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RESEARCH ARTICLE

# Characterization of PERV from Bama Miniature Pigs in Human Cells

Ling Ma[1, 2], Jun Lin[2], Haibo Tang[2], Anbin Bai[2], Fenglian Chen[2], Shaomin Qin[2], Jinfeng Liu[2], Shuying Qin[2] and Jianmin Wu[1, 2,*]

[1]College of Animal Science and Technology, Guangxi University, Nanning 530004, Guangxi, China
[2]Guangxi Veterinary Research Institute, Nanning, 530001, Guangxi, China
*Corresponding author: wu-jm20@163.com

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**ABSTRACT**

Bama miniature pig (BM) as the inbreed pigs is considered to be a potential donor for xenotransplantation. However, porcine endogenous retrovirus (PERV) integrated in pig's genome, is likely to infect human cells in vitro. PERV infection from BM remains unknown. This study aimed to investigate PERV infection in BM in comparison with that of PK15 cells and further to identify the integration sites of PERV from BM (PERV-BM) in human genome. Two full-length PERV-BMs were cloned from 15 isolates of PERV from Bama miniature pigs and sequence analysis indicated that PERV-BM had limited infection to human cells. Subsequently, PBMC from the BM and PK15 cells were co-cultured with HEK293 cells, respectively. Using nested RT-PCR assay and TEM observation, one PERV-BM was observed to infect HEK293 cells with lower transcriptional level than PERV from PK15 cells (PERV-PK) did, while the others showed no sign of transmission of PERV to human cells. Furthermore, 4 and 157 integration sites were identified from PERV-BM-infected HEK293 cells (HEK293-PERV-BM) and PERV-PK-infected HEK293 cells (HEK293-PERV-PK), respectively, which displayed variations of PERV integration. For further verification of the PERV-BM integration, the expression level of *cyclin D1*, *c-myc*, *p53* and *p16* genes of HEK293-PERV-BM decreased significantly while the *CDK4* genes were upregulated dramatically in comparison with those of the HEK293-PERV-PK. It is the first time that PERV-BM has been confirmed to be low infection to human cells and highly integration into the PK15 cells. Therefore, BM is a potential xenograft donor due to less PERV transmission to human beings.



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## INTRODUCTION

Xenotransplantation is proposed to be a promising solution alleviating the shortage of human donor organs, and pigs are considered to be the suitable donor, especially the inbred miniature pigs (Zhang *et al*., 2017; Fishman, 2018). However, PERV which integrates in all species of pigs as provirus is a potential infection barrier to xenotransplantation. PERV can infect human cell lines *in vitro* and severe combined immunodeficient (SCID) mice *in vivo* (Patience *et al*., 1997; Van der Laan *et al*., 2000; Lopata *et al*., 2018).

PERV is a gammaretroviruse with three subtypes, including PERV–A, PERV-B and PERV–C. Whereas PERV-A and –B are polytropic, capable of infecting a wide spectrum of human and other mammalian cells, while PERV-C is ectopic, infecting only pig cells, but the recombinant PERV-A/C viruses highlight the risk of infection to human cells and a 500-fold infectivity than PERV-A, which are attracting many researches to investigate the mechanism of PERV from pigs to human cells (Oldmixon *et al*., 2002). Due to pig species and territories, the findings of reports in literature are divergent. PERV from islets of inbred XENO-1 miniature pigs and miniature porcine kidney (MPK) cells do not infect HEK293 cells (Patience *et al*., 1997; Guo *et al*., 2014); PERV from SNU and Yucatan miniature pigs as well as recombinant PERV-A/C viruses from some National Institute of Health miniature pigs (NIH) could infect human cells (Oldmixon *et al*., 2002; Tacke *et al*., 2003; Jung Heon Kim, 2010). HEK293 cells were the favorable human cells by the PERV infection.

PK15 cells, characterized as *APOBEC3F* gene expression, are widely used to study PERV, and PERV released from PK15 cells (PERV-PK) can infect pig, mink and several human cells *in vitro*. Although no pathology is associated with PERV in its natural host, however, the pathogenic potential might differ in the case of cross-species transmission (Moalic *et al*., 2006). In addition, PERV-PK displays the integration preferences around the transcriptional star sites and near CpG islands of transcriptional active genes in the human chromosomes (Moalic *et al*., 2006; Moalic *et al*., 2009), which might activate or inactivate the flanking genes.

Bama miniature pig (BM) is a particular species of pigs in Guangxi, China. After 30 years inbreeding management, BM has developed into a mature pig subspecies and an important animal model used in several biomedical analyses. Also, BM shares anatomical and physiological resemblance to human, high genetic homozygosity, clear genetic background and high inbreeding endurance and as such widely used in biomedical analyses in China. Free of PERV-C expression and low in copy numbers of PERV, BM is considered as a potential safe donor for xenotransplantation (Wu *et al*., 2008; Xia *et al*., 2015; Yang *et al*., 2016; Zhang *et al*., 2018). However, the infection, integration features of PERV from BM (PERV-BM) are still unknown.

Therefore, this study is intended to compare the difference between PERV-BM and PERV-PK in terms of the infection of PERV-BM to human cells and to identify the integration sites in human chromosomes. It is anticipated that the results will spur other researchers to further study the safety issue of BM used in xenotransplantation.

## MATERIALS AND METHODS

**PBMC isolated and cloning of full-length provirus PERV-BM:** The study was approved by the Ethics Committee of Guangxi University (Protocol number GXU2015-08). Peripheral blood mononuclear cells (PBMC) were isolated from fifteen inbred Bama miniature pigs (BM1~15).Full-length DNAs of PERV were cloned from 15 BMs and marked from the *PERV-BM1* to *PERV15* genes as the previously described (Ma *et al*., 2010).

**Cell culture and PERV-BM infection:** HEK293 cells and PK-15 cells (China Center for Type Culture Collection, CCTCC) were cultured in DMEM (Thermo Fisher Scientific, USA), supplemented with 10% and 5% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) at 37°C with 5% $CO_2$.

HEK293 were co-cultured with PBMC of BMs and PK-15 cells to harvest the virus genes of the *HEK293-PERV-BM* and *HEK293-PERV-PK* following the previous procedures with the presence of 2.5 μg/mL of phytohemagglutinin (Solarbio, China) (Patience *et al*., 1997; Wilson *et al*., 1998; Oldmixon *et al*., 2002; Semaan *et al*., 2013; Guo *et al*., 2014). All cells were harvested for extraction of DNA and RNA post cultivation on day 18 and on day 80, using a QIAamp DNA Mini Kit (Qiagen, Germany) and MiniBEST universal RNA Extraction Kit (Takara, China), respectively. Both PERV *gag* gene and *α-1,3-galactotransferase (α-1,3-GT)* gene were used to determine PERV transcription and monitor removal of PBMC and PK-15 cells in the co-culture systems (Table 1).

**Transmission electron microscopy (TEM):** After 18-day co-culture, the HEK293-PERV-BM cells were washed 3 times to make ultrathin sections for the observation by transmission electron microscope (TEM, Hitachi H-7650, Japan).

**Identification of integration sites:** Genomic DNA samples extracted from *HEK293-PERV-BM* and *HEK293-PERV-PK* post 80-day co-culture were applied as the templates in the modified ligation-mediated PCR (LM-PCR) to clone the flanking sequences of the 3'LTR of the genomics of the PERV-BM and PERV-PK integrated in HEK293 cells. Subsequently, more integration sites of PERV-BM were cloned from the flanking sequences of the 5'LTR of the PERV-BM using the thermal asymmetric interlaced PCR (TAIL-PCR) as previously descripted (Chen *et al*., 2016).Briefly, the genomic DNA of *HEK293-PERV-BM* and *HEK293-PERV-PK* were cleaved with *Nhe*I instead of *Avr*II/*Nhe*I, respectively to lessen the artificial ligation of the digested genomic DNA and then ligated to a double-strand linker. Afterwards, the ligation products were cleaved with the *Eae*I. The specific first-round PCR products were concentrated by streptavidin-coated beads (Thermo Fisher Scientific, USA) and used as the template of the second-round PCR with another 3'LTR-specific primer (TTGGAATAAAA ATCCTCTTGCTGT), after which the second round PCR products were cloned and sequenced. The sequences just next to the PERV LTRs were mapped to the human genome using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the judgement of integration site was based on the criterion previously reported (Moalic *et al*., 2006).

**Table 1:** The primers of *gag* gene and α-1,3-GT gene

| Genes | Names | Sequences (5' - 3') |
|---|---|---|
| gag | gagF1 | ATGGGACAGACRGTGACKACCC |
| | gagR1 | ATCTTTATCTTCTTCTAGAGC |
| | gagF2 | CCCGATCAGGAGCCCTATATCCTTACGTG |
| | gagR2 | CGCAGCGGTAATGTCGCGATCTCGT |
| α-1,3-GT | E3 | TCCGAGCTGGTTTAACAATGG |
| | E4 | TCTTCTTCGTGGTAACTGTGAGTC |

**Table 2:** The primers used in the Real-time PCR

| Genes | Names | Sequences (5' - 3') |
|---|---|---|
| cyclin D1 | Cyclin D1 ORF 448U20 | TGGAACCTGGCCGCAATGAC |
| | Cyclin D1 ORF 673L22 | AGCGTGTGAGGCGGTAGTAGGA |
| CDK1 | CDK1 ORF 552U24 | AGTTGACATTTGGAGTATAGGCAC |
| | CDK1 ORF 807L22 | AATTCGTTTGGCTGGATCATAG |
| CDK4 | CDK4 ORF 170U20 | TGGCTTTACTGAGGCGACTG |
| | CDK4 ORF 380L22 | TTGGCATGAAGGAAATCTAGGC |
| c-Myc | c-Myc ORF 214U21 | CCCTCCTACGTTGCGGTCACA |
| | c-Myc ORF 394L22 | AGAAGCCGCTCCACATACAGTC |
| K-ras | K-ras ORF 26U18 | TTGGAGCTTGTGGCGTAG |
| | K-ras ORF 210L21 | CCCTCCCCAGTCCTCATGTAC |
| p53 | p53 orf 991U18 | CAGATCCGTGGGCGTGAG |
| | p53 orf 1121L22 | TTATGGCGGGAGGTAGACTGAC |
| p16 | p16 ORF-1 304U15 | GCGCGGCTGGACGTG |
| | p16 ORF-1 435L17 | GACCTTCCGCGGCATCT |
| Human β-actin | Human β-actin 657U20 | CGGGAAATCGTGCGTGACAT |
| | Humanβ--actin 905L21 | GCGTACAGGTCTTTGCGGATG |

**Bioinformatic analysis of integration sites:** Sequences of the integration sites, the transcriptional direction of the host genes, the distance from the transcription start sites and CpG islands were determined while the functions of the main RNA-Seq expression tissues, and the related diseases of host genes were annotated by Genome Brower of UCSC (http://www.genome.ucsc.edu/).

**Real-time PCR:** To determine the expressions of *cyclin D1*, *CDK4*, *c-myc*, *p53*, *p16* and *β-actin* genes, total RNA of HEK293, *HEK293-PERV-BM* and *HEK293-PERV-PK* were extracted, and 1μg RNA from each sample was detected the targeted genes by three dependent SYBR Green real-time PCR (Table 2). The results were presented according to the $2^{-\triangle\triangle Ct}$ method. Comparisons in different groups were performed by Student's *t*-tests, and $P<0.05$ was considered significant.

## RESULTS

**Analyses of the full-length PERV-BM:** PERV-BM2 and PERV-BM4 with the intact *gag*, *pol* and *env* ORF genes produced 8860 bp and 8850 bp bands, respectively, which were high homology to the PERV-A isolates from Wuzhishan miniature pigs (WZS) (GU980187, EF133960), BM (HM159246) and NIH (HQ536005). Based on the PCR products and phylogenetic tree (data not shown) analysis, both PERV-BM2 and PERV-BM4 belonged to subtype PERV-A.

To estimate the infection of PERV-BM for HEK293 cells, the diversity analyses were performed based on *env* gene and long terminal repeat (LTR) sequence. The 5'LTR of PERV-BM2/4 isolates comprised 2.5 copies of 39bp repeats and two 18bp-21bp sub-repeats followed by a 18 bp sub-repeat in U3 region (Fig. 1A), which conferred promoter activities in HEK293 cell line (Scheef *et al*., 2001). Meanwhile, one 21bp-24bp-39bp repeat sequence was found in 3'LTR (Fig. 1B), with the exception of 1~3 bases identical to the former ones (Denner *et al*., 2003). Additionally, a 22 bp fragment (TGAGATAACAGGGAAAAAGGTT) was found in the upstream of the repeat sequences in 3'LTR, and the same insertion was also detected in the 3'LTR of GU980187.

A curtailment of 12 nt (CCTTGTGGACAG) in the *env* gene of PERV-BM4 showed non-frameshift. Two bases of mutations and one base insertion were identified in the packaging signal of BM4. One base of mutation of BM4 in the *env* gene led to a premature stop codon in the N terminal of SU protein.

**Establishment of HEK293-PERV-BM and HEK293-PERV-PK:** The presence of PERV *gag* gene and α-1,3-GT were detected by the nested PCR and PCR assay, and the results showed that *gag* gene was positive both in HEK293-PERV-BM2 and in HEK293-PERV-PK after 18-day and 80-day co-culture, no porcine genomic DNA could be detected as contaminant (Fig. 2A). The results showed that the PBMC of BM2 and PK15 cells were eliminated completely in the HEK293-PERV-BM2, the HEK293-PERV-PK, and the PERV-BM2 and the PERV-PK infected HEK293 cells, respectively. The PERV-BM2 and the PERV-PK were able to transmit from PBMC and PK15 cells to HEK293 cells. The transcriptional level of HEK293-PERV-BM2 was much lower than that of HEK293-PERV-PK after 18-day and 80-day co-culture, but no transcription was detected in HEK293-PERV-BM4 and other HEK293-PERV-BMs, with the same amount of RNA (Fig. 2B).

Under TEM observation, a virion with typical C retrovirus was observed in HEK293-PERV-BM2 (Fig. 2C), indicating that HEK293-PERV-BM2 (designated as HEK293-PERV-BM) and HEK293-PERV-PK were established successfully. Contrarily, BM4 failed to infect HEK293 cells.

**Integration characterization in human genome of HEK293-PERV-BM:** Among 3875 clones, 4 integration sites were identified from HEK293-PERV-BM (Table.3 and Fig. 3A). One was identified in the 5'LTR of PERV-BM2 by TAIL-PCR assay, integrating between exon2 and exon 3 of human protein tyrosine phosphatase receptor type N2 (PTPRN2) in chromosome 7 and the junction sequences of PERV-BM were the N terminal of the leader sequence which included splicing donor site (SD), while the 5'LTR and PBS at the upstream of the leader sequence were completely lost. The other three sites were obtained in the 3'LTR of PERV-BM by LM-PCR method, in which two joined a 231 bp fragment and a 197 bp fragment comprised of PBS and partial leader sequence (including SD and parts of packaging signal) downstream 3'LTR of PERV-BM2 before integrating the plexin A4 (PLXNA4) gene of chromosome 7 and molybdenum cofactor synthesis 1 (MOCS1) on chromosome 6, respectively; the last integration site was in human roundabout guidance receptor 2 (ROBO2) on chromosome 3 with the end of 3'LTR integrating directly.

All the integration sites were over±5 kb from transcriptional start sites to CpG islands.

**Integration characterization in human genome of HEK293-PERV-PK:** Out of 1483 clones, 157 PERV-PK integration sites were identified in the HEK293-PERV-PK. Among these, 151 integration sites showed PERV directly integrated in different chromosomes while other 6 integration sites displayed variations in formation, including 2 direct integration of two truncated 3'LTR in the chromosome 8 and 19, respectively; 3 sites with a 38 bp fragment of U5 insertion after 3'LTR of PERV then integration in chromosome 1, 11 and X, respectively. The remaining one was observed to integrate in chromosome X after the truncated 3'LTR connected with a 211 bp fragment (PBS+ partial leader sequence) and a 38bp U5 fragment. In addition, there was one common integration site of chromosome 3 between PERV-BM and PERV-PK as mentioned above (Table 4 and Fig. 3B).

The distance from the integration site of PERV-PK to the transcription start sites and CpG islands was calculated, and 35.03 and 40.13% of the integration events occurred near to transcriptional start sites and CpG islands, suggesting that PERV-PK tended to integrate in human genomic DNA directly around the transcriptional start sites and near CpG islands of transcriptional active genes in the human chromosome. In addition, more integration sites were discovered in chromosome 11, 12 and 20 and less in chromosome 1, 2, 5 and X than the integration sites previously reported.

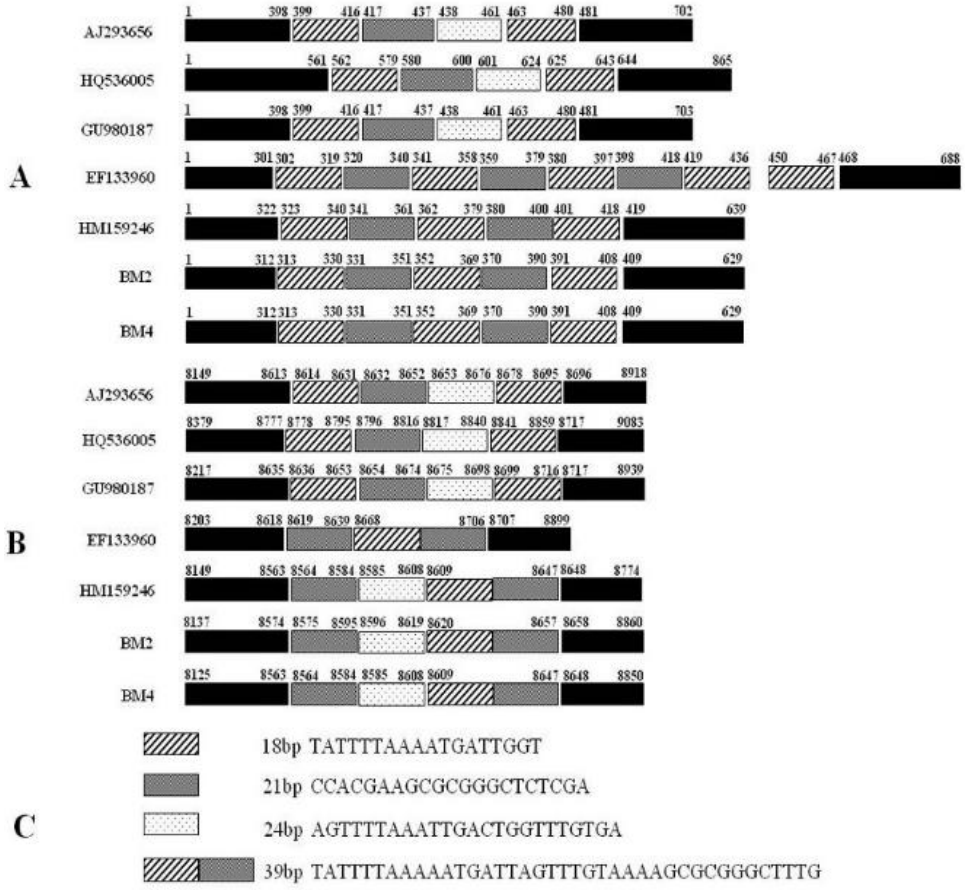


**Fig. 1:** Schematic representation of the 5'LTR and 3'LTR of PERV from different sources. (A) 5'LTR of PERV from PK15 cells, WZS and BM pigs. (B) 3'LTR of PERV from PK15 cells, WZS and BM pigs. (C) Different numbers of tandem repeated regions were characterized. The black boxes represented sequences outside of the repeats, and different boxes indicated different tandem repeated sequences.

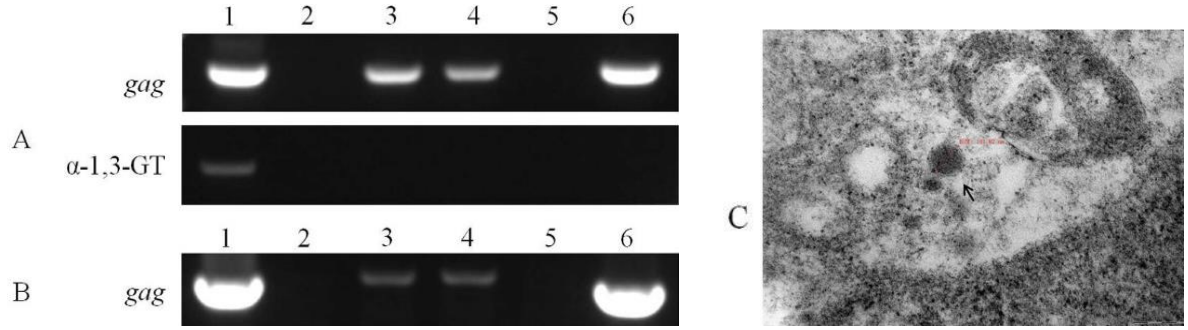


**Fig. 2:** Identification of HEK293-PERV-BM and HEK293-PERV-PK. (A) *gag* gene of PERV and α-1,3-GT of pigs using Nested PCR and PCR assay. 1: PK15 cells; 2: HEK293 cells; 3: HEK293-PERV-BM2 18 days post infection; 4: HEK293-PERV-BM2 80 days post infection; 5: HEK293-PERV-BM4; 6: HEK293-PERV-PK. (B) Transcriptional level of PERV by the Nested PCR and PCR assay. 1: PK15 cells; 2: HEK293 cells; 3: HEK293-PERV-BM2 at days 18 post infection; 4: HEK293-PERV-BM2 at days 80 post infection; 5: HEK293-PERV-BM4; 6: HEK293-PERV-PK. (C) The type C retrovirus particle was observed in HEK293-PERV-BM by TEM. (Scale bar=200 nm).

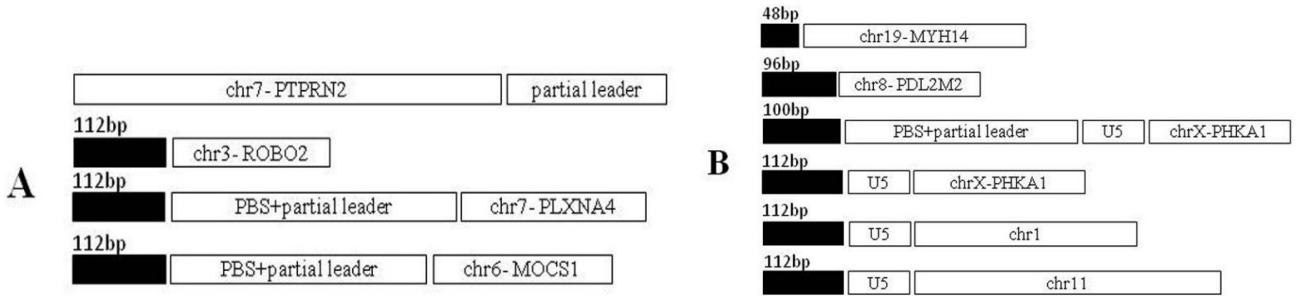


**Fig. 3:** Schematic representation of the integration sites of PERV-BM and PERV-PK in human genome. The black box indicated the end of 3LTR, the length of each black box was labeled, and the other boxes represented the partial leader sequence, PBS and partial leader sequence, truncated U5 of PERV and different Chromosome. (A) The junction of PERV-BM and human genome. Boxes of partial Leader sequence and PBS+partial leader sequence represented the 671~689bp fragment and 630~860bp, 630~826bp fragments of PERV-BM2. (B) The unusual integration of PERV-PK in human genome. PBS+partial leader sequence and U5 represented the 8639~8849bp fragment and 8812~8849bp fragment of PERV isolate (AJ133817), respectively.

**Table 3:** The integration sites of PERV-BM

| Chr | Position (GRCh38.P7) | Gene | Intron/ Exon | Related Diseases | Main Molecular Function | Main RNA-Seq expression tissue |
|---|---|---|---|---|---|---|
| 7 | 158360063-158360366 | PTPRN2 | intron | Diabetes mellitus, Anomalous left coronary artery | protein tyrosine phosphatase activity and etc | Brain- Frontal Cortex (BA9) |
| 3 | 77317934-77317967 | ROBO2 | Intron | vesicoureteral reflux | Protein binding and etc | Brain- Caudate (basal ganglia) |
| 7 | 132190330-132190442 | PLXNA4 | - | - | Semaphorin receptor activity and etc | Adipose- Visceral (Omentum) |
| 6 | 39912617-39912737 | MOCS1 | Intron | molybdenum cofactor deficiency | Nucleotide binding, GTP binding, lyase activity, metal ion binding | Adipose- Subcutaneous |

**Table 4:** Unusual integration sites of PERV-PK

| Chr | Position (GRCh38.P7) | Gene | Intron/Exon | Related Diseases | Molecular Function | Main RNA-Seq expression tissue |
|---|---|---|---|---|---|---|
| 19 | 50215713-50215873 | MYH14 | Intron | peripheral neuropathy, myopathy, hoarseness, and hearing loss | nucleotide binding, motor activity, microtubule motor activity | Muscle - Skeletal |
| 8 | 22597812-22597891 | PDLIM2 | Intron | Ogan-reese syndrome | Protein binding, metal ion binding | Spleen, skin, esophagus, lung |
| X | 72713969-72714098 | PHKA1 | Intron | Muscle glycogenosis | Catalytic activity, phosphorylase kinase activity, calmodulin binding | Muscle- skeletal |
| 1 | 90893701-90893830 | - | - | - | - | - |
| 11 | 65492228-65492514 | - | - | - | - | - |

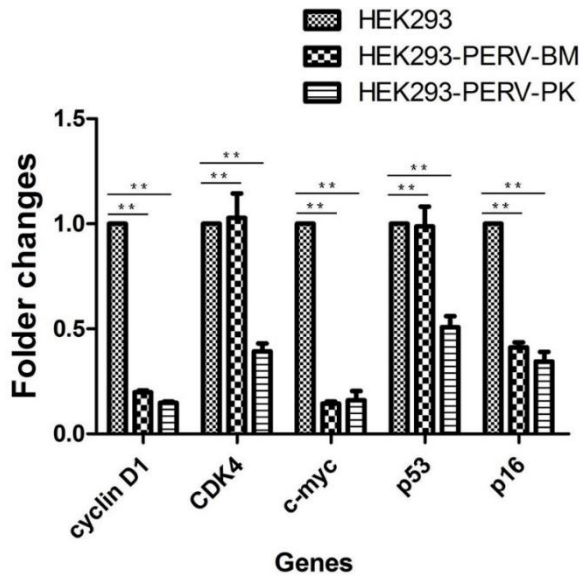


**Fig. 4:** Relative expression of *cyclin D1*, *CDK4*, *c-myc*, *p53* and *p16* genes in HEK293-PERV-BM and HEK293-PERV-PK.

**Expression of *cyclin D1, CDK4, c-myc, p53* and *p16* genes in HEK293-PERV-BM:** Expression levels of *cyclin D1*, *CDK4*, *c-myc*, *p53* and *p16* genes were detected using SYBR Green-based real-time PCR assays post PERV integrating into the chromosome of HEK293 cells (Altun *et al*., 2017; He *et al*., 2018). The expression levels of *cyclin D1*, *p16*, *c-myc* and *p53* decreased more subtly in the HEK293-PERV-BM than those in the HEK293-PERV-PK, while *CDK4* gene expression increased in HEK293-PERV-BM and decreased in the HEK293-PERV-PK.

## DISCUSSION

In this study, we discovered that the infection of PERV from different BM pigs varied in human cells. Moreover, our study for the first time identified the integration sites of PERV-BM in human genome, which was over ±5kb from the transcriptional start sites and CpG island.

Previous data on the infection of PERV from miniature pigs were diverse due to different species. PERV from the MPK cells were discovered to have no infection to human cells (Patience *et al*., 1997). However, PBMC and primary aorta endothelial cells (PAEC) from Göttingen miniature pigs showed different infection to HEK293 cells (Martin *et al*., 1998; Semaan *et al*., 2013). In our study, PERV from BM2 showed infectivity to human cells, and BM4 showed no signs of infection, indicating that PERV-BM from different pigs harbored different infectivity to human cells. The molecular characteristics of PERV-BM, including 2.5 copies of 39bp repeats in the 5'LTR, 12nt curtailment and the mutation in packaging signal of BM4, suggested limited infection of PERV in BM pigs ( Scheef *et al*., 2001; Lee *et al*., 2012; Choi *et al*., 2015). Whether these molecular characteristics could be used as the bio-makers needs further investigations in preliminary screening of pigs for xenotransplantation.

The integration sites of PERV-PK were found to have high similarities to those of murine leukemia virus (MLV) and other gammaretroviruses, suggesting that the integration strategies were specific to each retrovirus genus. In view of the lower transcriptional activity of PERV, there might be fewer integration sites. By comparing the integration features of PERV-BM and PERV-PK, 4 and 157 integration sites were identified in the HEK293-PERV-BM and HEK293-PERV-PK, respectively, displaying variations in position and formation of PERV integration. No integration sites of PERV-BM situated near the transcriptional start site or CpG island of human genome. In addition, the integration of PERV-BM contributed to a slighter decrease in the expression of *cyclin D1*, *c-myc*, *p53* and *p16* compared with those of PERV-PK, while the expression of *CDK4* increased slightly.

In conclusion, it is the first time that few PERV-BM has been integrated into HEK293 cells, suggesting the low possibility of PERV transmission from BM to human beings. However, the integration shows a diversity in 15

BMs. Bama miniature pigs might be a potential xenograft donor duo to low PERV integration into human genomics. Furthermore, more comprehensive assessments on the safety of BM for xenotransplantation are required in a long-term observation.

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**Authors contribution:** JL and HBT executed the experiment and ABB, FLC, SMQ, JFL and SYQ prepared the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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