial dilutions of MMuLV rRT against the concentration of MMuLV present (LOT number 11071). The equation for the curve is as follows: y = 2.671x + 0.092 (R^2 = 0.997). *Marks indicate the statistical differences among targeting site groups (SPSS program Kor 12.0.1 paired *t*-test, $P < 0.015$).

Knockdown of PERV mRNA expression in PK15 cells by miR expression vectors

The sequences of gag2, pol2, and multitargeting miRNA (gag2-pol2) were designed corresponding to those of siRNA targeting gag2 and pol2 of PERV mRNA. POL II miR RNAi Expression Vector (Invitrogen) was used for multitargeting for inhibition of PERV by cloning gag2 with pol2 (Fig. 3b). Two-step real-time qPCR was performed, showing mRNA expression level of PERV as relative RQ values. The multitargeting miRNA vector (gag2-pol2) could reduce the expression efficiency up to 42% when the target region was pol gene of PERV. However, each single targeting miRNA (gag2 and pol2) showed only 0.7% and 21% (Fig. 5b) reductions, respectively. In addition, when targeting the gag region, vectors containing both genes allowed a 31.9% reduction in efficiency, although each of single miRNA (gag2 and pol2) demonstrated only 0% and 7%, respectively (Fig. 5a). PK15 cells transfected with the miRNAs (gag2-pol2) plasmid selected through blasticidin resistance remained at stable inhibitions of 88.1% and 72% when targeting pol gene and gag gene, respectively, during 2 weeks compared to miR-neg control plasmid (Fig. 5c).

RT activity of miRNAs transfected into PK15 cells

According to the analysis of the RT activity, PERV pol2 miR RNAi Expression Vector transfected into PK15 cells induced 55.4% of RT-activity inhibition. Also, miR RNAi Expression Vectors (pol2-gag2) reduced activity further to 87.8% inhibition compared to miR-neg control plasmid. Gag2 miRNA showed up to 37.1% of RT-activity inhibition (Fig. 6). On the basis of the results of statistical analysis, it was found that gag2-pol2 miRNAs were better than either pol2 miRNA or gag2 miRNA in terms of RT-activity inhibition efficiency.

FISH assay of miRNA(gag2-pol2) transfected into PK15 cells

B2M (beta-2-microglobulin) gene probe was used for green fluorescence expression on PK15 cells membranes. The pol mRNA of PERV probe set is designed to specifically hybridize, expressing red fluorescence. Fluorescence in situ hybridization assay showed that gag2-pol2 miRNA caused significant decrease in red expression compared to neg-vector miRNA (Fig. 7). PK15 cells could be identified like the circle around these membranes because B2M gene probe was designed for expressing only in PK15cells.

Expression of PERV in HEK293 cell line after cocultivation with inhibition of PERV in PK15 cells in vitro

According to the analysis of RT-qPCR using PERV *pol* primer set, the amount of PERV numbers of proviral copies in the genomic DNAs and mRNAs from infected HEK293 cells was measured. As time has gone by, both genomic DNA and mRNA copy numbers have increased. In particular, the cocultivation incubation time change from 72 to 96 h was the biggest change of PERV amount in the control group. The amount of PERV genomic DNA changed from 3.03×10^3 to 1.52×10^4 (copies/μl) and mRNA changed from 2.73×10^3 to 1.20×10^4 (copies/μl). Despite increasing the amount of PERV, this did not result in a significant change at miRNA (gag2+pol2)-transfected groups in PK15 cell compared to the control. It

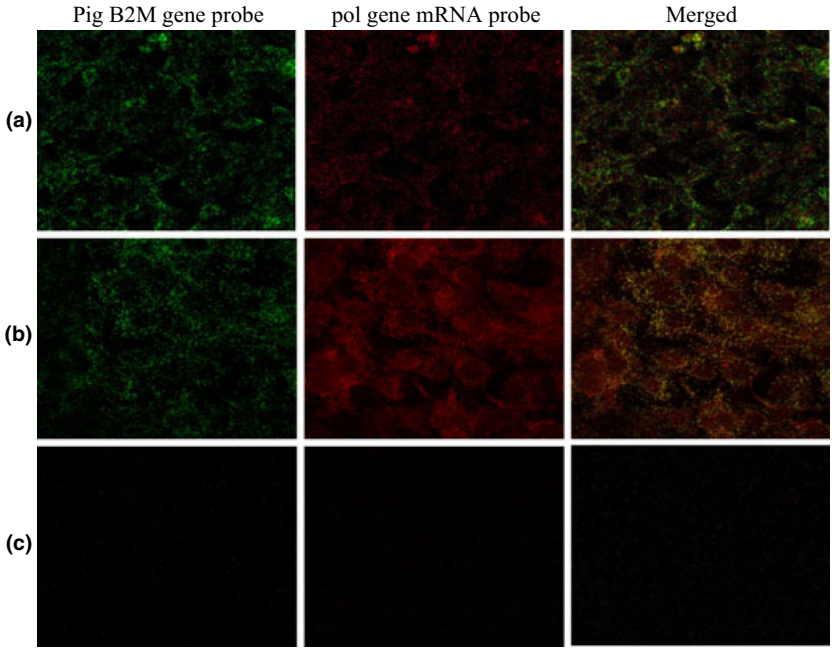

Figure 7 Fluorescence in situ hybridization assay for targeted pol gene mRNAs of porcine endogenous retroviruses in PK15 cells. (a) gag2-pol2 miRNAs vector transfected into PK15 cells. (b) neg-vector miRNAs transfected into PK15 cells. (c) negative control *Images were taken 1 h postmounting using a confocal laser scanning microscope.

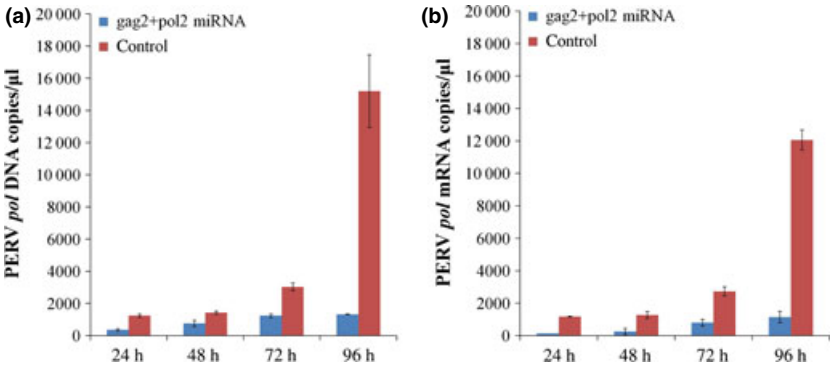

Figure 8 Expression of porcine endogenous retroviruses (PERV) in HEK293 cell line after cocultivation with inhibition of PERV in PK15 cells according to the change of incubation times (a) Genomic DNA, (b) mRNA was extracted from HEK293 cell and quantitative real-time RT-PCR assay was performed to measure the amount of PERV (copies/μl).

demonstrates that miRNA (gag2+pol2)-transfected groups in PK15 cells could reduce infected PERV to human cells (Fig. 8).

Discussion

This study is significant for reducing the expression of PERV by multitargeting both gag and pol genes of PERV, initially utilizing the most developed technique, RNAi system [22]. At first, two of the siRNAs (gag2 and pol2) could be found among four siRNAs through screening, and it enabled to show the reduction efficiency up to 88% by simultaneously targeting two regions of the best knockdown efficiency. However, this siRNA system had only transient knockdown efficiency as verified on Fig. 4. Therefore, miRNA was designed, which could persistently depress through shRNA vector system, encoding the same as those of siRNA target sequences. And we have chosen the human CMV promoter vector because they contain the human cytomegalovirus (CMV) immediate early pro-

moter to allow high-level [23], constitutive miRNA expression in mammalian cells .

According to Figs 3 and 5, up to 48 h after transfecting siRNA, siRNA was more effective than shRNA in terms of temporary knockdown efficiency. Although PK15 cells transfected with both gag and pol siRNAs were subsequently subcultured, it could not maintain knockdown efficiency eternally. One hundred and forty-four hours after transfection, siRNA knockdown efficiency disappeared (Fig. 4). In contrast, although miRNA showed the low knockdown efficiency during initial 48 h after miRNA transfection (Fig. 5b and c), this proved better knockdown efficiency than siRNA by selecting blasticidin-resistant colonies through miR expression vector system [24]. It could allow these PK15 cells to be used as control for other experiments and primary cells to be utilized for producing PERV-suppressed pigs [6,24,25]. According to the result, we used Pol II miR RNAi Expression Vector (Invitrogen), which was used to target multiple of several specific genes of PERV [25,26]. The vector could allow transient or stable expression of miRNA in mammalian cells, targeting multiple genes or increasing knockdown efficiency compared to a single target gene with one construct [18]. Ultimately, it was important to consider the appropriate transfection method for using this RNAi technique [27].

PK15 cells have been known for inducing efficient transfection only using transfection reagent among many various techniques [13,14]. In this study, three transfection reagents (Lipofectamine RNAiMAX, Lipofectamine RNAi2000 MAX, and Lipofectamine 2000) were used to deliver RNAi into PK15 cells. As a result, both miRNA and siRNA could be delivered into PK15 cells more efficiently by Lipofectamine 2000 compared to the other two transfection reagents, and high knockdown efficiency of target genes was obtained by Lipofectamine 2000 even at a low concentration of RNAi. Reverse transfection method showed higher efficiency than forward transfection. By fluorescence microscope analysis, miRNA was transfected with efficiency up to 70~80% into PK15 cells (Fig. 1), which was developed for highly efficient delivery. It proved that transfection reagents alone were efficient enough to induce transfection when compared to others, such as electroporation or viral system [28,29] transfection. The mean expression levels were calculated as RQ values by comparing the PERV gag, pol, both gag and pol, cellular GAPDH RNA expression through real-time two-step qRT-PCR [11,30]. Real-time PCR could be sensitive for detecting PERV mRNA. RT-activity analysis and FISH assay were performed for identifying pol gene which is critical for replication of PERV. Activity analysis confirmed the inhibition level of multitargeting PERV by miRNA in PK15 cells. Also, FISH assay showed that gag2-pol2 miRNA caused significant decrease in pol mRNA level expression compared to neg-vector miRNA (Fig. 7). Fluorescence in situ hybridization could verify how much mRNA, which was targeted by miRNA, could be suppressed using probe sets attaching specific sequences of mRNA [31,32].

On the basis of these results, the reduction in mRNA level of gag, which encodes capsid proteins essential for shedding, was due to the use of RNAi. Also, pol-targeting siRNA could prevent infection owing to reduced reverse transcription and PERV replication [22]. Among them, targeting both gag2 and pol2 mRNA by RNAi proved the most effective in reducing virus expression in PK15 cells. Through this research in advance, there was also the highest inhibition efficiency when both gag2 and pol2 genes were multitargeted. Furthermore, Pol II miR RNAi Expression Vector (Invitrogen) was utilized [25,26] for producing PERV-suppressed pigs [6,24,25]. Therefore, according to these results above, gene knockdown system (siRNA and shRNA) through multitargeting strategy could effectively inhibit PERV in PK15 cell, and using this designed vector might contribute to the mass production of the PERV-suppressed pigs with alleviating the concerns of pig to human infection of PERV [33].

Authorship

H-CC: participated in research design, writing of the paper, performance of the research, and data analysis. V-GN, H-JM, and H-KK: participated in research design, provided intellectual input. S-JP, J-HL, and M-GC: contributed important information. A-RK: edited the manuscript. B-KP: 'supervisor'; participated in research design, provided intellectual input, and edited the manuscript.

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References

1. Takeuchi Y, Patience C, Magre S, *et al.* Host range and interference studies of three classes of pig endogenous retrovirus. *J Virol* 1998; **72**: 9986.

2. Petersen B, Carnwath JW, Niemann H. The perspectives for porcine-to-human xenografts. *Comp Immunol Microbiol Infect Dis* 2009; **32**: 91.
3. Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. *Nat Med* 1997; **3**: 282.
4. Czauderna F, Fischer N, Boller K, Kurth R, Tonjes RR. Establishment and characterization of molecular clones of porcine endogenous retroviruses replicating on human cells. *J Virol* 2000; **74**: 4028.
5. Wilson CA, Wong S, VanBrocklin M, Federspiel MJ. Extended analysis of the in vitro tropism of porcine endogenous retrovirus. *J Virol* 2000; **74**: 49.
6. Kaulitz D, Mihica D, Plesker R, Geissler A, Tonjes RR, Denner J. Absence of infection in pigs inoculated with high-titre recombinant PERV-A/C. *Arch Virol* 2011; **156**: 707.
7. Miyagawa S, Nakatsu S, Nakagawa T, *et al.* Prevention of PERV infections in pig to human xenotransplantation by the RNA interference silences gene. *J Biochem* 2005; **137**: 503.
8. Li Z, Ping Y, Shengfu L, Youping L, Jingqiu C, Hong B. The lack of inhibition of porcine endogenous retrovirus by small interference RNA designed from the long terminal regions. *Transplant Proc* 2006; **38**: 2258.
9. Denner J. Infectious risk in xenotransplantation – what post-transplant screening for the human recipient? *Xenotransplantation* 2011; **18**: 151.
10. Kubo T, Yanagihara K, Takei Y, Mihara K, Sato Y, Seyama T. Lipid-conjugated 27-nucleotide double-stranded RNAs with dicer-substrate potency enhance RNAi-mediated gene silencing. *Mol Pharm* 2012; **9**: 1374.
11. Dieckhoff B, Petersen B, Kues WA, Kurth R, Niemann H, Denner J. Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs. *Xenotransplantation* 2008; **15**: 36.
12. Fishman JA, Scobie L, Takeuchi Y. Xenotransplantation-associated infectious risk: a WHO consultation. *Xenotransplantation* 2012; **19**: 72.
13. Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, Aebischer P. Lentiviral-mediated RNA interference. *Hum Gene Ther* 2002; **13**: 2197.
14. Semaan M, Kaulitz D, Petersen B, Niemann H, Denner J. Long-term effects of PERV-specific RNA interference in transgenic pigs. *Xenotransplantation* 2012; **19**: 112.
15. Martineau HM, Pyrah IT. Review of the application of RNA interference technology in the pharmaceutical industry. *Toxicol Pathol* 2007; **35**: 327.
16. Moon HJ, Park SJ, Kim HK, *et al.* Simultaneous detection and subtyping of porcine endogenous retroviruses proviral DNA using the dual priming oligonucleotide system. *J Vet Sci* 2010; **11**: 269.
17. Bittmann I, Mihica D, Plesker R, Denner J. Expression of porcine endogenous retroviruses (PERV) in different organs of a pig. *Virology* 2012; **433**: 329.
18. Zhang P, Yu P, Wang W, Zhang L, Li SF, Bu H. An effective method for the quantitative detection of porcine endogenous retrovirus in pig tissues. *In Vitro Cellular & Developmental Biology-Animal* 2010; **46**: 408.
19. Chan DC, Kim PS. HIV Entry and Its Inhibition. *Cell* 1998; **93**: 681.
20. Cullen BR. Derivation and function of small interfering RNAs and microRNAs. *Virus Res* 2004; **102**: 3.
21. Dalzell JJ, Warnock ND, Stevenson MA, Mousley A, Fleming CC, Maule AG. Short interfering RNA-mediated knock-down of drosha and pasha in undifferentiated Meloidogyne incognita eggs leads to irregular growth and embryonic lethality. *Int J Parasit* 2010; **40**: 1303.
22. Wall NR, Shi Y. Small RNA: can RNA interference be exploited for therapy? *Lancet* 2003; **362**: 1401.
23. Merkl C, Leuchs S, Saalfrank A, Kind A, Schnieke A. RNA Interference in Pigs: Comparison of RNAi Test Systems and Expression Vectors. *Mol Biotechnol* 2011; **48**: 38.
24. Takeuchi S, Hirayama K, Ueda K, Sakai H, Yonehara H. Blasticidin-S, a new antibiotic. *J Antibiot* 1958; **11**: 1.
25. Ge Q, Xu JJ, Evans DM, Mixson AJ, Yang HY, Lu PY. Leveraging therapeutic potential of multi-targeted siRNA inhibitors. *Future Med Chem* 2009; **1**: 1671.
26. Patience C, Switzer WM, Takeuchi Y, *et al.* Multiple groups of novel retroviral genomes in pigs and related species. *J Virol* 2001; **75**: 2771.
27. Scherr M, Eder M. Gene silencing by small regulatory RNAs in mammalian cells. *Cell Cycle* 2007; **6**: 444.
28. Dieckhoff B, Karlas A, Hofmann A, *et al.* Inhibition of porcine endogenous retroviruses (PERVs) in primary porcine cells by RNA interference using lentiviral vectors. *Arch Virol* 2007; **152**: 629.
29. Ramsoondar J, Vaught T, Ball S, *et al.* Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs. *Xenotransplantation* 2009; **16**: 164.
30. Ma Y, Yang Y, Lv M, *et al.* Real-time quantitative polymerase chain reaction with SYBR green i detection for estimating copy numbers of porcine endogenous retrovirus from Chinese miniature pigs. *Transplant Proc* 2010; **42**: 1949.
31. Weier HUG, Lucas JN, Poggensee M, Segraves R, Pinkel D, Gray JW. Two-color hybridization with high complexity chromosome-specific probes and a degenerate alpha satellite probe DNA allows unambiguous discrimination between symmetrical and asymmetrical translocations. *Chromosoma* 1991; **100**: 371.
32. Levsky JM, Singer RH. Fluorescence in situ hybridization: past, present and future. *J Cell Sci* 2003; **116**: 2833.
33. Semaan M, Kaulitz D, Petersen B, Kues WA, Niemann H, Denner J. Transgenic pigs with reduced PERV expression by RNA interference. *Xenotransplantation* 2012; **19**: 22.

