Originally published as:

Alexander Karlas, Markus Irgang, Jörg Votteler, Volker Specke, Mushin Özel, Reinhard Kurth, Joachim Denner.
Characterisation of a human cell-adapted porcine endogenous retrovirus PERV-A/C (2010) Annals of Transplantation, 15(2) pp. 45-54.

Characterisation of a human cell-adapted porcine endogenous retrovirus PERV-A/C

Alexander Karlas, Markus Irgang, Jörg Votteler, Volker Specke, Mushin Özel, Reinhard Kurth, Joachim Denner

Robert Koch Institute, Berlin, Germany

Summary

Background: *Porcine endogenous retroviruses (PERVs) pose a potential risk for xenotransplantation using pig cells, tissues or organs. A special threat comes from viruses generated by recombination between human-tropic PERV-A and ecotropic PERV-C.*
Serial passages of a recombinant PERV-A/C on human 293 cells resulted in increased infectious titers and a multimerization of transcription factor binding sites in the viral long terminal repeat (LTR).
In contrast to the LTR, the sequence of the env gene did not change, indicating that the LTR represents the determinant of high infectivity.

Material/Methods: *The virus was further propagated on human cells and characterized by different methods (titration, sequencing, infection experiments, electron microscopy).*

Results: *Further propagation on human 293 cells resulted in deletions and mutations in the LTR.*
In contrast to low-titer viruses, the high-titer virus was infectious for cells from non-human primates including chimpanzees. Scanning electron microscopy revealed clustering of budding virions at the cell surface of infected human cells and transmission electron microscopy indicated that the virus infects them via receptor-mediated endocytosis.

Conclusions: *After propagation of PERV on human cells without selection pressure, viruses with different LTR were generated. High titer PERV was shown to infect cells from non-human primates. The experiments performed here simulate the situation in vivo and give an extended characterization of human cell-adapted PERVs.*

Background

Xenotransplantation may develop into a medical technology able to save life or improve its quality. Porcine endogenous retroviruses (PERVs) are considered to be the main microbiological risk if pig cells tissues or organs are to be transplanted as they (i) are integrated in numerous copies in the genome of all pig strains [1–3], (ii) are produced by normal pig cells [4–7], (iii) infect human cell *in vitro* [2,8,9] and (iv) have immunosuppressive properties[10,11]. Three subclasses of infectious PERV have been described, polytropic, human-tropic PERV-A and PERV-B as well as ecotropic PERV-C [12]. Infection studies with PERV showed a productive infection of primary cells of different species including non-human primates [13].
However, evidence for PERV transmission was neither seen in patients who had received porcine xenotransplants nor in butchers frequently exposed to pig tissues [14–17]. Similarly, rats, rhesus macaques, pig tailed macaques and baboons inoculated with high doses of PERV and given strong daily immunosuppressive treatment failed to exhibit evidence of infection [18–22].
In guinea pigs a transient infection was observed, however with a low virus load unable to induce a specific antiviral immune response [23].
Recently, recombinant PERV-A/C able to infect human cells were described which *de novo* integrated into the genome of spleen cells of miniature pigs and melanoma-bearing pigs, but they were not found in the germ line of these animals [24–28].

Serial cell-free passaging of PERV-A/C on human cells, simulating the situation after xenotransplantation, resulted in an increase in the titer of the virus [29,30]. This increase was associated with genetic changes in the viral LTR, in particular with a multimerization of NF-Y transcription factor binding sites in the LTR. Similar changes in the LTR were observed when PERV-A was passaged [29,31]. On the other hand, comparison of highly replication competent PERV-A/C with the paternal PERV-A identified mutations in the *env* gene that may be responsible for higher titers [32].
To characterize the possible evolution of PERVs in xenotransplant recipients, high-titer PERVA/C with a longer LTR were obtained after rapid passages on human cells and then cultured for a long time without passaging. Deletions and mutations were observed after long term culture.
In addition, infection and release of high-titer PERV-A/C were studied by electron microscopy,determinants of high infectivity were screened for and infectivity for non-human primate cells was studied.

Material and Methods

Viruses, virus passages and long-term cultures

PERV/5° was obtained from PERV-NIH, 3rd passage (PERV-NIH/3°, kindly provided by C. Wilson, FDA, Washington, DC, USA) by cellfree passaging on human 293 cells in the presence of 8 µg/ml polybrene (Sigma, Deisenhofen, Germany) [29]. PERV-NIH/3° has been derived from 293 cells infected with PERV released from stimulated pig lymphocytes after two consecutive passages on 293 cells [7,33]. PERV-NIH/3° has been shown to represent a recombination between PERV-A and PERV-C [30]. After isolation, PERV/5° was propagated on 293 cells for three years.

Virus titration

Virus-containing supernatant produced by a confluent PERV-producing cell culture (1×107 293 cells) was taken three days after the last medium change. Cells were removed by centrifugation and sterile filtration (0.45 µm) and the supernatant was serially diluted before transfer to 96-well plates containing uninfected 293 cells (3×104/well) and incubated at 37°C with 5% CO2 and 98% humidity. Titers were determined measuring provirus integration by PCR or by real time PCR.

PCR and cloning of PERV LTRs

For direct amplification of a PERV 5' LTR sequence the primers PK34 (nt3 to 26) and PK26 (nt1134 to 1111) [31] (Table 1) were used.
Amplification was carried out using standard PCR conditions in a Biozym cycler (Biozym Diagnostic, Oldendorf, Germany): one initial cycle of 10 minutes denaturation at 95°C, 35 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 58°C, 1 minute elongation at 72°C, and one final cycle of 7 minutes elongation at 72°C. The amplicons were cloned into a pCR2.1-TOPO vector (Invitrogen). In addition, the primers LTR up and LTR down were used (Table 1).

PCR and cloning of env

DNA from PERV producing cells was extracted using the QIAamp DNA MiniKit (Qiagen).
The *env* gene was amplified using primers Env up and Env down (Table 1). The PCR product was purified, digested with the restriction enzymes SacI and PstI and cloned into the vector pQE30 (Qiagen). The sequence of the insert was determined by DNA sequence analysis (Medigenomix).

Transmission electron microscopy (TEM)

PERV/5° producing 293 cells were fixed with 2.5% glutaraldehyde in HEPES, pH 7.2, for 30 min or several days and investigated as described [34]. The samples were washed with HEPES and fixed with 1% osmium tetraoxide for 1 h. To increase the contrast, specimens were treated with 1% tannic acid for 1 h after osmification and block embedded by mixing equal amounts of cell sediment and low melting agarose (3% in PBS) in the tip of Pasteur pipette. After gelling, the agarose was cut into blocks 1 mm in length, stained for 1 h with 1% uranyl acetate, dehydrated in a graduated ethanol series and embedded in Epon.
Thin sections were cut on a Leica- Ultracut ultramicrotome, mounted on naked 300 mesh copper grids, and post-stained with lead citrate.
Sections were stabilised with a thin layer of carbon evaporation and evaluated using a Zeiss EM 902 at 80 kV.

Scanning electron microscopy (SEM)

PERV/5° producing 293 cells were grown on cover-glasses or after resuspension adsorbed to 1% alcian blue-treated cover-glasses for 20 min at room temperature and fixed with 2.5% glutaraldehyde in PBS for 30 min followed by 1% osmium tetraoxide for 1 h. Specimens were dehydrated in a graduated ethanol series, critical-point dried in carbon dioxide, and mounted on SEM specimen stubs. Samples were sputter coated with an 8 nm layer of gold and evaluated in a LEO 1530-SEM at 5 kV.
For cryo scanning electron microscopy the cover-glasses with PERV/5° producing cells were immediately cryofixed in liquid propane after glutaraldehyde fixation, mounted on the specimen holder of a BAL TEC cryopreparation unit MED 020 and freeze-dried for 3 h.
Specimens were than sputter coated with platinum with a thickness of up to 2–3 nm, transferred with the BAL TEC transfer-unit VCT into the same scanning electron microscope and evaluated under cryo conditions at –100°C and 1–3 kV.

Cloning and sequencing of PERV-A/C molecular clones

Proviral DNA was isolated from cells freshly infected with PERV/5° and cloned into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, USA). The *neo* resistance gene was removed from the vector using *Not* I and *Bst*Z17 I. First, amplification was performed using a primer in the 5' R region of the LTR (PERV-LTR-R *Spe* I) and in the *env* region (PERV-gp70-blunt down) (Table 1), the amplicon was digested at the single *Kpn I* restriction site. pcDNA3.1(+) was also digested using *Kpn I*. Second, an amplification was performed using the primers PERV-3'-LTR-*Kpn* I down and PERV-gp70-mid-*SacI* up, digested again with *Kpn I* and both were ligated to obtain a fulllength clone. For the first clone the CMV promotor was used, for the second both LTRs were amplified and cloned into the pCR-XL-TOPO vector.
Sequencing was performed using different primer pairs.

Infection of non-human primate cells

Rhesus LLCMK2 kidney cells (ATCC) and EBV transformed lymphoblastoid cells from a male chimpanzee (ECACC) were incubated in the presence or absence of 8 µg/ml polybrene.
To confirm and characterize infection, DNA and RNA were isolated at different time points and analyzed for provirus integration using primers specific for *pol, env-A* [9] and allowing to discriminate the expression of spliced *env* and unspliced full-length mRNA (Table 1).

Results

Influence of passaging and long-term culture on recombinant PERV-A/C

Previously we had shown that rapid passaging of PERV/NIH/3°, a recombinant between PERV-A and -C [7,30] on human 293 cells increased the infectious titer of the viruses from 5.3×101 (PERV/NIH/3°, third passage) to 8.3×103 (PERV/5°, fifth passage) and the length of the LTR increased due to a multimerization of 37 bp repeats containing transcription factor NF-Y binding sites [29].
Both viruses contained the receptor- binding domain of PERV-A (and therefore infect human cells) and the LTR of PERV-C, which increased in length during culture. The longest LTR contained 5 repeats (Figure 1A). However, when PERV/5° was cultured for months on human 293 cells without selection pressure, the length of the LTR decreased again (Figure 1B, Figure 2). This virus was called long-term passaged virus (PERV/LT).
Due to a deletion (aa 415–452) and one point mutation in position 508, PERV/LT has lost two NF-Y binding sites (ATTGG) and due to a mutation in position 478 it lost one CCAAT enhancer-binding protein (CEBP) binding site (GAAA). These data may reflect genetic changes in PERVs after infecting humans *in vivo*.

Sequencing the cloned virus

In order to clone a whole recombinant PERV-A/C, DNA and RNA from cells infected with PERV/5° and the cloning strategies described in Materials and methods were used.
Two full-length clones were prepared, one using a CMV promotor, another amplifying both LTR. The whole genome of the last clone was sequenced using different primer pairs (see AY953542).
One clone obtained carried an LTR corresponding to PERV/4° containing two NF-Y binding sites. This clone was later upgraded with an LTR from PERV/5° containing five repeats. These sequencing data confirm results shown in Figure 2B, indicating that after long term culture of cells previously infected
with PERV/5° proviruses with different LTRs were found expressed, mainly PERV/5° and PERV/LT, but obviously also a few PERV/4°.

Search for high-titer determinants

In order to identify determinants responsible for the enhanced titer of the human cell adapted virus, the sequences of the *env* genes (coding for the surface envelope protein gp70 and the transmembrane envelope protein p15E) from PERV-NIH/3°, PERV/5° and PERV/LT were compared.
No changes in the sequence of the receptor-binding site were found. Only a difference in two amino acids (G231R and E415G) had been observed when comparing the *env* genes of PERV/5° and PERV/LT.
Although a substitution at position I140V and another in the proline rich region were recently identified as two determinants of high infectivity of a high titer A/Crecombinant virus (PERV-A14/220), which is 500 fold more infectious than the parental PERV-A [32], here the I140V substitution was already found in PERV/NIH/3°, and therefore it could not be the reason for higher titers after passaging.
No alterations were observed in the proline rich region. The results indicate that the changes in the LTR are likely the determinants of high titer replication.

Morphological characterization of PERVs serially passaged on human cells

Since release of PERV particles from porcine PK-15 cells as well as from 293 cells infected with PERV-A is very low [10,19], the situation in the case of 293 cells infected with PERV/5° was analyzed.
Although no differences in the morphology, in the budding and infection process of PERV/5° compared with PERV derived from PK-15 cells or produced on human 293 cells were observed by transmission electron microscopy, a very high number of particles was observed (Figure 3).
These data were confirmed by scanning electron microscopy showing massive budding

of PERV/5° particles from human 293 cells (Figure 4A). By high quality cryo scanning electron microscopy budding in distinct areas of the cell surface was demonstrated (Figure 4B). It is likely that, as in the case of other retroviruses including HIV-1, the areas of the cell membrane where budding occurs are characterized by a particular lipid content, representing the so-called 'lipid rafts' [35,36].
These data demonstrate that massive budding takes place on the surface of human 293 cells infected with PERV/5°. The presence of "coated pits" during infection (Figure 3, infection, b) indicate that PERV/5° infects human cells via receptor-mediated endocytosis.

Generation of a real time RT-PCR measuring full-length and spliced mRNA

The presence of spliced *env* mRNA is a prerequisite for the translation of the Env protein and particle release. The full-length mRNA encodes for Gag and Pol, the spliced mRNA for Env.
To detect spliced mRNA in cells expressing PERV, a RT-PCR was developed.
To discriminate between full-length mRNA and spliced RNA specific primers were placed in front or behind the
splice donor as well as behind the splice acceptor (Figure 5). Utilization of this primer combination allowed detecting three different amplicons: Two amplicons (380 and 480 bp) corresponding to differently spliced mRNA detected in PK-15 cells releasing PERV-A and PERV-B, and another of 470 bp corresponding to PERV/5°.
This method allows detection of spliced mRNA and an estimation of the probability of protein expression and particle release.

Infection of non-human primate cells

PERV/5° has been shown to infect cells from numerous
species *in vitro* including primary PBMCs from rhesus monkeys and baboons [18,22]. To analyze, whether PERV/5° is able to infect rhesus and chimpanzee cell lines, rhesus LLCMK2 kidney cells and EBV transformed lymphoblastoid cells from chimpanzees (EB176 cells) were incubated with cell-free supernatant containing PERV/5°. Infection was detected in the absence or presence of polybrene one, two or three weeks after infection (Figure 6). To study virus expression in a long-term culture, rhesus LLCMK2 kidney cells and EBV transformed chimpanzee lymphoblastoid cells (EB176 cells) were infected with PERV/5° and cultured over weeks (Figure 7).
Full length mRNA was detected immediately, whereas spliced mRNA was detected only after one week. As mentioned above, the presence of spliced *env* mRNA is a prerequisite for the production of Env protein and release of virus particles.
Although low RT activity was observed in the supernatant, indicating release of virus particles, the released virus did not infect human cells (data not shown).

Discussion

The high-titer PERV-A/C (PERV/5°) was originally obtained by cell-free passaging of a PERV-A/C on human cells in short time intervals (simulating a high selection pressure) and it is characterized by an increase in the number of NF-Y transcription factor binding sites (Figure 1). When PERV/5o was cultured without passaging (absence of selection pressure), viruses with a lower number of NF-Y binding sites, with mutations and deletions accumulated.
This experiment simulates the possible evolution of PERV after infection of a human xenotransplant recipient.
This experiment also demonstrated that PERV-A/C recombinant viruses might represent a new risk for xenotransplantation due to the high replication titers [10,37]. Analyzing the reason for the high titers of PERV-A/C recombinants in comparison with parental PERV-A, an isoleucine to valine substitution at position 140 in the receptor-binding site and changes in the proline rich region had been reported in PERV/5° [32].
In addition, an enhanced reverse transcriptase (RT) activity of PERV-A/C viruses when compared with the RT activity of PERV-A was described [38]. Since in our case the I140V
substitution had been detected already in an early passage of PERV/5°, and no changes in the proline rich region were found, all changes leading to higher titers have to be attributed

to the alterations in the LTR. It is interesting to note, that in the genome of German landrace pigs and Yucatan micropigs, proviruses carrying PERV-C LTRs with five 37 bp repeats were not found (unpublished data).
To avoid the generation of high-titer PERV-A/C recombinants, it is recommended not to use pig strains carrying ecotropic PERV-C for breeding [39].
Whereas PERV-A and PERV-B are present in all pig strains, PERV-C is missing in some of them [12]. However, a recent screening for PERV-C-free animals in colonies of non-transgenic (including 18 wild boars) and multitransgenic animals generated to prevent hyperacute rejection, demonstrated that only 5 of 181 animals were PERV-C negative, indicating that this virus is widely distributed [40].
The results presented here are important for the evaluation of a non-human primate model for analyzing PERV transmission. Non-human primate cells carry the receptor for PERV [41] and can be infected *in vitro* [19,22,42,43], however no transmission was observed *in vivo* [22,44,45].
Not only cell lines from non-human primates such as baboon and rhesus monkey have been infected *in vitro*, even primary cells from these animals were infected using high-titer PERV [19]. A productive infection of cells from baboons and rhesus monkeys was demonstrated by increasing reverse transcriptase activity over time in the supernatant and viral genomic RNA in the pelleted virus.
Cells from pig-tailed monkeys could not be infected productively, only integrated proviral DNA, but no release of virus particles was observed [19], whereas Ritzhaupt et al. [42] showed that cell lines from African green monkeys, rhesus macaques, and baboons were infected with PERV as measured by viral DNA integration and RNA expression using PCR and RT-PCR assays, respectively. Virions released from these infected cells infected productively human 293 cells, but not cells from non-human primates.
This indicatesthat a productive infection, but no replication took place. The low replication rate of PERV in primate cells including most human cells may be explained by inhibition of virus replication by intracellular restriction factor such as TRIM5a and members of the APOBEC family [46,47].
However, it has been shown that cellular restriction factors can be neutralized by higher virus doses, enabling to achieve productive infection at high virus titers and that some PERVs are insensitive to mammalian TRIM5a [38].
In addition, there is evidence that the function of the non-human primate PERV-receptor is not optimal [48]. Based on these results there is an ongoing discussion whether the appropriateness of nonhuman primates as suitable animal models needs re-evaluation.
However, as shown by different laboratories, it was also difficult to infect productively most human primary cells and cell lines with exception of 293 kidney cells (which were transformed by adenovirus 5 and do not express APOBEC).
Of great interest is the mechanism of PERV infection shown here by electron microscopy. We demonstrate that the virus infected human cells by receptor-mediated endocytosis (Figure 3).
Only recently a similar mechanism has been described for HIV-1 [49]. The knowledge of this mechanism has important implications for the development of vaccines, especially for the generation of neutralizing antibodies directed against conserved domains of the transmembrane envelope protein [50].

Conclusions

Although not yet described, multimerization of transcription factor binding sites may also occur *in vivo*, in a virus released from the xenotransplant and adapting to human cells during experimental or clinical xenotransplantation.
However, *in vivo* transmission of PERV has been observed neither in first clinical xenotransplantations nor in experimental xenotransplantations with rats and monkeys using organs, cells or cell-free PERVs [for review see 33]. In monkeys undergoing triple immunosuppressive therapy to simulate the conditions during xenotransplantation no *in vivo* infection with PERV was observed even when high doses of high-titer PERV-A/C were inoculated [22].
The extensive genetic, biochemical and morphological characterization of PERVs being used in several *in vitro* and *in vivo* infection experiments is crucial for the safety evaluation of experimental and clinical xenotransplantations.

Acknowledgments

We would like to thank G. Holland and A. Schnartendorff (Robert Koch-Institute, Berlin) for excellent technical help and Dr. R. Weiss (Wohl Viron Center, London) and Dr. C. Wilson (CEBR, FDA, Bethesda, MD) for their generous gift of viruses and cells.
This work was supported the Deutsche Forschungsgemeinschaft (DE729/3).

References

1. Ericsson T, Oldmixon B, Blomberg J et al: Identification of novel porcine endogenous betaretrovirus sequences in miniature swine. J Virol, 2001; 75(6): 2765–70
2. Patience C, Takeuchi Y, Weiss R: Infection of human cells by an endogenous retrovirus of pigs. Nat Med, 1997; 3: 282–86
3. Patience C, Switzer W, Takeuchi Y et al: Multiple groups of novel retroviral genomes in pigs and related species. J Virol, 2001; 75: 2771–75
4. Le Tissier P, Stoye JP, Takeuchi Y et al: Two sets of human-tropic pig retrovirus. Nature, 1997; 389: 681–82
5. Martin U, Steinhoff G, Kiessig V et al: Expression of pig endogenous retrovirus by primary porcine endothelial cells and infection of human cells. Lancet, 1998; 352: 692–94
6. Tacke SJ, Specke V, Denner J: Differences in release and determination of subtype of porcine endogenous retroviruses (PERV) produced by stimulated normal pig blood cells. Intervirology, 2003; 46: 17–24
7. Wilson C, Wong S, Muller J et al: Type C retrovirus released from porcine primary peripheral blood mononuclear cells infects human cells. J Virol, 1998; 72: 3082–87
8. Martin U, Winkler M, Id M et al: Productive infection of primary human endothelial cells by pig endogenous retrovirus (PERV). Xenotransplantation, 2000; 7: 138–42
9. Specke V, Rubant S, Denner J: Productive infection of human primary cells and cell lines with porcine endogenous retroviruses. Virology, 2001; 285: 177–80
10. Denner J: Immunosuppression by retroviruses: implications for xenotransplantation. Ann NY Acad Sci, 1998; 862: 75–86
11. Tacke S, Kurth R, Denner J: Porcine endogenous retroviruses inhibit human immune cell function: risk for xenotransplantation? Virology, 2000; 268: 87–93
12. Takeuchi Y, Patience C, Magre S et al: Host range and interference studies of three classes of pig endogenous retrovirus. J Virol, 1998; 72: 9986–91
13. Denner J: Xenotransplantation: State of the art of biosafety. In: Jansen ES, Simon JW, (eds.), Xenotransplantation – ethic, economic, social, cultural and scientific background. Saarbrücken: VDM-Verlag; 2008; 7–78
14. Elliott RB, Escobar L, Garkavenko O et al: No evidence of infection with porcine endogenous retrovirus in recipients of encapsulated porcine islet xenografts. Cell Transplant, 2000; 9: 895–901
15. Garkavenko O, Croxson MC, Irgang M et al: Monitoring for presence of potentially xenotic viruses in recipients of pig islet xenotransplantation. J Clin Microbiol, 2004; 42(11): 5353–56
16. Paradis K, Langford G, Long Z et al: Search for crossspecies transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. Science, 1999; 285: 1236–41
17. Tacke S, Bodusch K, Berg A, Denner J: Sensitive and specific immunological detection methods for porcine endogenous retroviruses applicable to experimental and clinical xenotransplantation. Xenotransplantation, 2001; 8: 125–35
18. Denner J: Porcine endogenous retroviruses (PERVs) and xenotransplantation: screening for transmission in several clinical trials and in experimental models using non-human primates. Ann Transplant, 2003; 8: 39–48
19. Specke V, Tacke S, Boller K et al: Porcine endogenous retroviruses: *in vitro* host range and attempts to establish small animal models. J Gen Virol, 2001; 82: 837–44
20. Specke V, Plesker R, Coulibaly C et al: Productive infection of a mink cell line with porcine endogenous retroviruses (PERVs) and lack of transmission to minks *in vivo*. Arch Virol, 2002; 147: 305–19
21. Specke V, Schuurman HJ, Plesker R et al: Virus safety in xenotransplantation: first exploratory *in vivo* studies in small laboratory animals and nonhuman primates. Transplant Immunology, 2002; 9: 281–88
22. Specke V, Plesker R, Wood J et al: No *in vivo* infection of triple immunosuppressed non-human primates after inoculation with high titers of porcine endogenous retroviruses (PERVs). Xenotransplantation, 2009; 16: 34–44
23. Argaw T, Colon-Moran W, Wilson CA: Limited infection without evidence of replication by porcine endogenous retrovirus in guinea pigs. J Gen Virol, 2004; 85(1): 15-19
24. Bartosch B, Stefanidis D, Myers R et al: Evidence and consequence of porcine endogenous retrovirus recombination. J Virol, 2004; 78: 13880–90
25. Denner J: Is porcine endogenous retrovirus (PERV) transmission still relevant? Transplant Proc, 2008; 40(2): 587–89

26. Dieckhoff B, Puhlmann J, Büscher K et al: Expression of porcine endogenous retroviruses (PERVs) in melanomas of Munich miniature swine (MMS) Troll Vet Microbiol, 2007; 123: 53–68
27. Martin SI, Wilkinson R, Fishman JA: Genomic presence of recombinant porcine endogenous retrovirus in transmitting miniature swine. J Virol, 2006; 3: 91–96
28. Wood JC, Quinn G, Suling KM et al: Identification of exogenous forms of human-tropic porcine endogenous retrovirus in miniature swine. J Virol, 2000; 78: 2494–501
29. Denner J, Specke V, Thiesen U et al: Genetic alterations of the long terminal repeat of an ecotropic porcine endogenous retrovirus (PERV) during passage in human cells. Virology, 2003; 314: 125–33
30. Wilson C, Wong S, VanBrocklin M, Federspiel M: Extended analysis of the *in vitro* tropism of porcine endogenous retrovirus. J Virol, 2000; 74: 49–56
31. Scheef G, Fischer N, Krach U, Tönjes R: The number of a U3 repeat box acting as an enhancer in long terminal repeats of polytropic replication-competent porcine endogenous retroviruses dynamically fluctuates during serial virus passages in human cells. J Virol, 2001; 75: 6933–40
32. Harrison I, Takeuchi Y, Bartosch B, Stoye JP:
Determinants of high titer in recombinant porcine endogenous retroviruses. J Virol, 2004; 78: 13871–79
33. Wilson CA: Porcine endogenous retroviruses and xenotransplantation. Cell Mol Life Sci, 2008; 65(21): 3399–412
34. Ozel M, Pauli G, Gelderblom HR: The organization of the envelope projections on the surface of HIV. Arch Virol, 1988; 100: 255–66
35. Metzner C, Salmons B, Günzburg WH, Dangerfield JA: Rafts, anchors and viruses – a role for glycosylphosphatidylinositol anchored proteins in the
modification of enveloped viruses and viral vectors. Virology, 2008; 382(2): 125–31
36. Wang W, Fu YJ, Zu YG et al: Lipid rafts play an important role in the vesicular stomatitis virus life cycle. Arch. Virol, 2009; 154(4): 595–600
37. Denner J: Recombinant porcine endogenous retroviruses (PERV-A/C): A new risk for xenotransplantation? Arch Virol, 2008; 153: 1421–26
38. Wood A, Webb BL, Bartosch B et al: Porcine endogenous retroviruses PERV A and A/C recombinant are insensitive to a range of divergent mammalian TRIM5alpha proteins including human TRIM5alpha. J Gen Virol, 2009; 90: 702–9
39. Denner J, Schuurman H, Patience C: The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – Chapter 5: Strategies to prevent transmission of porcine endogenous retroviruses Xenotransplantation, 2009; 16: 239–48
40. Dieckhoff B, Kessler B, Jobst D et al: Distribution and expression of porcine endogenous retroviruses (PERV) in multi-transgenic pigs generated for xenotransplantation. Xenotransplantation, 2009; 16: 64–73
41. Ericsson TA, Takeuchi Y, Templin C et al: Identification of receptors for pig endogenous retrovirus. PNAS, 2003; 100: 6759–64
42. Ritzhaupt A, van der Laan LJW, Salomon DR, Wilson CA: Porcine endogenous retrovirus infects but does not replicate in nonhuman primate cells and cell lines. J Virol, 2002; 76: 11312–20
43. Blusch JH, Patience C, Takeuchi Y et al: Infection of nonhuman primate cells by pig endogenous retrovirus. J Virol, 2000; 74: 7687–90
44. Garkavenko O, Dieckhoff B, Wynyard S et al: Absence of transmission of potentially xenotic viruses in a prospective pig to primate islet xenotransplantation study. J Med Virol, 2008; 80(11): 2046–52
45. Loss M, Arends H, Winkler M et al: Analysis of potential porcine endogenous retrovirus (PERV) transmission in a whole-organ xenotransplantation model without interfering microchimerism. Transpl Int, 2001; 14: 31–37
46. Goff SP: Retrovirus restriction factors. Mol Cell, 2004; 16(6): 849–59
47. Takeuchi H, Matano T: Host factors involved in resistance to retroviral infection. Microbiol Immunol, 2008; 52(6): 318–25
48. Mattiuzzo G, Takeuchi Y: PERV-A receptors in non-human primates: implication for their employment as an animal model for xenotransplantation.
Xenotransplantation, 2009; 16(5): 383
49. Miyauchi K, Kim Y, Latinovic O et al: HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. Cell, 2009; 137(3): 433–44
50. Fiebig U, Stephan O, Kurth R, Denner J: Neutralizing antibodies against conserved domains of p15E of porcine endogenous retroviruses (PERVs): basis for a vaccine for xenotransplantation? Virology, 2003; 307(2): 406–13

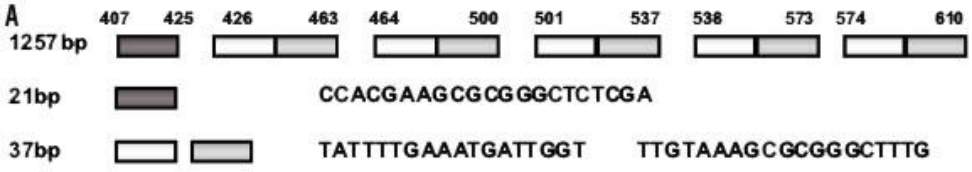
Tables and Figures

Table 1. Primers and probes used.

Primers/probes	**Sequence**
PK34	AAAGGATGAAAATGCAACCTAACC
PK26	ACGCACAAGACAAAGACACACGAA
LTR up	TCTTGGTGACAACATGTCTC
LTR down	AGTGTGGAGTCGGGACAGCT
Env up	TTATGAGCTCATGCATCCCACGTTAAGCC
Env down	CCCAACTGCAGTCTTTTTGGCCGATTATATCT
PERV-LTR-R Spe I	AATTACTAGTGCGTGGTGTACGACTGTGGG
PERV-gp70-blunt down	TCTTTTTGGCCGATTATATCT
PERV-3'-LTR-Kpn I down	TCTCGGTACCGATGCAAACAGCAAGAGGAT
PERV-gp70-mid-SacI up	AACTGAGCTCGATGGGAATTGGAAATGGCC
Pol up	TTGACTTGGGAGTGGGACGGGTAAC
Pol down	GAGGGTCACCTGAGGGTGTTGGAT
Env A up	TGGAAAGATTGGCAACAGCG
Env A down	AGTGATGTTAGGCTCAGTGG
P1 (In front of SD*)	TGCTGTTTGCATCAAGACCGC
P2 (Behind SD)	ACAGACACTCAGAACAGAGAC
P3 (Behind SA**)	ATGGAGGCGAAGCTTAAGGGGA

* SD – splice donor; **SA – splice acceptor.

Figure 1. (**A**) Schematic presentation of the repeat sequences in the LTR of PERV/5°. Dark grey indicates a single 21bp domain, white and light grey a 37bp repeat with the sequences shown. (**B**) Sequences of PERV/3°, PERV/5° and PERV/LT, the domains as shown in A are indicated above the sequence. Factor NF-Y bindings sites are underlined (ATTGG), putative binding sites for CEBP are in bold, the TATA box is framed.



B

```
PERV/3⁰ TCTTGGTGAC AACATGTCTC CCCCACCCCG AAACATGCGC AAATGTGTAA CTCTAAAACA ATTTAAATTA 400
PERV/5⁰ TCTTGGTGAC AACATGTCTC CCCCACCCCG AAACATGCGC AAATGTGTAA CTCTAAAACA ATTTAAATTA
PERV/LT TCTTGGTGAC AACATGTCTC CCCCACCCCG AAACATGCGC AAATGTGTAA CTCTAAAACA ATTTAAATTA

PERV/3⁰ ATTGGTCCAC GAAGCGCGGG CTCTCGATAT TTTGAAATGA TTGGTTTGTA AAGCGCGGGC TTTG------ 470
PERV/5⁰ ATTGGTCCAC GAAGCGCGGG CTCTCGATAT TTTGAAATGA TTGGTTTGTA AAGCGCGGGC TTTGTATTTT
PERV/LT ATTGGTCCAC GAAG------ ---------- ---------- ---------- --GCGCGGGC TTTGTATTTT

PERV/3⁰ ---------- ---------- ---------- -------- ---------- ---------- ---------- 540
PERV/5⁰ GAAATGATTG GTTTGTAAAG CGCGGGCTTT GTATTTTGAA ATGATTGGTT TGTAAAGCGC GGGCTTTGTA
PERV/LT GAAATGACTG GTTTGTAA-- ---------- -TATTTT-AA ATGATTGGTT TGTAAAGCGC GGGCTTTGTA

PERV/3⁰ ---------- ---------- ---------- ---------- ---------- ---------- ---------- 610
PERV/5⁰ TTTTGAAATG ATTGGTTTGT AAAGCGCGGG CTTTGTATTT TGAAATGATT GGTTTGTAAA GCGCGGGCTT
PERV/LT TTTTGAAATG ATTGGTTTGT AAAGCGCGGG CTTTGTATTT TGAAATGATT GGTTTGTAAA GCGCGGGCTT

PERV/3⁰ --TTGTGAAC CCCATAAAAG CTGTCCCGAC TCCACACT 646
PERV/5⁰ TGTTGTGAAC CCCATAAAAG CTGTCCCGAC TCCACACT
PERV/LT TGTTGTGAAC CCCATAAAAG CTGTCCCGAC TCCACACT
```

Figure 2. Analysis of PCR amplification using (**A**) env specifc and (**B**) LTR specific primers.
In A amplification was performed using primers located in the receptor binding site of PERV-A, 1 – water, 2 – uninfected 293, 3 – PERV/3°, 4 – PERV/5°, 5 – PERV/LT, 6 – env plasmid.
In B amplification was performed using LTR specific primers, 1 – uninfected 293 cells, 2–293 cells infected with PERV/3°, 3–293 cells infected with PERV/4°, 4–293 cells infected with PERV/5°, 5, 6–293 cells originally infected with PERV/5° at different time points (1 year, 2 years) of long-term culture (PERV/LT).

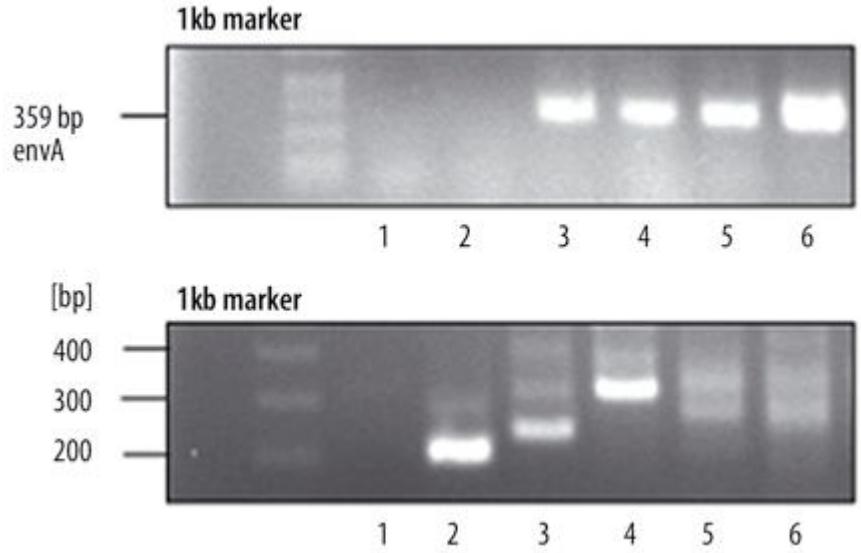


Figure 3. Budding on (top) and infection of (bottom) human 293 cells with PERV/5° (transmission electron microscopy).

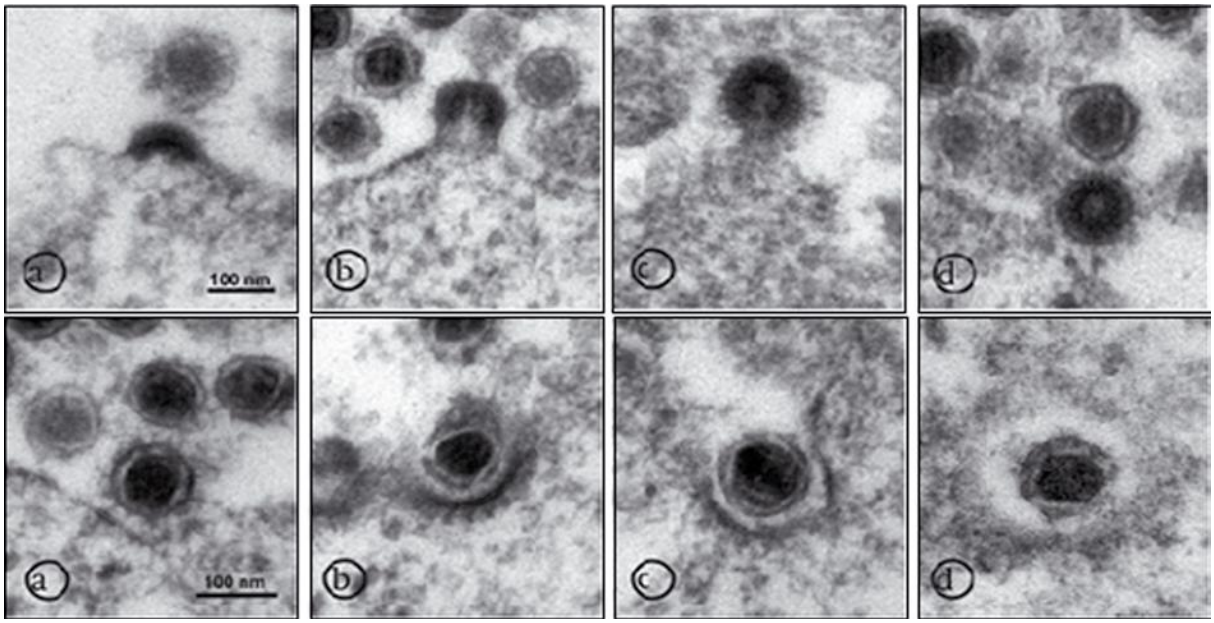


Figure 4. (**A**) Release of PERV/5° particles on the surface of human 293 cells (scanning electron microscopy) and (**B**) (cryo scanning electron microscopy), Insert: arrows indicate envelope glycoprotein spikes on the surface of virus particles.

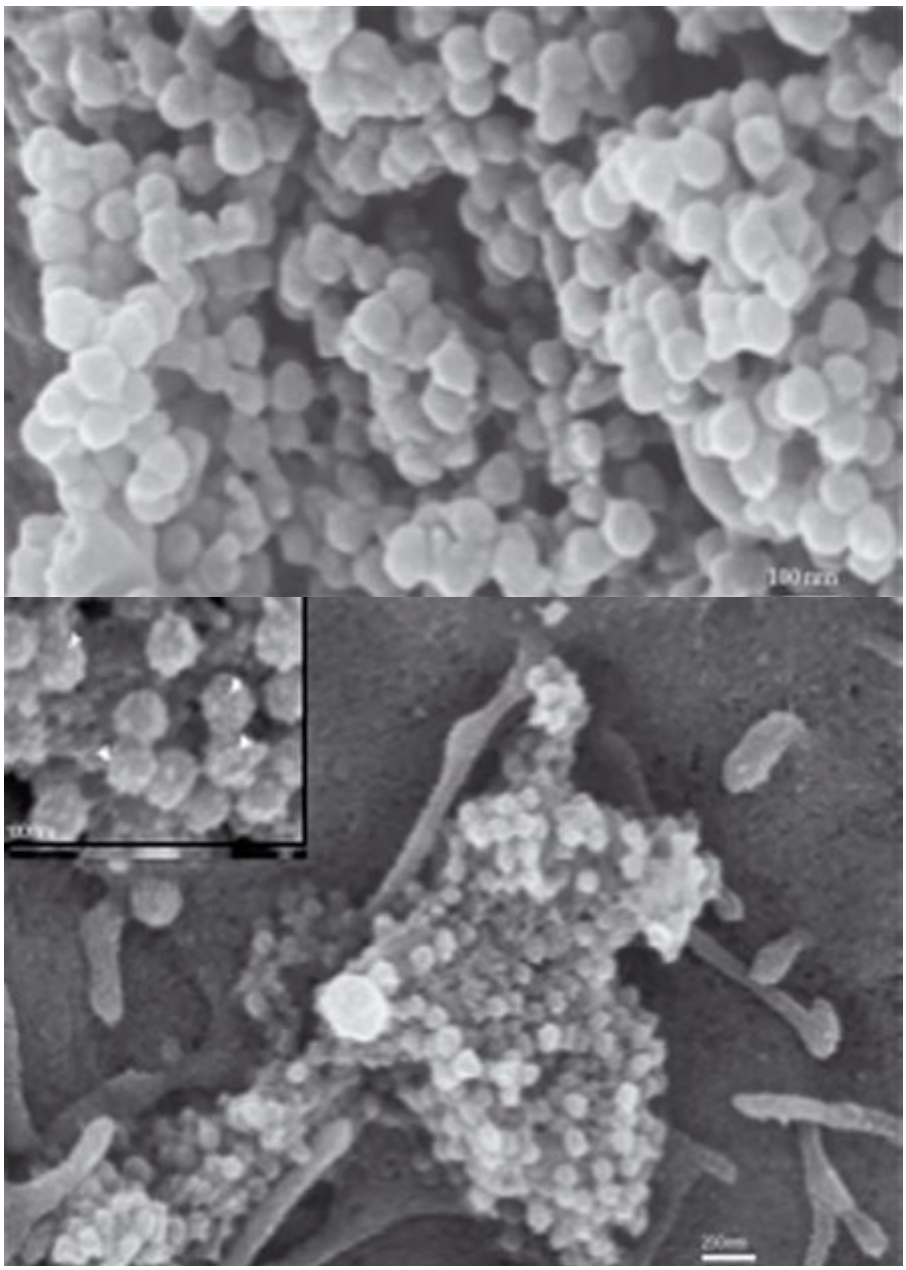


Figure 5. Development of a PCR assay detecting full-length and spliced mRNA of PERV using selected primers in the LTR (P1, in front of the splice donor SD, P2, behind the splice donor SD) and in the env region behind the splice acceptor SA (P3).
Full-length, spliced mRNA, β-actin and β-actin without reverse transcriptase indicating absence of DNA contamination were studied in 293 cells infected with PERV/5° (PERV-A/C), in pig kidney cells releasing PERV-A and PERV-B (PK-15), as well as in uninfected 293 cells.

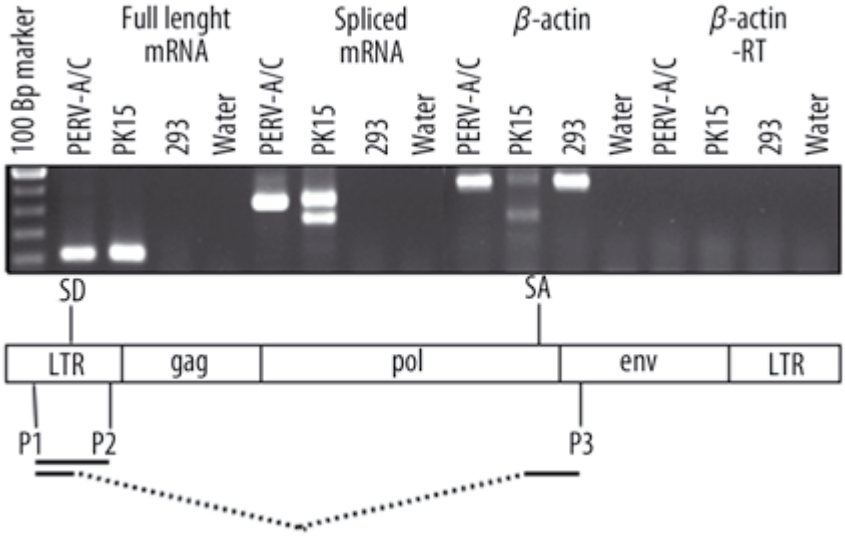


Figure 6. Infection of rhesus kidney cells LLCMK2 (**A**) and lymphoid chimpanzee cells (EB176) (**B**) with PERV/5° (PERVA/ C), (–) indicates absence, (+) presence of polybrene in the culture medium during infection, primers specific for pol, for env-A and β-actin were used.

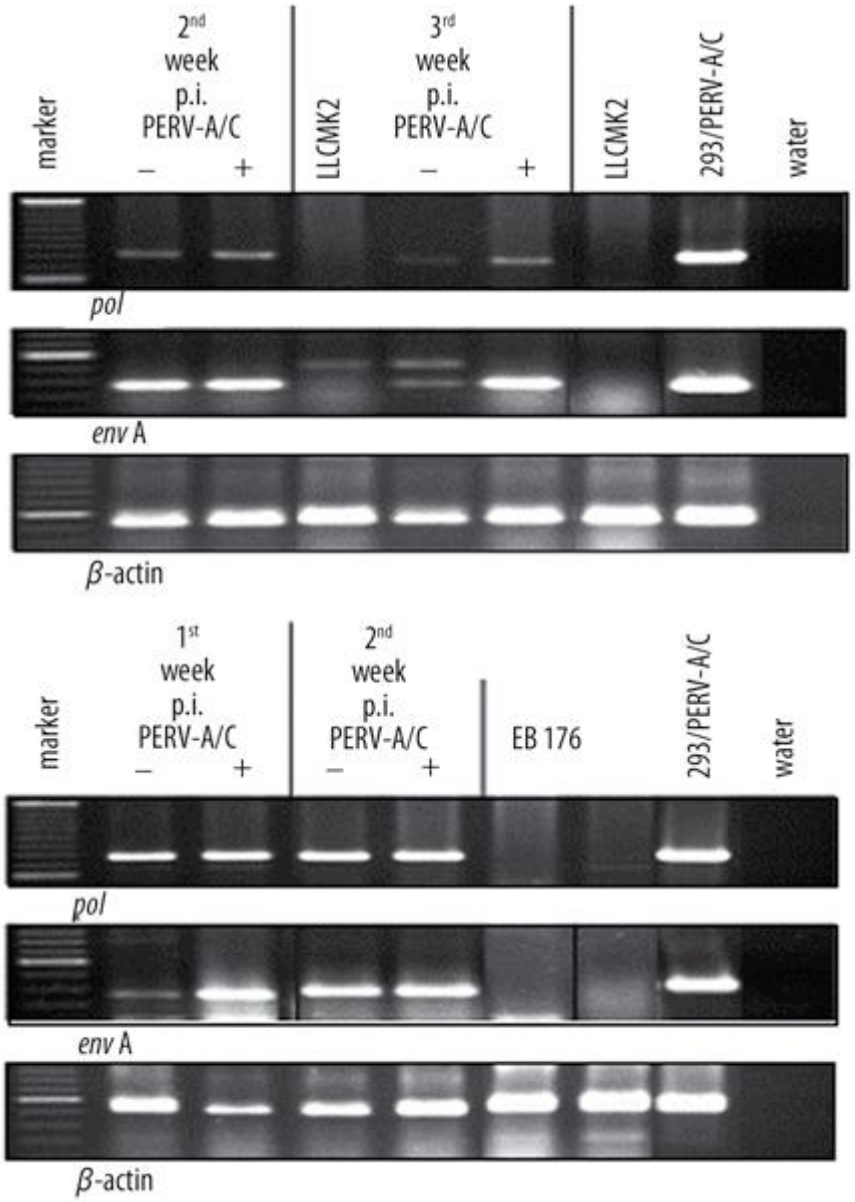


Figure 7. Long-term culture of PERV/5° (PERV-A/C) on rhesus kidney cells LLCMK2 (**A**) and lymphoid chimpanzee cells (EB176) (**B**). Using specific primers, full-length mRNA and spliced RNA as well as β-actin with and without reverse transcriptase (-RT) were studied in these cells over 7 to 8 weeks.