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Inhibition of porcine endogenous retrovirus by multi-targeting RNA interference in porcine cells

다중 표적 RNA 간섭에 의한 돼지 내인성 레트로바이러스 억제

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The Graduate School of

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Abstract

For xenotransplantation, pigs present numerous advantages as donor animals as compared with other non-human primates, and are recognized as recipient animals for organ xenotransplantation in preclinical studies. However, all pigs have the porcine endogenous retrovirus (PERV) genome inserted in their germ line, which is transmitted to offspring. Because of the potential risk of interspecies transmission, PERV is a major threat in xenotransplantation experiments. Ribonucleic acid interference (RNAi) technology is used to knockdown gene expression, which reduces risk and improves the overall safety of xenotransplantation. Herein, an RNAi strategy was employed to inhibit

PERV expression in porcine cells by targeting multiple PERV genes.

As stated in chapter 1, polymerase (*pol*)-targeting RNAi prevented infection through the reduction of reverse transcription and replication. The quantity of group-specific antigen (*gag*) mRNA, which encodes capsid protein essential for budding, was reduced by RNAi by blocking the shedding of viral particles. Therefore, if *gag* and *pol* genes of PERV can be suppressed simultaneously, it is an important strategy for significant inhibition of PERV gene expression. Among the four siRNAs targeting the *gag* and *pol* genes of PERV that were designed, two were more effective siRNAs (*gag*2, *pol*2) in reduction of the expression of PERVs. Concurrent treatment of porcine cells with these two siRNAs (*gag*2 + *pol*2) showed knockdown efficiency up to 88% as compared to the negative control. Despite the high initial knockdown efficiency by siRNA 48 hours after transfection, that effect may be a mere transient effect for PERV suppression. A multi-targeting vector was designed containing both *gag* and *pol* genes, and making use of the POL II microRNA (miRNA) expression vector, this vector allows for simultaneous targeting of multiple genes. The sequence of miRNA for the combined genes (*gag*2 + *pol*2) was designed in the same region as siRNAs which target *gag*2 and *pol*2 separately. Through the antibiotic-resistance characteristics of this vector, miRNA-transfected porcine kidney (PK) 15 cells (*gag*2 + *pol*2) were selected over 2 weeks. Reduction of mRNA expression for *pol* and *gag* gene targets was 88.1% and 72%, respectively. In addition, two assays were performed: 1) reverse transcriptase assay (RT) activity analysis and 2) fluorescence *in situ* hybridization (FISH) assay and it demonstrated the highest knockdown efficiency in the multi-target (*gag*2 + *pol*2) miRNA

group. According to the results above, using gene knockdown systems (siRNA and shRNA) through a multi-targeting (*gag*2 + *pol*2) strategy are effective methods to inhibit PERVs.

In chapter 2, the objective was to target the long terminal repeats (LTR) region with a dual LTR1 + LTR2 miRNA. The miRNA expression vector was designed using PERV LTRs sequences from porcine organs eligible for xenotransplantation. The targets for the LTR region of miRNAs were automatically selected via the online program BLOCK-iT RNAi Designer. The inhibition efficiency among the miRNAs was compared based on their inhibition of different PERV genes, LTR, *gag*, and *pol*. Relative quantitative real-time polymerase chain reaction (qPCR) and C-type reverse transcriptase activity were performed. The miRNA targeting the LTR region degraded the target sequence, but simultaneously inhibited the mRNA expression of both *gag* and *pol* genes of PERV. The LTR1, LTR2, and dual LTR1+LTR2 miRNA inhibited 76.2%, 22%, and 76.8% of *gag* gene expression, respectively. Similarly, miRNA knock-downed *pol* gene expression by 69.8%, and 25.5% for the single targeting miRNA (LTR1 and LTR2), respectively and 77.7% for the multi-targeting miRNA (LTR1 + LTR2). A stable PK15 clone constitutively expressed dual LTR1 + LTR2 miRNA and exhibited higher inhibition up to 82.8% and 92.7% of the expression for the *gag* and *pol* genes, respectively. Also, co-cultivation of dual LTR1 + LTR2 miRNA-transfected PK15 cells with a human cell line (HeLa cells) showed that dual LTR1 + LTR2 miRNA inhibited expression of LTR, *gag*, and *pol* genes of PERV mRNAs so that PERV infectivity was reduced in a human cell line.

Dual mRNA targeting of LTR produced a marked reduction in PERV expression. However, the experimental design for the study in chapter 2 did not include primary porcine kidney cells and or examine inhibition of the *env* gene. Therefore, in chapter 3, this study was performed in primary porcine kidney cells *in vitro* to determine whether miRNAs selected that target specific regions of the LTR could exert an inhibitory effect on the expression of LTR (the U3 promoter region of PERV), *gag*, *pol*, and *env* genes. Two miRNAs (LTR1 and LTR2), and dual LTR1 + LTR2, were selected to inhibit the expression of PERV in primary porcine kidney cells. The inhibition efficiency of the miRNAs was compared based on their inhibition of different PERV regions, specifically LTRs (U3 promoter region of PERV), *gag*, *pol*, and *env*. Gene expression was quantified using real-time polymerase chain reaction (RT-PCR) and the C-type reverse transcriptase (RT) activity was determined. The mRNA expression of the PERV LTR (U3 promoter region of PERV) and *env* region was determined in HeLa cells co-cultured with primary porcine kidney cells. The mRNA expression of the LTR (U3 promoter region of PERV), *gag*, *pol*, and *env* regions of PERV was dramatically inhibited by dual miRNA (LTR1 + LTR2) from 24 hours to 144 hours after transfection, with the highest inhibition observed for the LTR (U3 promoter region of PERV) and *pol* regions at 120 hours. Changing the co-culture incubation time from 48 hours to 120 hours resulted in the largest change in the PERV amount in the negative vector control group contrary to HeLa cells co-cultured with primary porcine kidney cells transfected with dual LTR1 +LTR2 miRNAs.

In conclusion, these three studies confirm that miRNA techniques targeting the regions of PERV can positively inhibit the expression of PERV in porcine kidney cells.

Multi-targeting miRNAs for LTR1 +LTR2 of PERV reduced gene expression for the LTR region as well as the expression of functionally important PERV genes such as *gag*, *pol*, and *env*. The methods on co-cultivation and gene expression profiling offer new approaches to generate data to help evaluate the risk/benefit balance for PERV inhibition for safer xenotransplantation, and ultimately for the future creation of transgenic pigs.

Keywords: Inhibition; miRNA vector; Porcine endogenous retrovirus; Transfection; Human cell; Multi-targeting; RNA interference

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transfected with dual LTR1 + LTR2 miRNA. Blue line represents naive HeLa cells.

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Abbreviations

APOBEC	Apolipoprotein B mRNA editing enzyme catalytic
AZT	Azidothymidine
CA	Capsid
Cas9	Associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
env	Envelope
ERV	Endogenous retrovirus
gag	Group specific antigen
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
HEK-293	Embryonic kidney cell
IN	Integrase
INDELs	Insertions and deletions
LTR	Long terminal repeat
MA	Matrix
miRNA	microRNA
NC	Nucleocapsid
NHDFs	Normal dermal human fibroblasts
NHEJ	Nonhomologous end joining
NHP	non-human primate
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERT	Product enhanced reverse transcriptase
PERV	Porcine endogenous retrovirus
PK-15	Porcine kidney-15
Pol	Polymerase
PPT	Poly purine tract
pri-miRNA	primary microRNA
pro	Protease
RNAi	RNA interference
RISC	RNA induced silencing complex
RT	Reverse transcription
SA	Splice acceptor
shRNA	Small hairpin RNA

siRNA	Small RNA
sgRNA	Single guide RNA
SU	Surface
TM	Trans membrane

General introduction

Pig organs and tissues are well suited for organ transplantation to humans (Petersen et al., 2009, Takeuchi et al., 1998) and organs are approximately the same size as human organs. However, all pigs have the porcine endogenous retrovirus genome inserted in the pig germ line and transmitted to offspring. Porcine endogenous retrovirus (PERV) is a member of the *Retroviridae* family. The virus is classified into an infectious group (PERV-A, -B, and -C) and a non-infectious group (γ2 to γ5, and β1 to β4) (Garcia-Etxebarria et al., 2014, Patience et al., 2001). The genomic structures of PERV are comprised of *gag* (group specific antigen), *pol* (polymerase), and *env* (envelope) genes which are flanked with 5' and 3' long terminal repeats (LTR) possessing regulatory elements (Lower et al., 1996). The expression of PERV genes and their adjacent genes is controlled by transcription regulatory elements in the LTR (Kowalski et al., 1999). The majority of PERVs are transcriptionally inactive, because deletions and point mutations interrupt the coding potential of the *gag, pol*, and *env* genes (Lee et al., 2008c).

Until now, the fact that PERV-A and PERV-B can infect human cells *in vitro* is recognized but without direct corroborating evidence (Czauderna et al., 2000, Patience et al., 1997). Furthermore, PERVs have wide range of hosts including mouse, chimpanzee, dog, cat, horse, mink, and cow as well as pig (Wilson et al., 2000). The recombination rate of PERV-A/C is much higher than that of PERV-A alone for infection of HEK293 cells. Elimination of PERV from pigs is difficult (Kaulitz et al., 2011b, Takeuchi et al., 1998), and PERV increases risk during organ xenotransplantation.

Recently, RNA interference (RNAi) technology was developed to knockdown gene

expression, and to potentially increase the safety of xenotransplantation (Denner, 2011; Li et al., 2006; Miyagawa et al., 2005). In one study using this technique, the expression of PERV was significantly inhibited by up to 94% in all organs tested in two transgenic piglets (Dieckhoff et al., 2008). In fact, short hairpin RNA (shRNA) vectors can be transfected into primary pig fibroblasts, allowing for production of PERV-controlled transgenic pigs, in which PERV expression is suppressed for prolonged periods. On that basis, primary porcine kidney cells, rather than immortalized cancer cell lines, are available for in vitro inhibition studies and for safer somatic nuclear transfer for the creation of transgenic pigs for xenotransplantation. Additionally, genome-wide inactivation of PERV was achieved using the CRISPR/Cas9 procedure (Yang et al., 2015), an essential technique for safe xenotransplantation. However, Joachim Denner (Denner et al., 2018) said current experiences with clinical xenotransplantation implies cell and tissue transplantation in the absence of immunosuppressed conditions (Güell et al., 2017). In support of the contention that complete PERV inhibition occurs and that there is no in vivo PERV delivery, clinical data are lacking, particularly information in immunocompromised patients with hypertension organ transplants (Denner, 2015). Furthermore, co-culture models (HEK293 or HUVEC cells) showed types of PERV infection still occur, and further investigation is required to demonstrate complete suppression of PERV (Denner, 2015, Denner et al., 2018). For the complete inhibition of PERV, an essential technique for safe xenotransplantation, further research is still needed.

The overall objective of this thesis was to investigate inhibition of PERV by

RNAi techniques in porcine cells. In order to achieve this objective, three sub-objectives were accomplished by three chapters of studies. The objective of chapters I, II, and III are inhibition of porcine endogenous retrovirus in PK15 cell line and primary porcine kidney cells by efficient multitargeting RNA interference whether miRNAs that target specific regions of the *gag* and *pol*, LTR could simultaneously exert an inhibitory effect on the expression of LTR, *gag*, *pol*, and *env* genes.

Taken together, these experiments show significant inhibition of PERV expression using an RNAi strategy which exploits multi-targeting of PERV genes.

Literature review

1. PERV in xenotransplantation

1.1 PERV historical perspective

In 1971 a porcine kidney cell line (PK 15 cells), was reported to spontaneously release retrovirus particles type C (Armstrong et al., 1971). Over the next 10 years indicated that PERV replication was limited to porcine induced cells (Lieber et al., 1975), and attempts to confirm causative associations between PERV and cancerous were unsuccessful (Strandström et al., 1974). Nearly 20 years later, few field of clinical research in transplantation medicine emerged that involved the use of living non-human cells or organs to treat human disease, termed xenotransplantation (Wilson, 2008, Ibrahim et al., 2006). In the 1990s, the field of xenotransplantation began moving away from the utility of non-human primates, towards the use of pigs as the primary source for xenotransplantation organs, based on a number of considerations: i) easier animal husbandry, ii) relatively similar to anatomic size of organs. physiologic compatibility (Ibrahim et al., 2006), and iii) the assumption that pigs would be safer from a microbiological point of view (Allan, 1998). During the 1970s, PERVs had a narrow host range exclusive of human cells, the increased risk of porcine to human xenotransplantation certified further study of this question based on modern tools of micro virology (Wilson, 2008). Robin Weiss et al., used the same cell line that Armstrong used, PK 15cells, and demonstrated that PERV could be transmitted to human cells *in vitro* (Patience et al., 1997). One year later, it was shown that primary porcine peripheral blood mononuclear cells (PBMCs), upon mitogenic stimulation, also

released virus that could be transmitted to and replicate in human cell lines (Wilson, 2008). Thus, PERV research returned to the mainstream with new goals that included the development of the following: i) baseline knowledge of PERV biology, replication and potential for pathogenesis to increase our comprehension of the risks in porcine to human xenotransplantation; ii) methods with improved sensitivity and specificity for detecting evidence of PERV transmission in xenotransplantation product recipients; and iii) means to prevent transmission or to treat disease, should it develop in xenotransplantation product recipients (Wilson, 2008).

To date, research of clinical xenotransplantation studies using pig cells, tissues, and organs have failed to demonstrate transmission of PERV to humans including transplantation of porcine pancreatic islets and over 200 individuals exposed to pig cells or tissues or *in vitro* perfusion of pig organs or pig cell–based bioreactors (Morozov et al., 2017).

1.2. Xenotransplantation trials

Porcine materials such as livers, splenic or kidney perfusion *ex vivo*, fetal pig neural cells, porcine islets, corneas, and skin have been used to treat different human diseases (Sasaki et al., 2009, Fink et al., 2000, Deacon et al., 1997). In addition, porcine heart valves have been widely used for many years in replacement cardiac valve surgery (Gu et al., 2008). However, nonliving animal biological materials are classified as medical devices, drugs,

or biological products, but not as xenotransplantation products. A few xenotransplantation clinical trials, such as the investigation of the safety and effectiveness of DIABECELL® (immunoprotected alginate-encapsulated porcine islets for xenotransplantation), have been conducted in patients with type 1 diabetes mellitus (O'Connell et al., 2013). Retrospective studies have also been conducted to assess possible PERV infection in human xenograft recipients (Heneine et al., 1998, Patience et al., 1998, Paradis et al., 1999). Paradis et al. (Paradis et al., 1999) collected peripheral blood mononuclear cells (PBMCs) and serum samples from 160 patients who underwent different xenotransplantation procedures, such as extracorporeal liver, splenic or kidney perfusion, pancreatic islet cell transplantation, and skin xenografts. While 81% of those samples were PERV DNA-negative, some samples were found to be positive for the presence of pig centromeric or mitochondrial DNA, indicating microchimerism. No PERV RNA was found in the serum or saliva. Similar results were obtained in other studies, suggesting a lack of PERV infection in patients exposed to various porcine materials, including pig islets, skin grafts, livers, kidney and splenic perfusions, and heart valves (Elliott et al., 2000, Cunningham et al., 2001, Patience et al., 1997). However, the ability of PERVs to infect human cells *in vitro* has led to the development of diagnostic tools to detect viral infection in patients exposed to pig cells and tissues (Specke et al., 2001, Li et al., 2006a).

1.3. PERVs and potential to cause xenozoonotic disease

The expression of PERVs may differ, depending on the breed of pig and the tissue (Clemenceau et al., 1999, Tacke et al., 2003, Sypniewski et al., 2005), but the PERV DNA copy number in the whole organism is about 50 copies per haploid genome (Patience et al., 2001, Klymiuk et al., 2006). Moreover, there are variations in PERV integration sites among breeds (Yu et al., 2011, Groenen et al., 2012). Groenen et al. (Groenen et al., 2012) analyzed the genome sequence of a domestic Duroc pig and compared it with the genomes of wild and Europe and Asia domestic pigs. The authors identified 20 almost intact PERV γ1 loci and four *β*-retroviral PERVs, but with defects in the *gag*, *pol*, or *env*, indicating that these proviruses are not replicable. Moreover, these loci were different in the studied pigs, which might suggest considerable PERV polymorphisms. Endogenous retroviruses are proviruses integrated into the germ line of the host and inherited by the offspring. PERVs can be activated to emerge as potentially infectious virus particles; therefore, the existence of PERVs in exogenous form has been proposed, and a PERV-A/C recombinant, which appears to exist *in vivo*, has been isolated from PBMCs but has not been found in the germ line of the same individuals (Scobie et al., 2004b, Wood et al., 2004, Scobie et al., 2004a). Martin et al. (Martin et al., 2006) demonstrated the presence of the recombinant PERV-A/C provirus in the genome of some porcine cells in some organisms. Some endogenous retroviruses can induce diseases, but they are generally nonpathogenic in their original hosts. Moreover, many endogenous proviral elements are transcriptionally silent or defective, carrying

deletions or point mutations, and are thus incapable of producing an infectious virus (Herring et al., 2001b, Machnik et al., 2005). However, some gamma retroviridae, such as feline leukemia virus, murine leukemia virus, gibbon ape leukemia virus, and koala retrovirus induce leukemia and immunodeficiency in the infected host (Denner, 2007). PERVs are not known to cause disease, although a recent work reported an increased incidence rate of PERV-A/C viraemia in pigs suffering from clinical conditions including diarrhea, wasting, and respiratory disease compared to healthy pigs (Pal et al., 2011).

1.4. PERV risk of xenotransplantation

The ability of PERVs to infect human cells *in vitro* raises afraid, especially in the context of the eventual use of porcine cells, tissues, and organs in xenotransplantation. The possibility of PERV transmission to various human cell lines such as PBMCs (Clemenceau et al., 2001, Cullen, 2004b), embryonic kidney cell (HEK-293) line, and normal dermal human fibroblasts (NHDFs) has been confirmed *in vitro* (Kimsa et al., 2013). However, PERV transmission *in vivo* has not been reported among patients with type 1 diabetes, after pancreatic islets xenografts (Heneine et al., 1998), recipients of pig's nerve cells, patients with porcine liver cell based on bio artificial liver (Di Nicuolo et al., 2010) porcine skin graft recipients, and butchers exposed to contact with pig tissues. It is possible, that in the case of *in vitro* studies, it's not able to reproduce the

involuted dependence networks that have a significant impact on the defense of cells against PERV infection *in vivo* (Garkavenko et al., 2004).

Currently, the risk of PERV transmission is considered to be low, assuming that the pigs are adequately and continuously monitored. To minimize the risk of PERV transmission during human xenotransplantation, donor pigs should be selected using the absence of PERV-C and the lowest expression of PERV-A and -B. Biological materials such as animal saliva or blood should be used for screening. However, if the number of PERV copies in the organ for xenotransplantation differs compared to the material used for screening, an investigation of the whole animal or of its sisters or brothers should be performed (Łopata et al., 2018).

Even a small change in the genetic code of the PERV may be dangerous (Gemeniano et al., 2006; Argaw et al., 2008). In addition, the barriers associated with the tropism of PERVs to human cells can be overcome with the use of other receptors or their corresponding domains from other viruses or by recombination with other PERV subtypes (Harrison et al., 2004).

Expression of the PERVs' *env* proteins plays an important activity in the development of the placenta. It provide fusogenic activity for the syncytiotrophoblast formation and regulate its homeostasis (Denner, 2016a). Their immunosuppressive properties contribute to preventing the rejection of the semi allotransplant embryo (Denner, 2016a). PERVs can influence the regulation of the innate immunity, and some may even protect their hosts against viral infection (Chuong et al., 2016).

It has been estimated that about 8% of the human genome consists of retroviral

sequences. Potential recombination of one of the three subtypes of PERVs with closely related human ERVs (HERVs), especially HERV-R or HERV-E, cannot be excluded, especially if both elements are located near to one another. Such a situation could theoretically give rise to a new virus with unknown pathogenic potential. Recombination could occur during packaging of PERV and HERV transcripts into a single retroviral particle. Changes in the expression of HERV genes have been observed in studies of the HEK 293 cell line exposed to PERVs. Both the mRNA and protein abundance of *env* were significantly higher than in the control cells (Machnik et al., 2005). Conversely, studies on infected HEK 293 cells indicated that the recombination potential of PERVs and HERVs was low (Suling et al., 2003). Moreover, the evolution gap of about 20 million years between the time of PERV and HERV incorporation into the host genome probably permitted primates to develop adequate defense mechanisms for inactivating foreign ERVs, for example, restriction factors such as apolipoprotein B mRNA editing enzyme catalytic subunit 3 (APOBEC3).

(Denner, 2016b). However, the final exclusion of PERV–HERV recombination would necessitate experiments *in vivo* studies (Suling et al., 2003).

1.5. The need to screen for PERVs in xenotransplantation

PERV DNA cannot be completely eliminated from materials used in xenotransplantation (Cyganek-Niemiec et al., 2012, Li et al., 2013). It is also difficult to eliminate PERV by

designated pathogen-free pig breeding, as their presence in the host genome means they are inherited by the offspring. Thus the need to monitor transplant recipients for PERV infection has long been recognized (Herring et al., 2001a). Moreover, there is a need to look for virus-human junction fragments to provide unambiguous evidence of infection of human cells. Moalic et al. (Moalic et al., 2006, Moalic et al., 2009), by cloning and mapping PERV integration sites in infected human embryonic kidney 293 cells (HEK293), revealed an integration preferences of the PERV DNA genome near the transcriptional start sites and CpG islands of transcriptional active genes in the chromosomes, similar to murine leukemia virus. These authors also revealed 224 hot spots in the human genome (Moalic et al., 2009). In fact, it is important that screening for PERVs be carried out in both donors and recipients using sensitive and specific methods, and that it be carried out at the genome, transcriptome, and proteome stages.

The methods described in these studies could be used to evaluate the risk of PERV transmission in human recipients, enhance the effectiveness and reliability of monitoring procedures, and stimulate discussion regarding the development of improved, more sensitive methods of detecting PERVs in the future.

2. PERV structure and biology

2.1. PERV molecular structure

The genomic RNA of PERV is composed of two identical single strands with positive polarity and includes both coding and noncoding genome sequences (Czauderna et al., 2000, Güell et al., 2017). The non-coding sequences are localized at both ends of the RNA, which includes the R and U5 regions at the 5'-end and the U3 and R regions at the 3'-end (Lower et al., 1996). Between noncoding sequences, there are sequences encoding the *gag*, *pol*, and *env* proteins, that is, the *gag* (group-specific antigen), *pol* (polymerase gene), and *env* (envelope gene) genes, respectively (Figure 1A). The *gag* gene encodes the structural proteins of the matrix (MA), the capsid (CA), and the nucleocapsid (NC) (Figure 1B).

MA is associated with the inner lipid bilayer that descends from a host cell during budding. CA is the main structural protein of PERV, with a molecular weight of about 27 kDa. NC is the third structural protein, with a molecular weight of about 10 kDa , and it is responsible for the efficient packaging of RNA in the virion (Akiyoshi et al., 1998). The *pol* gene has the information to encode reverse transcriptase (RT), and intergrase (IN) enzymes. RT is responsible for the transcription of viral ssRNA into dsDNA, which is subsequently incorporated into the genome of the host with the help of IN (Denner and Tönjes, 2012). The viral envelope glycoprotein is encoded by *env* gene and this glycol protein is made up with surface (SU) and transmembrane (TM) domains (Denner

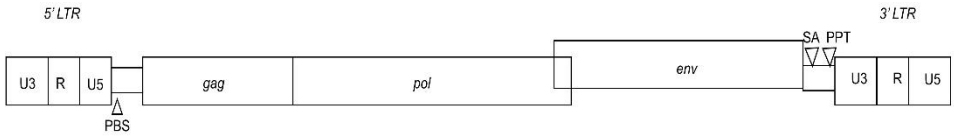
and Tönjes, 2012). *Env* glycoprotein has several glycosylation sites: about 10 in PERV-A, 6 in PERVB, and 8 in PERV-C. Glycosylation may influence the binding to the host receptor (Lee et al., 2008a, Lee et al., 2008b). The tropism of the retrovirus depends on the *env* proteins. The TM protein is buried in the lipid bilayer and anchors the SU protein to the surface of viral particles. The TM protein mediates the membrane fusion reaction (Watanabe et al., 2005).

The primer-binding site (PBS), the sequence responsible for starting the first RNA strand-reverse transcription (RT), is located between the U5 region and *gag*.

The splice acceptor (SA) site is located between the *pol* and *env* genes. The polypurine tract (PPT) is located between the *env* region and U3. PPT is required for RT as the primer for synthesis of the second strand of the DNA copy (Magre et al., 2003). The cap is situated on the 5'-side of the genomic RNA, while the 3'-end contains a poly A tail (Akiyoshi et al., 1998, Magre et al., 2003).

PERV, occurs mainly in the form of provirus integrated within the DNA of the host genome. The length of the provirus is about 9000 bp (Czauderna et al., 2000, Preuss et al., 2006, Tonjes et al., 2004). Just like the virus genome, the provirus contains coding sequences *gag*, *pol*, and *env*. These sequences are flanked by noncoding sequences, LTRs, with U3, R, and U5 regions at both the 5'- and 3'-ends. The length of these LTRs is about 600 to 800 bp (Akiyoshi et al., 1998, Magre et al., 2003) (Figure 1C).

A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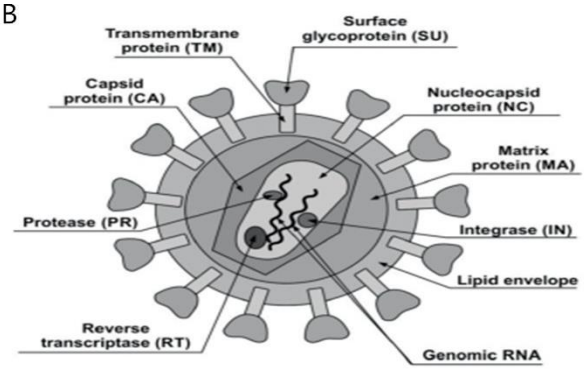
3' LTR
SA
PPT
U3
R
U5
gag
pol
env
U3
R
U5
PBS

B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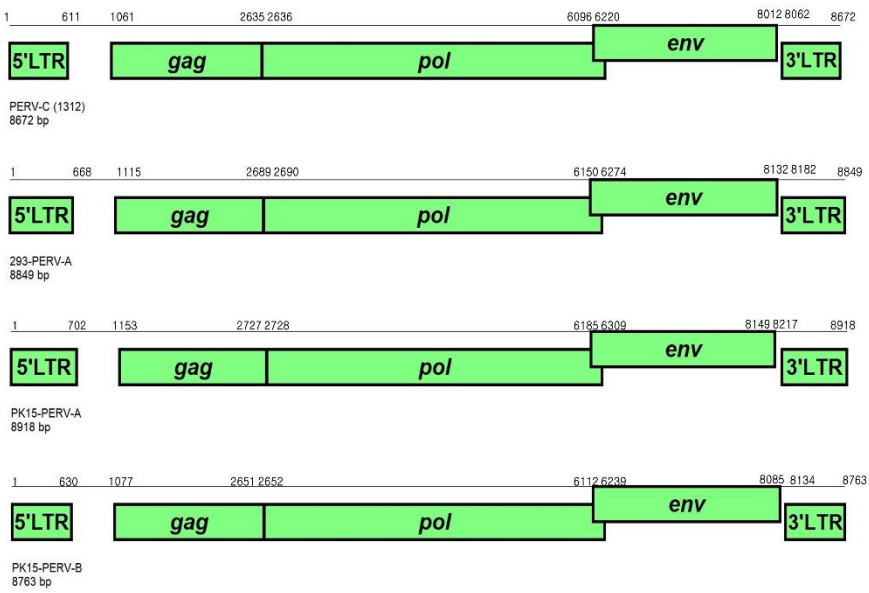
Surface glycoprotein (SU)
Capsid protein (CA)
Nucleocapsid protein (NC)
Matrix protein (MA)
Protease (PR)
Integrase (IN)
Lipid envelope
Reverse transcriptase (RT)
Genomic RNA

C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pol
env
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8672 bp
1
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2689 2690
6150 6274
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5'LTR
gag
pol
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8849 bp
1
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1153
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8918 bp
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630
1077
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6112 6239
8085 8134
8763
5'LTR
gag
pol
env
3'LTR
PK15-PERV-B
8763 bp

Figure 1. PERV. (A) Genomic RNA. (B) PERV structure. (C) Proviral DNA and nucleotide positions of the main elements (Łopata et al., 2018). PBS, primer-binding site; SD, splice donor site; %, packaging signal psi; SA, splice acceptor site; PPT, polypurine tract; MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface envelope protein; TM, transmembrane envelope protein; LTR, long terminal repeat; *gag*, group-specific antigen; *pol*, polymerase; *env*, envelope.

2.2. PERV biology

LTRs play an important role in the integration of the provirus within the host genome and the replication cycle of the virus. In addition, they contain promoter, enhancer, and other regulator sequences important for the subsequent proviral transcription. U3 appears to be the most heterogeneous region, with many binding sites for numerous transcription factors (Akiyoshi et al., 1998, Magre et al., 2003, Wilson et al., 2003).

The replication cycle of PERVs is similar to that of other *orthoretroviruses*, especially *gammaretroviruses* such as MLV, and can be divided into early and late phases (Łopata et al., 2018). The early phase includes adsorption onto the cell surface, entry into the cell, RT, and integration within the genome of the host cell (Figure 2). The late phase includes the expression of retrovirus genes, the release, and maturation of descendant virions.

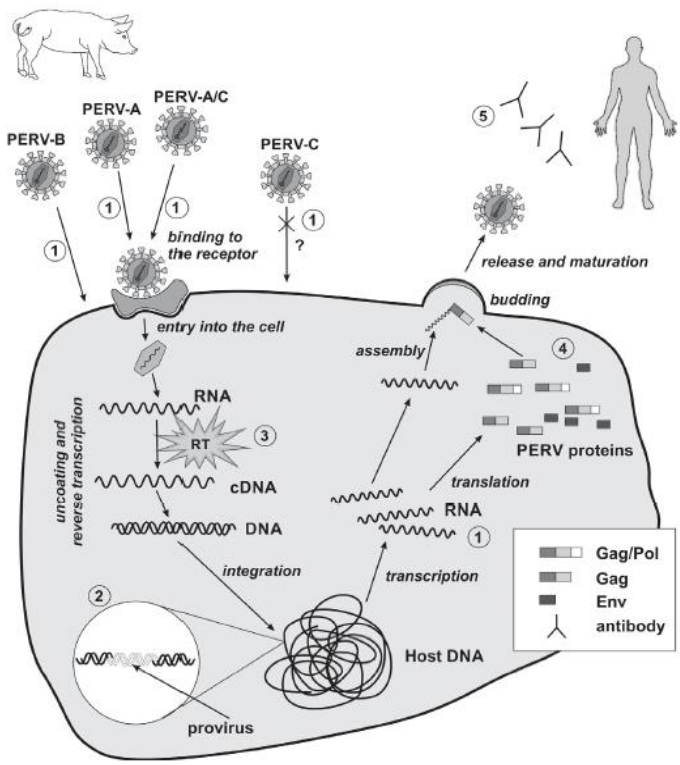

Figure 2. Replication cycle of PERV and strategies of PERV detection in xenotransplantation (Łopata et al., 2018). 1, detection of viral RNA; 2, detection of viral DNA; 3, evaluation of reverse transcriptase activity; 4, detection of PERVs proteins; 5, detection of PERVs antibodies. RT, reverse transcriptase; cross and question mark, PERV-C there is no body of evidence for the possibility of human cells infection *in vivo*.

3. Control of PERV

3.1. RNA inference (RNAi)

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 base pairs in length and operating within the RNAi pathway. It interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation (Fabian et al., 2010).

Over the past decade "RNA interference" has emerged as a natural mechanism for silencing gene expression (Wang et al., 2011). This ancient cellular antiviral response can be utilities to allow specific inhibition of the function of any chosen target genes, including those involved in causing diseases such as AIDS, cancer, and hepatitis. RNAi is already proving to be an invaluable research tool, allowing much more rapid characterization of the function of known several genes. In addition, the technology considerably bolsters functional genomics to help in the identification of novel genes involved in disease processes (Downward, 2004). RNAi is also probably important in maintaining order in the genome by suppressing the movement of mobile genetic elements such as transposons and repetitive sequences. The RNA interference machinery may also have a role in fine tuning normal cellular gene expression (Downward, 2004).

3.2. Small interfering RNA (siRNA)

Not content with just degrading the viral double stranded RNA, the cell uses an enzyme complex called RISC (RNA induced silencing complex) to use the short pieces of RNA produced by dicer as a template to seek out and destroy single stranded RNA with the same sequence, such as mRNA copies used by the virus to direct synthesis of viral protein. Together, dicer and RISC make up the RNAi system whereby double stranded RNA is recognized and used as a guide to prevent expression of similar sequences by destroying mRNA transcripts, a process sometimes termed post-transcriptional gene silencing (Downward, 2004).

Natural mechanism of RNA interference.

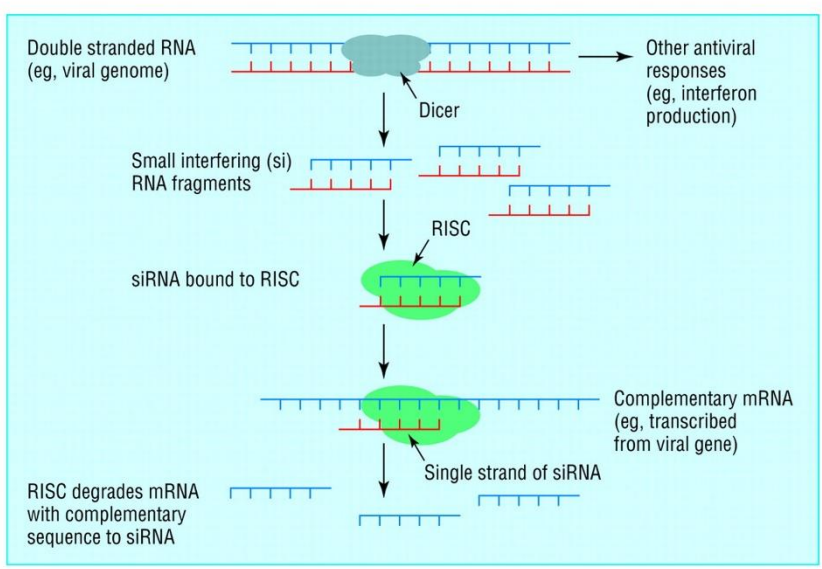

Figure 3. Natural mechanism of RNA interference (Downward, 2004) . The appearance of double stranded (ds) RNA within a cell—for example, as a result of viral infection—triggers an RNA interference response. The cellular enzyme dicer binds to the dsRNA and cuts it into short pieces of 20 or so nucleotide pairs in length known as small interfering RNAs or siRNAs. These bind to a cellular enzyme complex RISC (RNA induced silencing complex) that uses one strand of the siRNA to bind to single stranded RNA molecules such as mRNA of complementary sequence. RISC then degrades the mRNA, thus silencing expression of the viral gene. In mammals, other antiviral responses to dsRNA also exist.

3.3. Small hairpin RNA (shRNA)

A short hairpin RNA or small hairpin RNA (shRNA/ Hairpin Vector) is an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNAi (Brummelkamp et al., 2002, Paddison et al., 2002). Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors. shRNA is an advantageous mediator of RNAi in that it has a relatively low rate of degradation and turnover. However, it requires use of an expression vector, which has the potential to cause side effects in medicinal applications (Wang et al., 2011). The promoter choice is essential to achieve robust shRNA expression. At first, polymerase

III promoters such as U6 and H1 were used; however, these promoters lack spatial and temporal control (Wang et al., 2011). As such, there has been a shift to using polymerase II promoters to regulate shRNA expression.

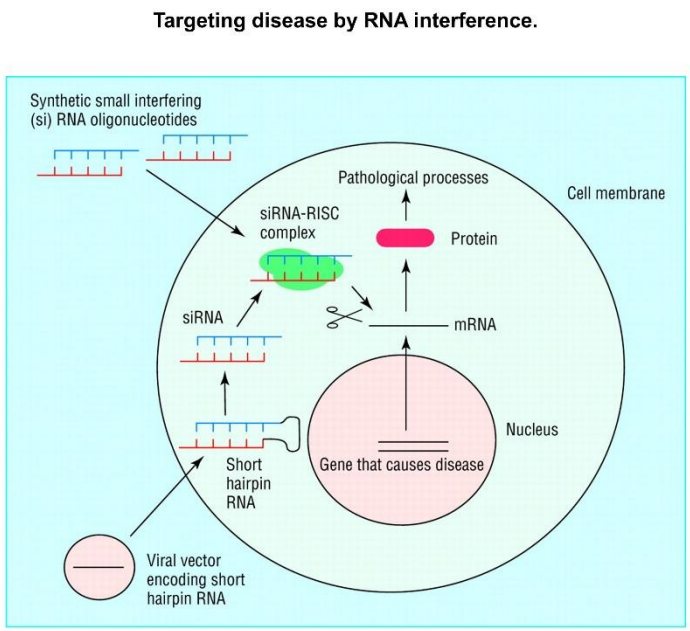

Figure 4. Targeting disease by shRNA (Wang et al., 2011). Diseases caused by aberrant gene expression include viral diseases and cancer. A gene implicated in causing the disease state can be silenced by RNA interference. Two of the most commonly used methods for artificially inducing RNA interference are shown here. Synthetic small interfering RNA molecules can be introduced into cells by using reagents such as cationic lipids to promote uptake across the cell membrane. Alternatively, engineered viral vectors can be used to deliver an expression construct to the cell, which will direct the production of a short hairpin RNA. This is then processed within the cell to form an

siRNA. The siRNAs from either route then use the cellular RNA machinery to degrade mRNA with complementary sequence, in this case chosen to target the gene that causes the disease.

3.4. microRNA (miRNA)

A microRNA (miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses, that functions in RNA silencing and post-transcriptional regulation of gene expression(Ambros, 2004) miRNAs function via base-pairing with complementary sequences within mRNA molecules (Bartel, 2009). As a result, these mRNA molecules are silenced, by one or more of the following processes: (i) cleavage of the mRNA strand into two pieces, (ii) destabilization of the mRNA through shortening of its poly (A) tail, and (iii) less efficient translation of the mRNA into proteins by ribosomes (Fabian et al., 2010). miRNAs resemble the siRNAs of the RNAi pathway, except miRNAs derive from regions of RNA transcripts that fold back on themselves to form short hairpins, whereas siRNAs derive from longer regions of double-stranded RNA (Fabian et al., 2010). miRNAs are abundant in many mammalian cell types (Lagos-Quintana et al., 2002) and appear to target about 60% of the genes of humans and other mammals (Lewis et al., 2005). Many miRNAs are evolutionarily conserved, which implies that they have important biological functions (Fabian et al., 2010). For example, 90 families of miRNAs have been conserved since at least the common ancestor of mammals and fish, and most of these conserved miRNAs have important functions, as shown by studies in which genes for one or more members of a family have been knocked out in mice (Fabian et al., 2010).

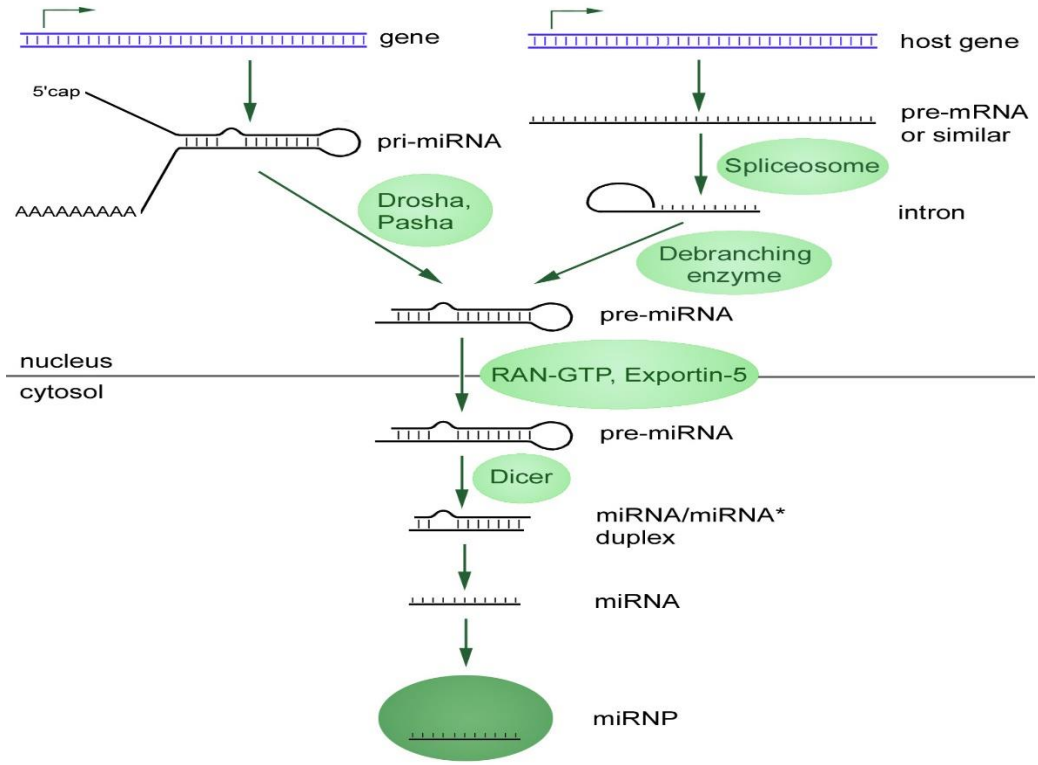

Figure 5. Overview of microRNA processing in animals, from transcription to the formation of the effector complex (Fabian et al., 2010). There are two pathways, one for microRNAs from independent genes and one for intronic microRNAs. Enzymes in the picture: Drosha, Pasha (pri-miRNA → pre-miRNA) Spliceosome (pre-mRNA → intron lariat) Debranching enzyme (intron lariat → RNA that can fold into pre-miRNA) RAN-GTP, Exportin-5 (export from nucleus) Dicer (pre-miRNA → miRNA)

3.5. Clustered regularly interspaced short palindromic repeats (CRISPR)

CRISPR (clustered regularly interspaced short palindromic repeats) is a family of DNA sequences found within the genomes of prokaryotic organisms such as bacteria and archaea (Barrangou, 2015). These sequences are derived from DNA fragments of viruses that have previously infected the prokaryote and are used to detect and destroy DNA from similar viruses during subsequent infections. These sequences play a important role in the antiviral defense system of prokaryotes (Barrangou, 2015).

Cas9 (or "CRISPR-associated protein 9") is an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence. Cas9 enzymes together with CRISPR sequences form the basis of a technology known as CRISPR-Cas9 that can be used to edit genes within organisms (Zhang et al., 2014). This editing process has a wide variety of applications including basic biological research, development of biotechnology products, and treatment of cancer or diseases (Zhang et al., 2014).

The CRISPR/Cas9 system has been harnessed to create a simple, RNA-programmable method to mediate genome editing in mammalian cells, and can be used to generate gene knockouts (via insertion/deletion) or knockins (Barrangou, 2015). To create gene disruptions, a single guide RNA (sgRNA) is generated to direct the Cas9 nuclease to a specific genomic location. Cas9-induced double strand breaks are repaired via the NHEJ DNA repair pathway. The repair is error-prone, and thus insertions and deletions (INDELs) may be introduced that can disrupt gene function (Zhang et al., 2014).

A single guide RNA (sgRNA), consisting of a crRNA sequence that is specific to the DNA target, and a tracrRNA sequence that interacts with the Cas9 protein, binds to a recombinant form of Cas9 protein that has DNA endonuclease activity. The resulting complex will cause target-specific double-stranded DNA cleavage. The cleavage site will be repaired by the nonhomologous end joining (NHEJ) DNA repair pathway, an error-prone process that may result in insertions/deletions (INDELs) that may disrupt gene function (Zhang et al., 2014).

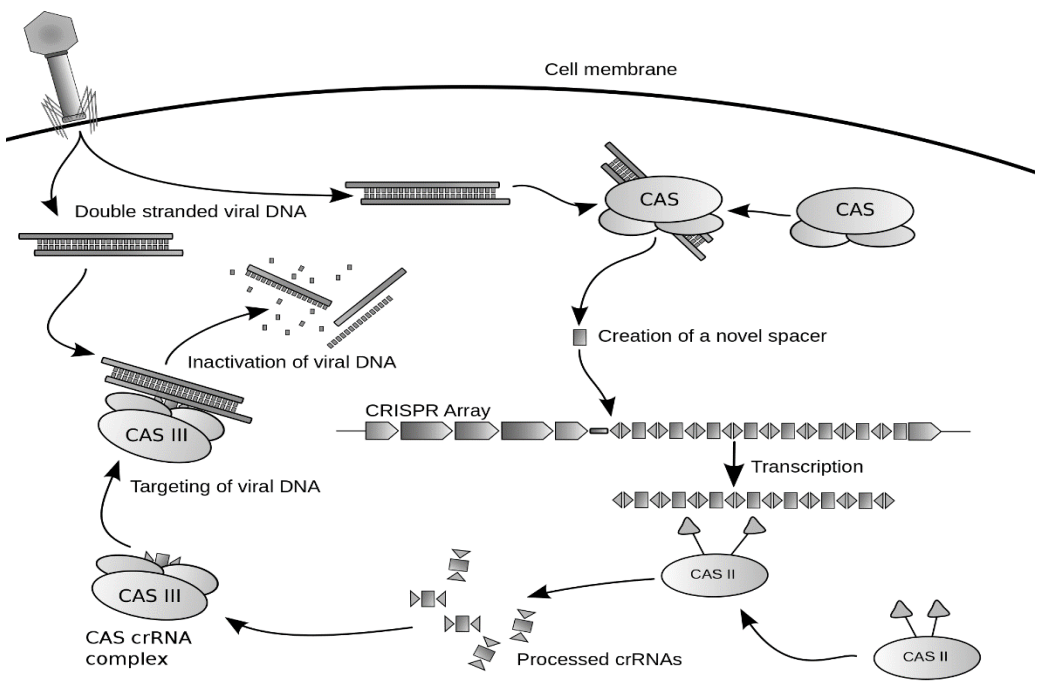

Figure 6. Diagram of the CRISPR prokaryotic antiviral defense mechanism (Zhang et al., 2014).

3.6. Latest strategy

The strategies of removal the potential problems related with PERVs rely on the search of specific vaccines strategy (Kaulitz et al., 2011a), the use of antiretroviral drugs (mainly azidothymidine; AZT) (Denner, 2017), attempts to suppress the PERVs expression by RNAi (Semaan et al., 2012a, Semaan et al., 2012b), or inactivation of all PERV proviruses in the pig germline genome by the CRISPR/Cas technique (Niu et al., 2017, Yang et al., 2015).

CRISPR is a remarkable technology that enables accurate and efficient tools of several mammalian genomes. Since its first application in xenotransplantation, it has confirmed the means to simultaneously knock down multiple porcine several genes that contribute to the xenoimmune response (Martens et al., 2017) to knock protective transgenes into detrimental loci, and even to eradicate all copies of PERV from the porcine genome (Yang et al., 2015). With the application of the CRISPR-Cas9 technique, 62 copies of PERV *pol* gene were deactivated, leading to a 1000 times reduction in the virus ability to infect human cells (Yang et al., 2015). Long-term studies are underway to monitor the impact of PERV inactivation and gene editing on PERV-inactivated pigs (Niu et al., 2017). Previously, the lengthy period required to generate and breed multi-modified donor pigs was a considerable brake on progress. Now, with the acceleration provided by CRISPR, there is a plethora of new pigs available, and the bottleneck has become the strict testing of these candidate pigs in non-human primate (NHP) models (Cowan, 2018). However, the question of whether such genetically engineered pigs could serve in the

future as a safe resource of tissues and organs for xenotransplantation remains open (Denner et al., 2018, Walters and Burlak, 2017).

Although the functionality has been shown in *in vitro* cell culture, with inherent low translation value to the pig-to- human clinical situation as outlined above, it needs to be shown in an *in vivo* situation that the inactivation of PERVs in the pig donor makes sense, also in relation to the off-target effects of the gene editing procedure (Denner et al., 2018). This aside, the possibility of gene editing resulting in inactivated PERVs raised the question whether conventional pigs can still be used for xenotransplantation, or whether only CRISPR/Cas9-inactivated pigs have to be used as source animals for future xenotransplantations (Scobie et al., 2017). Furthermore, off-target effects by CRISPR/Cas9 may happen, but they will be detected when analyzing the health of the animals, and animals with defects will be eliminated (Scobie et al., 2017, Denner et al., 2018).

Chapter I

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

Abstract

To effectively suppress porcine endogenous retroviruses (PERVs), 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, the 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.

Key words: *inhibition, multi-targeting, PERVs, PK15 cells, RNA interference*

1.1. Introduction

Pig organs and tissues are well suited for transplantation of human (Petersen et al., 2009, Takeuchi et al., 1998) 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 (Czauderna et al., 2000, Patience et al., 1997). Furthermore, PERVs are known to have wide host range including mouse, chimpanzee, dog, cat, horse, mink, and cow as well as pig (Wilson et al., 2000). 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 (Kaulitz et al., 2011b, Takeuchi et al., 1998). 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 (Denner, 2011, Li et al., 2006b, Miyagawa et al., 2005). 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 (Kubo et al., 2012). Also, short hairpin RNAs (shRNA) could target multiple of specific genes (Dieckhoff et al., 2008). This vector system could be transfected into primary pig's fibroblast and allow to produce PERV-controlled transgenic pigs (Abbas-Terki et al., 2002, Fishman et al., 2012) in

which PERV expression would be suppressed for a long time (Semaan et al., 2012b). Therefore, shRNA vector-based system should be considered for long-term inhibition of PERV (Dieckhoff et al., 2008, Semaan et al., 2012b). Through these technologies, PK15 cell lines derived from pigs that have PERV-A and PERV-B (Kaulitz et al., 2011b) will be available on the several inhibition studies through *in vitro* experiments for safety xenotransplantation (Martineau and Pyrah, 2007). 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 (Bittmann et al., 2012, Moon et al., 2010). The copy number of *pol* gene of PERV was higher than *gag*, *env*A, *env*B, and *env*C (Zhang et al., 2010). 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 (Chan and Kim, 1998, Patience et al., 1997). 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 multi-targeting of PERV *gag* and *pol* genes in PK15 cells.

1.2. Materials and methods

1.2.1. 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, these got 70% transfection efficiencies into PK15 cell for both siRNA and miRNA (Figure 7). Transfected PK15 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), and without antibiotic, in 5% CO2 incubator (Dieckhoff et al., 2008).

1.2.2. 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.

1.2.3. 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 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) (Figure 8a).

1.2.4. 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 targetgene knockdown, comparing uninduced cells with cells stably transfected with negative control plasmid (Cullen, 2004a).

1.2.5. 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 A405 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).

1.2.6. 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 (Moon et al., 2010). The remaining reagents including 59 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´- CACCCTGTTGCTGTAGCCAAA, GAPDH rev: 5´-CGACCACTTCGTCAAGCTCAT) the shRNA expression cassette (*pol* for:

5´- CATCCTCTTAC CTTCCACCACAT, *pol* rev: 5´- GACTGGAGAAGTGCTAA CCTGGTT) and (*gag* for: 5´-CCTACCTTCAGCCGTGTTG TAGT, *gag* rev: 5´-AGATTGACATGGGATTTCCCTTAA) 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) (Dalzell et al., 2010, Semaan et al., 2012b).

1.2.7. 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.

1.2.8. 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 CO2 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.

1.3. Results

1.3.1. 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 1517 bp of PERV *gag* mRNA sequence. *Pol2*-targeting siRNA between nucleotides 4093 to 4111 bp 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 *gag*1-targeting and *pol*1-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 (Figure 9a). Also, targeting *pol* regions of PERV certainly proved that *pol*2 siRNA is more effective than *pol*1 siRNA in terms of inhibition efficiency (Figure 9b).

1.3.2. 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 efficiency for up to 96 h was maintained on some extent. However, 144 h after transfection, siRNA knockdown efficiency disappeared when compared to negative control (Figure 10). They originally exceeded the amount of PERV mRNA expression of the control. It was confirmed that using siRNA just showed a transient inhibition effect.

1.3.3. 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* (Figure 9b). 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% (Figure 11b) 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 (Figure 11a). 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 (Figure 11c).

1.3.4. 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 (Figure 12). 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.

1.3.5. 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 negvector miRNA (Figure 13). PK15 cells could be identified like the circle around these membranes because B2M gene probe was designed for expressing only in PK15 cells.

1.3.6. 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 x 10^3 to 1.52 x 10^4 (copies/μl) and mRNA change from 2.73 x 10^3 to 1.20 x 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 demonstrates that miRNA (*gag2*+*pol2*)-transfected groups in PK15 cells could reduce infected PERV to human cells (Figure 14).

1.4. 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 (Wall and Shi, 2003). 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 Figure 10. Therefore, miRNA was designed, which could persistently depress through shRNA vector system, encoding the same as those of siRNA target sequences. And the vector have chosen the human CMV promoter vector because they contain the human cytomegalovirus (CMV) immediate early promoter to allow high-level (Merkl et al., 2011), constitutive miRNA expression in mammalian cells. According to Figure 9 and Figure 11, 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 (Figure 10). In contrast, although miRNA showed the low knockdown efficiency during initial 48 h after miRNA transfection (Figure 11 b and c), this proved better knockdown efficiency than siRNA by selecting blasticidin resistant colonies through miR expression vector system (Takeuchi et al., 1958). 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 (Ge et al., 2009, Kaulitz et al., 2011b, Takeuchi et al., 1958). According to the result, POL II miR RNAi Expression Vector (Invitrogen), which was used to target multiple of several specific genes of PERV (Ge et al., 2009, Patience et al., 2001). 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 (Zhang et al., 2010). Ultimately, it was important to consider the appropriate transfection method for using this RNAi technique (Scherr and Eder, 2007). PK15 cells have been known for inducing efficient transfection only using transfection reagent among many various techniques (Abbas-Terki et al., 2002, Semaan et al., 2012b). 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 (Figure 7), 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 (Dieckhoff et al., 2007, Ramsoondar et al., 2009) 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 (Dieckhoff et al., 2008, Ma et al., 2010). 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 (Figure 13). 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 (Levsky and Singer, 2003, Weier et al., 1991). 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 (Wall and Shi, 2003). 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 (Ge et al., 2009, Patience et al., 2001) for producing PERV-suppressed pigs (Ge et al., 2009, Kaulitz et al., 2011b, Takeuchi et al., 1958). 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 (Semaan et al., 2012a).

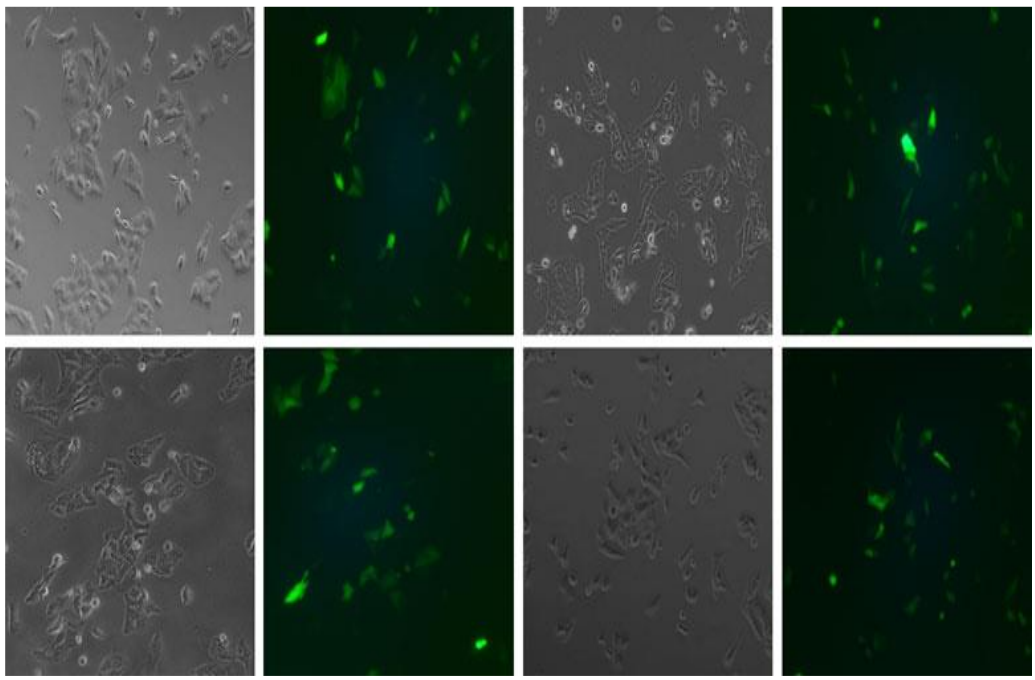

Figure 7. The miRNAs transfection. Lipofectamine2000 (Invitrogen) Transfection reagent was used to transfect the plasmid into PK15 cells (a) *gag2* miRNA transfection (b) *pol2* miRNA transfection (c) *gag2-pol2* miRNA transfection (d) Neg-vector transfection. *Images were taken 24 h post-transfection using fluorescence microscope x 50 (4:1 reagent-to-DNA ratio).

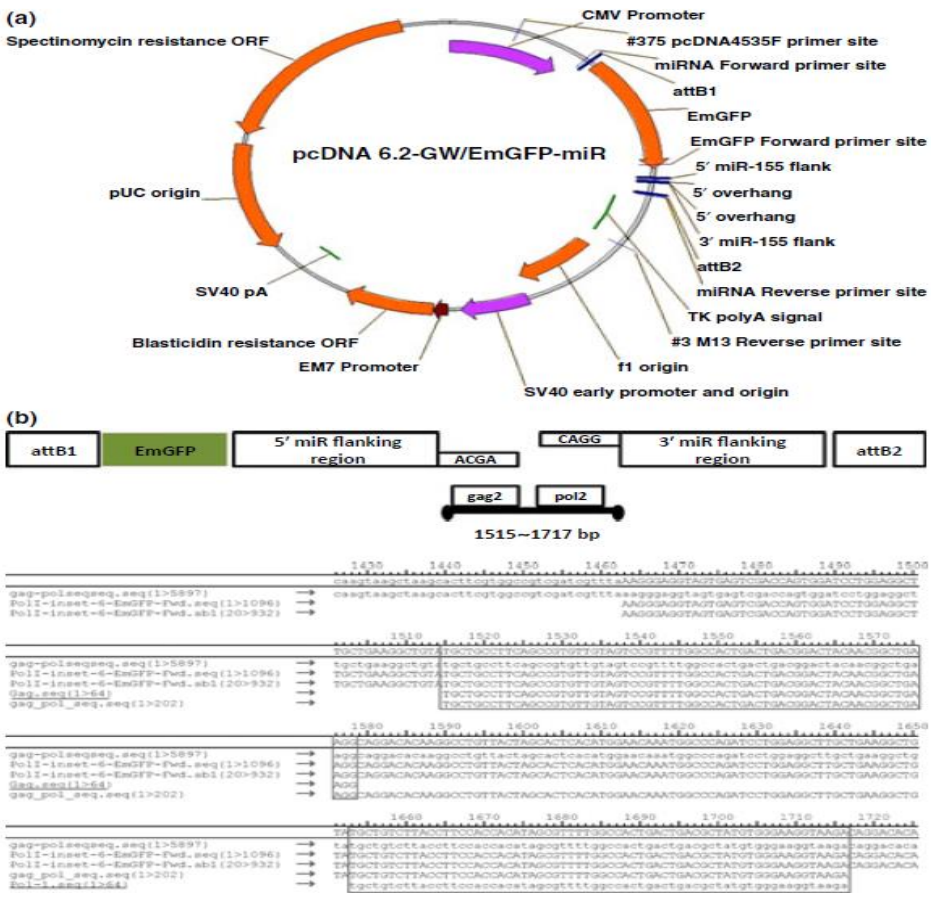

Figure 8. Constructs design. (a) Map and features: pcDNA 6.2-GW/EmGFP-miR Expression Vector for targeting of porcine endogenous retroviruses (PERV). CMV promoter, green fluorescent protein coding sequence (GFP), PERV multitargeting genes inserted into the attB2 sites, EM7 promoter, and blasticidin resistance 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).

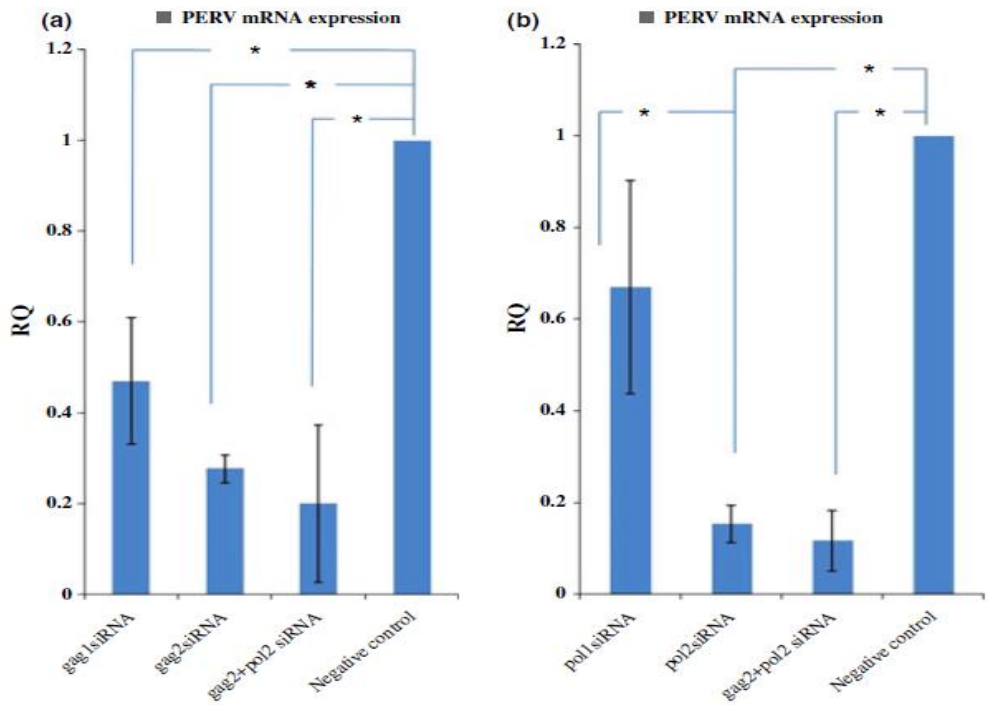

Figure 9. Efficiency of siRNA in reduction of porcine endogenous retroviruses (PERV) mRNA expression in PK15 cells. The control did not affect any experiment results through Invitrogen Web because the sequence of siRNAs did not target any gene product as standard control (RQ = 1), comparisons showed 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, $P < 0.05$).

*RQ represents relative quantity.

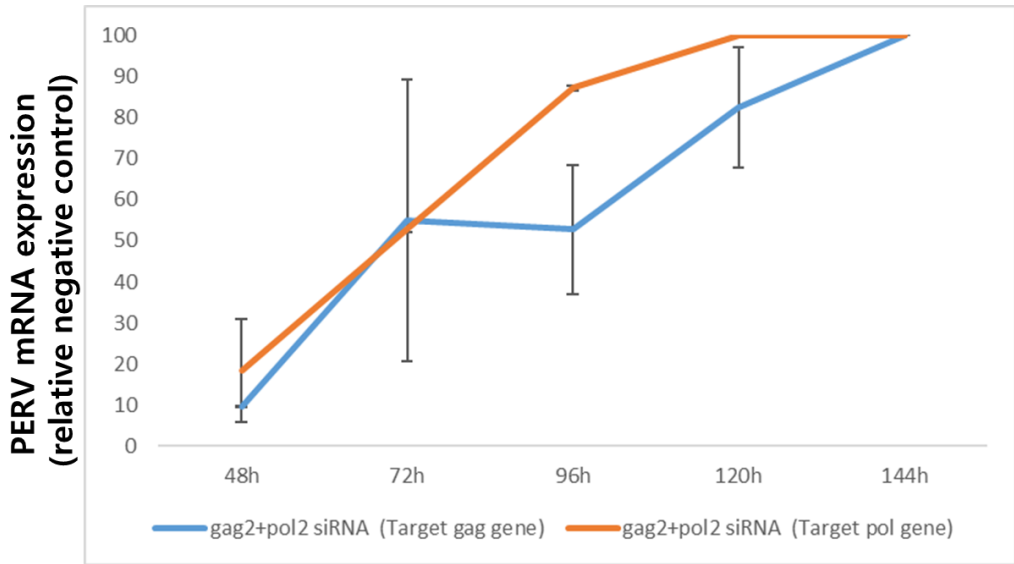

Figure 10. Knockdown efficiency of siRNAs (*gag2+pol2*) expression in transfected PK15 cells. Real-time PCR was used to detect the expression level of PERV mRNA in PK15cells relative to standard negative control (RQ = 100). *RQ represents relative quantity. Knockdown efficiency of siRNAs (*gag2+pol2*) 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 = 100). *RQ represents relative quantity.

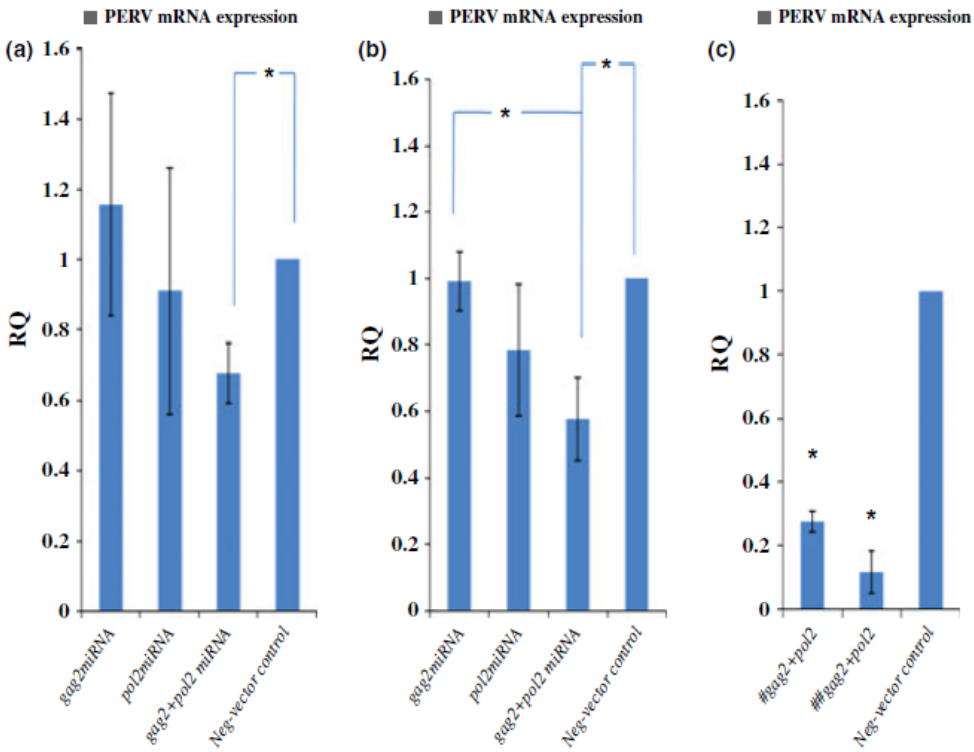

Figure 11. Suppression efficiency among miRNAs targeting different sites (a) *gag*, (b) *pol*, and (c) after transfection, selected blasticidin-resistant colonies; *gag2-pol2* miRNAs (#) targeting *gag* region, (##) targeting *pol* region. The neg-vector-transfected control is regarded as standard control (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.

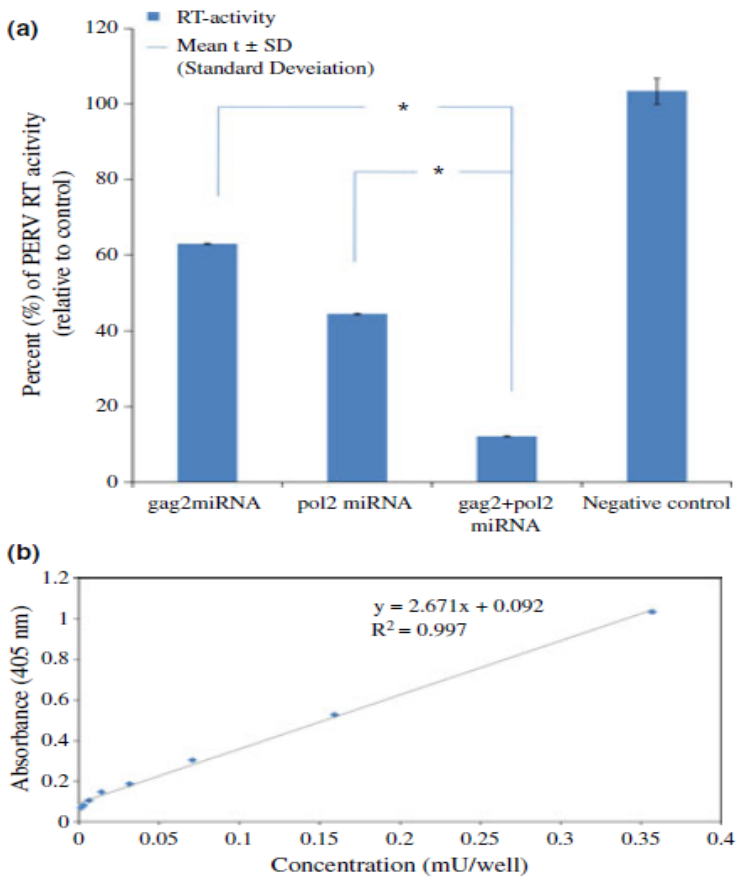

Figure 12. Porcine endogenous retroviruses reverse transcriptase activity among miRNA targeting different sites. (a) Indicating a practical value of negative control as a standard, each of sample values was presented as above by % level. (b) Standard curve for C-type RT activity kit was obtained with 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 (R2 = 0.997). *Marks indicate the statistical differences among targeting site groups (SPSS program Kor 12.0.1 paired t-test, $P < 0.015$).

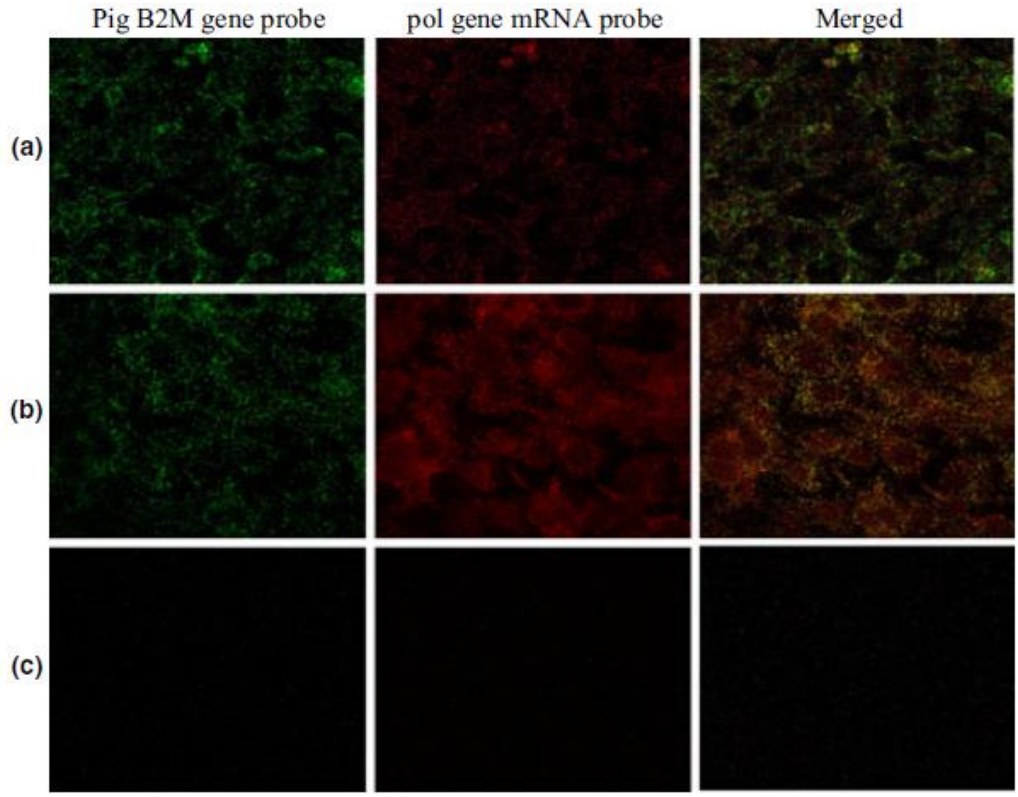

Figure 13. Fluorescence in situ hybridization assay for targeted *pol* gene mRNAs of porcine endogenous retroviruses in PK15 cells. (a) *gag2-pol2* miRNAs vector transfected into PK15 cells. (b) neg-vector miRNAs transfected into PK15 cells. (c) negative control *Images were taken 1 h postmounting using a confocal laser scanning microscope.

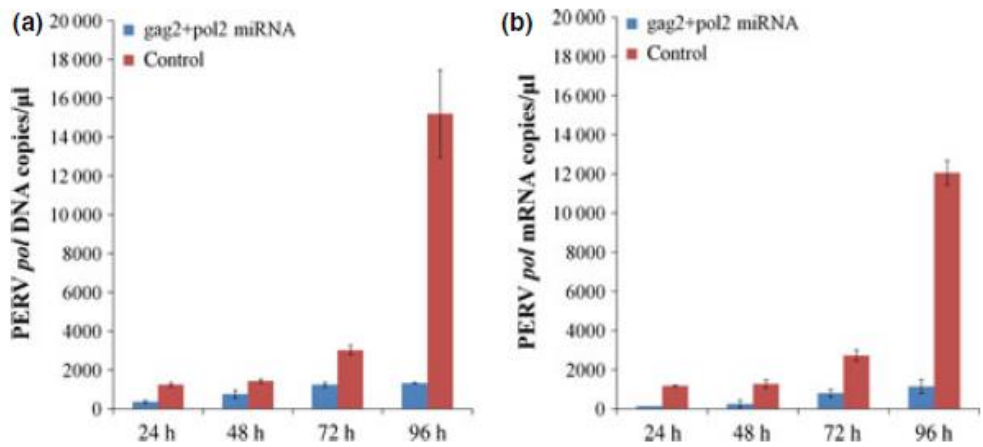

Figure 14. Expression of porcine endogenous retroviruses (PERV) in HEK293 cell line after cocultivation with inhibition of PERV in PK15 cells according to the change of incubation times (a) Genomic DNA, (b) mRNA was extracted from HEK293 cell and quantitative real-time RT-PCR assay was performed to measure the amount of PERV (copies/ µl).

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

** desingned 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

No	Gene*	GenBank**	Target site***	Pre-miRNA oligo		
				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

** desingned 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.*

Chapter II

Inhibition of porcine endogenous retrovirus by multi-Targeting microRNA against long terminal region

Abstract

There might be much benefit in xenotransplantation, however, the risk of infections across species barriers remains, especially porcine endogenous retrovirus (PERV). To date, many attempts have been made to knock down active PERVs by inhibitory RNA (RNAi) and micro RNA (miRNA), which target different genes of PERV. There are a few studies that have explored whether targeting promoter regions of PERV could exert an inhibition effect. miRNAs were automatically selected based on an online program BLOCK-iT RNAi Designer. The inhibition efficiency between miRNAs was compared based on their inhibition of different PERV genes: long terminal repeats (LTR), *gag*, and *pol*. Both relative quantitative real-time polymerase chain reaction (PCR) and C-type reverse transcriptase activity were performed.

The results demonstrated that miRNA targeting the LTR region degraded the target sequence, and simultaneously inhibited the mRNA expression of both *gag* and *pol* genes of PERV. The LTR1, LTR2, and dual LTR1+LTR2 miRNA inhibited 76.2%, 22%, and 76.8% of *gag* gene expression, respectively. Similarly, the miRNA was found to knock down the *pol* gene expression of 69.8%, 25.5%, and 77.7% for single targeting miRNA (LTR1 and LTR2) and multi-targeting miRNA (LTR1 + LTR2), respectively. A stable PK15 clone constitutively expressed dual LTR1 + LTR2 miRNA and exhibited higher inhibitory up to 82.8% and 92.7% of the expressions of the *gag* and *pol* genes, respectively. Also, the result of cocultivation of dual LTR1 + LTR2 miRNA transfected PK15 cell with a human cell line inhibited expression of LTR, *gag*, and *pol* genes of PERV. In conclusion, this study suggested that the LTR might be an alternative target for

gene silencing of PERV, and that multi-targeting miRNA had better inhibitory effect than single- targeting miRNA. In an *in vitro* model, the presence of miRNA was able to reduce PERV infectivity in a human cell line.

Key words: *inhibition, PERV, miRNA, long terminal region*

2.1. Introduction

Porcine endogenous retrovirus (PERV) is a member of the *Retroviridae* family. The virus is classified into infectious group (PERV-A, -B, and -C) and non-infectious group (γ2 to γ5, and β1 to β4) (Garcia-Etxebarria et al., 2014, Patience et al., 2001). The genomes of PERV consist of three major viral genes (*gag*, *pol*, and *env*), which are flanked by two long terminal repeats (LTR) (Czauderna et al., 2000, van de Lagemaat et al., 2003).

The LTR of PERV acts as a viral promoter, having an important effect on the integration, replication, and regulation of retrovirus expression (Cohen et al., 2009, Ha et al., 2007, Wilson et al., 2003). PERV is known to integrate different sites in the genome of all pig breeds (Lee et al., 2002), and is expressed in different organs of pigs (Bittmann et al., 2012). PERV's copy number varies between individuals and breeds (Garkavenko et al., 2008), ranging from 9 to 50 copies (Lee et al., 2011). Generally, PERVs are not pathogenic to its natural host, but may cause diseases if they cross a species barrier (Denner and Tönjes, 2012). Therefore, PERV is one of the major threats in xenotransplantation (Blusch et al., 2002, Niemann, 2001, Patience et al., 1997). To date, several methods have been studied in an attempt to knock down active PERVs in the porcine genome, as seen in inhibitory RNA (RNAi) (Chung et al., 2014, Karlas et al., 2004, Miyagawa et al., 2005), short hairpin RNAs (shRNA) (Dieckhoff et al., 2007, Dieckhoff et al., 2008), and the genome-editing technique CRISPR/Cas9 (Yang et al., 2015). The inhibition effect of the above-mentioned studies is nearly based on the mechanisms of post-transcriptional regulation by microRNA (miRNA), which occur in the cytoplasm (Filipowicz et al., 2008). In detail, miRNAs are found embedded,

sometimes in clusters, in long primary transcripts (pre-miRNAs) of several kilobases in length containing a hairpin structure and are driven by RNA Polymerases II (Lee et al., 2004), the polymerase also responsible for messenger RNA (mRNA) expression. Drosha, a nuclear RNasa III, cleaves the stem-loop structure of the pre-miRNA to generate small hairpin precursor miRNAs (pre-miRNAs), which are ~ 70 nucleotides in length (Zeng et al., 2005). The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5, a nuclear transport receptor (Bohnsack et al., 2004, Yi et al., 2003). Following the nuclear export, the pre-miRNAs are proceeded by Dicer into a ~ 22-nucleotide miRNA (mature miRNA) molecule and incorporated into an miRNA-containing RNA-induced slicing complex (Cullen, 2004b).

The application of the gene silencing techniques, which targeted *pol* gene, inhibited 80% - 90% of PERV infectivity (Dieckhoff et al., 2008), even under the condition of long-term expression of the shRNA (Lee et al., 2004). These vector systems have been designed into pig's primary fibroblast and allow production of PERV-controlled transgenic pigs (Semaan et al., 2012a) in which PERV expression would be suppressed for a long time. Through these technologies, PK15 cell lines derived from pigs that have PERV-A and PERV-B (Kaulitz et al., 2011b) will be available on the several knock-down of PERV studies through *in vitro* experiments for safe xenotransplantation. In addition, for the complete inhibition of PERV, an essential technique for safe xenotransplantation, further research is still needed (Salmanidis et al., 2014). To our knowledge, there are a few studies exploring whether targeting promoter regions of PERV could exert an inhibition effect. In this communication, the report a study result in which RNAi

technique was applied to inhibit the expression of PERV *in vitro* by targeting the promoter region of PERV LTRs.

2.2. Materials and methods

2.2.1. Designing miRNA and expression vector

Because the main purpose of this study was to investigate the usefulness of LTR in inhibition of PERV *in vitro*, the design of miRNAs relied on the algorithm of an online program BLOCK-i RNAi Designer (https://rnaidesigner.thermofisher.com/rnaiexpress/). Based on the input sequences of the LTR of PERV subtype B (Genbank: FJ357768, FJ357772, FJ357767, FJ357773, AB280720, and AB274943), two miRNAs (LTR1 and LTR2) targeting the 5' LTR unique 3 (U3) region were automatically selected (Table 3). To prove the specificity of the chosen miRNAs against PERV contained in the PK15 cells used in this study (Korean Cell Line Bank No. 10033), the sequences of LTR from PERV-infected PK15 cells were obtained by the usage of LTR DF/DR primers (Table 4) and were then aligned to the selected miRNAs. In this study, three miRNA expression vectors, which deliver single miRNA (either LTR1- or LTR2- miRNA) and dual miRNA (LTR1 + LTR2 miRNA), were evaluated. For negative control, miRNA expression vector containing no targeting sequence was also included in all runs of the experiment.

2.2.2. miRNA transfection of PK15 cells

Lipofectamine 2000 (Invitrogen, Carlsbad, Calif, United States) was used to transfect the miRNAs in to the PK15 cells according to the manufacturer's reverse transfection method. The transfection condition was set as follows: overnight PK15 cells seeded at a density of 10,000 cells/well (12-well plate), miRNA expression vector (1.6 mg), and 4

mL lipofectamine. Transfected PK15 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), 5% CO2, and without antibiotics.

2.2.3. Generating a stable PK15 clone constitutively expressing miRNA

Because the miRNA expression vector contained blasticidin-resistant gene, this reagent was used to positively select only PK15 cells, which contain expression vector. In brief, transfected PK15 cells were plated at 60% confluence in a 24-well plate. Cells were allowed to adhere to the plate overnight. The next day, the culture medium was replaced with DMEM containing 8 mg/mL blasticidin (sufficient to kill nontransfected PK15 cells). The DMEM containing blasticidin was replenished every 2 - 3 days, and the percentage of surviving cells was observed until blasticidin-resistant colonies could be identified (generally at 14 -16 days after selection). At least 10 blasticidin-resistant colonies (stably transfected) per construct were selected and analyzed to evaluate the level of target gene knock down.

2.2.4. RNA extraction

Total RNA was extracted from transfected PK15 and HeLa cells using the RNA Plus Kit (Qiagen Ltd., Manchester, UK) according to the manufacturer's protocol. Then, RNA concentration was measured with an ultraviolet (UV) spectrophotometer at 260 nm. Total RNA concentration was adjusted to be 500 ng/20 mL. All of the contaminated

genomic DNA was destroyed by adding DNase I (Invitrogen, Carlsbad, Calif, United States). Extracted RNA was converted to complementary DNA (cDNA) by using oligo dTs primer (10 pmol) and MMuLV cDNA synthesis kit (Invitrogen), following the manufacturer's protocol.

2.2.5 Measuring the level of gene inhibition through mRNA expression

Knock down efficiency induced by single miRNA (either LTR1 or LTR2 miRNA) and dual miRNA (LTR1 + LTR2 miRNA) was evaluated using real-time quantitative PCR at the RNA level of the LTR, *gag*, and *pol* genes. Table 4 lists specific primers used to calculate relative quantity (RQ) values between LTR, *gag*, *pol* and cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Quantitative PCR (qPCR) was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Pittsburgh, Pa, United States). All qPCR runs were performed with StepOnePlus Real-Time PCR System (Applied Biosystems). All experiments were repeated three times. Statistical comparisons were made using paired t test ($P < 0.05$) and performed using SPSS program version 15.0.0 (Chicago, Ill, United States). Asterisk marks (*) showed the statistical significance among targeting site groups.

2.2.6. Measuring the level of gene inhibition through reverse transcriptase activity

The knock down efficiency induced by single miRNA (either LTR1 or LTR2 miRNA)

and dual miRNA (LTR1 + LTR2 miRNA) was further evaluated using reverse transcription (RT) activity of the PERV *pol* gene. C-type RT activity kit (Cavidi, Uppsala, Sweden) was used to quantify RT activity of the PERV *pol* gene according to the manufacturer's protocol. Briefly, 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 reverse transcriptase (RT) against the concentration of MMuLV present (lot number 11071). All experiments were repeated three times. Statistical comparisons were made using paired t test ($P < 0.034$) and performed using SPSS program version 15.0.0 (Chicago, Ill, United States). Asterisk marks (*) showed the statistical significance among targeting site groups.

2.2.7. Determining the inhibition effect in a human cell line

The inhibition effect of miRNA on gene expression of PERV was demonstrated on HeLa cells (Korean Cell Line Bank No. 10002). To do so, HeLa cells was grown on a Nunc Carrier Plate (Thermo Scientific) until a monolayer was formed. These cells were then cocultured with PK15 cells (transfected with either dual LTR1 + LTR2 miRNA or only expression vector), which were grown in an insert plate. The co-cultivation of HeLa and PK15 cells were maintained for 24, 48, 72, 96, 120, and 144 hours. At each indicated period, the insert plate and the supernatant were removed. After being thoroughly washed 3 times with phosphate-buffered saline (PBS), the HeLa cells were harvested by trypsinization. Total RNA was extracted from HeLa cells. Relative quantitative real-time

RT-PCR (as described in Materials and Methods section) was performed to demonstrate the reduction in expression of LTR, *gag*, and *pol* genes. All experiments were repeated 3 times.

2.3. Results

2.3.1. Selection of miRNA targeting LTR

Figure 15 A mapped important regions of LTR. It was shown that the miRNAs target the unique 3 region, which is a cis acting promoter for gene expression of PERV (Yi et al., 2003). Compared with the sequences of LTR of PERV recovered from the PK15 cells used in this study (accession numbers KY752830 to KY752832 for clone A-C), the LTR1 miRNA and LTR2 miRNA differed as to the target sequence at 3 and 4 positions, respectively (squares, Figure 15B).

2.3.2. miRNA-mediated degradation of LTR

In this study, miRNAs were designed to target specific regions of the LTR of PERV. Therefore, the miRNA check whether the binding of specific miRNA results in degradation of the target sequence or not. The degradation of LTR was also shown at the mRNA level by comparing the reduction in transcribed RNA of LTR between the miRNA- and mock- transfected PK15 cells. As shown in Figure 16, the transcribed RNA of LTR was reduced 77.1% by LTR1 miRNA, 25.6% by LTR2 miRNA, and 69.8% by LTR1+ LTR2 miRNA each.

2.3.3. Knock down of PERV mRNA expression in vitro

Depicted in Figure 17, it was observed that all 3 miRNAs targeting LTR could

significantly inhibit the mRNA expression of PERV's *gag* and *pol* genes. The level of gene knockdown was different for each miRNA. Measuring the expression of *gag* gene, although LTR1 miRNA inhibited 76.2%, LTR2 miRNA inhibited only 22%. The dual LTR1 + LTR2 miRNA knocked down 76.8% of the *gag* gene expression. The miRNA targeting the LTR of PERV was also found to knock down the *pol* gene expression. Of which, single-target miRNA (LTR1 and LTR2) inhibited 69.8%, and 25.5%, respectively. In contrast, multi-target miRNA (LTR1 + LTR2) showed 77.7% inhibition. Those study results suggested that miRNA targeting promoter region of PERV (the LTR) could knock down several genes of PERV. It appeared that dual miRNA, which targets different locations of the LTR, enhanced gene silencing more than single miRNA. In the following experiment, only dual miRNA (LTR1 + LTR2) was evaluated. Measuring the long-term inhibition effect of dual miRNA was demonstrated on a PK15 clone selected by blasticidin. For the PK15 clone with stable transfection, as shown in Figure 18, multi-targeting LTR1 + LTR2 miRNA resulted in higher decreasing gene expressions of the LTR (89.7%), *gag* gene (82.8%), and *pol* gene (92.7%) in comparison with the PK15 cells transfected with expression vector.

2.3.4. RT activity of PERV

RT activity of the *pol* gene of PERV was measured. The result presented in Figure 19 showed a clear different between the RT activity of miRNA- and mock-transfected PK15 cells. The RT activity was knocked down by 74%, 49.5%, and 91.1% in the presence of

LTR1, LTR2, and dual LTR1 + LTR2 miRNA, respectively.

2.3.5. inhibition of PERV in a human cell line

A co-cultivation system (between PK15 and HeLa cells) was applied to determine whether or not dual LTR1 + LTR2 miRNA could block infectivity of PERV. For the HeLa cells co-cultured with PK15 cells, which were transfected by expression vector only (red lines, Figure 20), the amount of mRNA of LTR, *gag*, and *pol* genes (Figure 20A-C, respectively) clearly increased overtime. The result implied that PERV was released from PK15 cells and infected HeLa cells. Of the HeLa cells co-cultured with PK15 cells, which were transfected by dual LTR1 + LTR2 miRNA, the amount of mRNA of LTR, *gag*, and *pol* genes was always significantly lower than that of the cells co-cultured with vector-transfected PK15 cells (Figure 20A-C). Collectively, in the presence of dual miRNA, PERV infectivity was reduced in a human cell line.

2.4. Discussion

In the literature, the studies of knocking down active PERVs by RNAi were implemented with different genes (Chung et al., 2014, Karlas et al., 2004, Miyagawa et al., 2005). It was shown that the highest inhibition of miRNA against PERV was shown in the *gag* and *pol* genes (Karlas et al., 2004). To our knowledge, there are a few studies that investigated whether the LTR (acts as viral promoter) could be an alternative target for PERV inhibition. The first publication in 2006 indicated a lack of inhibition of PERV expression by small interfering RNA (siRNA) targeting LTR (Li et al., 2006b). However, because these measured the inhibition effect by using a less sensitive method (observing the density of electrophoretic bands), the conclusion might have an error. In contrast, by the usage of quantitative measurement (luciferase assay), a siRNA specifically targeting the LTR of PERV subtype D (the Hand1:E47 transcription factor) was demonstrated to decrease around 60% of the promoter activity of the virus (Jung et al., 2013).

The main purpose of this study was further exploring the inhibition effect of miRNA targeting the LTR region of PERV(the U3 region, which is the key cis-acting promoter for gene expression of PERV (Jung et al., 2013)). The first result of this study seems unusual because miRNA targeting the LTR region degraded the target sequence at mRNA levels. It was known that beside the role of controlling gene expression post-transcriptionally (Filipowicz et al., 2008), miRNA can directly regulate gene expression over promoter regions through miRNA-DNA•DNA triplex structure formation (Paugh et al., 2016, Salmanidis et al., 2014, Toscano-Garibay and Aquino-Jarquin, 2014). To

date, this direct transcriptional regulation is due to the decreased association of RNA polymerase-II, the increased association of hetero chromatising proteins (Salmanidis et al., 2014). In the previous study done by the same authors, the gene expressions of PERV were long-term inhibited by a dual vector expressing both miRNAs that targeted *gag* and *pol* genes (Chung et al., 2014). In that experiment, the mRNA level expression of *gag* and *pol* genes was reduced up to 72% and 88.1%, respectively. Additionally, the RT activity was inhibited by 87.8%. In this present study, by designing miRNA targeting the U3 region at the 50 of the PERV LTR, the dual LTR1 + LTR2 miRNA simultaneously inhibited the expression of *gag* and *pol* genes up to 76.8% and 77.7%, respectively. Dual miRNA was able to repress the RT activity up to 91.1%. From this point of view, the results reported in this communication were comparable with those of the previous study (Chung et al., 2014). However, this study made a step forward as the inhibition efficiency of dual LTR1 + LTR2 miRNA was higher (inhibiting up to 82.8% and 92.7% the expressions of the *gag* and *pol* genes, respectively) in a stable miRNA expression clone of PK15. Although both LTR1 miRNA and LTR2 miRNA targeted the LTR of PERV (at different sites predicted by BLOCK-i RNAi Designer), their knock down efficacies were far different (76.2% vs 22% for *gag* gene; 69.8% vs 25.5% for *pol* gene). In the literature, it had been reported that siRNA targeting the same gene might have suppressed PERV's gene expression differently (Karlas et al., 2004). These might indicate off-target effect of miRNA and/or a role of non-seed factors in modulating down-regulation of miRNA (Chen et al., 2015). Additionally, that result might be explained by imperfect matching between sequences of LTR1/LTR2 miRNA and the LTR sequences of PERV infected

PK15 cells used in this study (Figure 7). Fortunately, because miRNA-RNA interactions were mediated by several modes of binding (Cloonan, 2015), the inhibition effect was still achieved. However, the difference in knock down efficiencies between the LTR miRNAs left a weak point of this study, and thus a thorough investigation is required for the most suitable targets of LTR.

In conclusion, this study suggested that the LTR might be an alternative target for gene silencing of PERV, and that multitargeting miRNA had better inhibitory effect than single- targeting miRNA. In an *in vitro* model, the presence of miRNA was able to reduce PERV infectivity in a human cell line.

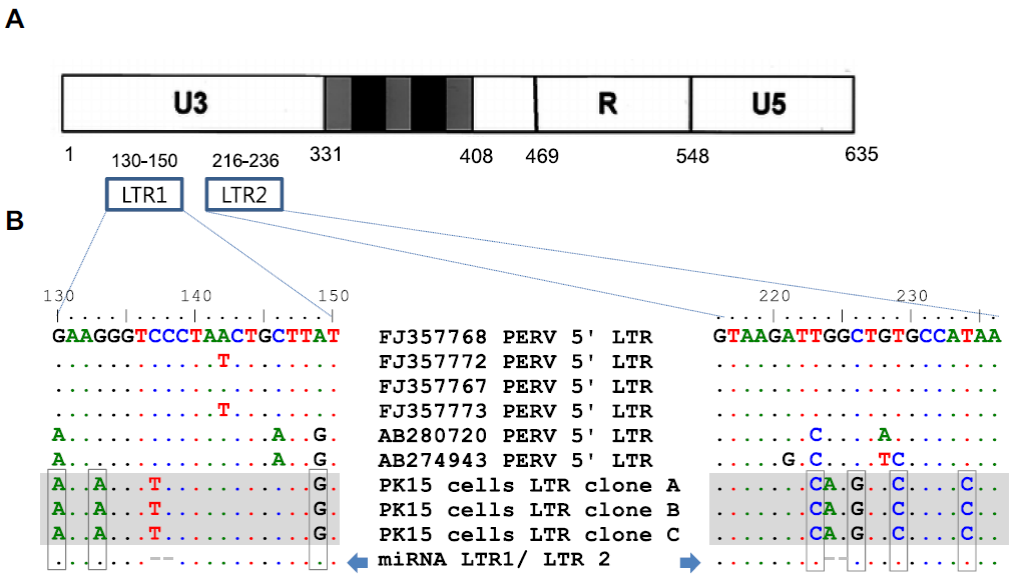

Figure 15. Map of miRNAs. (A) Both of the LTR1 and LTR2 miRNAs are located at the U3 region of the LTR of PERV. The positions of miRNAs were based on GenBank accession number FJ357768. (B) Alignment between miRNAs and the LTR sequences of PERV infected PK15 cells (shaded areas). Mismatches between LTR1 and LTR2 miRNAs and the LTR sequences of PERV were indicated by squares. Gaps in sequences of miRNA were introduced due to the algorithm of BLOCK-iT RNAi Designer.

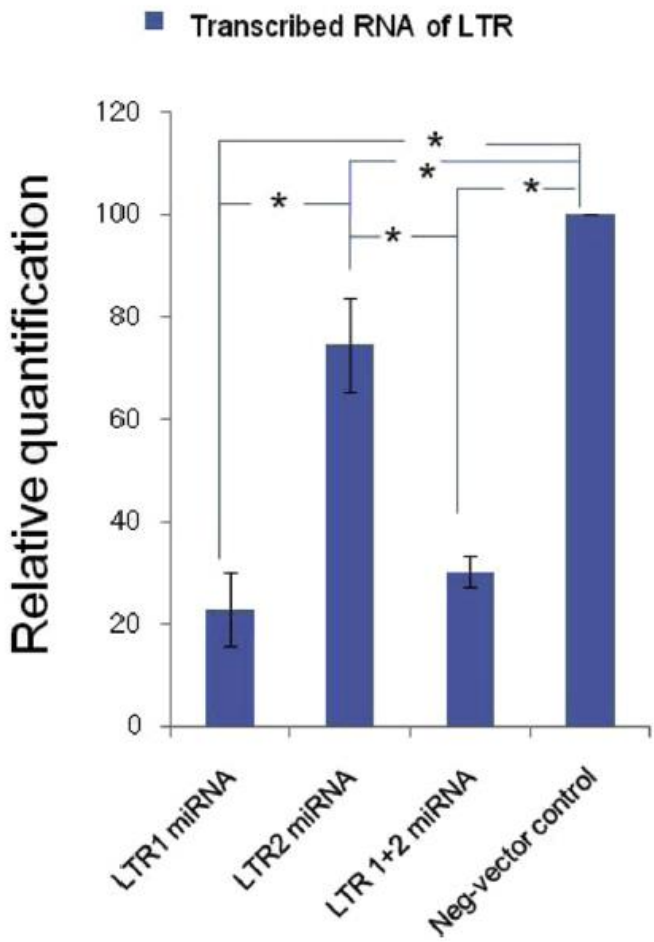

Figure 16. miRNA-mediated degradation of LTR at RNA level. Column indicated level of transcribed RNA of LTR in PK15 cells transfected by single miRNA (LTR1, LTR2), dual miRNA (LTR1 + LTR2), and negative miRNA expression vector.

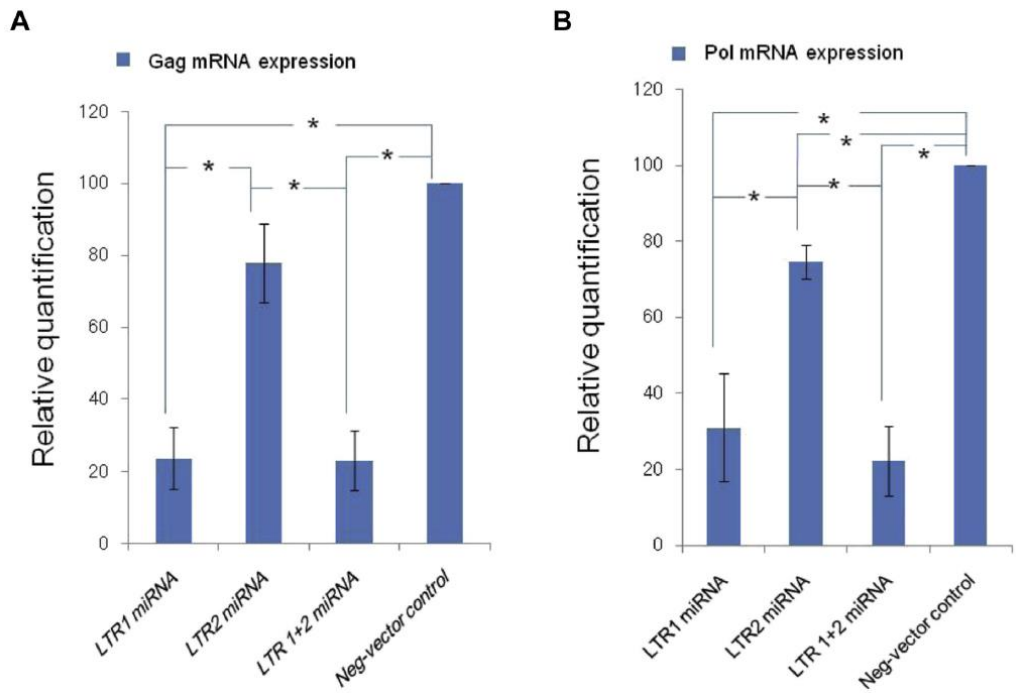

Figure 17. LTR miRNA induced suppression of the *gag* gene (A) and the *pol* gene (B). Column indicated level of gene inhibition by single miRNA (LTR1, LTR2), dual miRNA (LTR1 + LTR2), and negative miRNA expression vector.

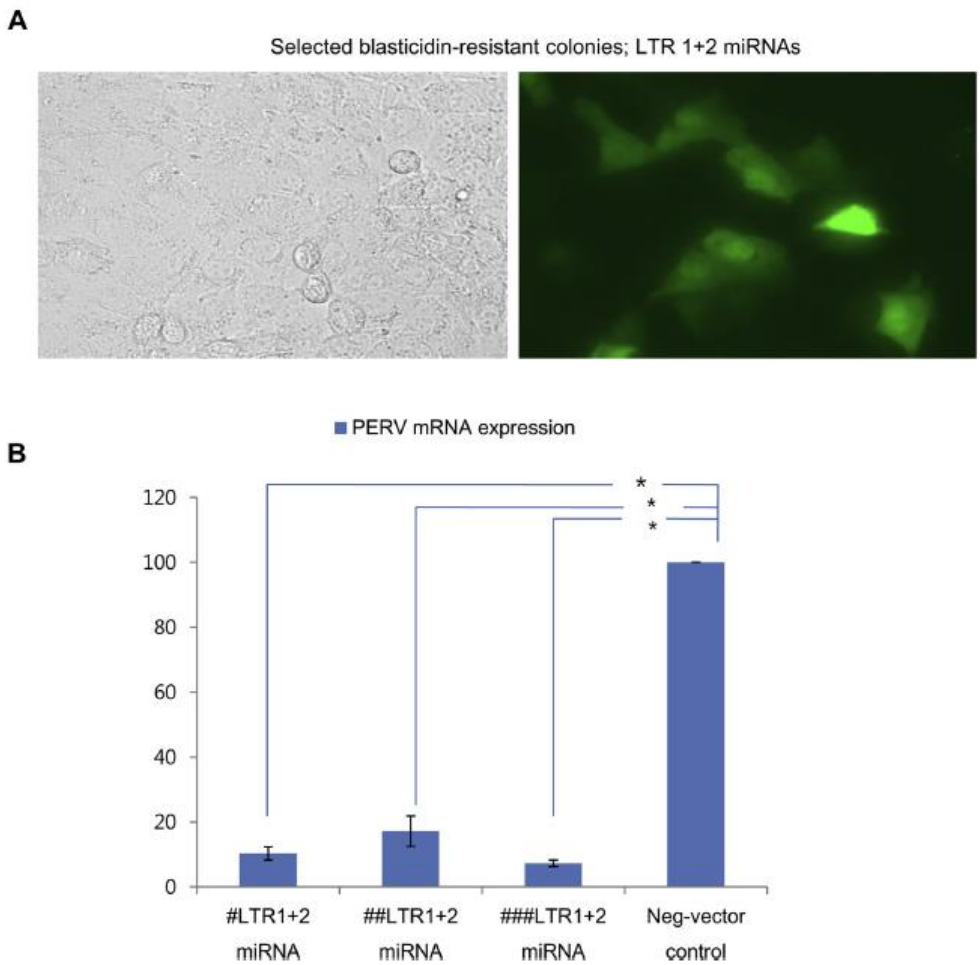

Figure 18. (A) After transfection; LTR1+2 miRNA, selected blasticidin-resistant colonies during 14 days (original magnification 200X; fluorescence microscope). (B) LTR1+2 miRNAs (#) targeting LTR region, (##) targeting *gag* region, and (###) targeting *pol* region. The negvector- transfected control is regarded as standard control (RQ = 100).

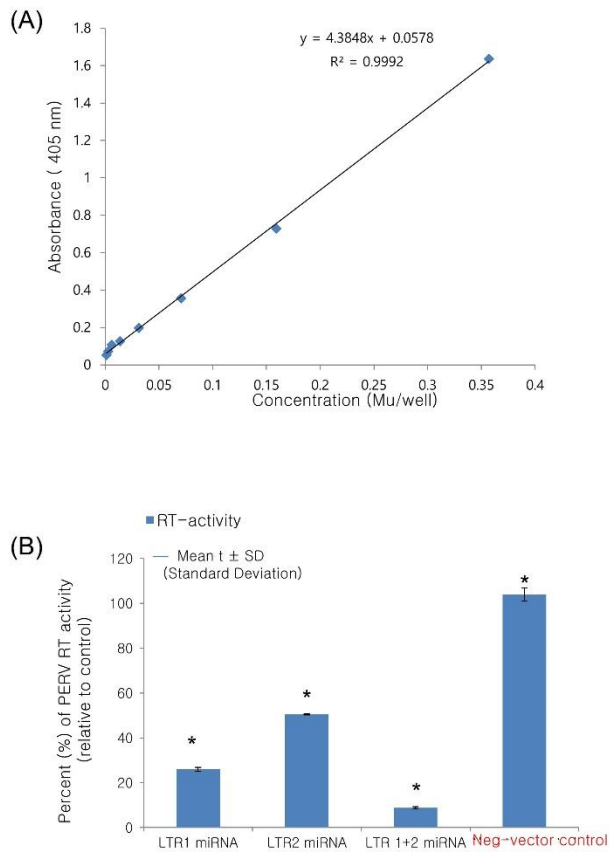

Figure 19. RT activity of PERV in PK15 cells transfected by single miRNA, multi-miRNA, and negative expression vector. (A) Standard curve for C-type RT activity kit was obtained with the serial dilutions of MMuLV rRT against the concentration of MMuLV present (lot number 11071). The equation for the curve is as follows: y = 4.384x + 0.057 (R2 = 0.999). (B) Indicating a practical value of negative control as a standard, each sample value was presenteds above by percentage.

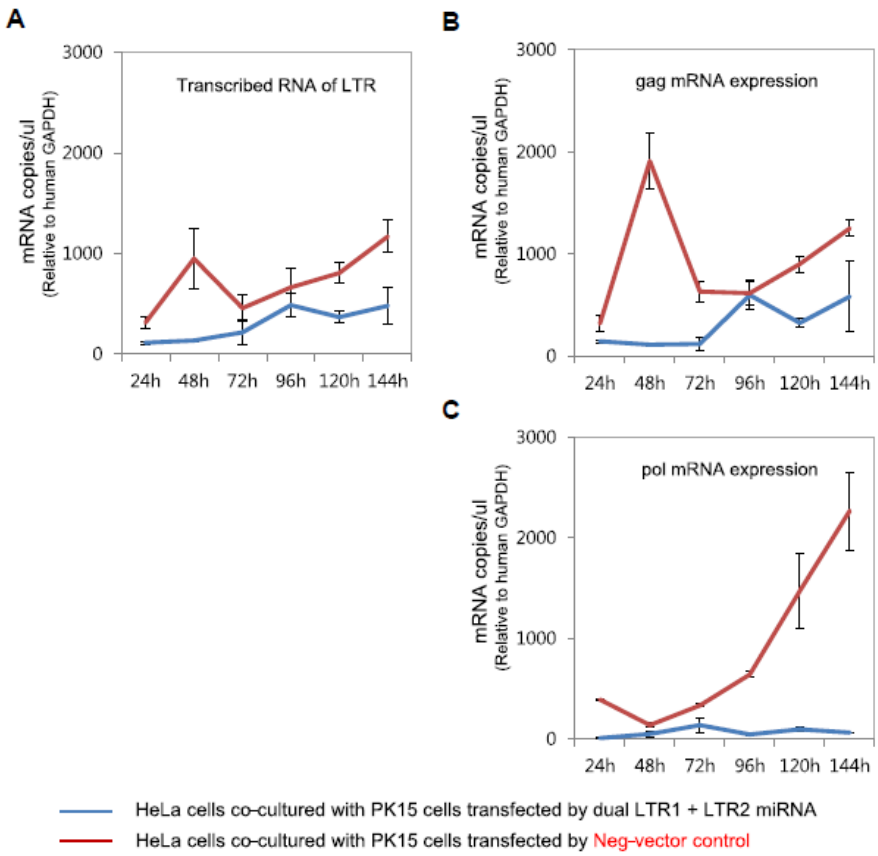

Figure 20. Gene expression of PERV in HeLa cells after co-culture in PK15 cells. Red line was HeLa cells co-cultured with PK15 cells transfected by off-target expression vector. Blue line was HeLa cells co-cultured with PK15 cells transfected by dual LTR1 + LTR2 miRNA.

Table 3. The sequence of miRNA

miRNA	Target site	Pre-miRNA oligo		
		Antisense target sequence	**Loop Sequence**	**Sense target Sequence (nucleotide 1-8 and 11-21)**
LTR1	130-150	GATAAGCAGTTAGGGACCCTTC	GTTTTGGCCACTGACTGAC	GAAGGGTCTAACTGCTTAT
LTR2	216-236	GTTATGGCACAGCCAATCTTAC	GTTTTGGCCACTGACTGAC	GTAAGATTCTGTGCCATAA

Note: The sequence of miRNA was designed by BLOCK-iT™ RNAi Designer, according to PERV's LTR sequence (Genbank accession number FJ357768).

Table 4. Primers for quantitative PCR measuring the level of gene inhibition

Primer name	GenBank	Detection method	Sequence (5'-3')	Location (nt)	Region/ Gene*	Size (bp)
Gapdh F	AF017079	qPCR	F: CAGCAATGCCTCCTGTACCA	776-845	GAPDH	70
Gapdh R			R: GATGCCGAAGTTGTCATGGA			
LTR F	AB535596	qPCR	F: CCCATAAAAGCTGTCCCAACTC	523-600	LTR	78
LTR R			R: GCTGGTGCCCACAGTCGTA			
Gag F	AF038600	qPCR	F: GGTTGCAAAATGAGATTGACATG	1450-1525	*gag*	76
Gag R			R: TCCCTACCTTCAGCCGTGTT			
Pol F	AF038600	qPCR	F: GATCCATGCATCCCACGTTAA	5615-5684	*pol*	70
Pol R			R: ATTTTCAGTCTTTTCGGCTTTCC			
HGapdh F	NM_002046	qPCR	F: GCGCCCCCGGTTTCTATA	70-148	GAPDH	79
HGapdh R			R: GATGCGGCTGACTGTCGAA			

Abbreviations: qPCR, quantitative polymerase chain reaction; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Chapter III

Regulation of porcine endogenous retrovirus by dual LTR1+2 (Long Terminal Region) miRNA in primary porcine kidney cells

Abstract

Porcine endogenous retroviruses (PERVs) integrate into germline DNA as proviral genome that enables vertical transmission from parents to their offspring. The provirus usually survives as part of the host genome rather than as an infectious agent, but may become pathogenic if it crosses species barriers. Therefore, replication-competent PERV should be controlled through selective breeding or knockout technologies. Two microRNAs (miRNAs), dual LTR1 and LTR2, were selected to inhibit the expression of PERV in primary porcine kidney cells. The inhibition efficiency of the miRNAs was compared based on their inhibition of different PERV regions, specifically long terminal repeats (LTRs), *gag*, *pol*, and *env*. Gene expression was quantified using real-time polymerase chain reaction and the C-type reverse transcriptase (RT) activity was determined. The messenger RNA (mRNA) expression of the PERV LTR and *env* regions was determined in HeLa cells co-cultured with primary porcine kidney cells. The mRNA expression of the LTR, *gag*, *pol*, and *env* regions of PERV was dramatically inhibited by dual miRNA from 24 to 144 h after transfection, with the highest inhibition observed for the LTR and *pol* regions at 120 h. Additionally, the RT activity of PERV in the co-culture experiment of porcine and human cells was reduced by 84.4% at the sixth passage. The dual LTR 1+2 miRNA efficiently silences PERV in primary porcine kidney cells.

Key words: *PERV, inhibition, miRNA, long terminal region, primary porcine kidney cell*

3.1. Introduction

Xenotransplantation is defined as the process of transplanting organs, tissues, or cells between different species. Porcine organs and tissues have been employed for human organ transplantation in recent years, prompting the use of designated pathogen-free (DPF) pigs as an alternative source for xenotransplantation (Cascalho and Platt, 2001). However, the presence of bacteria and viruses in porcine tissue continues to pose challenges, with porcine endogenous retrovirus (PERV) being one of the most problematic pathogens (Hering et al., 2009, Denner and Tönjes, 2012).

PERV, a member of the *Retroviridae* family and Gammaretrovirus genus, contains approximately 8 kb of homodimeric RNA genomes similar to other gammaretroviruses (Patience et al., 1997). Organization of the PERV viral gene is similar to that of simple retroviruses, consisting of three major viral genes (*gag*, *pol*, and *env*). These structural viral genes are flanked by long terminal repeats (LTRs), which act as viral promoters (Niebert and Tönjes, 2003). The *gag* gene codes the proteins for matrix, capsid, and nucleocapsid while the *pol* gene codes reverse transcriptase (RT) and integrase enzymes. The viral envelope glycoprotein is encoded by the *env* gene, which is made up of surface transmembrane domains (Niebert and Tönjes, 2003). The major sequences of typical retroviral replication can be summarized as infection, formation of proviral DNA, integration to genome, transcription to RNA, packaging, and budding (Blusch et al., 2002). The repeated sequence blocks harbored in LTRs demonstrate exceptionally strong

promotional activity, whereas repeatless LTR can show a reduced effect of viral replication (Niebert and Tönjes, 2003).

PERV is integrated at approximately 30 to 50 sites in the genome of different pig breeds (Le Tissier et al., 1997) and three receptor classes are known, namely PERV-A, -B, and -C (Patience et al., 2001, Takeuchi et al., 1998). These classes present high sequence homology in the genes for *gag* and *pol*, but differ in the genes encoding the envelope proteins (*env*), which determine the host ranges of the classes (Akiyoshi et al., 1998, Patience et al., 2001, Takeuchi et al., 1998). The *env* gene has the motifs or variable regions A and B along with a proline-rich region in the gp70 protein responsible for host tropism (Le Tissier et al., 1997, Patience et al., 2001). PERV-A and -B were reported to display tropism for the human cell line *in vitro* (Le Tissier et al., 1997), while PERV-C-enveloped vectors were shown to have a 'pig-tropic' or ecotopic host range (Akiyoshi et al., 1998, Takeuchi et al., 1998, Denner, 2008). Furthermore, recombinant PERV-A/C demonstrated higher infectivity (500-fold) to human cells than PERV-A *in vitro* (Denner, 2008). PERV-A and B can infect human cells *in vitro* as well as immunosuppressed mice transplanted with pig islets. Fortunately, no PERV transmission has been reported in humans to date; however, no long-term studies in patients under immunosuppression have been reported either (Denner and Tönjes, 2012). Thus, the risk of infection remains and the deletion or knockdown of PERV sequences could be a solution (Denner, 2015).

Until now, strategies to control replication-competent PERV have involved targeting multiple PERV genes with inhibitory RNA (RNAi) methods (Chung et al., 2014, Chung et al., 2017). Although several PERV target genes have been successfully inhibited both

at the messenger RNA (mRNA) and protein levels, none have been shown to inhibit PERV infectivity beyond 80%–90% (Dieckhoff et al., 2007, Miyagawa et al., 2005), even when using lentiviral vectors to stably express short hairpin RNAs (Dieckhoff et al., 2007, Dieckhoff et al., 2008). Pigs transgenic for short hairpin RNA (shRNA) targeting the *pol* gene were generated through somatic cell nuclear transfer, with the live-born piglets showing normal weight and no malformations (Dieckhoff et al., 2008). The transgene was present in all 6 piglets and the shRNA was detected in all organs. PERV expression was significantly inhibited by up to 94% in all organs of the two transgenic piglets tested (Dieckhoff et al., 2008). These shRNA vectors can be transfected into primary pig fibroblasts, allowing for the production of PERV-controlled transgenic pigs in which PERV expression can be suppressed for prolonged periods. Additionally, genome-wide inactivation of PERV has been achieved using CRISPR/Cas9 (Yang et al., 2015), an essential technique for safe xenotransplantation. In a previous study, the same authors (Chung et al., 2017) attempted to target the LTR region with a dual LTR 1+2 microRNA (miRNA), which was designed based on the sequences of pigs whose organs were eligible for xenotransplantation. In our previous study (Hering et al., 2009), these observed a dramatic reduction in PERV expression following LTR inhibition. However, the experimental design for the previous study did not include primary porcine kidney cells and inhibition of the *env* gene was not investigated (Chung et al., 2017). Therefore, this study was performed on primary porcine kidney cells to determine whether miRNAs that target specific regions of the LTR could simultaneously exert an inhibitory effect on the expression of LTR, *gag*, *pol*, and *env* genes.

3.2. Materials and methods

3.2.1 miRNA and expression vector design

The primary porcine kidney cells (from the kidneys of 5-day-old piglets) used in this study are the raw materials used for manufacturing vaccines at Green Cross Veterinary Products Co., Ltd. The used the LTR DF and DR primers (Table 5) to identify the LTR sequence for the primary porcine kidney cells. The polymerase chain reaction (PCR) product identified DNA fragments corresponding to 600–650 bp. These DNA samples were used for TA cloning, utilizing a commercial TA cloning kit (TOPcloner™ TA kit; Enzynomics, Korea), and subsequently transformed into competent Escherichia coli cells (DH5a) (Chung et al., 2014). Additionally, primary porcine kidney cells were genotyped using the targeting enveloped gene primer sets shown in Table 5 (*env* AF, *env* AR, *env* BF, *env* BR, *env* CF, *env* CR) (Wu et al., 2008).

The LTR sequence of the primary porcine kidney cells was used as the template for designing the miRNAs. Based on our previous report (Chung et al., 2017), dual-targeting (LTR1 + LTR2) miRNAs have better inhibitory effects on the mRNA expression of LTR, *gag*, and *pol* genes than single targeting miRNAs (LTR1 and LTR2). Therefore, these designed a dual LTR 1+2 miRNA based on the methods used in our previously reported study (Chung et al., 2017), in which these had designed a miRNA vector targeting the 5'LTR U3 region of PERV.

3.2.2. Primary porcine kidney cell culture and transfection

Primary porcine kidney cells were cultured in T-75 flasks with Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics for 5 days at 37ºC under 5% CO2. Reverse transfection of miRNAs into primary porcine kidney cells was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The transfection condition was as follows: primary porcine kidney cells were seeded overnight at a density of 80,000 cells/well (6-well plates). The transfection complex consisted of the miRNA expression vector (1.8 μg) and 12 μL Lipofectamine. Transfected primary porcine kidney cells were maintained in 1X Opti-MEM (Gibco, UK) supplemented with 10% FBS without antibiotics at 37ºC and 5% CO2. The transfection efficiency was automatically measured using the cell transfection program from Bertine Instruments (InCellis®, France).

3.2.3. Generating a stable primary porcine kidney cell clone for constitutive miRNA Expression

Blasticidin was used to positively select primary porcine kidney cells containing the miRNA expression vector with a blasticidin-resistant cassette. Briefly, transfected primary porcine kidney cells were seeded in six-well plates and allowed to grow overnight to 60% confluence.

The culture medium was replaced the next day with Opti-MEM media containing 12 μg/ mL blasticidin (sufficient to kill non-transfected primary porcine kidney cells). Blasticin resistant cells were sub-cultured every three to four days and blasticidin-resistant colonies were obtained after 20 days and 6 passages. At least 10 blasticidin-resistant colonies (stably transfected; green fluorescent protein continuous expression) per construct were selected to evaluate the knockdown of the target genes.

3.2.4. Measurement of the inhibition of mRNA expression

The knockdown efficiency induced by dual LTR 1+2 miRNA was evaluated based on the expression of LTR, *gag*, *pol*, and *env* regions using real-time quantitative PCR. Total RNA was extracted from transfected primary porcine kidney cells using the RNA Plus Kit (Qiagen Ltd., UK) according to the manufacturer's instructions, followed by measurement of the RNA concentration with a ultraviolet spectrophotometer at 260 nm. The total RNA concentration was adjusted to 500 ng/20 μL. Genomic DNA contamination was removed using DNase I (Invitrogen). Total RNA was converted into cDNA using 10 pmol oligo dTs primer and the MMuLV cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) in combination with specific primers (Gapdh F and Gapdh R; LTR F and LTR R; *Gag* F and *Gag* R; *Pol* F and *Pol* R; *Env* F and *Env* R) (Table 5). Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was used as the internal control. All qPCR experiments were performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, USA).

3.2.5. Measurement of inhibition through RT activity

The effect of miRNA on the expression of PERV was also measured based on the RT activity of the primary porcine kidney cells maintained in blasticidin medium (using passages 2, 4, and 6). The C-type RT activity kit (Cavidi, Sweden) was used to quantify the RT activity of the PERV *pol* gene according to the manufacturer's protocol. Briefly, RT activity was determined for wells with a reading of A405 within the linear range. Additionally, a standard curve for the C-type RT activity kit was obtained through serial dilutions of MMuLV rRT against the concentration of MMuLV present (LOT number 34213).

3.2.6. Inhibition of PERV in primary porcine kidney cells co-cultured with HeLa cell lines

To further investigate the inhibition efficiency of the dual miRNA, co-cultured the stably expressed miRNA from the primary porcine kidney cells with HeLa cells free of PERV. If the dual miRNA worked properly, the level of PERV gene expression in the HeLa cells

would be negligible. Primary porcine kidney cells and HeLa cells (Korean Cell Line Bank No. 10002) were respectively cultured overnight in insert and 24-well carrier plates (Nunc Cell Culture Inserts and Carrier plate, Thermo Scientific) using 10% FBS in Opti-MEM and incubated at 37°C in a 5% CO2 incubator. The primary porcine kidney cells used in this study were transfected with LTR 1+2 miRNA at the sixth passage and showed stable inhibition of PERV.

Additionally, primary porcine kidney cells transfected with negative vector were used as the negative control. After one night (a monolayer formed), the insert plate was transferred to a 24-well carrier plate for co-cultivation. The co-cultivation of HeLa and primary porcine kidney cells was maintained for 24, 48, 72, 96, and 120 h. After incubation for the above time periods, the insert plate with primary porcine kidney cells and the supernatant were completely removed from the 24-well carrier plate and the HeLa cells were harvested with trypsin-ethylenediaminetetraacetic acid (0.05%). RNA was extracted from the HeLa cells and RT-PCR was performed to identify the PERV inhibitory effect according to the mRNA level in the co-cultivated human cells. The expression of the target genes was calculated using relative standard curve values between LTR, *gag*, *pol*, and *env* genes and cellular human GAPDH genes using the specific primers shown in Table 5 (HGapdh F and HGapdh R; LTR F and LTR R; *Gag* F and *Gag* R; *Pol* F and *Pol* R; *Env* F and *Env* R). Each gene-expression value was normalized to human GAPDH and presented as copies/μL. All qPCR runs were performed on the StepOnePlus Real-Time PCR System (Applied Biosystems).

3.2.7. Statistical analysis

All experiments were repeated 3 times. Statistical comparisons were performed using paired T-Test ($p < 0.05$) within the SPSS program (version 15.0.0) (SPSS Inc., USA). An asterisk (*) denotes statistical significance among the mean data.

3.3. Results

3.3.1. Transfection efficiency

The first part of this study evaluated whether the designed LTR 1+2 miRNA works properly in primary porcine kidney cells. As shown in Figure 21A, the newly designed miRNA had no mismatches with the LTR sequence of the primary porcine kidney cells. Dual LTR 1+2 miRNAs (targeting nucleotides 130–150 and nucleotides 216–236) for the conserved regions of both primary porcine kidney cell types (GenBank accession numbers FJ357767 and FJ357768) (Figure 21A) were obtained. With the transfection method used, which were able to achieve 60%–70% transfection efficiency for the LTR 1+2 miRNA vector in primary porcine kidney cells (Figure 22).

3.3.2. In vitro knockdown of PERV mRNA in primary porcine kidney cells

The primary porcine kidney cells used in this study were confirmed to harbor PERV-B through genotype-specific PCR (Figure 21B). After the dual LTR 1+2 miRNA was successfully transfected, a decrease in the gene expression level from approximately 20% to 80% (compared to the negative control) was observed in the LTR, *gag*, *pol*, and *env* genes after 24 h (dashed lines, Figure 23). For the same genes, the level of gene expression inhibition fluctuated over time from 24–120 h post-transfection (Figure 23).

However, in the homologous population of LTR 1+2 miRNA-transfected primary porcine kidney cells (selected with blasticidin), a significant trend of reduced gene expression was observed from passage 2 to passage 6 (Figures 24 and 25). At the sixth passage, compared to the primary porcine kidney cells transfected with the negative miRNA expression vector, the clone of primary porcine kidney cells that stably expressed dual LTR 1+2 miRNA significantly inhibited the LTR (86.9%), *gag* (61.2%), and *env* genes (85.2%) (Figure 24).

3.3.3. RT activity and pol gene expression

We measured the RT activity and mRNA expression of the PERV *pol* gene. The results showed a clear difference between the RT activity of miRNA- and mock-transfected primary porcine kidney cells (Figure 25). The RT activity was reduced by 58.7%, 79.2%, and 84.4% in the presence of blasticidin in sub-cultured cells at passages two, four, and six, respectively. Statistical significance was confirmed in passages 2 and 6 with paired t-tests. The mRNA expression level of the *pol* gene showed patterns that corresponded with RT activity in passage 6, which was the most efficient at 86.9%. Passages 2 and four had RT activity of 53.8% and 76.9%

3.3.4. Expression of PERV in HeLa cells after co-culture with PERV-inhibited primary porcine kidney cells

HeLa cells (human origin) are known to be free of PERV. To determine the inhibition efficiency of dual miRNA, which measured the gene expression of PERV LTR, *gag*, *pol*, and *env* in HeLa cells after co-culture with stably expressed miRNA from primary porcine kidney cells.

PERV expression in the infected HeLa cells was measured with real-time PCR using primers against the PERV LTR, *gag*, *pol*, and *env* regions. Compared to the negative control (Figure 26, gray lines), the expression of the LTR, *gag*, *pol*, and *env* genes was significantly lower (Figure 26A-D) in the dual LTR 1+2 miRNA transfected cells, measured by copies/μL. The mRNA copy numbers increased progressively with time. In particular, changing the co-culture incubation time from 48 to 120 h resulted in the largest change in the PERV amount in the negative vector control group (gray lines) contrary to HeLa cells co-cultured with primary porcine cells transfected with dual LTR 1+2 miRNA (orange lines).

3.4. Discussion

Previous experiments in PK15 cells, which are porcine-derived cells, showed inconsistent results (3–5 nt) at the target sites. However, I observed a dramatic reduction in PERV expression following LTR inhibition. In this study, I wanted to determine whether LTR inhibition could result in the reduction of PERV in primary porcine kidney cells. Therefore, the design for this study was based on primary porcine kidney cells without mismatches for the LTR 1+2 miRNA targeting region (Figure 21). Genotyping results, as shown in Figure 21B, indicated that the primary porcine kidney cells have the PERV-B type. These focused on targeting the same LTR region described in a previous report (Chung et al., 2014).

I confirmed that between the mRNA and RT activity levels, the highest inhibition efficiency was obtained at the sixth passage, similar to the results in our previous study (Chung et al., 2014). Over time, untransfected (without blasticidin-resistant gene) LTR 1+2 miRNA in the primary porcine kidney cells were killed, leaving blasticidin-resistant colonies with continuous expression of green fluorescent protein (GFP) and PERV inhibition. In a previous study (using the PK15 cell line), the mRNA expression decreased for LTR (89.7%), *gag* (82.9%), and *pol* (92.7%). In addition, the RT activity was inhibited by 91.1%. In this study (using primary porcine kidney cells), the dual LTR 1+2 miRNA simultaneously inhibited the expression of the LTR (86.9%), *gag* (61.2%), *pol* (84.4%), and *env* genes (85.2%). Dual miRNA was able to inhibit the RT activity by up to 86.9%. Therefore, I observed inhibition

efficiency up to 86.9%, similarity to the results in our previous study (Chung et al., 2017) in which PERV genes were inhibited in primary porcine kidney cell colonies after 20 days at the sixth passage. Overall, our results suggested that the LTR 1+2 miRNA targeting the promoter region of PERV (LTR) could knock down several PERV genes in primary porcine kidney cells. In addition, I confirmed the *env* region of PERV was inhibited as well.

As shown Figure 26 of the HeLa cells co-cultured with primary porcine kidney cells, which were transfected by dual LTR1 + LTR2 miRNA, the amount of mRNA of LTR, *gag*, *pol*, *env* genes was always significantly lower than that of the cells co-cultured with vector-transfected primary porcine kidney cells. The study, final goal is to develop PERV-inhibited pig using nuclear replacement technique within primary cells. In order to achieve this goal, the current study used only cancer cell line (HeLa), but in future studies, it is necessary using the human primary cells such as fibroblasts, epithelial, and endothelial cells co-cultured with primary porcine kidney cells to confirm the expression of the PERV.

In conclusion, this study confirmed that miRNA targeting the LTR region of PERV could positively inhibit the expression of PERV in primary porcine kidney cells. Dual LTR 1+2 miRNA reduced gene expression for the LTR region as well as the expression of functionally important PERV genes such as *gag*, *pol*, and *env*.

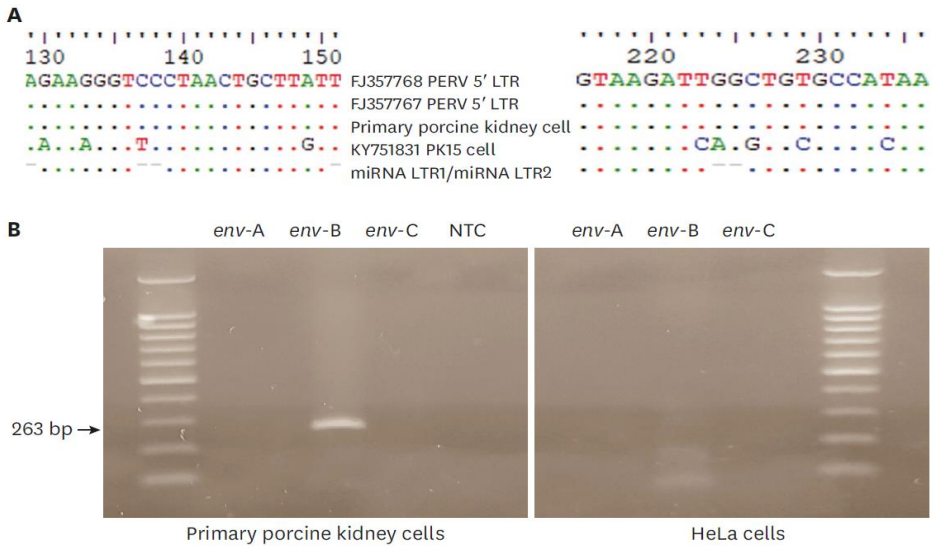

Figure 21. Confirmation of miRNA targeting site in primary porcine kidney cells and HeLa cells (A) Both LTR1 and LTR2 miRNAs are located at the U3 region of the LTR of PERV (Chung et al., 2017). Alignment between miRNAs and the LTR sequences (GenBank accession numbers FJ357767 and FJ357768) was confirmed and primary porcine kidney cells were used for this study. Matches between the LTR1 and LTR2 miRNAs and the LTR sequences of PERV are indicated. (B) Genotyping by RT-PCR in primary porcine kidney cells and HeLa cells using envelop primer sets (Wu et al., 2008). No template control.

miRNA, microRNA; LTR, long terminal repeat; PERV, porcine endogenous retrovirus; RT-PCR, reverse transcriptase polymerase chain reaction.

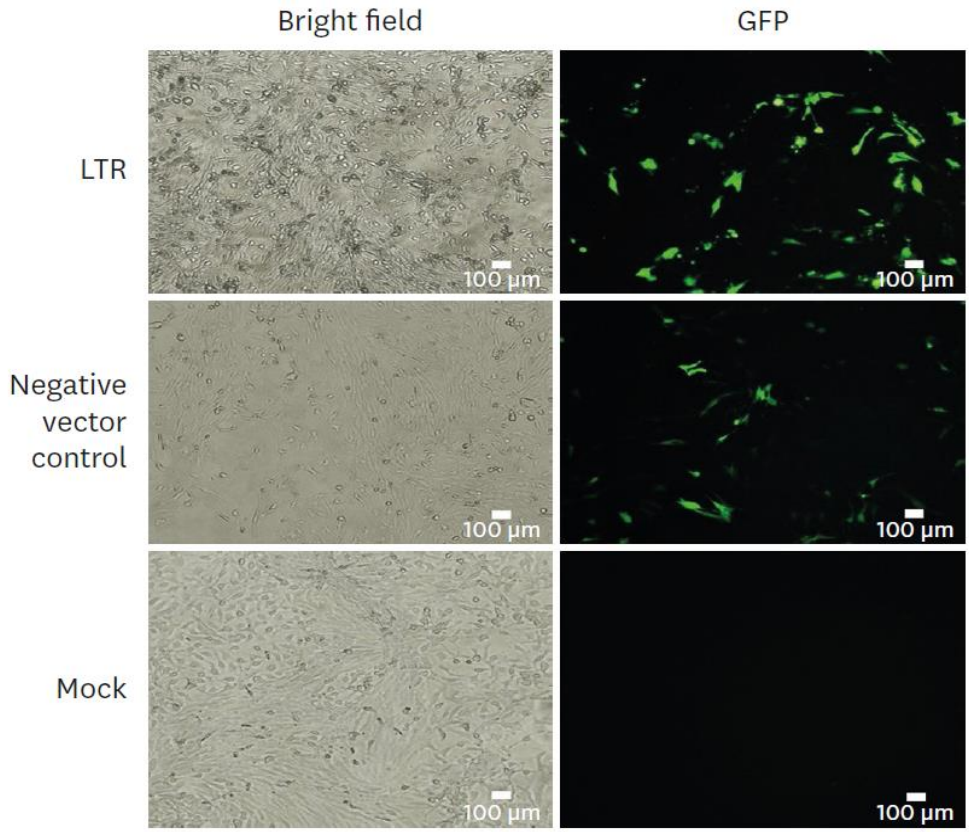

Figure 22. Determination of miRNA transfection efficiency. Lipofectamine 2000 (Invitrogen) reagent was used to transfect the plasmids (dual LTR 1+2 miRNA, and negative vector control miRNA) into primary porcine kidney cells. Mock-transfected cells were subjected to the transfection process without the addition of miRNA (cells were treated with transfection reagent only). Bright field images (optical microscopy) on the left side and the GFP images (fluorescence microscopy) on the right were taken 24 h post-transfection. The scale bar in the micrographs = 100 µm.

LTR, long terminal repeat; GFP, green fluorescent protein; miRNA, microRNA.

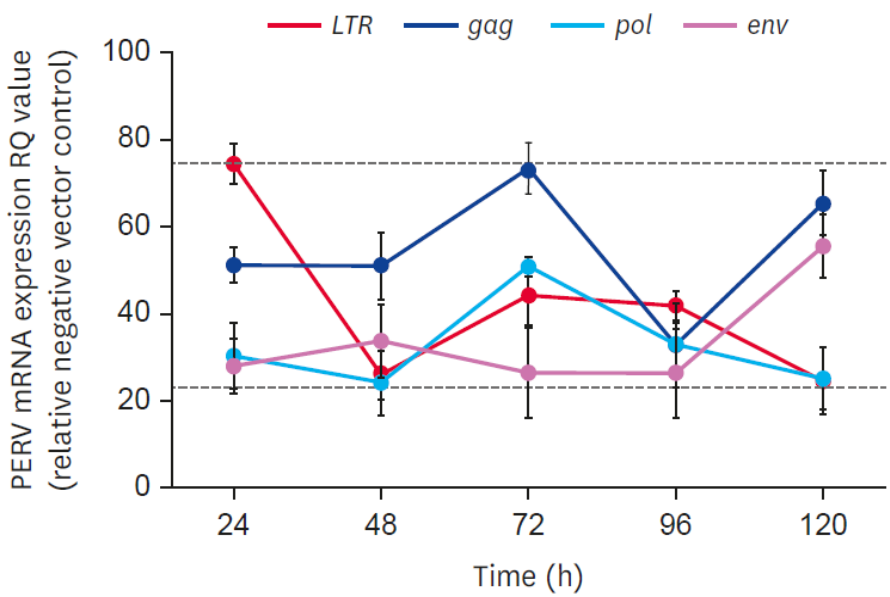

Figure 23. Knockdown efficiency of miRNA (dual LTR1+LTR2)-transfected primary porcine kidney cells. Real-time PCR was used to detect the expression level of PERV mRNA in primary porcine kidney cells relative to the standard negative vector control (RQ = 100).

miRNA, microRNA; PCR, polymerase chain reaction; PERV, porcine endogenous retrovirus; mRNA, messenger RNA; RQ, relative quantity; LTR, long terminal repeat; SD, standard deviation.

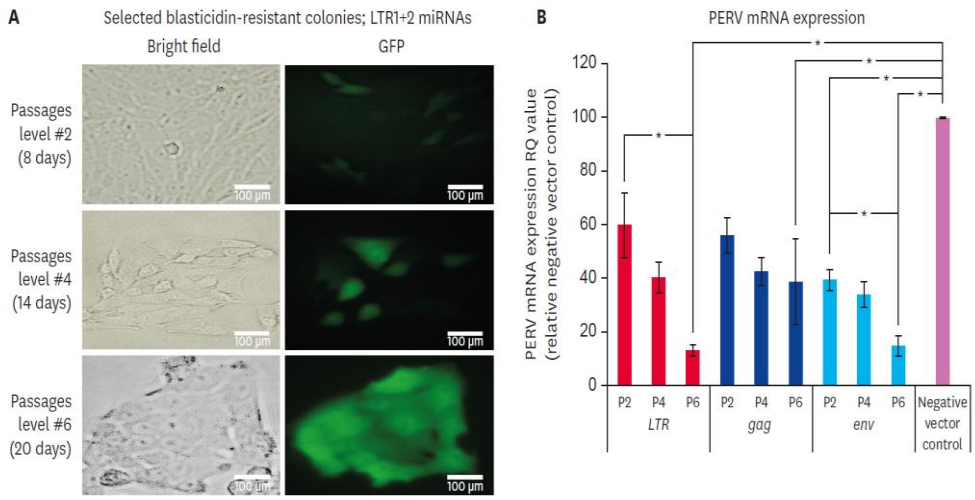

Figure 24. Selected blasticidin-resistant colonies and analysis of PERV *pol* gene mRNA levels based on passage numbers. (A) Following transfection with dual LTR 1+2 miRNA, blasticidin-resistant colonies were selected after 20 days at passage 6. (B) Dual LTR 1+2 miRNA targeting the LTR, *gag*, and *env* regions. The negative vector-transfected control is regarded as the standard control (RQ = 100 value). Bright field images (optical microscopy) are shown on the left and fluorescence microscopy images (GFP filter) are shown on the right. The scale bar in the micrographs = 100 µm.

PERV, porcine endogenous retrovirus; mRNA, messenger RNA; LTR, long terminal repeat; miRNA, microRNA; GFP, green fluorescent protein; RQ, relative quantity.

*Asterisk marks indicate statistical significance between targeting site groups and negative vector control group.

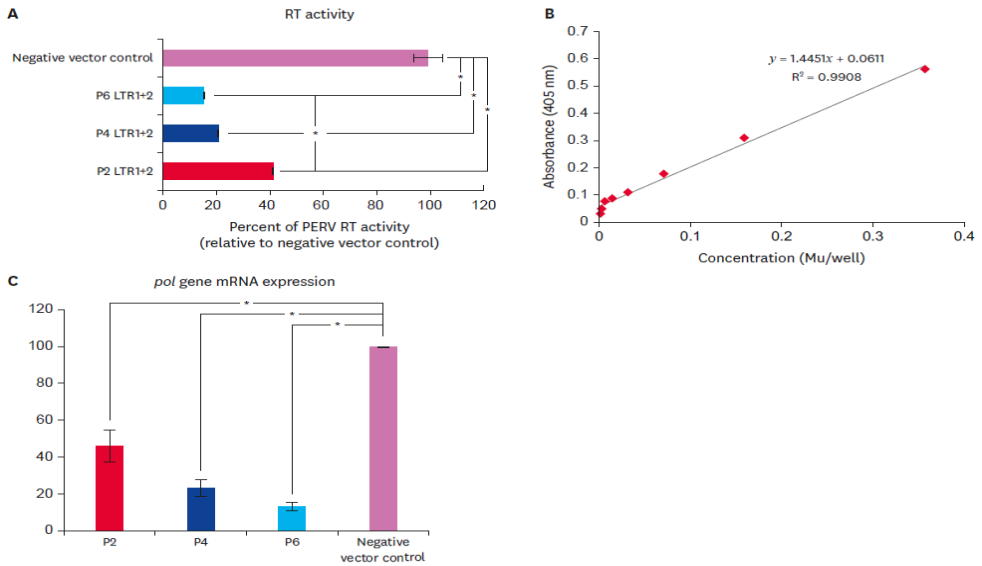

Figure 25. PERV *pol* gene analysis by 2 methods: reverse transcriptase (RT) activity and mRNA levels in blasticidin-resistant primary porcine kidney cells over 20 days. (A) Plots show the negative vector control as the standard and sample values are represented by percentage for passages 2, 4, and 6. (B) A standard curve for the C-type RT activity kit was obtained with serial dilutions of MMuLV rRT with respect to the concentration of MMuLV present. The equation for the curve is as follows: y = 1.4551x + 0.0611 (R2 = 0.99). (C) Real-time PCR was used to detect the expression level of the PERV *pol* gene mRNA in primary porcine kidney cells relative to the standard negative vector control (RQ = 100).

PERV, porcine endogenous retrovirus; RT, reverse transcriptase; mRNA, messenger RNA; PCR, polymerase chain reaction; RQ, relative quantity.

*Asterisk marks indicate statistically significant differences among targeting site groups ($p < 0.05$) by paired t-test.

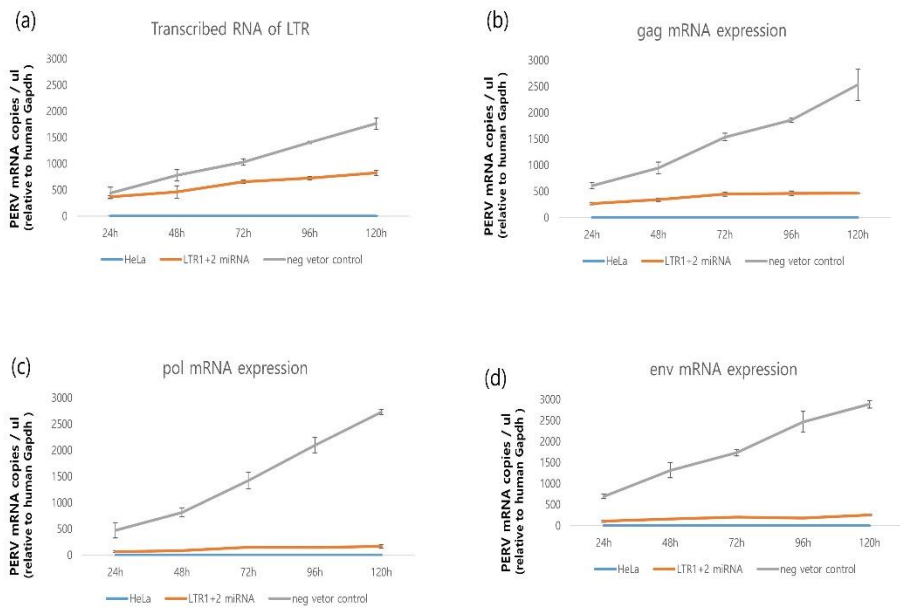

Figure 26. Gene expression of PERV in HeLa cells after co-culture with primary porcine kidney cells stably expressing miRNA. Gray line indicates HeLa cells cocultured with primary porcine kidney cells transfected with off-target expression vector. Orange line denotes HeLa cells co-cultured with primary porcine kidney cells transfected with dual LTR1 + LTR2 miRNA. Blue line represents naive HeLa cells. PERV, porcine endogenous retrovirus; miRNA, microRNA; mRNA, messenger RNA.

Table 5. Primers used for the detection of PERV regions

Primer	Method	Sequence (5'-3')	Location † (nt)	Region (Genes)	Size (bp)	Reference
Gapdh F	qPCR	CAGCAATGCCTCCTGTACCA	776-845	GAPDH	70	(Chung et al., 2017)
Gapdh R		GATGCCGAAGTTGTCATGGA				
LTR F	qPCR	CCCATAAAAGCTGTCCCAACTC	523-600	LTR	78	(Chung et al., 2017)
LTR R		GCTGGTGCCCACAGTCGTA				
Gag F	qPCR	GGTTGCAAAATGAGATTGACATG	1450-1525	*gag*	76	(Chung et al., 2017)
Gag R		TCCCTACCTTCAGCCGTGTT				
Pol F	qPCR	GATCCATGCATCCCACGTTAA	5615-5684	*pol*	70	(Chung et al., 2017)
Pol R		ATTTTCAGTCTTTTCGGCTTTCC				
Env F	qPCR	AAGGCACCTGCATAGGAAAGG	6671-6741	*env*	71	*This study*
Env R		TTGATTAAAGGCTTCAGTGTGGTTA				
HGapdh F	qPCR	GCGCCCCCGGTTTCTATA	70-148	GAPDH	79	(Chung et al., 2017)
HGapdh R		GATGCGGCTGACTGTCGAA				
env AF		TGGAAAGATTGGCAACAGCG		*env*	359	

env AR	RT-PCR	AGTGAATGTTAGGCTCAGTGG				(Wu et al., 2008)
env BF	RT-PCR	TTCTCCTTTGTCAATTCCGG		*env*	263	(Wu et al., 2008)
env BR		TACTTTATCGGGTCCCACTG				
env CF	RT-PCR	CTGACCTGGATTAGAACTGG		*env*	281	(Wu et al., 2008)
env CR		ATGTTAGAGGATGGTCCTGG				
LTR DF	PCR	TTCTTGCTGTTTTAGGGCTTG	1-635	LTR	635	*This study*
LTR DR		AATGAAAGGCCAGTAGAAAGA				

PERV, porcine endogenous retrovirus; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcriptase polymerase chain reaction.

*GenBank: AF038600.1; Sus scrofa porcine endogenous retrovirus PERV-MSL mRNA, complete sequence.

General conclusions

All pigs have the porcine endogenous retroviruse (PERV) genome inserted in the pig germline and it is transmitted to offspring. To reduce the release of PERVs by porcine transplants, a new approach, RNA interference, was applied. For confirm of PERV expression relative quantitative real-time polymerase chain reaction (PCR), RT-activity analysis, and fluorescence in situ hybridization assay, were performed.

The most inhibitory sequences were selected and expressed as short hairpin RNAs (shRNAs) by a POL II vector system leading to persistent suppression of PERV replication. Here, the data's show significant reduction of PERV expression by multi-targeting miRNA corresponding to different parts of the viral genes LTR, *gag*, *pol*, and *env*.

In the first study, multi-targeting the *gag* and *pol* genes of PERV in PK15 cells showed the inhibition of PERV mRNA and RT-activity. The second study focused on LTR region of PERV by multi-targeting (LTR1 + LTR2) miRNA, and results found dramatic regulation PERV mRNA and RT-activity. In the final study, results suggested that the LTR1 + LTR2 miRNA targeting the U3 promoter region of PERV (LTR) could knock down several PERV genes (LTR, *gag*, *pol*, *env*) in primary porcine kidney cells. These study results confirmed that miRNA targeting the *gag*, *pol*, and LTR regions of PERV could positively inhibit the expression of PERV in primary porcine kidney cells and PK15 cells.

1. Inhibition of PERV in PK15 cell by multitargeting (gag2 + pol2) miRNA

RNAi is a technique used for gene knockdown which target multiple genes simultaneously, such as both *gag* and *pol* genes critical for replication of PERVs. Previously, two effective siRNAs (*gag*2, *pol*2) reduced the expression of PERVs. Concurrent treatment of these two siRNAs (*gag*2 + *pol*2) showed knockdown efficiency of up to 88% as compared to negative control. However, despite the high initial knockdown efficiency noted at 48 hours after transfection, this effect may only be for transient suppression of PERVs. A multi-targeting vector was designed, which contained both *gag* and *pol* genes, and making use of POL II mRNA expression vector, allowed for persistent and multiple targeting.

In addition, an shRNA technique, such as miRNA, can be used for targeting of gene expression. Manipulating the antibiotic resistance characteristics of this vector, miRNA transfected PK15 cells (*gag*2 + *pol*2) were selected over 10 days. An 88.1% reduction in the 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 multi target (*gag*2 + *pol*2) miRNA group. Therefore, the gene knockdown systems (siRNA and shRNA) using multiple targeting effectively inhibited PERVs.

2. Inhibition of PERV in PK15 cell by multi targeting (LTR1 + LTR2) miRNA

The results demonstrated that miRNA targeting the LTR region degraded the

target sequence, but simultaneously inhibited the mRNA expression of both *gag* and *pol* genes of PERV. The LTR1, LTR2, and dual (LTR1 + LTR2) miRNA inhibited 76.2%, 22%, and 76.8% of *gag* gene expression, respectively. Similarly, miRNA knocked down *pol* gene expression of LTR1 (69.8%), LTR2 (25.5%), and LTR1 + LTR2 (77.7%), respectively. A stable PK15 clone constitutively expressed the dual LTR1 + LTR2 miRNA and exhibited higher inhibition up to 82.8% and 92.7% of the expressions of the *gag* and *pol* genes, respectively. The result of co-cultivation of dual LTR1 + LTR2 miRNA PK15 cell transfected with a human cell line inhibited expression of LTR (U3 promoter region of PERV), *gag*, and *pol* genes of PERV. This results suggests that the LTR (U3 promoter region of PERV) might be an alternative target for gene silencing of PERV, and that multi-targeting miRNA had a better inhibitory effect than single targeting miRNA. Using a human cell line in vitro model, the presence of miRNA reduced PERV infectivity.

3. Inhibition of PERV in primary porcine kidney cells by multi targeting (LTR1 + LTR2) miRNA

Two microRNAs, dual LTR1 and LTR2, were selected to inhibit the expression of PERV in primary porcine kidney cells. The inhibition efficiency of the miRNAs was compared based on their inhibition of different PERV regions, specifically long terminal repeats (LTRs), *gag*, *pol*, and *env*. Gene expression was quantified using real-time polymerase chain reaction and the C-type reverse transcriptase (RT) activity was

determined. The mRNA expression of the PERV LTR and *env* regions was determined in HeLa cells co-cultured with primary porcine kidney cells. The mRNA expression of the LTR, *gag*, *pol*, and *env* regions of PERV was dramatically inhibited by dual miRNA from 24 hours to 144 hours after transfection, with the highest inhibition observed for the LTR and *pol* regions at 120 hours. Additionally, the RT activity of PERV in the co-culture experiment of porcine and human cells was reduced by 84.4% at the sixth passage. The dual LTR 1+2 miRNA efficiently silences PERV in primary porcine kidney cells.

In conclusion, while multi targets have been identified that lead to decreases in several PERV genes of mRNA and RT activity, have been shown to inhibit PERV infectivity beyond 80 – 90%, when using miR expression vectors to stably express the shRNA. Although these studies generating PERV-specific muti-targeting miRNAs has shown decreased PERV mRNA expression, the study have not yet examined whether this correlates with a decrease in infectious PERV, a critical element to demonstrate whether the genetic modification will reduce the risk of transmission. In the face of still uncertain risks, efforts should continue *in vitro* and *in vivo* of the area of identifying a means to prevent transmission, development of improved co-culture system with primary human cells, animal models and improved assays to monitor for PERV transmission in xenotransplantation product recipients. These works are needed before xenotransplantation becomes a safe medical procedure.

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국문 초록

다중 표적 RNA 간섭에 의한 돼지 내인성 레트로바이러스 억제

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수의미생물학 전공

세포, 조직 또는 종 사이의 이종 이식에서 돼지의 장기는 장기 부전 및 치료를 위한 인간 장기 부족에 대한 해결책으로 제안된다. 그러나 모든 돼지는 항상 돼지 생식 계열에 돼지 내인성 레트로바이러스 (PERV) 게놈이 삽입되어 자손에게 전파된다. 따라서 PERV는 잠재적인 위험으로 인한 이종 이식의 주요 위협 중 하나이다. 최근에, RNA 간섭 기술은 유전자 발현을 억제 시키기 위해 개발되었으며, 이종 이식의 안전성을 증가시키는 좋은 대안이 될 수 있다.

이 연구에서, PERV 유전자의 다중 표적화를 통한 RNA 간섭 전략을 사용하여 PERV 발현의 억제를 돼지 세포에서 수행하였다.

첫 번째 챕터에서 PERV의 *pol* 과 *gag* 유전자를 동시에 억제하는 전략을 세웠는데, *pol* 유전자 타깃의 RNA 간섭은 PERV 복제에서 가장 중요한 역할을 수행하기 때문에 역 전사 및 복제를 저해하고 성장에 필수적인 캡시드 단백질을 암호화하는 *gag* 유전자의 억제는 바이러스 입자가 만들어지는 것

을 차단하여 PERV의 *gag* 및 *pol* 유전자가 동시에 표적화되어 억제될 수 있다면, 이는 PERV 발현의 현저한 억제를 위한 훌륭한 전략이 될 것이라는 가설을 세우게 되었다.

siRNA 연구에서는 *gag*의 *gag1*과 *gag2* siRNA, *pol*의 *pol1*과 *pol2* siRNA 중 가장 효과적인 2 지역의 siRNA (*gag2*, *pol2*)가 PERV의 발현을 감소시키는 것으로 밝혀졌다. 이들 2 개의 siRNA (*gag2*+*pol2*)의 다중 표적화된 그룹은 음성 대조 군과 비교하여 최대 88 %의 억제 효율을 나타냈다. 그러나 siRNA로 인한 형질 감염 48 시간 후 높은 초기 억제 효율에도 불구하고, PERV를 일시적으로만 억제하였다. 그래서, *gag2* 및 *pol2* 유전자를 모두 클로닝하고 POL II miR 발현 벡터를 사용하여 다중 표적화 벡터를 설계하여, 지속적인 억제를 가능하게 하였다. 이 벡터에서 항생제 내성 특성을 이용하여, miRNA 형질 감염된 PK15 세포 (*gag2*+*pol2*)가 2주 동안 선택되었다. mRNA 발현 수준에서 표적화된 *pol* 과 *gag* 유전자에서 88.1%와 72%의 감소가 발견되었다. 또한, 역전사 효소 활동 (Reverse Transcriptase activity) 분석 및 형광동소보합법 (Fluorescence in situ Hybridization) 분석을 수행하였고, 다중 표적화(*gag2*+*pol2*) miRNA 그룹에서 가장 높은 억제 효율을 입증하였다.

따라서, 상기 결과에 따르면, 다중 표적화 전략을 통한 유전자 발현 억제 시스템 (siRNA 및 shRNA)은 PERV를 효과적으로 억제할 수 있었다.

두 번째 챕터 연구는 다중 표적 LTR1+2 miRNA를 사용하여 LTR 영역을 표적하려고 시도하였다. LTR 영역이 표적화된 실험 결과는 miRNA가 표적 서열을 저하시키고 동시에 PERV의 *gag* 및 *pol* 유전자의 mRNA 발현을 억제함을 입증하였다. LTR1, LTR2 및 다중 표적 LTR1+LTR2 miRNA는 각각 *gag* 유전자 발현의 76.2 %, 22 % 및 76.8 %를 억제하였다. 유사하게, miRNA는 단일 표적 miRNA (LTR1 및 LTR2) 및 다중 표적 miRNA (LTR1+LTR2)에 대해 각각 69.8 %, 25.5 % 및 77.7 %의 *pol* 유전자 발

현을 억제 시키는 것으로 밝혔다. 항생제 내성으로 선택된 PK15 클론은 다중 표적화된 LTR1+LTR2 miRNA를 연속적으로 발현하였고, 각각 *gag* 및 *pol* 유전자 발현의 최대 82.8 % 및 92.7 %의 높은 억제율을 나타냈다. 또한, 인간 세포주 (HeLa 세포)와 다중 표적화된 LTR1+LTR2 miRNA 형질 감염된 PK15 세포의 공동 배양 결과 PERV의 LTR, gag 및 pol 유전자의 발현이 효과적으로 억제되어 HeLa 세포로의 PERV 감염이 억제됨을 확인하였다.

세 번째 챕터 연구에서는 실질 돼지 신장 세포에 대해 수행되어 LTR의 특정 영역을 표적하는 miRNA가 LTR, *gag*, *pol* 및 *env* 유전자의 발현에 동시에 억제 효과를 발휘할 수 있는지를 확인하였다. 실질 돼지 신장 세포에서 PERV의 발현을 억제하기 위해 2 개의 마이크로 RNA (miRNA), 다중 표적화를 위한 LTR1 및 LTR2가 선택되었다. PERV의 LTR, *gag*, *pol* 및 *env* 영역의 mRNA 발현은 형질 감염 후 24시간에서 144 시간까지 다중 표적화된 miRNA에 의해 극적으로 억제되었으며, 120 시간에서 LTR 및 *pol* 영역에 대해 가장 높은 억제가 관찰되었다. 또한, 돼지와 인간 세포의 동시 배양 실험에서 PERV의 RT 역 전사 효소 활성은 여섯 번째 계대배양에서 84.4 % 감소했다. 결과적으로, 다중 표적 LTR 1+2 miRNA는 실질 돼지 신장 세포에서도 PERV 유전자 발현을 효과적으로 억제 시켰다. 결론적으로, 위의 세 가지 연구는 PERV 주요 유전자 및 LTR 영역을 표적으로 하는 miRNA가 돼지 신장 유래 및 실질 돼지 신장 세포에서 PERV의 발현을 억제할 수 있음을 확인하였다. 다중 표적 miRNA는 LTR 영역에 대한 유전자 발현뿐 만 아니라 *gag*, *pol* 및 *env*와 같은 기능적으로 중요한 PERV 유전자의 발현을 감소시켰다. 인간 세포 동시 배양 실험을 통해 PERV 유전자 발현의 억제는 결과적으로 PERV 감염능의 억제까지 이어질 수 있음을 확인하였다. 따라서, 상기 기술을 사용하여, 실질 돼지 신장 세포를 이용하여 PERV 억제 연구 및 이종 이식을 위한 형질 전환 돼지를 만

들기 위한 방법에 도움을 줄 수 있기를 기대한다.

주요어: 억제, miRNA 벡터, 돼지 내인성 바이러스, 다중 표적화, 인간 세포, RNA 간섭

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감사의 글

공부를 계속하는 것이 내 적성이 맞는지 흥미가 있어서 공부의 끈을 놓지 않았던 것인지 고민을 많이 하였었는데 대학원 입학 후에 끼니도 거르며 밤도 새며 연구를 하고 아플 때 도 논문을 쓰고 읽고 있는 나 자신을 돌이켜 보노라면 공부하고 연구하는 일이 즐거웠던 것 같고 내 인생에서 제일 보람도 많이 느꼈었고 미래에 제가 스스로 발전하는데 소중한 자양분이 되었던 시간인 것 같습니다.

많이 부족한 저에게 학위를 할 수 있는 기회를 주셨고, 기초를 가르쳐 주셨으며 실수하지 않도록 혼도 많이 내시며 잘 되라고 좋은 말씀과 격려를 아끼지 않고 해 주셨고 저를 옆에서 가장 오랫동안 지도해 주신 박봉균 교수님께 고마움을 표현하려면 끝이 없지만 이렇게라도 제일 먼저 감사의 말씀 드립니다. 그리고 제가 운이 정말 좋게도 인자하시고 학문적으로도 깊고 넓은 지식으로 항상 학생들을 지도해 주시는 박용호 교수님께 박사과정 지도를 받아 학위 논문을 잘 마무리를 할 수 있어서 정말 감사드립니다.

바쁘신 와중에도 항상 자기 일처럼 학위 논문을 완성하도록 꼼꼼히 논문을 봐주시며 수정해 주시고 도와주신 유한상 교수님, 논문 심사 중에 새로운 아이디어와 연구 방향을 제시해 주신 송대섭 교수님께 역시 감사의 말씀 드립니다.

제 선배이지만 때론 지도 교수처럼 새로운 아이디어와 연구 방향 및 학문적으로 제가 가장 많은 성장을 할 수 있게 도와주었고 현재도 같이 많은 연구를 같이 수행하고 있고 가르침을 주고 계신 베트남

대학교에 계시는 지압 교수님께 정말 많은 감사드립니다. 또한 항상 바쁘신 와중에도 후배가 잘 되도록 아끼지 않고 자기 일처럼 먼저 나서서 도와주신 문형준 박사님과 이지훈 박사님, 자상하시고 항상 많은 논문에 도움을 주시며 좋은 아이디어와 격려를 해 주셨던 김혜권 교수님과 박성준 박사님께도 또한 감사드립니다.

특히, 처음 실험실 생활을 하면서 제가 연구에 매진할 수 있게 도움을 주시며 부족한 것을 가르쳐 주셨고, 많은 가르침을 주신 세 분의 김정아, 김은옥, 양혜정 선생님과 학위과정 동안 친구 같이 많은 것을 배우고 연구할 수 있게 도와준 최민경, 김아름, 이찬희, 이가은, 김성재 학생과 제 사수로 많은 가르침과 도움을 주신 노세미 선생님, 어머니 같이 자상하시고 학위과정 동안 많이 도와주신 신숙 선생님께도 감사드립니다.

마지막으로 항상 저를 뒤에서 응원해 주시며 공부를 할 수 있게 힘이 되어 주시며 적극적으로 도와주신 아버지, 어머님께 항상 감사하며 은혜를 잊지 않고 열심히 노력하여 꼭 훌륭한 아들이 되겠습니다.

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정희천