Expression analysis of Porcine Endogenous Retroviruses (PERVs) in Korean native pig organs

Hyung-Gil Oh[1], Woo-Young Jung[1], Seung-Lan Yu[1], Jun-Heon Lee[1]*

[1]Department of Animal Science & Biotechnology, Chungnam National University, Daejeon 305-764, Korea

한국재래돼지의 장기조직에서 PERVs의 발현 특성 분석

오형길[1] · 정우영[1] · 유성란[1] · 이준헌[1]*

[1]충남대학교 동물자원생명과학과

Received on 6 February 2011, revised on 3 March 2011, accepted on 9 March 2011

Abstract : Pigs have anatomically and physiologically very similar to human and because of this, pigs are the possible xenotransplantation donors for human organs. PERVs (Porcine Endogenous Retroviruses) are known to be one of the possible obstacles for using porcine organs regardless of the immunological barriers. In order to understand the expression patterns of PERVs in Korean native pigs, we investigated PERV expressions in porcine liver, heart, spleen, and lung samples. After RNA extraction, two types of specific PERV envelope genes (ENV-A and ENV-B) were amplified using specific primers by RT-PCR. The results indicated that the variable PERV expressions were observed in inconsistent patterns among animals and tissues. The PERV expressions were verified with semi-quantitative real-time PCR with three replicates. Even though, these results confirm the previous findings that the PERVs were differentially expressed between animals and tissues. These results also give some valuable information for xenotransplantation when using the Korean native pigs as the organ donor.

Key words **:** Xenotransplantation, PERVs, Korean native pigs, RT-PCR

I. Introduction

Xenotransplantation, transferring cells, organs, and tissues from one species to another, can offer a potential solution for shortage of human organs and tissues for medical transplantation. For the xenotransplantation to human, pigs are thought to be the best source of organ donors than primates for the various reasons including safety, economical and practical applications. Also, pigs have little ethical problems than primates because large number of pigs was slaughtered every day for providing pork for human consumption (Lee and Moran, 2001). Recent advancement of genetic modifications in pig including Gal knock-out made the possibility of decreasing the xenograft rejections in the recipient immune system (Weiss et al., 1998). Using the samples from these pigs, pre-medical trials have been previously carried out. For example, pig livers were used for perfusion of human blood and porcine hepatocytes were used for the overcome of hepatic failure in a short period of time. Also, pancreatic islet cells were used to cure type I diabetes and fetal neuronal tissues were implanted as a treatment for Parkinson's disease (Deacon et al., 1997).

Porcine endogenous retroviruses (PERVs) are provirus status of retroviruses and are inherited to the next generation in a Mendelian fashion (Patience et al., 1997), as well as being acquired via maternal infection.

***Corresponding author:** Tel: +82-42-821-5779
E-mail address: junheon@cnu.ac.kr

Previously, Akiyoshi et al. (1998) have been estimated that about 30 to 50 PERVs are present in the pig genome. Three main types of PERVs, PERV-A, -B and -C based on the envelope proteins, have been extensively investigated. Within the *env* gene, the region encoding the ligand of the host cellular receptor has high sequence variations among the PERV types (Akiyoshi et al., 1998; Le Tissier et al., 1997). These three types of viruses have distinct receptor specificity and in vitro cell tropism (Wilson et al., 2000). Meanwhile, *gag* and *pol* genes in the PERV genome have less nucleotide variations and could not distinguish the type of PERVs only using the sequence information from the *gag* and *pol* genes.

Previously, human cell lines were infected by PERVs when co-culture with porcine PK-15 cell lines (Patience et al., 1997). Also, there is an evidence of PERV infection in SCID mouse tissue in vivo (van der Laan et al., 2000). This indicates that the PERVs can be transmitted from pig to human and at the same time, it gives very high incident of transmitting harmful diseases from animal to human. However, the percentage of the outbreaks of a new disease in human is very low since the PERVs were hardly developed to a disease, even in pigs. However, until now, no PERV infection has been recorded in vivo in baboon monkeys (Martin et al., 1998; Valdes-Gonzalez et al., 2010) or in humans (Paradis et al., 1999). The PERV expression analyses indicate that they can be expressed in most of the porcine tissues (Klymiuk et al., 2006).

With the controversy between the development of disease from PERV infection in relation to xenotransplantation, PERVs are one of the key point in the xenotransplantation and worth to investigate in a variety of pig breeds. Martin et al. (1998) have founded PERVs that primary endothelial cells, hepatocytes, lung and skin from minipigs, Yucantan micropigs and German landbreed pigs. Moreover, PERV-A and PERV-B genomic locations have been identified in the several pig breeds such as Korean native pig, Large White pig and Westran pig (Regel-Gaillared et al., 1999; Regel-Gaillared et al., 2001; Lee et al., 2002; Jung et al., 2010) and Fujimura et al. (2008) reported that Large White, Landrace, Duroc, Berkshire, miniature pigs and genetically modified triple-cross breed pigs in Japan can express both PERV-A and PERV-B. Recently, recombination between PERV types has been reported. The recombinant PERV-A/C had roughly 500-fold more infectious into human cells than PERV-A (Harrison et al., 2004, Denner et al., 2010). Furthermore, the possibility of recombination between PERVs and HERVs (cross-species recombination) can be happened when the xenotransplantation is performed. Therefore, so many researchers have been trying to eliminate PERVs using knock-out and knock-down skills. Especially, RNA interference, which may prevent expression of viral genes by using small interfering RNAs (siRNAs), has been developed (Li et al., 2006; Dieckhoff et al., 2009; Ramsoondar et al., 2009). However, this method could not perfectly block the PERVs expression.

In this study, in order to understand the molecular characteristics of PERVs in Korean native pigs, the possible organ donors in Korea, PERV expression patterns were investigated in liver, heart, spleen and lung using semi-quantitative real-time PCR. The results presented in this theses can give a valuable information for understanding of PERV expressions in different porcine tissues.

II. Materials and methods

1. Experimental samples

Korean native pigs (KNPs) were almost extinct until 1980 and new breeding program were established since then, mainly by National Institute of Animal Science (NIAS) in Korea. In order to investigate the PERV expression levels in liver, heart, spleen and lung tissues that were compared the RNA expression profiles, 10 KNPs

(J1001, J1006, J1016, J1025, J1029, J1033, J1034, J1038, J1053 and J1058) were used in this study. Samples were provided from National Institute of Subtropical Agriculture in Jeju province. Tissue samples from the above four organs were excised from the pigs, immediately frozen in liquid nitrogen, and stored at −70℃ until use.

2. RNA extraction from tissues

Total RNAs were isolated from frozen tissue samples. Initially, the tissue samples were subjected to homogenization and total RNAs were extracted using a QIAgen RNeasy midi kit according to the manufacturer's instruction (Qiagen, USA). Total RNAs were treated with RNAs-free DNaseI enzyme (Invitrogen, USA) at 37℃ for 1 hr to remove possible contamination with DNA. The extracted RNA concentrations were measured by NanoDrop 2000C spectrophotometer (Thermo Scientific, USA) at wavelength of 260 nm (OD260). After finishing all the procedures, extracted RNA samples were stored at −70℃ freezer until use.

3. RT-PCR analysis and PCR amplification

Total RNAs were employed for the synthesis of first-strand cDNA by reverse transcriptase using cDNA synthesis kit (Promega, USA). Initially, contaminating DNA was digested by DNase (RQ1 RNase-Free DNase) (Promega, USA). After alcohol precipitation, RNA purity and integrity were determined by spectrophotometry. RNA was performed in reactions containing RT reaction mixture 0.5 ug oligo $(dT)_{15}$ Primer, Reverse Transcription 10X buffer (1.4 ml), $MgCl_2$ (25 mM), dNTP Mixture (10 mM), Recombinant RNasin Ribonuclease Inhibitor (2500 units), AMV Revers Transcriptase (1500 units) at 42°C for 1 hr by AMV Reverse Transcription System (Promega, USA). The PERV-A and PERV-B specific primers were designed on the basis of PERV-A and PERV-B sequences (GenBank accession number Y-12238 for PERV-A; Y-12239 for PERV-B). The PCR amplification was performed in order to investigate the PERVs expression between the tissue samples and individual expression variations within the same tissue samples. The primer information for the PCR reaction is shown in Table 1.

Table 1. The primer sequence information for amplifying PERV-A and PERV-B. The positive control primers, β-actin and GAPDH were also shown.

Primer name	F/R	Primer sequence (5'-3')	Length (bp)
PERV-A	F	TCCGTGCTTACGGGTTTTAC	224
	R	TTGCCAATCTTTCCATCTCC	
PERV-B	F	TAAAAGCACACCTCCCAACC	192
	R	CCGGAATTGACAAAGGAGAA	
β-actin	F	AAGGAGAAGCTCTGCTACGT	283
	R	ATGTCCACGTCGCACTTCAT	
GAPDH	F	GGTCATCATCTCTGCCCCTT	267
	R	ATGATGTTCTGGAGAGCCCC	

Using the cDNA as a template, PCR reaction was performed. PCR reactions contained 10X PCR gold buffer (Applied Biosystems, USA), 25 mM $MgCl_2$ (Applied Biosystems, USA), 10 mM of dNTP (Genetbio, Korea), 10 pmol of forward and reverse primers, 0.5 unit of Amplitaq Gold (Applied Biosystems, USA) and cDNA in a total volume of 25 μl. Thermal cycling was performed in a GeneAmp PCR system 2700 (Applied Biosystems, USA).

Reaction profiles included predenaturation step at 10 min at 94°C followed by 35 cycles, each consisting of 30 sec of denaturation at 94℃, 30 sec of annealing step at 56℃, 30 sec of final step at 72℃, after a final extension of 10 min at 72℃. The PCR products were separated using 2% standard TAE agarose gel electrophoresis.

4. Semi-quantitative real-time PCR

Semi-quantitative real-time PCR reactions were performed using Roter-Gene (Corbett Research, USA) and the PCR reactions were employed according to the manufacturer's instructions (Corbett Research, USA).

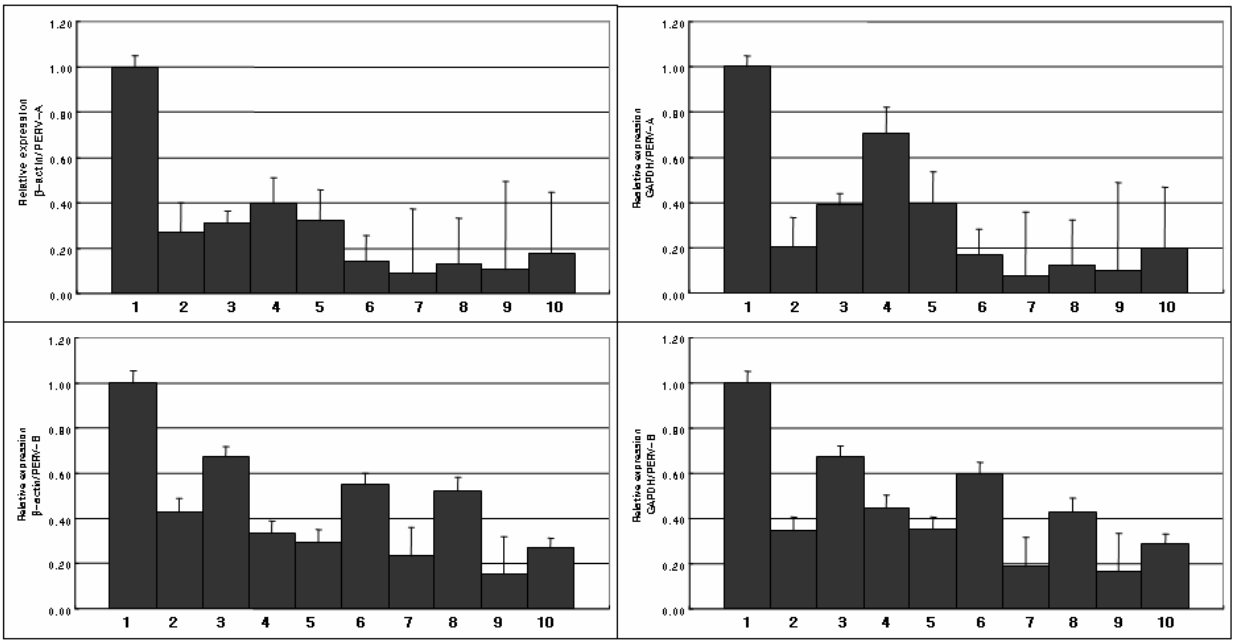

Fig. 1. Expression patterns of PERV-A and PERV-B in liver samples from 10 different Korean native pig individuals. The expression control genes, β-actin and GAPDH, are also indicated.

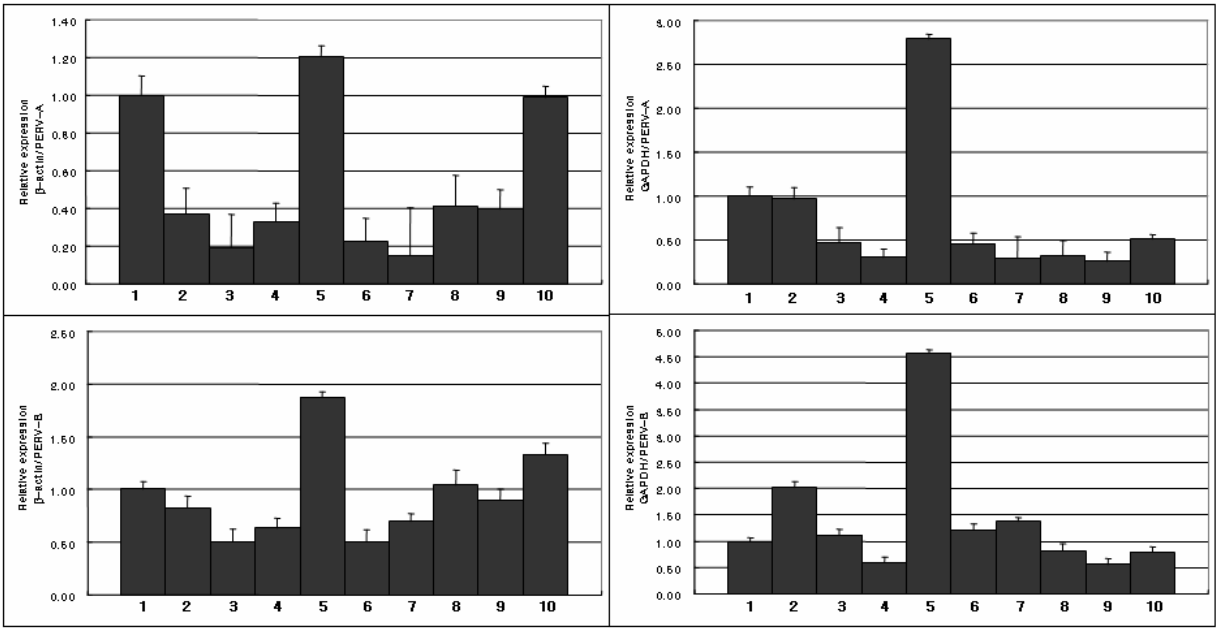

Fig. 2. Expression patterns of PERV-A and PERV-B in heart samples from 10 different Korean native pig individuals. The expression control genes, β-actin and GAPDH, are also indicated.

The primers used for the semi-quantitative real-time PCR were the same as RT- PCR reactions (Table 1). Both β-actin and GAPDH mRNA expressions were used as the internal standards. For the fluorescence incorporation, SYBR green I Prime Q-Mastermix (Genetbio, Korea) was used and this mastermix contains *Taq* DNA polymerase and 10X reaction mix (contains reaction buffer, SYBR green, optimized PCR buffer, 5 mM $MgCl_2$, and a dNTP mixture that includes dUTP). The PCR involved an initial denaturation program (95℃ for 10 min), followed by 45 cycles of amplification and quantification (95℃ for 10 sec, 56℃ for 30 sec, 72℃

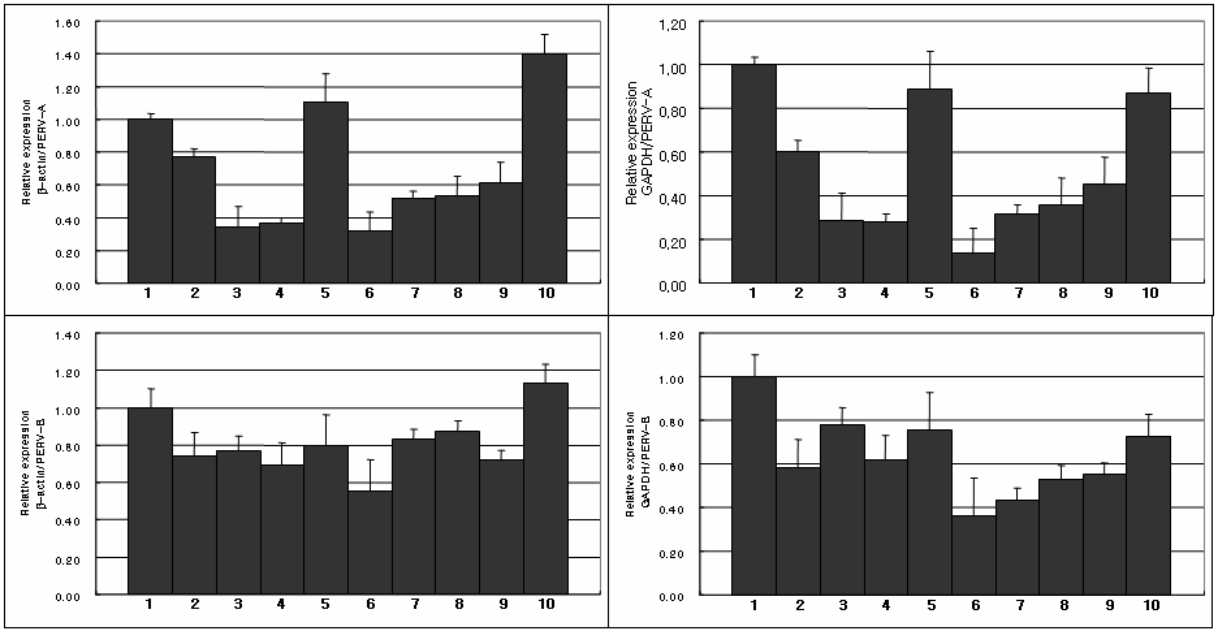

Fig. 3. Expression patterns of PERV-A and PERV-B in spleen samples from 10 different Korean native pig individuals. The expression control genes, β-actin and GAPDH, are also indicated.

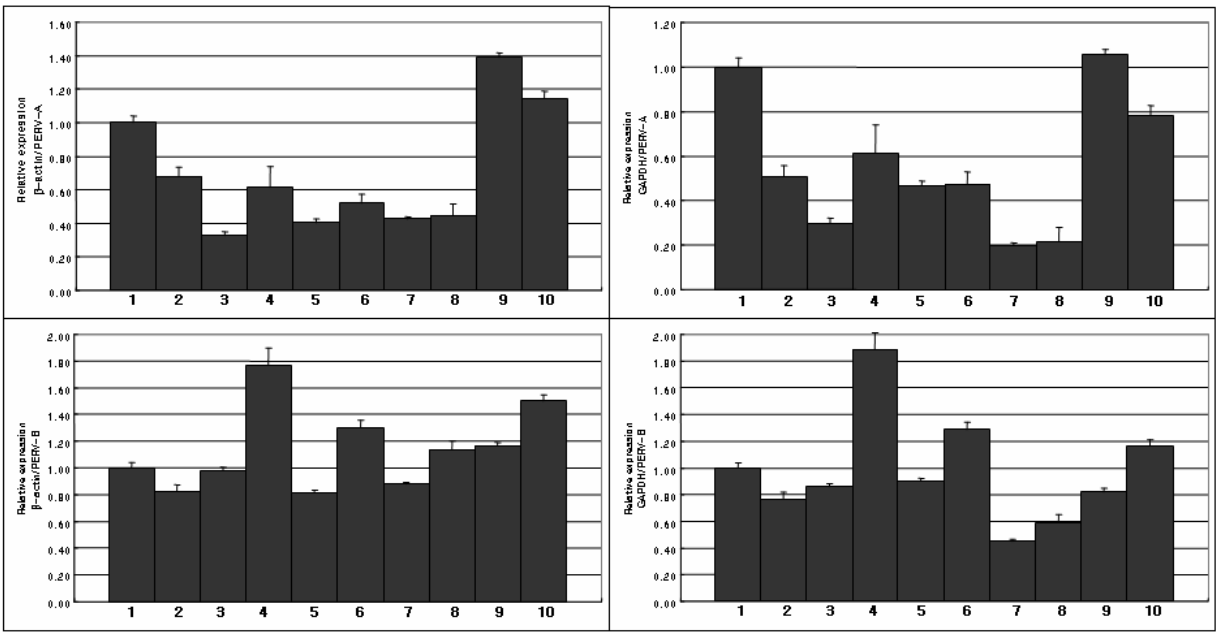

Fig. 4. Expression patterns of PERV-A and PERV-B in lung samples from 10 different Korean native pig individuals. The expression control genes, β-actin and GAPDH, are also indicated.

for 30 sec with a single fluorescence measurement), The melting curve of each sample was carefully checked and the fluorescence data were used for quantification. Since the melting curve of a product was sequence-specific, it can be used to distinguish between non-specific and specific PCR products. Data were analyzed using the mathematical model and for doing this, the determination of the crossing points (CP) for each transcript is needed. The CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed using the ddCt method (Livac et al., 2001).

III. Results and discussion

1. Partial envelope gene sequences for PERV-A and PERV-B

The PERV expression levels were investigated in four tissues, namely, liver, heart, spleen and lung samples from 10 KNPs. In order to confirm the specificity of the primers that we used, PCR products using PERV-A and PERV-B specific primers were sequenced. The results indicated that the primers are PERV-A and PERV-B specific, respectively, and therefore these primers were used for the further experiments for the semi-quantitative real-time PCR (Table. 1).

2. RT-PCR Analysis

Using PERV-A and PERV-B specific primers, RT-PCR reactions were performed and the PERV expression levels were measured. Initially, the PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel stained with Ethidium Bromide (EtBr) and visualized under ultraviolet light. All the PCR products gave a single band with the expected size of 224 bp for PERV-A and 192 bp for PERV-B, respectively.

3. Semi-quantitative real-time PCR analysis of PERVs

The PERV expression levels were measured using semi-quantitative real-time PCR. For the normalization of the PCR reactions β-actin and GAPDH gene were used as the internal control. After normalization, the PERV expression levels in liver, heart, spleen and lung samples were investigated. The real-time PCR reactions were validated by repeating these procedures three times. As the results, relatively high PERV expressions were observed in spleen, heart and lung tissues (Fig. 2, 3, and 4). However, in case of the liver samples, relatively low expression patterns were found (Fig. 1).

Based on the overall expression patterns in four tissues in this study, PERV-B expression was appeared to be higher than PERV-A expression. This may indicates that the PERV-B copy numbers in the KNPs were relatively higher than that of PERV-A. Previously, Klymiuk et al. (2006) investigated the PERV expression in liver and spleen tissues. They showed that PERV expressions in pregnant Landrace sows were lower than that of their fetuses. This may indicates that the PERV expressions are higher in early developing stages and the PERV expression was decreased when the animals become mature. Also, Clemenceau et al. (1999) investigated the relative PERV expressions among PERV-A, -B and -C in French SPF and conventional Large White pigs. They found that relatively small PERV-A expression was observed in these pigs. However, there were large PERV-B expression differences between the SPF and conventional Large White pigs.

This study confirms the previous findings that the PERV expression had variation among animals and tissues (Akiyoshi et al., 1998; Clemenceau et al., 1999). Also, the different PERV expression was observed among the pig breeds (Tacke et al., 2000).

Until now, a number of studies have shown the possible PERV transmission in relation to xenotransplantation. Also, large number of researches have been carried out the elimination of PERV expressions in pigs. This study gives the basic information in PERV expression in Korea native pigs which will be useful for the understanding the expression nature of PERVs and possibly give some clues as the xenotransplantation donor.

IV. Conclusions

In this research, we have investigated PERV-A and -B expressions in liver, heart, spleen and lung samples from ten Korean native pigs. The PERV expressions in spleen, heart and lung tissues were relatively higher than those in liver samples. Also, PERV-B expression was appeared to be higher than PERV-A expression. This

indicated that the PERV-B copy numbers in the KNPs were higher than those of PERV-A. These results will give some valuable information for xenotransplantation studies using these pigs.

References

1. Akiyoshi D, Denaro M, Zhu H, Greenstein J, Banerjee P, Fishman J. 1998. Identification of a full-length cDNA for an endogenous retrovirus of miniature swine. J. Virol. 72: 4503-4507.
2. Clémenceau B, Lalain S, Martignat L, Saï P. 1999. Porcine endogenous retroviral mRNAs in pancreas and a panel of tissues from specific pathogen-free pigs. Diabetes Metab. 25: 518-525.
3. Deacon T, Schumacher J, Dinsmore J, Thomas C, Palmer P, Kott S, Edge A, Penny D, Kassissieh S, Dempsey P, Isacson O. 1997. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. Nat. Med. 3: 350-353.
4. Dieckhoff B, Kessler B, Jobst D, Kues W, Petersen B, Pfeifer A, Kurth R, Niemann H, Wolf E, Denner J. 2009. Distribution and expression of porcine endogenous retroviruses in multi-transgenic pigs generated for xenotransplantation. Xenotransplantation. 16: 64-73.
5. Denner J. 2010. Recombinant porcine endogenous retroviruses (PERV-A/C): a new risk for xenotransplantation? Xenotransplantation. 17: 120-120.
6. Fujimura T, Miyagawa S, Takahagi Y, Shigehisa T, Murakami H. 2008. Prevalence of porcine endogenous retroviruses in domestic, minature and genetically modified pigs in Japan. Transplant Proc. 40: 594-595.
7. Harrison I, Takeuchi Y, Bartosch B, Stoye J. 2004. Determinants of high titer in recombinant porcine endogenous retroviruses. J. Virol. 78: 13871-13879.
8. Jung WY, Kim JE, Jung KC, Jin DI, Moran C, Park EW, Jeon JT, Lee JH. 2010. Comparison of PERV genomic locations between Asian and European pigs. Anim. Genet. 41: 89-92.
9. Klymiuk N, Muller M, Brem G, Aigner B. 2006. Phylogeny, recombination and expression of porcine endogenous retrovirus c2 nucleotide sequences. J. Virol. 87: 977-986.
10. Lee JH, Webb GC, Allen RDM, Moran C. 2002. Characterizing and mapping porcine endogenous retroviruses in Westran pigs. J. Virol. 76: 5548-5556.
11. Le Tissier P, Stoye J, Takeuchi Y, Patience C, Weiss R. 1997. Two sets of human-tropic retrovirus. Nature. 389: 681-682.
12. Li Z, Ping Y, Shengfu L, Youping L, Jingqiu C, Hong B. 2006. The lack of inhibition of porcine endogenous retrovirus by small interference RNA designed from the long terminal regions. Transplant. proc. 38: 2258-2260.
13. Livak K, Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. Methods. 25: 402-408.
14. Lee JH, Moran C. 2001. Current status of xenotransplantation - a review. Asian-Australas. J. Anim. Sci. 14: 1497-1504.
15. Martin U, Steinhoff G, Kiessig V, Chikobava M, Anssar M, Morschheuser T, Lapin B, Haverich A. 1998. Porcine endogenous retrovirus (PERVs) was not transmitted from transplanted porcine endothelial cells to baboons in vivo. Transplant Int. 11: 247-251.
16. Martin U, Kiessig V, Blusch J, Haverich A, von der Helm K, Herden T, Steinhoff G. 1998. Expression of pig endogenous retrovirus by primary porcine endothelial cells and infection of human cells. Lancet. 352: 666-667.
17. Paradis K, Langford G, Long Z, Heneine W, Sandstrom P, Switzer W, Chapman L, Lockey C, Onions D, Otto E. 1999. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. Science. 285: 1236-1241.
18. Patience C, Takeuchi Y, Weiss RA. 1997. Infection of human cells by an endogenous retrovirus of pigs. Nat. Med. 3: 282-286.
19. Rogel-Gaillard C, Bourgeaux N, Billault A, Vaiman M, Chardon P. 1999. Construction of a swine BAC library: application to the characterization and mapping of porcine type C endoviral elements. Cytogenet. Cell Genet. 85: 205-11.
20. Rogel-Gaillard C, Hayes H, Bourgeaux N, Chardon P. 2001. Assignment of two new loci for gamma 1 porcine (c1 PERV) to pig chromosome bands 2q21 and 11q12 by in situ hybridization. Cytogenet. Cell Genet. 95: 112-113.
21. Ramsoondar J, Vaught T, Ball S, Mendicino M, Monahan J, Jobst P, Vance A, Duncan J, Wells K, Ayares D. 2009. Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs. Xenotransplantation. 16: 164-180.
22. Tacke S, Specke O, Stephan E, Seibold K, Bodusch K, Denner J. 2000. Porcine endogenous retrovirus: diagnostic assays and evidence for immunosuppressive properties. Transplant. proc. 32: 1166.
23. Van der Laan L, Lockey C, Griffeth B, Frasier F, Wilson, Onions D, Hering B, Long Z, Otto E, Torbett B, Salomon D. 2000. Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice. Nature 407: 90-94.
24. Valdes-Gonzalez R, Dorantes LM, Bracho-Blanchet E, Rodriguez-Ventura A, White DJG. 2010. No evidence of porcine endogenous retrovirus in patients with type 1 diabetes after long-term porcine islet xenotransplantation. J. Med. Virol. 82: 331-334.
25. Weiss R. 1998. Transgenic pigs and virus adaptation. Nature 391: 327-328.
26. Wilson C, Wong S, Van Brocklin M, Federspiel M. 2000. Extended analysis of the in vitro tropism of porcine endogenous retrovirus. J. Virol. 74: 49-56.